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Production of sugars, ethanol and tannin from spruce bark and recovered fibres

Katariina Kemppainen

VTT

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Abstract

The production of lignocellulosic biofuels and chemicals is driven by the increasing global consumption of energy, food and feed, the depletion of fossil oil reservoirs and efforts to slow down climate change. On the other hand the forest industry faces challenges derived from the digitalization of media, increasing global competition and rising costs of energy and raw materials. Valorisation of the industry-related side- and waste streams in a biorefinery context could help to reduce dependence on fossil resources and introduce new value chains and sources of income for the forest industry. This thesis examined two abundant and underutilized biomass streams spruce bark and recovered fibres as biorefinery feedstocks for the production of sugars, ethanol and tannin.

Spruce bark was found to contain 11-12% tannin and 48-51% carbohydrates, mainly cellulose, pectin and non-cellulosic glucose. The effects of temperature, solids content and the use of selected chemicals on the extraction yield of bark components were investigated. Up to 21% of spruce bark dry matter could be solubilised by hot water extraction at 60-90°C, and an increase in temperature was found to have a significant positive effect on extraction yield. However, the results indicated that a selective extraction of only tannins or carbohydrates is not possible in these conditions. The resulting spruce bark extracts were characterized in detail and found to contain up to 58% tannin and 22-32% carbohydrates, which were mainly present as oligo- and polysaccharides and as glycosylated compounds. Enzymes having hemicellulase, pectinase and β-glucosidase activities could be used to hydrolyse a minimum of 55% of the carbohydrates in the extract to monosaccharides, which could possibly enable their size-based separation by ultrafiltration from larger tannin molecules.

The effects of hot water extraction, steam explosion, and sequential combination of the two on the composition of the insoluble solids, and enzymatic digestibility and fermentation of spruce bark carbohydrates were studied. Steam explosion solubilised pectin and hemicellulose, and increased the enzymatic digestibility of spruce bark carbohydrates from 36% to 75%. A treatment at 190°C without an acid catalyst was found to improve the hydrolysis yield more than a treatment at 205°C or a treatment using an acid catalyst. Hot water extracted bark could be hydrolysed efficiently (80% hydrolysis yield) without steam explosion when an enzyme mixture containing pectinase activity was used, indicating that an additional pretreatment

step is not needed. Furthermore, the results indicated that enzymatic hydrolysates from hot water extracted and steam exploded bark can be fermented to ethanol even at 15% solids loading.

Recovered fibres were fractionated in pilot scale from solid recovered fuel (SRF), a standardised combustion fuel composed mainly of packaging waste, and the composition and enzymatic digestibility of the material were determined. A fibre yield of 25-45% was obtained in the pilot-scale fractionation of three different SRF samples, but it was estimated that a higher yield could be obtained from high quality SRF with industrial scale equipment. The recovered fibres contained at least 46% hexose polysaccharides and 12-17% ash partially derived from non-biomass sources such as inks and coating materials. The enzymatic digestibility of recovered fibres was found to be high without pretreatment (>82% hydrolysis yield in 24 h).

The hydrolysis yield of recovered fibres in high consistency conditions (15-25% dry matter content) was found to be higher than the hydrolysis yield of steam pretreated wheat straw and spruce. Non-ionic surfactants improved the hydrolysis yield of recovered fibres, and the effect was higher at low solids loading. The effects of surfactants on the three feedstocks were compared over a wide range of solids loadings (1-25%), and the results indicated that their effect is dependent on the botanical source, pretreatment and lignin content of the feedstock as well as the mixing regime.

Selected steps for processing spruce bark and recovered fibres were scaled up from laboratory- to small pilot scale. Up to 22 kg of crude tannin powder was produced from spruce bark representing a 9% yield from dry bark. Ethanol production was demonstrated from recovered fibres in seven up to 12-day long continuous pilot experiments according to the FibreEtOH concept. The results of the work carried out in this thesis indicate that the biorefinery concepts presented for spruce bark and recovered fibres have technical potential for industrial application.

Keywords spruce bark, recovered fibres, ethanol, tannin, enzymatic hydrolysis, biorefinery, lignocellulose

Tiivistelmä

Kasvava globaali energian, ruoan ja rehun kulutus, fossiilivarantojen ehtyminen ja etenevä ilmastonmuutos tukevat polttoaineiden ja kemikaalien tuotantoa lignoselluloosasta. Toisaalta median digitalisoituminen, maailmanlaajuisen kilpailun kiristyminen sekä energian ja raaka-aineiden hinnan kasvu asettavat haasteita perinteiselle metsäteollisuudelle. Metsäteollisuuden sivu- ja jätevirtojen hyötykäyttö biojalostamossa voisi vähentää riippuvuutta öljystä ja luoda uusia arvoketjuja ja tulonlähteitä tälle teollisuuden alalle. Tässä väitöstyössä selvitettiin kahden mittavan biomassavirran, kuusen kuoren ja jätekuidun käyttöä biojalostamossa sokerien, etanolin ja tanniinin tuotannon raaka-aineena.

Kuusen kuori sisälsi 11–12 % tanniinia ja 48–51 % hiilihydraatteja, pääasiassa selluloosaa, pektiiniä ja ei-selluloosaperäistä glukoosia. Työssä selvitettiin lämpötilan, sakeuden ja uuttokemikaalien käytön vaikutuksia kuoren ja sen komponenttien uuttosaantoon. Jopa 21 % kuoresta liukeni kuumavesiuutossa (60–90 °C), ja lämpötilan nosto vaikutti positiivisesti uuttosaantoon. Tulosten perusteella voitiin kuitenkin todeta, ettei tanniinien tai hiilihydraattien selektiivinen uutto ole mahdollista tällä tekniikalla. Uutteet sisälsivät vähintään 58 % tanniinia ja 22–32 % hiilihydraatteja, jotka olivat enimmäkseen oligo- tai polysakkaridimuodossa tai osana glykosyloituneita yhdisteitä. Vähintään 55 % uutteen hiilihydraateista pystyttiin entsymaattisesti hydrolysoimaan monosakkarideiksi, mikä voisi mahdollistaa niiden molekyylikokoon perustuvan kalvoerotuksen suuremmista tanniinimolekyyleistä.

Kuumavesiuuton, höyryräjäytyksen ja näiden yhdistelmän vaikutusta kiintoaineen koostumukseen ja hiilihydraattien hydrolyysiin sekä fermentointiin selvitettiin labratoriokokeilla. Höyryräjäytys liuotti pektiiniä ja hemiselluloosaa ja paransi kuoren hydrolysoitavuutta 36 %:sta 75 %:iin. Käsittely 190 °C:ssa oli tehokkaampi kuin käsittely 205 °C:ssa tai käsittely happokatalyytin kanssa. Kuumavesiuutettu kuori hydrolysoitui tehokkaasti (80 % saanto), kun käytettiin pektinaasia sisältävää entsyymiseosta. Tämän perusteella voitiin todeta, ettei erillistä esikäsittelyvaihetta tarvita. Tulosten perusteella kuumavesiuutettu ja/tai höyryräjäytetty kuusen kuori voidaan polysakkaridien hydrolysoinnin jälkeen fermentoida etanoliksi jopa 15 % kuiva-ainepitoisuudessa.

Jätekuitu fraktioitiin pilot-mittakaavassa erilleen standardoidusta kierrätyspolttoaineesta (SRF, solid recovered fuel), joka sisältää pääasiassa pakkausjätettä. Kuitusaanto kolmesta kierrätyspolttoainenäytteestä oli 25–45%, mutta korkeampi saanto on todennäköinen teollisen mittakaavan erotuslaitteissa. Jätekuituerät sisälsivät vähintään 46 % heksoosipolysakkarideja ja 12–17 % tuhkaa, josta osa on peräisin mm. musteista ja päällystemateriaaleista. Jätekuidun havaittiin hydrolysoituvan tehokkaasti ilman erillistä esikäsittelyä (> 82 % hydrolyysisaanto 24 tunnissa).

Jätekuidun hydrolyysisaanto korkeassa sakeudessa (15–25% sakeus) oli korkeampi kuin esikäsitellyn oljen ja kuusen. Varauksettomat surfaktantit paransivat jätekuidun hydrolyysisaantoa suhteessa enemmän matalassa kuin korkeassa sakeudessa. Surfaktantin vaikutusta hydrolyysiin tutkittiin laajassa sakeusskaalassa (1–25

%) ja tulosten perusteella voitiin todeta, että ilmiöön vaikuttavat myös materiaalin alkuperä, esikäsittely, ligniinipitoisuus ja sekoitustapa hydrolyysin aikana.

Valikoidut vaiheet kuusen kuoren ja jätekuidun prosessoinneista vietiin laboratoriosta pilot-mittakaavaan. Kuusen kuoresta tuotettiin enimmillään 22 kg raakatanniinijauhetta, joka vastasi 9 % saantoa kuivasta kuoresta. Etanolin tuotanto jätekuidusta osoitettiin viidessä jatkuvatoimisessa 5–12 päivän pituisessa pilot-kokeessa Fibre-EtOH-konseptin mukaisesti. Työn tulosten perusteella kuusen kuorella ja jätekuidulla on teknistä potentiaalia teollisen mittakaavan biojalostamon raaka-aineiksi.

Avainsanat kuusen kuori, jätekuitu, tanniini, etanoli, entsymaattinen hydrolyysi, biojalostamo, lignoselluloosa

Preface

This thesis work was carried out at VTT Technical Research Centre of Finland during the years 2009-2014 and I am deeply grateful for the opportunity to combine doctoral studies with my work in project management and sales during these years at VTT. Financial support from Tekes – the Finnish Funding Agency for Innovation and sponsor companies considering the projects Probark and BioFoamBark, EU 7th framefork programme considering the project FibreEtOH, and VTT is gratefully acknowledged. The BIOREGS Graduate School for Biomass Refining (Academy of Finland) is acknowledged for providing travel funds and excellent peer-support, seminars and courses for me as a matching funds student.

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Espoo, February 2015

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List of publications

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

I Kemppainen, K., Ranta, L., Sipilä, E., Östman, A., Vehmaanperä, J., Puranen, T., Langfelder, K., Hannula, J., Kallioinen, A., Siika-aho, M., Sipilä, K., von Weymarn, N., 2012. Ethanol and biogas production from waste fibre and fibre sludge – The FibreEtOH concept. Biomass Bioenergy 46, 60-69.

II Kemppainen, K., Inkinen, J., Uusitalo, J., Nakari-Setälä, T., Siika-aho, M., 2012. Hot water extraction and steam explosion as pretreatments for ethanol production from spruce bark. Bioresour. Technol. 117, 131-139.

III Kemppainen, K., Siika-aho, M., Pattathil, S., Giovando, S., Kruus, K., 2014. Spruce bark as an industrial source of condensed tannins and non-cellulosic sugars. Ind. Crops Prod. 52, 158-168.

IV Kemppainen, K., Siika-aho, M., Östman, A., Sipilä, E., Puranen, T., von Weymarn, N., Kruus, K., 2014. Hydrolysis and composition of recovered fibres fractionated from solid recovered fuel. Bioresour. Technol. 169, 88-95.

Author's contributions

I The author planned and carried out the laboratory-scale experiments related to liquefaction at high consistency conditions. She was the leader of the continuous pilot experiments being responsible for their planning, execution and interpreting the results. She had the main responsibility for writing the article and is the corresponding author.

II The author planned and carried out the hydrolysis experiments related to comparing steam explosion and hot water extraction as pretreatments, and interpreted the composition, pretreatment and hydrolysis results together with co-authors. She helped interpret the results concerning the fermentation of pretreated bark. She had the main responsibility for writing the article and is the corresponding author.

III The author planned the composition analysis and hot water extraction experiments. She prepared the extracts for glycome profiling, planned and helped execute the scale-up of extraction, and interpreted the results with co-authors. She had the main responsibility for writing the article and is the corresponding author.

IV The author planned the fractionation experiments together with co-authors and interpreted the results. She planned the composition analysis, hydrolysis experiments and enzyme activity assays and together with co-authors interpreted results. She had the main responsibility for writing the article and is the corresponding author.

Contents

List of abbreviations

AFEX	ammonia fibre expansion
CBH	cellobiohydrolase
CBM	carbohydrate-binding module
CBP	consolidated bioprocessing
CHP	combined heat and power
d.m.	dry matter
DNS	3,5-dinitrosalicylic acid
DP	degree of polymerization
EG	endoglucanase
ELISA	enzyme-linked immunosorbent assay
FPU	filter paper unit
GHG	greenhouse gas
HPAEC-PAD	high-pressure anionic exchange chromatography with pulsed amperometric detection
ILUC	indirect land-use change
MUL	4-methylumbelliferyl-β-D-lactoside
n.a.	not analysed
n.d.	no date
NMR	nuclear magnetic resonance
PEG	polyethylene glycol
SEC	size exclusion chromatography
SHF	separate hydrolysis and fermentation
SRF	solid recovered fuel

SSF	simultaneous saccharification and hydrolysis
TMP	thermomechanical pulp

1. Introduction

1.1 Drivers for lignocellulosic biofuels and chemicals

The production of lignocellulosic biofuels and chemicals is influenced by a number of environmental and economic drivers. Global energy consumption is on the increase due to population growth and the improvement of living standards in emerging economies (United Nations, 2012). Fossil oil and gas are the number one sources of energy accounting for 82% of overall global energy production (International Energy Agency, 2013). The fossil resources were formed millions of years ago from living organisms by anaerobic decomposition, but are not considered renewable because they cannot be replenished on a human time frame. Combustion of these fossil fuels results in a net release of carbon dioxide (CO_2), whereas the combustion of biomass is generally considered to be carbon neutral due to its quickly renewable nature. Carbon dioxide and other greenhouse gas emissions cause irreversible climate change resulting in atmospheric warming, changes in precipitation, and rising sea level (Solomon *et al.*, 2009). Despite efforts in improving energy efficiency, reduction of fossil fuel subsidies and charging for carbon emissions, the energy-related CO_2 emissions are expected to increase 20% by 2035 resulting in a long-term average temperature increase of 3.6°C (International Energy Agency, 2013). Climate change is a major driver for the development of renewable forms of energy such as lignocellulosic biofuels.

Another key driver for the change towards renewables is the price of fossil oil. This affects our lives because it is connected to, for example, the price of food and consumer goods made out of different chemicals and materials as well as the price of electricity and transport fuels. Modern day technology allows the unlocking of new sources of fossil oil such as light-tight oil (shale oil) and ultra-deepwater fields, but their exploitation is more costly. The International Energy Agency (2013) estimated that oil prices will steadily increase by 16% by the year 2035, which will undoubtedly affect the preferences of the energy and fuel markets.

The International Energy Agency defines renewable energy as energy derived from natural processes such as sunlight and wind, which is replenished at a faster rate than it is consumed. Solar and geothermal heat, hydropower, wind and biomass are sources of renewable energy. Of these, only biomass could potentially replace fossil oil-based materials such as plastics. Electricity may partially replace

transport fuels, but certain types of transportation, like aviation, will require liquid fuels for decades or even centuries to come. However, as only 16% of fossil oil goes to non-energy use, such as petrochemicals and materials (International Energy Agency, 2011), the replacement of oil in power generation and transport fuels is of utmost importance. The importance of renewables in the world energy mix will grow, and their share in total power generation is estimated to increase from 20% to 31% by 2035 (International Energy Agency, 2013). Simultaneously, the use of biofuels is estimated to triple by 2035, by which time it will account for 8% of the road transport fuel demand.

There are various types of biomass available in the world such as agricultural products and their residues and forest biomass. Although they by definition are renewable, their use in energy production needs to be assessed using sustainability criteria in order to ensure that the net effect of their use is positive compared to respective fossil alternatives. Important aspects include land availability and land use practices, economic and energy efficiency, equity issues such as geographic distribution of resources, the "food versus fuel" debate, socio-economic issues, environmental effects and emissions (Solomon, 2010). Biofuels should only be used if their large-scale production is environmentally sound and compatible with the socio-economic structure of society (Giampietro and Ulgiati, 1997). The availability of fresh water, soil erosion, air and water pollution from crop production and biofuel refining, biodiversity loss, and labour requirements must be accounted for before a decision on the large-scale production of biofuels can be made. Accounting for indirect land use change (ILUC) is currently debated in international forums as it is one of the possible secondary effects of switching to biofuels from fossil fuels. Indirect land use change occurs when the use of a certain feedstock for the production of biofuels increases the pressure to clear forested land for the production of agricultural crops elsewhere, and thus results in a net emission of carbon from biomass and soil to the atmosphere (Gnansounou *et al.*, 2008).

Lignocellulosic biomass has significant environmental advantages over the food-chain crops wheat and corn which are used for the production of biofuels today. A large amount of lignocellulosic biomass is formed as a waste or by-product of agriculture, forestry and industry, whereas dedicated starchy biofuel crops require planting, fertilization and harvesting. These factors are in part responsible for the greater greenhouse gas (GHG) reduction attributed to biofuels produced from lignocellulosic biomass compared to those produced from starch originating from agricultural crops. The calculation of GHG reductions is complex and depends on system boundaries and allocation principles. Solomon (2010) calculated that the reduction in GHG emissions in advanced cellulosic ethanol concepts is 85-95% compared to fossil fuels, whereas the reduction from typical corn-based ethanol is only 10-20%. An exception is sugarcane, which produces sucrose suitable for human consumption, but which is also used to produce biofuels with significant GHG emission reductions. The major food and biofuel species corn, wheat and sugarcane all produce a lignocellulosic residue (stover, straw and bagasse respectively), which offers immediate and sustained GHG advantages in the production of biofuels (Fargione *et al.*, 2008). Waste biomass is not necessari-

ly free from the effect of ILUC, but for example woody residues have been shown to have a substantially low ILUC potential (Spöttle *et al.*, 2013).

Both consumers and governments have been alerted by the effects of climate change. The increasing demand for renewable energy and other products from renewable resources is supported by national and international policies, subsidies and regulations, as well as the shifting consumer viewpoint in favour of environmentally sustainable production. The EU Renewable Energy Directive (2009/28/EC) requires that the share of energy from renewable sources in the transport sector must amount to at least 10% of overall energy consumption by 2020. This piece of regulation has been the key driver for increasing biofuel production in Europe. Companies are motivated to go green because of legitimation and ecological responsibility, but also because they believe it gives them a competitive edge in the marketplace (Bansal and Roth, 2000). NExBTL, a renewable diesel fuel produced by Neste Oil, and the PlantBottle technology by Coca-Cola Company are examples of this development.

Lignocellulose, an alternative source of fuels and chemicals, can help the world to tackle the challenges of increasing energy demand, prevention of climate change, increasing price of fossil oil, guaranteeing sustainable food supply and reducing environmental risks related to land use practices. It is the task for the global research and development community to solve the scientific and technical challenges in order to unlock the full potential of lignocellulosic biomass.

1.2 Drivers for forest industry renewal

In addition to environmental factors, there are market forces arising from globalization and digitalization of our society that drive the production of renewable fuels and chemicals. The forest-based sector is a major industrial sector in Finland and in Europe. It produces 8% of the manufacturing added value, manages 35% of the landmass and provides income for about 16 million forest owners and 3-4 million workers in the EU (Forest-based Sector Technology Platform, 2013). The traditional forest industry faces harsh competition from emerging low-cost producer nations and is impacted by increasing costs of energy and wood. The strategy of many pulp and paper companies has been to reduce costs, engage in mergers and acquisitions, and implement quality-based innovations (Chambost and Stuart, 2007). The competitive edge of several companies has by tradition been process and systems optimization, which alone is no longer sufficient. An increasing share of the global population is abandoning the daily newspaper and print magazines for digital media. The circa 5% average annual decline of the production of graphic grades in Europe (Confederation of European Paper Industries, 2014) is one of the biggest challenges that the forest industry must tackle. The drivers for change in the forest-based sector are widely recognized but pathways forward remain obscure (Näyhä and Pesonen, 2014). The industry may settle for cutting costs, increasing the value-added of the paper product or moving into niche markets, but many routes have already been exploited (van Heiningen, 2000). One direction

that could create growth for the forest-based sector is the adoption of an enlarged role in the future bioeconomy, in which various biorefining process concepts will be employed to sustainably produce fuels, chemicals, materials, food and feed.

The European Forest-based Sector Technology Platform (2013) has announced that the forest-based sector wants to be a key actor in and an enabler of the bio-based society in which resources are drawn from living nature. This target means diversifying the industry scope outside current markets. The forest biorefinery concept has been proposed as a means to increase the competitiveness of the forest-based sector. Biorefinery is a facility that integrates biomass conversion processes and equipment in order to simultaneously produce a variety of products such as fuels, materials, chemicals, heat and power in proportions that maximize economic return (Ragauskas *et al.*, 2006). Figure 1 presents the range of feedstocks, processes, products and markets available for consideration for a forest biorefinery. A biorefinery integrated to an existing pulp or paper/board manufacturing unit could valorise its own by-products such as sludges and bark, but could also take in additional feedstocks outside the current forest industry value chain such as industrial and agricultural wastes. Partnering with the new customer segments may be needed in order to guarantee the success of a market entry with a new product.

The forest-based sector is already heavily involved in the energy business, as it is the biggest producer and user of bio-based energy in Europe. In Finland, more than 70% of total renewable energy is generated from forest biomass, by-products and residues (Finnish Forest Industries, 2010). The Borregaard facility in Sarpsborg, Norway, is an example of a multiproduct forest biorefinery that produces fuels (ethanol) and chemicals (lignosulfonate, vanillin) in addition to fibre-based products. The forest industry has competence, functional infrastructure and logistical solutions for refining biomass into a multitude of products (Finnish Forest Industries, 2010). Therefore, it makes sense to invest in research and technology related to the field of forest biorefineries – success would be beneficial both to the environment and to the industry.

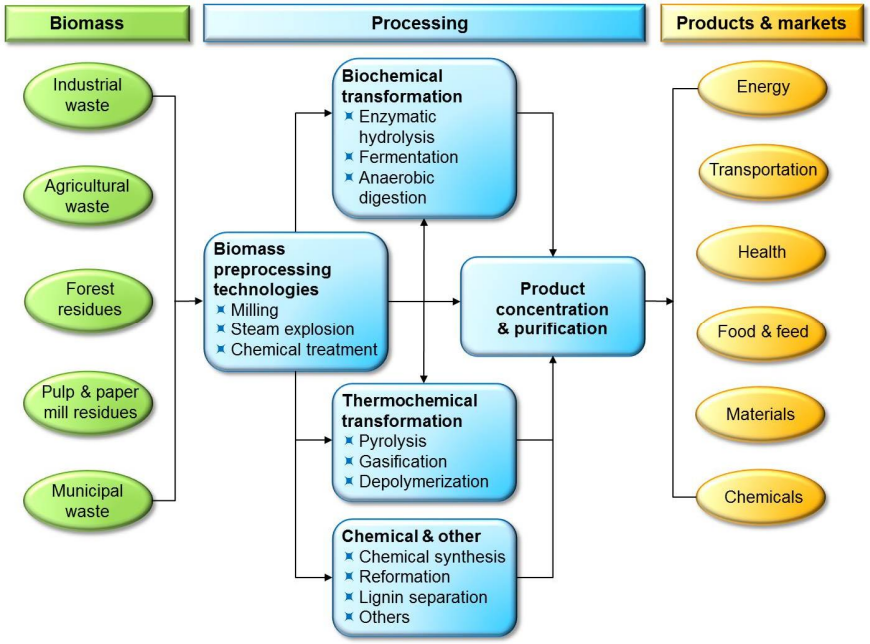

Figure 1. Schematic diagram of biomass options, processes and products and markets available for a forest biorefinery (modified from Chambost and Stuart, 2007).

1.3 Forest biorefinery concepts and technologies

A modern pulp and paper/board mill is in many ways already a biorefinery, since it produces materials (pulp and paper/board), chemicals (tall oil and turpentine) and energy from biomass. In this section however, the focus is on novel forest biorefinery concepts which are for the most part yet to be realised. As presented in Figure 1, there are many potential raw materials, process routes and products to be considered for a forest biorefinery concept. The easiest way to venture into the field is to consider keeping the current product portfolio and process configuration in place and finding new opportunities from side- and waste streams that are currently utilised as energy or put to landfill. This option is available at well-operating and profitable existing sites, where there is no need to question the main fractionation method (kraft cooking) or consider changing the main product. Totally new sites and sites under threat of closure can consider the situation from a wider perspective and contemplate switching into totally new fractionation methods, which extends the range of products that can be produced.

Table 1 categorizes several published forest biorefinery concepts and technologies based on the amount of change required at the mill site. The first category emphasizes concepts that can be implemented without interfering with the production of kraft pulp, the most common pulping method. The LignoBoost process is a

biorefinery concept that exploits the energy surplus of a kraft pulp mill by removing lignin from black liquor and simultaneously increasing the pulp production capacity of the mill (Tomani, 2010). Lignin is extracted at the black liquor evaporation plant, precipitated by acidification and washed and dried, producing a sulphur-containing lignin product with low ash and carbohydrate content. The company Valmet has commercialised this specific version of the technology originally developed by Innventia and Chalmers University of Technology. The first industrial-scale unit was taken into use by Domtar in USA in 2013 (Wimby, 2014). UPM is in the process of commercializing another add-on type of technology, the production of renewable diesel from tall oil, which is another by-product of the kraft pulping process. These two biorefinery concepts do not require changes in the main process of the pulp mill, but produce a new product stream for markets outside the pulp and paper industry, which paves the way for other more radical concepts.

Table 1. Forest biorefinery concepts and the amount of change required at a kraft pulp mill site.

Amount of changes required	Concept/technology
No or small changes to existing products or processes	LignoBoost and other similar kraft lignin concepts
	Production of diesel from tall oil
	Concepts valorising bark, screening dust, sludges and external biomass streams
Moderate to large changes to existing products and processes, option for retrofitting	Pre-extraction of hemicellulose before kraft pulping
	Ethanol production from chips pretreated with green liquor or autohydrolysis
	Organosolv fractionation of wood
Processes requiring heavy investment in new equipment and creation of totally new products	Production of pyrolysis oil from wood
	Gasification of wood for fuels and chemicals
	Fractionation of wood components with ionic liquids

Pre-extraction of hemicelluloses from wood chips affects the quality and the yield of the pulp, which makes the introduction of the extraction step into a pulping process a greater change to the mill compared to for example the production of diesel from tall oil. Dissolving grade pulp with over 90% cellulose content can be made by sulphite pulping or by pre- or post-extraction of hemicelluloses from chips or pulp in a kraft process. A part of the hemicellulose degrades into isosaccharinic acids in the kraft process and is combusted in the recovery boiler, which is the largest single capital investment at the mill (Ragauskas *et al.*, 2006). Thus, a mild pre-extraction of these carbohydrates could also be used to increase the pulp mill's pulp production capacity when the capacity of the recovery boiler is the bot-

tleneck. Hemicellulosic sugars are already used for ethanol production at sulphite mills and through pre- or post-extraction could be used for the production of fuels or sugar platform chemicals at kraft mills as well. The extraction can be carried out with an external acid catalyst, or with only hot pressurized water relying on the acetic acid released from wood (Ragauskas *et al.*, 2006). Alkaline pre-extraction is more suitable for hardwood, as hardwood xylan is more stable under alkaline conditions than softwood galactoglucomannan (Simonson, 1965). The recycled kraft process cooking chemicals white liquor (Helmerius *et al.*, 2010) and green liquor (Walton *et al.*, 2010) offer a source of alkalinity for hemicellulose pre-extraction.

There are a number of methods proposed in the literature which require switching from kraft pulping to other types of fractionation methods. One example is the organosolv concept enabling the production of sulphur-free lignin, which may become a highly valuable raw material for applications such as carbon fibres. In the organosolv process wood chips are cooked with organic solvents such as acetic acid, formic acid or ethanol, which requires effective and potentially costly recycling of the solvent. These concepts are studied both for the production of pulp, and as a pretreatment prior to enzymatic hydrolysis of carbohydrates to sugars. The Alcell process, which later evolved to be known as the Lignol process, is an example of an organosolv cooking of chips with 50% water-ethanol solution. It was demonstrated for pulp production at the Repap Enterprises pulp mill in Canada in the 1990s (Pye and Lora, 1991), but since then the focus has been shifted towards producing ethanol instead of pulp. The Avap technology by the company American Process is another example of an organosolv process, which uses ethanol and sulphur dioxide for biomass fractionation (Nelson *et al.*, 2013).

Repurposing or retrofitting pulp mills to produce ethanol instead of pulp has attracted considerable interest because of the heavy competition in the production of chemical pulps (Hytönen *et al.*, 2010). It is a compromise between add-on concepts and greenfield projects, because it requires switching to new products but allows the use of existing equipment. Biomass fractionation using green liquor is interesting in the repurposing context for a kraft pulping mill, whereas autohydrolysis techniques could be employed at a mechanical pulping unit (Phillips *et al.*, 2013). New equipment is needed in any case for enzymatic hydrolysis, fermentation and distillation. Many other technologies have been developed for the production of ethanol from woody raw materials, such as the SPORL process based on sulphite cooking (Zhou *et al.,* 2013), but not all of them can take advantage of the kraft pulping process or its equipment.

Examples of biorefining technologies at early research stages include the fractionation of wood using ionic liquids. Ionic liquids are salts with a melting point below 100°C, typically below room temperature. Wood can be dissolved in for example imidazolium-based ionic liquids, and lignin and amorphous cellulose may be recovered separately (Kilpeläinen *et al.*, 2007, Fort *et al.*, 2007). Ionic liquid treatment can be used as a lignocellulose pretreatment to improve the digestability of cellulose for enzymatic hydrolysis, or as a fractionation method to produce enriched fractions of cellulose, lignin and hemicellulose. For example the ION-

CELL process targets at producing dissolving pulp, or further along the line, textile fibres, from wood using more benign chemicals than the viscose process (Sixta *et al.*, 2013). The high costs of ionic liquids and the challenges in their efficient separation from the biomass and reuse still require further attention (da Costa Lopes *et al.*, 2013).

Gasification of biomass produces primarily a gaseous mixture of hydrogen and carbon monoxide called syngas, whereas another thermochemical method flash pyrolysis produces primarily a liquid product, pyrolysis oil. Both syngas and pyrolysis oil require heavy processing in order to be used as transport fuels or chemicals (Wright and Brown, 2007). The direct combustion of syngas and pyrolysis oil to energy is the first step to be realized on the route to their efficient conversion to transport fuels and chemicals. One of the first large-scale biomass-based pyrolysis plants has been commissioned in Joensuu, Finland, by the energy company Fortum (Fortum, 2013). It converts harvest residues and other woody biomass into pyrolysis oil. Crude pyrolysis oil is sold to make electricity and district heat in oil boilers. Several on-going demonstration scale projects employ Fischer-Tropsch technology, whereby syngas is converted to diesel, naphtha, ethanol, methanol or methane (Balan *et al.*, 2013). A thermal treatment of lignin in a hydrogen environment could also be used to convert it to phenols and from there to other aromatic chemicals (Pandey and Kim, 2011).

The break-even point for unconventional forest biorefineries has perhaps not yet been reached, especially regarding the production of fuels and bulk chemicals from roundwood. The conversion of Canadian hardwood kraft mills to biorefineries producing commodity chemicals and fuels has been calculated to bring less revenue than the current kraft pulp production (Browne, 2011). It has been estimated that the capital costs of advanced biochemical and thermochemical biorefineries producing the same volume of gasoline-equivalents are on the same level, but five times the cost of comparably sized grain ethanol plants (Wright and Brown, 2007). Studies have shown that a unique supply chain coupled with manufacturing flexibility may be the key to success, rather than competitive advantages related to technology (Chambost and Stuart, 2007). Sharing profits in the supply chain will be challenging and cooperation is needed for success (Näyhä and Pesonen, 2014). The current conservative organization culture in the Scandinavian and North American forest industries may hinder the innovativeness of individual employees (Näyhä and Pesonen, 2014). Market-driven mentality and product-centric research and development must be accompanied by changes in company culture in order to implement the first biorefinery opportunities (Chambost and Stuart, 2007).

1.4 Lignocellulosic feedstocks from the forest industry

Although product-centred orientation is important in developing biorefinery concepts, it can be argued that selection of the raw material is the most important decision to be made. Forest industry related biomass represents a major portion of

the total available lignocellulosic biomass in the world. OECD has estimated that forest residues account for 40% of the total available biomass energy potential, which is more than the potential from crop residues or from energy crops on additional available land (Doornbosch and Steenblik, 2007). Lignocellulosic feedstocks from the forest industry appear in many forms from solids to sludges and solutions. They cover the stumps and needles left at the harvest site, the bark removed from the log on-site, the side-streams produced in the processes, and the products recovered after consumer use. Table 2 summarizes the main forest industry related biomass-containing side- and waste streams, their main chemical components and current uses. Only streams that are currently combusted or remain completely unused are covered. The streams can be divided into native solid streams, processed solid streams and liquid process streams. Some of the native streams such as bark are currently collected or produced in one place, but some such as needles and leaves are not collected at all. The process streams are locally produced and vary in their nature from a waste to a valuable source of energy. Recovered wood and paper and solid recovered fuel (SRF) consisting mainly of packaging waste represent streams that fully or mostly originate from the forest industry but have to be gathered from the consumers post-use. The largest single chemical component of these streams is carbohydrates, namely cellulose and hemicellulose, black liquor being the exception. Fibre sludge, deinking sludge and SRF contain a high amount of ash, whereas thermomechanical pulping (TMP) and debarking effluents contain high amounts of water soluble extractives. Most processed and recovered streams contain some components not originating from biomass; plastics, stickies and printing inks in SRF and deinking sludges being the extreme examples of this. The price of the streams in Table 2 is calculated based on their density, energy density and dry matter content as reviewed by Alakangas (2000) and their price per MWh in 2014 in Finland as given confidentally by FOEX Indexes Ltd.

This thesis examines the valorisation of two lignocellulosic streams from the forest industry: spruce bark and recovered fibres derived from SRF. Their availability is briefly reviewed in the following sub-sections as it is the starting point which largely determines the biorefinery location and capacity.

Table 2. Lignocellulosic feedstocks from the forest industry. Prices calculated on the basis of data by Alakangas (2000) and confidential information from FOEX Indexes Ltd.

Stream	Type	Main components	Current use	Price
Softwood bark	Solid native streams	Carbohydrates, extractives, lignin	Energy production, landscaping	47-133 €/t d.m.
Logging residues		Carbohydrates, lignin, extractives	Energy production or no use	71-191 €/t d.m.
Needles, leaves		Carbohydrates, extractives, protein	No use	Collection costs
Sawdust		Carbohydrates, lignin, extractives	Energy production, wood products, pulp	37-112 €/t d.m.
Recovered wood		Carbohydrates, lignin, extractives, potentially toxic chemicals	Energy production, landfill	38-156 €/t d.m.
Fibre sludge	Solid processed streams	Carbohydrates, ash, lignin	Energy production, landfill	Negative to low
Deinking sludge		Carbohydrates, lignin, ash, inks, stickies	Landfill, energy production	Negative
Solid recovered fuel (SRF)		Carbohydrates, plastics, ash, lignin, inks, stickies	Energy production	From negative to moderate
Debarking effluent	Liquid process streams	Soluble carbohydrates, extractives	No use	Negative
TMP process waters		Soluble carbohydrates, extractives	No use	Negative
Kraft mill black liquor		Lignin, carboxylic acids, ash, extractives, soluble carbohydrates	Recycling of the chemicals, energy production	Low

1.4.1 Availability of spruce bark

Spruce is the most common or the second most common tree species in the Nordic forests (Yrjölä, 2002). The Finnish forest industry consumed 20.7 Mm^3 spruce roundwood (solid cubic metres including bark) in 2012, of which 58% was used by the wood products industry and the rest by the pulp and paper industry. Consequently, there is slightly more spruce bark available from the wood products industry than from the pulp industry in Finland. The wood products industry used large spruce logs for sawmill products (85%) and for plywood and veneer production (15%). The pulp industry used spruce for mechanical pulp production (66%) and for chemical pulp production (34%) (Finnish Forest Industries, 2013).

The relative amount of bark in spruce logs depends on the age and thickness of the tree, volume- and mass-based estimates being both typically in the range of 10-15% (Jensen *et al.*, 1963, Fengel and Wegener, 1984; Zhang and Gellerstedt, 2008). In narrow trees and in the top part of the tree there is more bark relative to wood compared to thick trees and the lower part of the tree. The dry density of fresh spruce bark is 365 kg/m^3 for normal bark and 410 kg/m^3 for the thick bark in old trees (Kärkkäinen, 1976). Thus the amount of spruce bark generated at mills in Finland is 0.8-1.2 Mt/a (d.m.) calculated with an estimated average dry density of 380 kg/m^3. As a comparison the Finnish forest industry used 2.8-3.6 Mt sawmill chips and sawdust (7.4 Mm^3 volume, 380-480 kg/m^3 average dry density) in 2012 (Finnish Forest Industries, 2013).

The debarking units at saw mill and pulp mill yards vary greatly in capacity in Finland. On average a large pulp mill like UPM Kaukas, Stora Enso Imatra or Metsä Fibre Joutseno uses annually 3-5 Mm^3 wood producing 110 000-170 000 t bark per year (assuming similar amounts of bark in all tree species). On the other hand a large Finnish saw mill like Metsä Wood Lappenranta sawmill, or UPM Savonlinna plywood mill uses 500 000 m^3/a wood producing 20 000-30 000 t/a bark. Small saw mills use only 10 000 m^3 wood per year producing 390-580 t/a bark. Spruce may be debarked wet in large drums where the logs rub against each other, or dry in rotary debarkers that take in one log at a time. Wet debarking uses 0.6-2 m^3 water per solid m^3 of wood, whereas only 0.1-0.5 m^3/m^3 is used in dry debarking (Kylliäinen and Holmbom, 2004). Rotary debarking is typically used in the sawmill and veneer industry, whereas drum debarking is used in pulp mills. Spruce bark from sawmills comes from relatively older and thicker trees and is often more recently felled. In addition, due to the preferred debarking method, it may have been less in contact with water and thus be better suited to an extraction process using water as a solvent.

Bark is typically combusted at industrial, and to a lesser extent, municipal heat and power plants (Alakangas, 2000). High moisture and ash content, inhomogeneity and softness make softwood bark a less preferred combustion fuel for power plants. Coniferous bark has a net calorific heating value of circa 9 MJ/kg in its typical moisture content (Alakangas, n.d.). Spruce bark has been used in chip boards and panels, but its addition reduces the strength of the products compared

to wood (Schmidt-Vogt, 1986). The use of bark in composting, mulching and gardening has a long history (Schmidt-Vogt, 1986). Bark coverage prevents the growth of weeds and eventually bark composts into nutritious soil for the plants. These competing uses affect the price and the availability of bark on a local level. They must also be accounted for in the sustainability assessment of a bark biorefinery concept. Nevertheless, bark, being already available at industrial sites and having a relatively low combustion value, is a strong candidate as a new raw material stream for the production of renewable fuels and chemicals.

1.4.2 Origin and availability of solid recovered fuel

Solid recovered fuel (SRF) is defined as a solid fuel prepared from non-hazardous waste meeting the classification and the specification requirements laid down in the standard EN15359 (European Recovered Fuel Organisation, n.d.). SRF is typically municipal solid, industrial, or commercial waste, which is homogenised and upgraded to a quality that can be traded amongst producers and users. Packaging waste composed mainly of paper, board and plastics, comprises a major part of SRF, especially when it is prepared from commercial and retail waste. SRF is a heterogeneous fuel with a net calorific value from 3 to above 25 MJ/kg depending on the original source. The lignocellulose content of SRF is a key attribute as it is the climate neutral part of the fuel (Flamme and Geiping, 2012). Astrup *et al.* (2009) estimated the paper content of SRF to be 54% of dry content, and reviewed the biogenic carbon content to be from 45 to 85% of the total carbon, which accounts for 45% of SRF dry weight. The average biomass content was calculated to be 86% for the ash-free portion of Finnish SRF produced from commercial and industrial waste, and 66% for the ash-free portion of Finnish SRF produced only from industrial waste (Vesanto *et al.*, 2007).

The production of SRF in 2010 was estimated to be circa 12 Mt/a in the EU, although the potential is much higher, estimated at circa 70 Mt/a (Straetmans, 2010). The production of refuse-derived fuel (RDF), which is shredded and sorted but not a standardized fuel, is even higher (Rotter *et al.*, 2011). SRF is utilised for energy production in cement kilns, coal-fired power plants, lime kilns, industrial boilers and combined heat and power (CHP) plants, which reduces the amount of waste going to landfill. The largest European CHP and power plant capacities for SRF are currently in Germany, Finland and Sweden (European Recovered Fuel Organization, n.d.). The Lahti Energia power plant in Finland combusts annually 250 000 t SRF using energy-efficient gasification technology. Their fuel is produced from so-called “energy waste” collected in Finland from households and the commercial sector (Lahti Energia, n.d.). The price of SRF depends very much on the local situation and can in some cases be negative (Department for Environmental, Food and Rural Affairs, 2008).

SRF is best available in highly populated areas, which makes it different from most other lignocellulosic biomass streams and makes it possible to consider setting up a biorefinery in an urban environment. SRF is most probably the cheap-

est standardized source of pulp and paper fibres, since recovered paper grades in the paper recycling system are relatively expensive. Due to the high local availability and low cost of SRF, it is a very potential lignocellulose-containing raw material for a forest biorefinery.

1.5 Structure and chemistry of lignocellulose

Lignocellulose is a matrix of natural polymers arranged and aligned from macro- to micro- and nanoscale. The three main components, cellulose, hemicellulose and lignin, form the basic composite structure of the plant cell wall. Minor components such as pectin, extractives, protein and ash are present in varying proportions depending on the botanical source. Out of the typical biorefinery feedstocks, cellulose is most abundant (>43%) in sugarcane, switchgrass, and hardwood (as reviewed by Pauly and Keegstra 2008). Many important feedstocks such as corn stover, switchgrass, sugarcane bagasse, rice straw and wood contain approximately one third of hemicellulose (31-33%). Wheat straw is an exception as it contains only about 35% cellulose and 23% hemicellulose (Pauly and Keegstra, 2008). The variation in the amount of lignin is the greatest ranging from 12% to 28% in the common feedstocks. Softwood contains the highest and switchgrass and rice straw the lowest amounts of lignin (Pauly and Keegstra, 2008). These natural polymers are in contact with each other through covalent bonds such as those between lignin and hemicellulose and through hydrogen bonds, which keep individual cellulose chains packed into microfibrils.

The plant cell wall is arranged in layers (Figure 2). Between the cells is the middle lamella, which is rich in pectin. The primary cell wall is an extendable layer that grows when the cell is growing. It is rich in cellulose, hemicellulose and pectin. The thick secondary cell wall forms in some species such as trees when the cell is fully grown. In a mature tree it can account for up to 89% of the dry mass of the tree (Rowell *et al.*, 2005). Water in the secondary cell wall is replaced by lignin, which makes the structure impermeable for water and enzymes (Pauly and Keegstra, 2008). The secondary cell wall is divided into three layers S1, S2 and S3 with a varying fibril orientation.

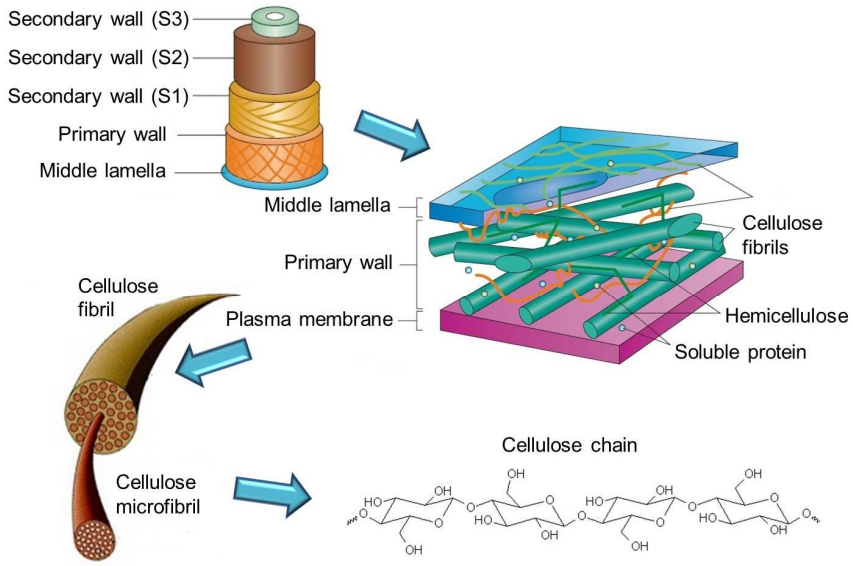

Figure 2. Structure of a plant cell wall and the orientation of cellulose chains into fibrils. Modified from Sticklen (2008) and Moore *et al.* (1998).

1.5.1 Polysaccharides

The basic structure of cellulose is a chain of glucose units, systematically named as unbranched 1,4-linked β-D-glucan (Figure 2). These glucose units alternate in their orientation making cellobiose, a dimer of glucose, the actual repeating unit. The glucan chains interact with each other via hydrogen bonds forming rigid crystalline microfibrils that typically consist of 36 chains and are 2-4 nm wide (Guerriero *et al.*, 2010). Interestingly these fibrils appear to adopt a helical twist in their native state (Lichtenegger *et al.*, 1999). Between the tightly arranged sections there are amorphous regions where the chains are less organized.

Hemicelluloses are branched amorphous heteropolysaccharides having a backbone formed of neutral sugars. They do not form microfibrils themselves but they may attach on the surface of cellulose microfibrils via hydrogen bonding and link cellulose microfibrils to each other (Somerville *et al.*, 2004). Hemicelluloses have a lower degree of polymerization (DP) than cellulose, only 80-200 compared to 6000-14 000 (Sjöström, 1981; Harris and Stone, 2008). The most common hemicellulose in hardwoods is glucuronoxylan, whereas in softwoods galactoglucomannan and arabinoglucuronoxylan are the dominant types (Pu *et al.*, 2011). Xylans have a backbone consisting of xylose with varying patterns of substitution and degree of acetylation. Hardwood xylans are partially acetylated (3.5-7.0 acetyl groups per 10 xylose units) and contain only a small amount of glucuronic acid,

whereas softwood xylans are not acetylated and contain more glucuronic acid (Pu *et al.* 2011; Ragauskas *et al.*, 2006). Galactoglucomannan backbone is built up of a random pattern of glucose and mannose and it contains galactose side groups linked to mannose. Softwood galactoglucomannans are either highly branched containing relatively more of galactose or less branched with relatively less galactose (Ragauskas *et al.*, 2006). Xyloglucan is another important class of hemicelluloses. It is also a 1,4-linked β-D-glucan like cellulose, but is branched with xylose residues which are further appended with galactose and L-fucose. Xyloglucan self-associates with cellulose microfibrils and may link neighbouring strands (Cosgrove, 2005; Kaida *et al.*, 2009). In addition to glycosidic bonds and acetylation, non-cellulosic plant polysaccharides may contain other units linked with ester bonds. Examples include glucuronoarabinoxylan which can contain ester-linked ferulic acid (Harris and Trethewey, 2010).

Pectin is a polysaccharide mainly consisting of galacturonic acid, an acidic sugar. It has been claimed to be structurally and functionally the most complex polysaccharide in plant cell walls (Mohnen, 2008). Uronic acids are the components responsible for creating the negative charge in native wood and mechanical pulp (Sjöström, 1989). Heterogeneity and branching determine whether pectin is classified as homogalacturonan, rhamnogalacturonan I or rhamnogalacturonan II. Homogalacturonan, which accounts for more than 60% of pectins in plant cell walls, is a linear polymer of α-1,4-linked D-galacturonic acid (Ridley *et al.,* 2001). Some of the carboxyl groups in homogalacturonan are methyl esterified and the O-2 and O-3 groups may also be acetylated. The negatively charged unmethylated carboxyl groups may interact with Ca^{2+} to form a stable gel in cell walls (Liners *et al.*, 1989). Rhamnogalacturonan I backbone is constructed of a repeating disaccharide of galacturonic acid and L-rhamnose, in which the rhamnose is substituted with neutral and acidic oligosaccharide side chains containing L-arabinose and galactose (Lerouge *et al.*,1993) and also ferulic and coumaric acids (Saulnier and Thibault, 1999). Rhamnogalacturonan II is also heavily substituted but has only galacturonic acid in its backbone and rhamnose only in the side chains.

1.5.2 Lignin

Lignin, the third main natural polymer in biomass, is structurally and chemically very different from cellulosic and non-cellulosic polysaccharides. It is a very interesting source of renewable chemicals as it is the only major biomass component that has aromatic structures in its monolignol building blocks, namely coniferyl, sinapyl and *p*-coumaryl alcohol. The respective lignin subunits guaiacyl (G), syringyl (S) and p-hydroxyphenyl (H) are bound to each other by various types of bonds including ester and ether bonds producing what is commonly thought of as a complex and branched matrix. However, Crestini *et al.* (2011) suggested that milled wood lignin of spruce may be composed of linear oligomers with 6-12 phenolic units. Lignin links covalently to hemicellulose and pectin, forming lignin-carbohydrate complexes (Iversen, 1985). In softwood, lignin is associated with at

least arabinose- and galactose-containing hemicelluloses (Laine *et al.*, 2004). In spruce, lignin is linked to xylan and glucomannan (Lawoko *et al.*, 2005).

1.5.3 Wood extractives including tannin

Non-carbohydrate low molecular weight components that can be extracted with different solvents are commonly named extractives. They range from lipophilic compounds such as fatty acids to hydrophilic compounds such as stilbene glycosides, and may also be volatile in nature. Extractives take care of different functions in the tree. Fats form an energy store whereas terpenoids and resin acids protect the tree from microbial attack (Amidon *et al.*, 2011). Tall oil, mainly composed of resin and fatty acids, and turpentine, mainly composed of the terpenes α- and β-pinene, are currently commercially the most important non-energy by-products of the kraft pulp industry (Amidon *et al.*, 2011).

Tannins are polyphenols that show phenolic reactions and are able to precipitate proteins. Their molecular weight ranges typically from 500 to 3000 Daltons (Bate-Smith and Swain, 1962) and they have a degree of polymerization between 2 and 10. Nevertheless, higher degrees of polymerization close to 100 have been reported for tannins corresponding to a molecular weight of 28 000 Daltons (Sanoner *et al.*, 1999). Most tannins are water-soluble, but some high-molecular weight tannins or tannins complexed with proteins or polysaccharides may be insoluble (Serrano *et al.*, 2009). Certain types of tannins form insoluble phlobaphenes and red-coloured anthocyanidins at low pH (Serrano *et al.*, 2009). Tannins can be structurally divided into hydrolysable tannins and condensed tannins (Wilson and Thomas, 1927). Phlorotannins composed of phloroglucinol units can be distinguished as a third group of tannins found solely in marine brown algae (Ragan and Glombitza, 1986). Hydrolysable tannins have a sugar core, which is bound to gallic or ellagic acids by easily hydrolysable ester bonds. Condensed tannins, also called proanthocyanidins, are composed of three-ring flavonoid units bound to each other by stable C-C bonds (Figure 3). The B-type proanthocyanidins have C-C bonds in the 4-6 and 4-8 position whereas A-types have additional C2-C7 ether-linkages (Serrano *et al.*, 2009). The number of hydroxyl groups on the B-ring determines whether a tannin is a procyanidin (catechin units with two hydroxyl groups) or a prodelphinidin (gallocatechin units with three hydroxyl groups). Furthermore, the stereochemistry around the central carbon C3 determines whether the tannin is catechin (trans form) or epicatechin (cis form). Gymnosperms, including softwood, produce only condensed tannins (Bate-Smith, 1977).

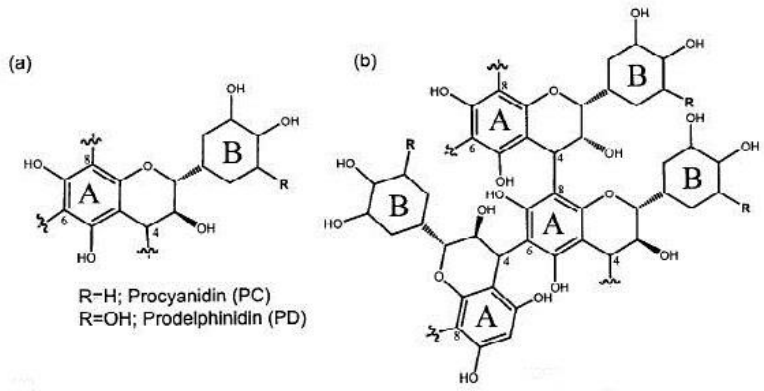

Figure 3. Structures of condensed tannin: (a) a trans monomer unit and (b) a trimer showing different intermonomer linkages (Kraus *et al.*, 2003).

1.5.4 Structure and chemistry of spruce bark

Wood bark consists of all tissues outside the cambium, which is the growing section of the tree. Bark can be separated into inner and outer layers called phloem and periderm (Chang, 1954; Figure 4). Nutrients travel in the phloem of the tree between different parts of the plant, whereas periderm, composed of phellem, cork cambium and phelloderm, protects the tree from environmental elements. Spruce bark contains the same main biomass components cellulose, hemicellulose and lignin as spruce stemwood, but has a more complex profile and a larger quantity of extractives. The inner and the outer bark have a chemically distinct composition, which complicates the composition analysis of bark (Norin and Winell, 1972; Krogell *et al.*, 2012; Zhang and Gellerstedt, 2008; David and Atarhouch, 1987). Moreover, the mass ratio between inner and outer bark appears to vary significantly. Zhang and Gellerstedt (2008) determined that there is more outer than inner bark in spruce (from 1:1 to 2:1 mass ratio), whereas Krogell *et al.* (2012) reported that there was twice as much inner than outer bark in their spruce sample. David and Atarhouch (1987) concluded that whole spruce bark contains 40% of inner bark and 60% of outer bark. In the next sub-sections the main features of spruce bark composition are summarized reviewing in detail especially its carbohydrate composition and phenolic components.

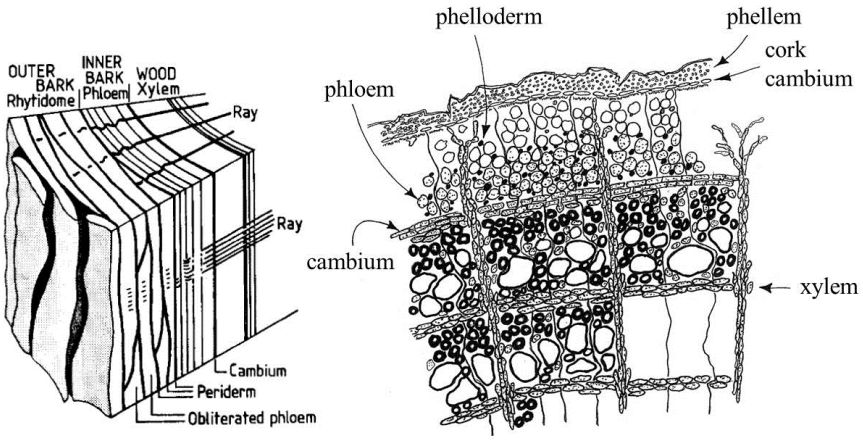

Figure 4. Two schematic pictures of a tree trunk showing the location of xylem (the wood), cambium (the separating layer), phloem (the inner bark) and rhytidome or periderm (the outer bark), which consists of phelloderm, cork cambium and phellem. Modified from University of Florida (2011) and Fengel and Wegener (1984).

Polysaccharides and simple sugars

Table 3 reviews the literature on the carbohydrate profile of *Picea abies* spruce bark including mono-, di-, oligo- and polysaccharides and glucosidic sugars. Spruce bark contains less cellulose than spruce stem wood. The cellulose content of spruce bark is ca. 15%, and the cellulose is more concentrated in the inner bark, which may contain up to 23% cellulose (Le Normand *et al.*, 2012; Krogell *et al.*, 2012). The inner bark of *Picea abies* contains in general more carbohydrates, also non-cellulosic polysaccharides (Zhang and Gellerstedt, 2008, Krogell *et al.*, 2012). The most abundant monosaccharides of non-cellulosic carbohydrates in spruce bark are glucose, galacturonic acid and arabinose (Krogell *et al.*, 2012; Painter and Purves, 1960). Xylose, galactose and mannose are present in lower amounts. Glucose, arabinose and galacturonic acid are present in structures that are easily extracted with hot water, whereas the extraction yield of mannose, xylose and galactose by hot water is lower (Le Normand *et al.*, 2012). The sugar profile of bark reveals the presence of pectic polysaccharides, which contain mainly galacturonic acid but also rhamnose and arabinose. The high amount of pectin differentiates spruce bark most distinctly from spruce stemwood. For comparison, the main non-cellulosic polysaccharides in spruce stemwood are galactoglucomannan and arabino-4-O-methylglucuronoxylan (Timell, 1967). Painter and Purves (1960) showed that the inner bark of White spruce (*Picea glauca*) contains chemically linked glucose and xylose as “glucoxylans”. Hemicellulose in bark appears to be partially acetylated releasing acetic acid (ca. 1% of bark dry matter)

in alkaline conditions (Krogell *et al.*, 2012). The non-cellulosic glucose in the bark of spruce species may at least partially be starch. The bark of autumn-felled trees usually contains considerable amount of starch granules in the parenchyma cells functioning as a food reserve (Chang, 1954; Painter and Purves, 1960). Another source of polymeric non-cellulosic glucose may be callose, which is a 1,3-linked β-D-glucopyranose (Painter and Purves, 1960; Fu *et al.*, 1972). Spruce bark callose fills the sieve pores in non-functioning or dead sieve cells. It is also produced when the bark is wounded (Fu *et al.*, 1972).

Table 3. The carbohydrate composition of *Picea abies* spruce bark presented as anhydrosugars (% of bark dry matter). Simple sugars and glycosidic sugars are included in the table. C-Glc = cellulosic glucose, NC-Glc = non-cellulosic glucose.

Sample	C-Glc	NC-Glc	GalA	Ara	Xyl	Gal	Man	Rha	MeGlcA	Total	Reference
Inner bark	22.5	13.5	6.4	5.4	2.5	2.3	1.4	0.9	0.6	33.0	Krogell *et al.*, 2012
Outer bark	10.7	6.1	4.5	3.9	4.5	1.4	2.3	0.6	1.1	24.4	Krogell *et al.*, 2012
Whole bark	14.9	9.2	3.7	6	2.8	2.3	3.1	0.9	0.4	28.4	Le Normand *et al.*, 2012
Whole bark	18.5	14.1	n.a.	4.8	4.5	2.4	3.4	0.7	n.a.	49.1	von Dietrichs *et al.*, 1978
Whole bark	38.5		n.a.	7.1	3.9	3.7	3.1	n.a.	n.a.	56.3	Eskilsson and Hartler, 1973

The high content of extractable simple sugars is another main difference between spruce bark and stemwood. The mono- and disaccharides glucose, fructose and sucrose are dominant components in spruce bark water extracts (Kylliäinen and Holmbom, 2004). These free simple sugars consist of 7-12% of bark dry matter (Zhang and Gellerstedt, 2008). The free sugars are at their maximum level after the growing season and at their lowest in May/June (Weissman, 1984). It is interesting that although many authors referred to in this section (Krogell *et al.*, 2012; Weissmann, 1984; von Dietrichs, 1978; Kylliäinen and Holmbom, 2004) analysed fructose in bark extracts, none report the presence of fructose-containing polysaccharides in spruce bark.

Considering the high amount of extractives in bark, one must account for the glycosidic sugars being part of total carbohydrates. For long it has been known that some polysaccharides may be bound to non-carbohydrate materials such as tannin, lignin and other substances (Painter and Purves, 1960). This topic is discussed in more detail in the following sub-section.

Lignin, tannin and stilbenes

The main phenolic components in spruce bark are lignin, tannin and stilbenes. The pyrolysis-gas chromatography-mass spectrometry profile of spruce bark lignin was similar to that of spruce milled wood lignin indicating a similar structure (Krogell *et al.*, 2012). However, the determination of the lignin content of bark is more complex because of the high amount of extractives. Tannin and stilbene glucosides may condense into insoluble material at low pH and show up as Klason lignin (*i.e.* acid insoluble material) in the gravimetric lignin analysis (Painter and Purves, 1960; Zech *et al.*, 1987; Krogell *et al.*, 2012). Suberin, an aliphatic-aromatic cross-linked polymer present in outer spruce bark (Ekman and Reunanen, 1983; Gandini *et al.*, 2006) may also be determined as Klason lignin. For example the amount of acid insoluble material in inner spruce bark was 32%, whereas after the removal of lipophilic and hydrophilic extractives it was only 12% (Krogell *et al.*, 2012). Typical lignin structures such as dimers of coniferyl alcohol have been shown to coexist with condensed tannin in spruce outer bark (Zhang and Gellerstedt, 2008) making the analytical separation of lignin and tannin difficult. The outer bark is richer in lignin-type structures than inner bark. Zhang and Gellerstedt (2008) detected 34.8% Klason lignin in extractives-free outer bark and only 3.1% in extractives-free inner bark. The amount of acid insoluble material appears to increase during long-term storage. Lehtikangas (2001) stored additive-free pellets made from 60% spruce and 40% pine barks containing 20% woody impurities, and found that the amount of Klason lignin (no pre-extraction) increased from 37.4% to 48.3% in 6 months. Jirjis and Theander (1990) reported the Klason lignin content of mixed bark (70% spruce bark) to increase from 35% to 51% in 24 months of uncompacted indoor storage.

The reported tannin content of spruce bark varies between 4% and 15% (Grassmann *et al.*, 1956; Surminsky, 2007; Peltonen, 1981; Krogell *et al.*, 2012; Schmidt-Vogt, 1986). The highest tannin content is assumed to be in the bark of 30-60 year old trees (Surminsky, 2007). On the other hand, fresh undried spruce bark contains more soluble tannins than dried bark (Grossmann *et al.*, 1956). Zhang and Gellerstedt (2008) reported a higher abundance of tannins in the outer bark whereas Krogell *et al.* (2012) found more tannin in the inner bark of spruce. Grossmann et al. (1956) stated that tannin in the inner bark is colourless, completely water soluble and lower in molecular weight, whereas tannin in the outer bark is coloured, has a higher molecular weight and is not completely water soluble. The polyphenolic content of spruce bark shows only small seasonal differences (Weissman, 1984).

Spruce tannins have a B-type proanthocyanidin structure with interflavonoid linkages between C4 and C8. The tannin units are mainly procyanidins with two hydroxyl groups in the B ring, but a very small amount of prodelphinidin (0.08% of bark dry matter) with three hydroxyl groups has also been detected in the bark of *Picea abies* (Matthews *et al.*, 1997). Zhang and Gellerstedt (2008) found only trans isomers (catechin) in terms of the configuration of the two chiral carbons in the dihydropyran ring, but Matthews *et al.* (1997) found 80% epicatechin, the cis

form, and 20% catechin in spruce bark. The traditional view has been that spruce tannins are solely composed of interlinked flavonoid units. However, Pan and Lundgren (1995) detected 3'-*O*-methylcatechin 7-*O*-β-D-glucopyranoside in root bark of *Picea abies*, showing that condensed tannin subunits can be covalently linked with glucose. Zhang and Gellerstedt (2008) concluded on the basis of their 1D and 2D nuclear magnetic resonance (NMR) data that spruce tannin (acetone-water 2:1 extract) is mainly composed of flavonoid units (53% of extract dry weight), but also contains stilbene units (33%), a high degree of glucose substitution and minor amounts of lignin-related structures (Figure 5). They estimated that every second flavonoid monomer in the condensed tannin of spruce bark is covalently bound to a glucose unit. They also detected flavonoid structures in the insoluble fraction of bark indicating the presence of tannin-lignin copolymers. Their later work has shown the presence of stilbene polymers or stilbene-procyanidin co-polymers in spruce bark acetone-water extracts (Zhang, 2011).

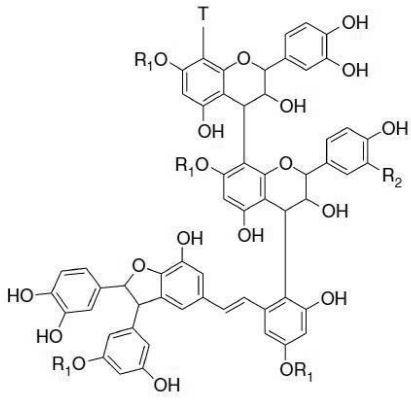

Figure 5. Proposed chemical structure of a segment of condensed tannins in spruce bark. R1 = glucose or H, R2 = OH or OCH_3, T = tannin. (Zhang and Gellerstedt, 2008).

Stilbene glycosides account for 5-10% of spruce bark dry weight and are significantly more abundant in the inner bark compared to outer bark (Kylliäinen and Holmbom, 2004; Zhang and Gellerstedt, 2008; Mannila and Talvitie, 1992; Krogell *et al.*, 2012). They are suspected to have a role in the antifungal defence of the tree (Hammerbacher *et al.*, 2011). Isorhapontin and astringin are the most abundant types accompanied by minor amounts of piceid (Figure 6). Astringin concentration was found to increase from the cambium towards the cortex tissue in Sitka spruce bark, whereas the trend for isorhapontin was the opposite (Toscano-Underwood and Pearce, 1991). The amount of free stilbenes is typically low as they are less water soluble compared to the corresponding glycosides (Kylliäinen and Holmbom 2004; Krogell *et al.*, 2012). Isorhapontin and piceatannol, the free

form of piceid, have been shown to possess antileukaemic properties (Mannila and Talvitie, 1992).

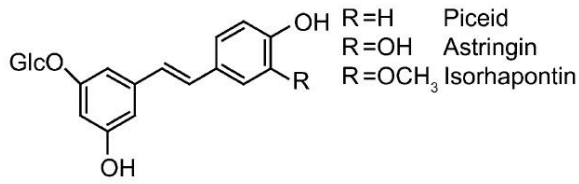

Figure 6. The structure of abundant spruce bark stilbene glycosides (Hammerbacher *et al.*, 2011).

1.6 Production of fermentable sugars from lignocellulose

Lignocellulose is a recalcitrant but abundant source of carbohydrates, which can be processed into fermentable sugars. In the following sections different aspects of the production of fermentable sugars from lignocellulose using enzymes are discussed including pretreatment, hydrolytic enzymes, combining hydrolysis and fermentation in biorefinery concepts, hydrolysing lignocellulose at high consistency and using surfactants to improve hydrolysis yield. Research concerning the enzymatic hydrolysis of the two raw materials in the experimental part of the thesis, spruce bark and recovered fibres, is reviewed in the end of the section. Carbohydrates can also be hydrolysed to monosaccharides using acids, but this route is not further elaborated.

1.6.1 Pretreatment

The crystalline nature of cellulose and its close association with hemicellulose and lignin makes the structure of lignocellulose resistant to degradation (Sarkar *et al.*, 2009). Pretreatment of lignocellulos is a process step performed to make cellulose more amenable to enzymatic hydrolysis (Kumar *et al.*, 2009). Pretreatment should preferably address all the key factors that make cellulose recalcitrant to enzymatic hydrolysis: the presence of lignin and hemicellulose, the crystallinity of cellulose, and the particle and pore size of biomass. The choice of pretreatment, whether it is chemical, physical or biological, affects all other processes of the biorefinery entity (da Costa Sousa *et al.*, 2009). Pretreatments can be classified in many ways. In this summary the following division is used: thermal and thermochemical methods, chemical methods including acid, alkaline and oxidative treatments, mechanical methods, and other methods including solvent-based and biological methods.

Thermal and thermochemical methods

This category comprises a number of methods employing steam or water, pressure and high temperature. Commonly some mechanical impacts are combined with the thermal and thermochemical effects. Small amounts of acid catalysts may be used to enhance the digestion, but the role and the concentration of the acid is smaller compared to acid pretreatments. Steam pretreatment has been the preferred method in the first wave of new lignocellulosic ethanol plants (Balan *et al.*, 2013). It, especially with H_2SO_4 or SO_2 as a catalyst, appears to be a popular choice for the pretreatment of softwoods (as reviewed by Galbe and Zacchi, 2007). The pretreatment employs direct saturated steam at 160-240°C increasing the process pressure to 0.7-4.8 MPa (Agbor *et al.*, 2011). The pressure may be released in an explosive step, in which case the method is called steam explosion. Treatment time is commonly from 5 to 15 minutes. Steam pretreatment partially solubilizes hemicellulose and may degrade the labile pentose sugars to furfural and hydroxymethylfurfural. The chemical structure of lignin changes in steam pretreatment as it becomes more condensed, its α-reaction sites become partially blocked, and its tendency to non-productively adsorb enzymes increases (Shevchenko *et al.*, 1999; Rahikainen *et al.*, 2013a). Hemicellulose removal is considered to be the most important effect of steam pretreatment, but the particle size reduction and the increase of pore volume also affect the digestibility of cellulose in a positive manner (Mosier *et al.*, 2005). In liquid hot water treatment biomass is treated with pressurized water for up to 20 min at temperatures of 140–230°C in a co-current, counter current or a flow through reactor (Agbor *et al.*, 2011; Mosier *et al.*, 2005). The risk of yield loss of sugars as degradation products is lower compared to steam pretreatment, because the concentration of solubilized hemicellulose and lignin products is lower (Hendriks and Zeeman, 2009).

Chemical and mechanical methods

Chemical methods include those in which acidic, alkaline or oxidative reagents have a crucial role in the process. Dilute acid treatment has its origins in the production of furfural from biomass using sulphuric acid (Root *et al.*, 1959; Mosier *et al.*, 2005). Later, the use of nitric, hydrochloric and phosphoric acids has also been demonstrated for pretreatment purposes (Agbor *et al.*, 2011). The main target of the pretreatment is to remove hemicelluloses from biomass in order to improve the accessibility of cellulose. It is considered more suitable for hardwoods and herbaceous biomass than more recalcitrant softwoods (Zhu *et al.,* 2010). Neutralization agents are needed in dilute acid methods, which may lead to problems with excessive production of gypsum if H_2SO_4 is used as the acid. Dilute acid pretreatment can be carried out in batch or continuous reactors or in a continuous refiner (Fang *et al.*, 2011). Acidic conditions can be used to boost the efficiency of pretreatments based on sulphite cooking such as the Bali pretreatment method of the company Borregaard (Rodsrud *et al.*, 2010) and the SPORL pretreatment (Zhu *et*

al., 2009). Relatively mild conditions in the SPORL pretreatment generate less fermentation inhibitors than dilute acid pretreatment but produce good hydrolysis yields even from softwoods (Zhu *et al.,* 2010).

Ammonia fibre expansion, the AFEX treatment, is the most studied alkaline pretreatment method. It uses liquid ammonia to treat biomass, and has a similar explosion stage to steam explosion, but operates at lower temperatures, in the range of 60-140°C (Holtzapple *et al.*, 1991; da Costa Sousa *et al.*, 2009). Ammonia becomes gaseous when pressure is released, and can be efficiently recovered leaving the biomass dried and ready for enzymatic hydrolysis. Crystallinity of cellulose decreases and its crystal structure changes from cellulose I to cellulose II (Mosier *et al.*, 2005). Hemicellulose and lignin mostly remain in the biomass but they are altered so that the digestibility of the material is increased (Agbor *et al.*, 2011). Ammonia treatments are more suitable to feedstocks with low lignin content. In addition to ammonia, alkaline pretreatments may also use lime or sodium hydroxide. Full recycling of the alkaline agent is in practice not possible because the biomass always consumes some of the alkali (Mosier *et al.*, 2005; Hendriks and Zeeman, 2009).

In an oxidative pretreatment the biomass is treated with an oxidizing compound such as oxygen gas, ozone, hydrogen peroxide or peracetic acid, which reacts with lignin, but may also have unwanted side-reactions with cellulose and hemicellulose (da Costa Sousa *et al.*, 2009; Hendriks and Zeeman, 2009). Combining pressurized oxygen with alkaline chemicals produces easily digestible biomass (Kallioinen *et al.*, 2013). Treating wet biomass with air or oxygen at high temperature is called wet oxidation, which results in partial dissolution of hemicellulose and lignin (Schmidt and Thomsen, 1998).

Mechanical methods are related to the reduction of the particle size of biomass. Different types of chipping, grinding and milling such as ball milling reduce the crystallinity of cellulose, which improves its accessibility to enzymes (Kumar *et al.*, 2009; Millett *et al.*, 1979). Woody biomass requires more energy for size reduction than herbaceous biomass (Zhu *et al.,* 2010). Refining with a disk-refiner, a PFI mill or a valley beater, which are laboratory-scale equipment used for refining of paper pulps, further improves the hydrolysis of pretreated substrates (Chen *et al.*, 2013; Jones *et al.,* 2013). Hydrolysis yields of both mechanical and bleached chemical softwood pulps can be increased significantly by stone grinding (Hoeger *et al.,* 2013). However, mechanical size reduction alone is rarely sufficient to dramatically increase cellulose conversion to sugars, and it is considered not to be economically feasible due to high energy requirements. However, it should be noted that most thermal and chemical pretreatment methods require some particle size reduction to overcome mass and heat transport problems (da Costa Sousa *et al.*, 2009; Hendriks and Zeeman, 2009).

Other pretreatment methods

Organosolv and ionic liquid treatments briefly described in Section 1.3 are among the most efficient lignin fractionation methods, which at the same time make the

remaining cellulose highly accessible to enzymes. Organosolv pulping is especially promising for woody feedstocks (Zhu et al., 2010), but the addition of SO_2 appears to be needed for softwoods (Iakovlev et al., 2009). Biological processes using fungi and actinomycetes have been proposed as very low energy pretreatment methods. The organisms are able to secrete extracellular enzymes that remove or modify lignin making the biomass more digestible for hydrolytic enzymes. Very long treatment times are the downside of this option (da Costa Sousa *et al.*, 2009). The enzymatic digestability of biomass has also been shown to improve using supercritical CO_2 (Zheng *et al.*, 1995) and pulsed electric fields (Kumar *et al.*, 2011). An alternative to using enzymes for the hydrolysis of cellulose is to continue the acid pretreatment in harsher conditions so that cellulose is also hydrolysed. Strong acid hydrolysis using concentrated hydrochloric or sulphuric acid can reach near-theoretical sugar yields (Moe *et al.*, 2012) but requires good corrosion-resistance from the process equipment. The Plantrose process developed by the company Renmatix claims to hydrolyse both hemicellulose and cellulose in lignocellulosic biomass using only supercritical water (Renmatix, n.d.).

1.6.2 Enzymes for lignocellulose degradation

A variety of enzymes working in synergy is needed for the complete hydrolysis of the carbohydrates in lignocellulose (Van Dyk and Pletschke, 2012). Filamentous fungi, such as the industrially exploited *Trichoderma reesei* and *Aspergillus niger,* and many aerobic bacteria secrete free enzyme systems, whereas complex bound enzyme systems called cellulosomes are found on the surface of anaerobic bacteria. Lignocellulose-degrading enzymes that degrade or modify glycosidic bonds are listed as structurally-related families in the CAZy database (CAZy, n.d.).

The enzymes responsible for hydrolysing cellulose into gluco-oligosaccharides, cellobiose and glucose monomers are exo-1,4-β-glucanases EC 3.2.1.91 and EC 3.2.1.176 (cellobiohydrolase), endo-1,4-β-glucanases EC 3.2.1.4, and β-glucosidases EC 3.2.1.21 as illustrated in Figure 7 (see reviews by Van Dyk and Pletschke, 2012, Lynd *et al.*, 2002, Bayer *et al.*, 1998 and Wilson, 2011). Endoglucanases attack cellulose in the middle of the chain preferably in amorphous regions, and reduce the degree of polymerization of cellulose. They typically have an open active site and a non-processive manner of action (Srisodsuk *et al.,* 1998). Cellobiohydrolases cleave cellobiose from the crystalline part of cellulose with a preference for either the reducing or nonreducing end of the chain (Teeri, 1997). Cellobiohydrolases have a tunnel-shaped active site and a processive manner of action along the chain length. Their rate-limiting action starts by adsorption of the enzyme on cellulose surface and the location of the chain end on the surface. The chain end becomes threaded into the catalytic tunnel and the hydrolysis of every second β-glycosidic bond begins (Bansal *et al.*, 2009). If the processive enzyme becomes stuck on cellulose surface, it must desorb and readsorb in order to continue the reaction. Family 7 cellobiohydrolases are the most sensitive glycoside hydrolases towards end product inhibition by cellobiose (Teugjas and

Väljamäe, 2013), which makes them a potential bottleneck in the hydrolysis reaction. Endoglucanases are needed to create new starting sites for cellobiohydrolase; otherwise the hydrolysis cannot proceed (Wood and McCrae, 1979). β-Glucosidases, which also have a pocket-shaped active site, finish the chain reaction by releasing glucose from soluble substrates cellobiose or cellotriose (Varghese *et al.,* 1999). An important structural feature of cellulases is the carbohydrate-binding module (CBM), which is linked to the core enzyme by an extended linker region (Boraston *et al.*, 2004). It promotes the association of the enzyme with the substrate. In high solids environment the advantage of having a CBM is diminished, and its omission may improve the recyclability of the enzymes in the process (Varnai *et al.*, 2013).

In addition, some non-hydrolytic enzymes may have an assisting role in the hydrolysis of cellulose. Expansins and expansin-like proteins can loosen or disrupt the packaging of the cellulose fibril network and work in synergy with cellulase enzymes (see review by Arantes and Saddler, 2010). Lytic polysaccharide monooxygenases, formerly called Family GH61 enzymes, generate oxidized and non-oxidized chain ends in crystalline cellulose thus boosting the efficiency of common cellulases (reviewed by Dimarogona *et al.*, 2012).

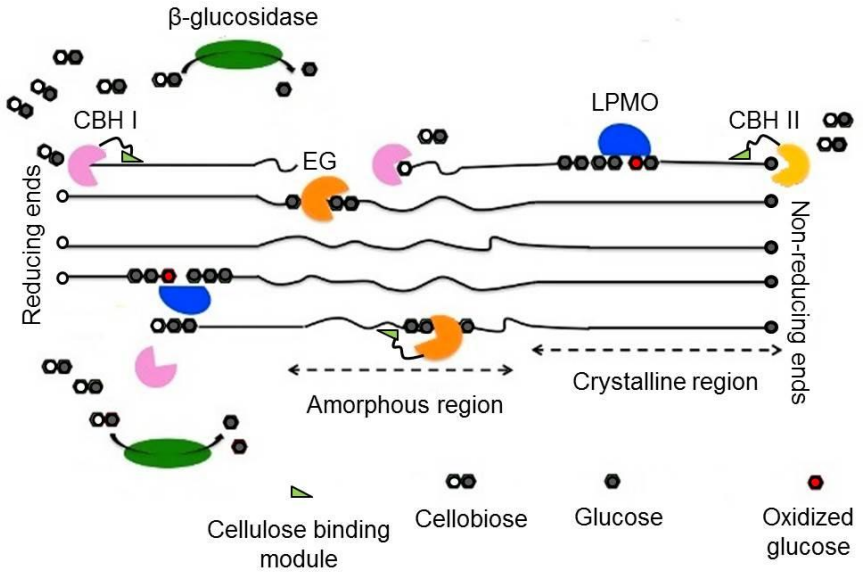

Figure 7. An overview of enzymatic hydrolysis of cellulose with endoglucanase (EG), cellobiohydrolases (CBH I and CBH II), β-glucosidase and lytic polysaccharide monooxygenases (LPMO) (modified from Dimarogona *et al.*, 2012).

Hemicellulases outnumber the different cellulases because of the wide range of different types of hemicelluloses and their branched structures present in biomass. Some hemicellulases cleave the backbone of hemicelluloses, whereas others

remove substituents from the main chain, which pose steric hindrances to other enzymes (as reviewed by Van Dyk and Pletschke, 2012). Xylanases participate in the hydrolysis of different types of xylan. Endo-1,4-β-xylanase (EC 3.2.1.8) hydrolyses the xylan backbone, and β-xylosidase (EC 3.2.1.37) cleaves the released xylobiose units producing xylose. The complete hydrolysis of different types of xylan also requires accessory enzymes such as α-D-glucurunosidase (EC 3.2.1.139), α-L-arabinofuranosidase (EC 3.2.1.55), acetylxylan esterase (EC 3.1.1.72), ferulic acid esterase and *p*-coumaric acid esterase (EC 3.1.1.73) (Polizeli *et al.*, 2005). Similarly, the complete hydrolysis of galactoglucomannan requires the synergistic action of endo-1,4-β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), α-galactosidase (EC 3.2.1.22) and acetyl mannan esterase (Dhawan and Kaur, 2007). Pectin is degraded by various enzymes including polygalacturonases (EC 3.2.1.15 and EC 3.2.1.67) and pectin methyl esterases (EC 3.1.1.11) (Pedrolli *et al.*, 2009).

1.6.3 Combining enzymatic hydrolysis and fermentation

Figure 8 presents a schematic view of certain hydrolysis and fermentation strategies available at a biorefinery setting, especially for the production of ethanol. The simplest route in terms of parameter optimization is to carry out separate hydrolysis and fermentation (SHF), in which hydrolysis is continued to its maximum level before fermentation is begun. The process temperatures are chosen on the basis of the optimum of each biological system separately: typically 45-50°C for the hydrolytic enzymes and around 30°C for the fermenting organism (Philippidis, 1996). SHF permits fermentation without insoluble solids since no further hydrolysis of insoluble substrates needs to take place during fermentation. The drawback of this strategy is that the end-product inhibition of the cellulolytic enzymes may slow down and limit the rate of hydrolysis and require a higher enzyme loading. The strategy of simultaneous saccharification and fermentation (SSF) combines the two processes in one vessel, which requires a compromise to be made in terms of the process temperature. Typically the optimal temperature is found around 35-38°C making hydrolysis the rate-limiting step in SSF (Philippidis, 1996; Philippidis and Smith, 1995). End-product inhibition is relieved and the growth of contaminants is restricted as the concentration of sugars is lower in the reactor (reviewed by Sun and Cheng, 2002). In addition, the total process time is typically reduced and less reactor volume is needed. To make the most of the two strategies, the process may be started with a hydrolysis stage at a higher temperature (a prehydrolysis or a liquefaction step), and continued by SSF once the end-product concentration starts to inhibit the enzymes.

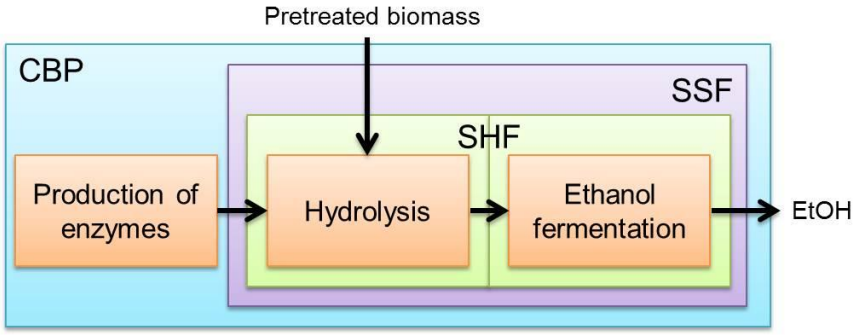

Figure 8. A simplified schematic view of alternative process configurations for the production of ethanol from lignocellulose. SHF = separate hydrolysis and fermentation, SSF = simultaneous saccharification and fermentation, CBP = consolidated bioprocessing.

Consolidated bioprocessing (CBP), also called direct microbial conversion (DMC), combines the production of hydrolytic enzymes, the hydrolysis reaction and the fermentation of the hydrolysis products into one process step (Lynd *et al.*, 2002; Balat, 2011). The process can be carried out by a microbial consortium or by a single yeast, bacterial or fungal strain (Lynd *et al.*, 2002; Lynd *et al.*, 2005; Xu *et al.*, 2009). The at least partial elimination of the separate production of enzymes reduces the processing costs, but research efforts are needed to achieve a high rate and high conversion of biomass with high yield and titre of the end product (Olson *et al.*, 2012; Lynd *et al.*, 2005). Some additional enzyme activities not produced in the CBP step may need to be added in order to achieve total hydrolysis.

Further consideration must be given to the principle of operation: whether the process is batch, fed-batch or continuous. A batch process requires the most from the process equipment since it must be able to handle great changes in viscosity and in the content of insoluble solids during the process. Fed-batch operation can be used to keep the concentration of insoluble solids more constant in the system, which helps in designing an optimal stirring system and results in an increased final concentration of sugars and ethanol (Hodge *et al.*, 2009). Fully continuous systems require the lowest equipment volume and may be more resistant to contamination due to lower substrate concentration in the fermentation broth but loss of unreacted substrate must be minimized (Guidoboni, 1984; Olsson and Hahn-Hägerdal, 1996).

1.6.4 High consistency hydrolysis

Several economic considerations support carrying out the hydrolysis step at high consistency. The separation of ethanol from the other soluble and insoluble components in the fermentation broth by distillation requires a considerable amount of energy and is thus a major cost factor. To reduce this energy consumption, a high

solids content in the beginning of hydrolysis should be employed as this leads to a high sugar and ultimately a high ethanol concentration. Additionally, high processing consistency decreases capital and operating costs through reduced vessel volumes, reduces other heating and cooling needs, and reduces water consumption and waste water production (Mohagheghi *et al.*, 1992, Modenbach and Nokes, 2013). However, high solids loading leads to a high concentration of potential inhibitors and makes agitation and pumping more difficult. Synonyms commonly utilized in the literature for high consistency hydrolysis include hydrolysis at high solids loading and at high dry matter concentration.

High solids processing generally refers to systems in which in the beginning there is very little or no free water present in the slurry. The dry matter content at this stage is typically >15%, depending on the water retention capability of the feedstock (Hodge *et al.*, 2009; Modenbach and Nokes, 2013). For many decades the laboratory work on enzymatic hydrolysis of lignocellulose was conducted only at low dry matter content (<5%). In recent years there has been an increasing number of publications reporting the phenomena occurring when the solids loading in the system is increased. Working in high consistency conditions is beneficial for ethanol recovery, but poses challenges for enzyme performance, equipment design, and the selection of the overall process concept. A general trend of reduction in the hydrolysis rate and yield, the so-called “solids effect” has been observed by many as reviewed by Kristensen *et al.* (2009a). Water has a role as a solvent being responsible for the transfer of the reactants and reaction products in the system, and as a substrate in the hydrolysis reaction (Roberts *et al.*, 2011). Although end-product inhibition can be alleviated by the SSF approach, the enzymes may become inhibited by increasing concentration of other soluble inhibitors such as organic acids, phenolic compounds, xylose, xylan and xylo-oligomers (Qing *et al.*, 2010). The ratio of insoluble solids and liquid in the system may also affect the adsorption kinetics of hydrolytic enzymes (Kristensen *et al.*, 2009b; Varnai *et al.*, 2013). The rheological behaviour of the material determines the stage of hydrolysis at which the material at high dry matter content becomes pumpable and allows mixing in a stirred-tank reactor. The choice of pretreatment, particle size distribution, particle aspect ratio and flexibility, and the water retention capability affect the rheological behaviour of the slurry at a certain consistency (Zhu *et al.,* 2011; Knutsen and Liberatore, 2009; Modenbach and Nokes, 2013). Fed-batch feeding strategy can be used to reach high final substrate loadings in enzymatic hydrolysis (Rosgaard *et al.*, 2007).

1.6.5 Effect of surfactants on enzymatic hydrolysis

Surfactants have surface active properties due to their amphiphilic nature. The hydrophilic head of a surfactant molecule can be cationic, anionic, or nonioninc, e.g. alcohol, whereas the hydrophobic tail is commonly an alkene chain. Polyethylene glycol (PEG) is a polymerized ethylene oxide chain that is frequently used as the hydrophilic part of surfactants, but has surface active properties alone as

well. For the sake of convenience, both the true amphiphilic surfactants and PEG are referred to as surfactants in this work.

Surfactants have been shown to be able to improve the rate and yield of enzymatic hydrolysis of pure cellulose and lignocellulose (Castanon and Wilke, 1981; Ooshima *et al.,* 1986; Helle *et al.,* 1993; Sewalt *et al.,* 1997; Eriksson *et al.,* 2002). Proposed mechanisms for pure cellulose include the disruption of the physical structure of cellulose leading to increased availability of the substrate, and the assistance of enzyme desorption from substrate surface (Helle *et al.,* 1993; Ooshima *et al.,* 1986). Surfactants have been suggested to affect the adsorption-desorption balance of endo- and exoglucanases by preventing the non-productive attachment of endoglucanases to the cellulose surface after reaction, which prevents the access of the saccharifying exoglucanase enzymes to the newly formed cellulose chain ends (Ooshima *et al.,* 1986, Mizutani *et al.,* 2002). For lignocellulosic substrates the prevention of non-productive adsorption of enzymes on lignin appears to be the most prominent mechanism of effect (Eriksson *et al.,* 2002; Kristensen *et al.,* 2007; Zheng *et al.,* 2008). In addition, the reduction of the viscosity of the reaction medium improving mass transfer may play a role (Knutsen and Liberatore, 2010). Mechanisms unrelated to the substrate include the protection of enzymes from thermal denaturation (Kaar and Holtzapple, 1998; Eriksson *et al.,* 2002) and from denaturation due to shear forces at the gas-liquid interface (Kim *et al.,* 1982). The capability of also other soluble compounds such as lignosulfonates to stabilize cellulases in solution and thus improve hydrolysis has also been reported (Lou *et al.,* 2014).

Several groups have studied the effect of the structure of the surfactant on the degree of hydrolysis improvement. Nonionic surfactants appear to be the most efficient type of surfactants whereas cationic and anionic surfactants typically denature cellulases and therefore have less use in enzymatic hydrolysis of lignocellulose (Ooshima *et al.*, 1986; Kaya *et al.*, 1995; Eriksson *et al.*, 2002). Figure 9 presents the structure of a typical non-ionic surfactant Tween 80, which is an ester of oleic acid and polyethoxylated sorbitan. Some investigators have found that shorter ethylene oxide chains produce a better effect (Kaar and Holtzapple, 1998), whereas others argue that a longer chain length is better (Börjesson *et al.*, 2007a; Ouyang *et al.*, 2010). A PEG product and a non-ionic surfactant having an ethylene oxide chain of similar length appear to produce a positive effect of similar magnitude on steam pretreated spruce (Börjesson *et al.*, 2007a), indicating that the polyethylene oxide part of the non-ionic surfactant is more important than the alkyl chain. However, the alkyl chain appears to have an effect as Park *et al.* (1992) found lauric and oleic acids to perform better than stearic acid as the hydrophobic part of a polyethylene oxide surfactant.

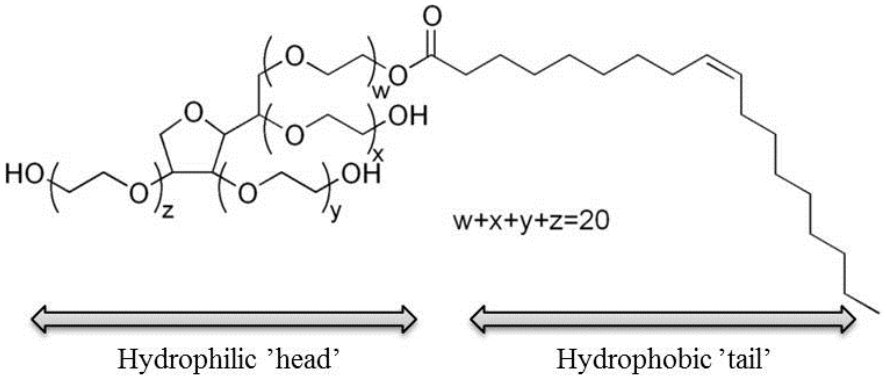

Figure 9. Structure of a non-ionic surfactant Tween 80 composed of polyethoxylated sorbitan and oleic acid. W, x, y and z relate to the number of ethylene oxide units in the structure of the surfactant.

The composition of the substrate affects the hydrolysis-improving effect of surfactants. The nature of lignin is one of the key factors in the system as the prevention of non-productive enzyme adsorption on lignin is one of the major mechanisms behind the positive effect of surfactants. Steam pretreated biomass has exposed lignin on its surface which is why surfactants have a more pronounced effect on this material compared to delignified biomass (Eriksson *et al.*, 2002). Kristensen *et al.* (2007) concluded that surfactants have a greater positive effect on the hydrolysis of acid and steam pretreated straw compared to ammonia and hydrogen peroxide treated straw. Improvements in hydrolysis yield and rate were also shown on steam pretreated spruce but not on delignified spruce (Börjesson *et al.*, 2007a). Sipos *et al.* (2011) suggested that the effect of PEG depends on the amount of unsubstituted phenolic hydroxyl groups in the substrate lignin. Ouyang *et al.* (2010) and Mizutani *et al.* (2002) found non-ionic surfactants and PEG to increase the conversion of Avicel, but Wang *et al.* (2011b) did not detect a positive effect on Avicel. Börjesson *et al.* (2007b) found PEG not to adsorb on Avicel but nevertheless to improve its hydrolysis rate slightly. In general it appears that PEG improves the hydrolysis yield most efficiently on materials with good enzyme accessibility and a relatively high content of lignin. The positive effect of surfactants on lignin-free substrates more probably arises from enzyme related rather than substrate related effects.

The effects of surfactants on enzyme adsorption and desorption have been studied by measuring free enzyme activities (Ouyang *et al.*, 2011; Börjesson *et al.*, 2007b) and protein concentrations (Castanon and Wilke, 1981; Sipos et al., 2011) from the liquid phase and by following radiolabelled surfactants and enzymes (Börjesson *et al.*, 2007a). Surfactants typically increase the concentration of desorbed enzymes, which correlates with an increase in hydrolysis rate and yield. Park *et al.* (1992) reported significant increases in hydrolysis yields when non-ionic surfactants were used, and correlated the improvement to decreased en-

zyme adsorption, suggesting that the effect comes from the ability of the surfactant to improve enzyme desorption. Börjesson *et al.* (2007b) reported a decrease in enzyme adsorption when surfactants were used on pretreated spruce. They found 10.0% of the original *T. reesei* Cel7A (CBHI) and 13.0% of Cel7B (EGI) activity (measured by ρ-nitrophenyl-β-D-cellobioside) in the solution after 6 h adsorption to steam pretreated spruce on a well plate. The addition of PEG increased the amount of free Cel7A activity to 13.0% and the amount of free Cel7B activity to 21.1%. The positive effect of PEG on free enzymatic activity in the presence of spruce hydrolysis lignin was even greater compared to that observed with pretreated spruce. The effect on adsorption and desorption explains why the particle size and thus the available surface area and surfactant dosage are related. According to Duff *et al.* (1995), saturation of the solid surfaces is a necessary prerequisite for an optimal effect. The effect of surfactants and the solids loading may be related, as Ma *et al.* (2011) reached 30% yield improvement at 25% solids loading but only 5% yield improvement at 10% solids loading.

The desorption-promoting effect of surfactants can be utilized for enzyme recycling. Tu *et al.* (2007) found Tween 80 to increase the free enzyme concentration from 71% to 96% of the initial protein loading in enzymatic hydrolysis of delignified lodgepole pine, and were able to recycle the enzymes for four successive rounds. Tu and Saddler (2010) calculated that it would be possible to save 60% of the total enzyme cost in the hydrolysis of organosolv cooked lodgepole pine and 9% of ethanol production costs from steam exploded lodge pole pine by enzyme recycling assisted by the use of a surfactant (Tu and Saddler, 2010).

Interestingly, non-ionic surfactants and polyethylene glycol have been shown to mitigate the inhibitory effect of tannins in enzymatic hydrolysis probably by preventing detrimental tannin adsorption onto the cellulose surface or by disrupting inactive tannin-enzyme complexes (Goldstein and Swain, 1965; Tejirian and Xu, 2011). Surfactants also help to detach printing ink from fibre surfaces and thus improve the accessibility of printed materials to enzymes (Kim and Chun, 2004). Tween improved the hydrolysis yield of newsprint both in preincubation followed by washing of the substrate and when used during hydrolysis (Kim *et al.*, 2006).

The effect of surfactants on enzymatic hydrolysis is not yet fully clarified, or at least forecasting the magnitude of the effect on different substrates is still difficult. Surfactants may offer a way to reduce the dosage of hydrolytic enzymes in an industrial biorefinery process, but they may also help us to understand the complex interaction of enzymes, their substrates and other components in the hydrolysis reaction medium.

1.6.6 Enzymatic hydrolysis of bark

Bark is an interesting but less studied source of fermentable sugars. There are only a few published papers on bark hydrolysis. The few plant sources studied include eucalyptus (Matsushita *et al.*, 2010; Lima *et al.*, 2013), paper bark tree (Ahmed *et al.*, 2013), aspen and poplar (Torget *et al.*, 1991), beech (Walch *et al.*,

1992), pine (Vazquez *et al.*, 1987; Salehian and Karimi, 2013) and spruce bark (David and Atarhouch, 1987). Bark is commonly considered as a challenging feedstock because of its high content of lignin and extractives (Robinson *et al.*, 2002; Torget *et al.*, 1991; Vazquez *et al.*, 1987). Tannins found in barks are known to bind and precipitate proteins and may thus adversely affect hydrolytic enzymes (Haslam, 1988; Walch *et al.*, 1992). For example Walch *et al.* (1992) demonstrated the almost complete inhibition of xylanase enzymes at 20 g/l tannin concentration. In the same study the addition of 0.9 mM oligomeric proanthocyanidins decreased the enzymatic hydrolysis of pretreated corn stover by 70-80%.

The comparison of hydrolysis results from different studies is difficult because varying enzyme mixtures and experimental set-ups have been used. However, the comparison of maximal hydrolysis yields reached in different publications provides some indication of the enzymatic digestibility of bark in general. David and Atarhouch (1987) obtained a 58% hydrolysis yield for spruce inner bark glucan after extraction with boiling water, whereas the hydrolysis yield of hot water extracted outer bark glucan was only 18%. Higher lignin content in the outer bark may have been the cause for the reduced yield, as delignifying treatments with NaOH or NaClO after boiling with hot water improved glucan hydrolysis significantly reaching yields up to 99% for the whole bark. For pine bark a pretreatment with NaOH (95°C, 15 min) was found to be inefficient and $NaClO_2$ was needed to produce reasonable hydrolysis yields (Vazquez *et al.*, 1987). Nevertheless, Salehian and Karimi (2013) reported ca. 53% hydrolysis yield for pine bark pretreated with NaOH (100°C, 10 min). Hardwood barks appear to be less recalcitrant towards enzymatic hydrolysis. Matsushita *et al.* (2010) reached a 60% hydrolysis yield for eucalyptus bark pretreated hydrothermally with CO_2, whereas Lima *et al.* (2013) reported a 79-99% hydrolysis yield after 4% NaOH pretreatment (120°C, 1 h). Close to 100% hydrolysis yields were published for dilute acid pretreated paper bark tree whereas a 85% glucan hydrolysis was reported after supercritical water pretreatment (Ahmed *et al.*, 2013).

The high content of phenolic extractives in bark could not only inhibit the hydrolysis enzymes, but also result in inhibition of the fermenting organism. Grain sorghum varieties with a high content of condensed tannins are known to be fermented more slowly than varieties with less tannin apparently due to nitrogen deprivation (Mullins and Lee, 1991). However, the few published results suggest that this is not the case with softwood bark. Salehian and Karimi (2013) obtained a 42% ethanol yield for NaOH pretreated pine bark which was in line with the hydrolysis yield (~53%) and lacked any signs of drastic inhibition of the fermenting yeast. Robinson *et al.* (2002) found that Douglas fir bark prehydrolysates from SO_2-catalyzed steam explosion could be efficiently fermented with complete hexose consumption and a 92% ethanol yield.

1.6.7 Enzymatic hydrolysis of pulp and paper derived materials

The pulp and paper industry produces many interesting side- and waste streams which have potential as a source of fermentable sugars. Processed wood fibres and materials based on them comprise a large group of lignocellulosic feedstocks varying from almost lignin- and hemicelluloses-free chemical pulps to thermomechanical pulps (TMP) that chemically resemble native wood, and further to various grades of recovered paper and board containing printing inks and inorganics in addition to the wood-based components. The latter materials have gone through considerable thermal, mechanical and chemical treatments and should therefore not require the exhaustive pretreatments developed for agro- and forest residues (Kim and Moon, 2003). The presence of inks and inorganic fillers, which may affect enzymatic hydrolysis, differentiates many of these materials from other lignocellulosic feedstocks (Kim and Moon, 2003).

The two types of wood fibres in paper and board products are mechanical pulp and chemical pulp fibres. Groundwood and TMP are very poorly digestible due to their close resemblance to native wood, which is not accessible to hydrolytic enzymes. Mooney *et al.* (1998) reached a ca. 25% hydrolysis yield for mechanical pulp, whereas Li *et al.* (2012) reported only a 11% hydrolysis yield for TMP and concluded that the hydrolysis was hindered by the presence of hemicellulose and lignin. Chemical kraft pulp, on the other hand, is very susceptible towards enzymatic hydrolysis. Yields in the range of 85 to 100% are commonly reported (Mooney *et al.*, 1998; Li *et al.*, 2012; Chen *et al.*, 2012). Drying of pulps, however, results in hornification of the fibres and reduces their enzymatic digestibility (Luo and Zhu, 2011).

Different paper and board grades vary in their cellulose, hemicellulose, lignin and ash contents (Table 4), which greatly affects their enzymatic digestibility. Office papers should be almost free of lignin (Eleazer *et al.*, 1997), whereas the lignin content of newsprints is high. Newspaper, magazine and cardboard contain more hemicellulose than office paper but less than native wood. Magazine papers contain the highest amount of ash as kaolin and clay.

Newsprint is mostly made of virgin mechanical pulp or recycled paper which has a high content of mechanical pulp, and a relatively high content of lignin (Kim and Moon, 2003). The high content of softwood lignin masks the carbohydrates and prevents swelling of the fibre (Mooney *et al.*, 1998), and causes non-productive adsorption and possible denaturation of the enzymes (Eriksson *et al.*, 2002). Untreated newsprint has been reported to produce varying hydrolysis yields ranging from <40% to 68% (Xin *et al.*, 2010; Kim and Moon, 2003; Wang *et al.*, 2012b). Office paper is more susceptible towards enzymatic hydrolysis producing yields typically close to and above 80% (Chu *et al.*, 2013; Wang *et al.*, 2012b; Elliston *et al.*, 2014; Park *et al.*, 2002). However, lower yields (34-55%) for office paper have also been reported (Chen *et al.*, 2012). Corrugated cardboard is also made of kraft pulp (Rivers and Emert, 1988) and is thus digested relatively well producing close to 70% hydrolysis yields (Wang *et al.*, 2012b). Kinnarinen *et al.*

(2012a) reached 55% hydrolysis yield for shredded cardboard waste. Magazine papers are typically even less digestible than newsprints. Wang *et al.* (2012b) reached a 57% yield with glossy supermarket catalogues.

Table 4. Chemical composition of different pulps and paper and board grades as % of d.m.

Material	Cellulose/Glucan	Hemicellulose	Lignin	Ash including $CaCO_3$	Reference
Kraft pulp	83.3	n.a.	3.7	n.a.	Aleksandrova *et al.*, 2000
	63.8	31.9	3.3	n.a.	Shackford, 2003
	72.5	20.7	6.2	n.a.	Shackford, 2003
	76.9	n.a.	3.4	n.a.	Mooney *et al.*, 1998
	87.0	16.0	1.0	n.a.	Lahtinen *et al.*, 2014
	87.0	16.8	2.6	n.a.	Lahtinen *et al.*, 2014
Mechanical pulp	56.0	22.2	25.6	n.a.	Lahtinen *et al.*, 2014
	42.7	21.5	28.4	n.a.	Hafren, 2007
	43.6	n.a.	27.9	n.a.	Mooney *et al.*, 1998
Newspaper	54.7	30.1	14.2	1.0	Rivers and Emert, 1988
	55.4	20.7	17.0	2.2	Kuhad *et al.*, 2010
	45.9	31.7	22.3	n.a.	Sangkharak, 2011
	47.2	18.2	18.1	10.5	Wang *et al.*, 2012b
	48.5	9.0	23.9	n.a.	Eleazer *et al.*, 1997
	48.3	18.1	22.1	n.a.	Wu *et al.*, 2001
	43.8	16.9	16.8	11.5	Wang *et al.*, 2012a
Office paper	46.6	24.2	19.3	n.a.	Sangkharak, 2011
	58.6	14.7	6.1	8.0	Wang *et al.*, 2012b
	87.4	8.4	2.3	n.a.	Eleazer *et al.*, 1997
	64.7	13.0	0.9	n.a.	Wu *et al.*, 2001
	55.7	13.9	5.8	15.3	Wang *et al.*, 2012a
	48.0	12.4	1.0	33.0	Elliston *et al.*, 2014
Magazines	35.9	14.2	14.8	30.1	Wang *et al.*, 2012b
	34.4	13.6	14.2	31.3	Wang *et al.*, 2012a
Cardboard and old corrugated containers	40.7	39.2	20.1	n.a.	Sangkharak, 2011
	52.6	16.7	15.8	9.9	Wang *et al.*, 2012b
	57.3	9.9	20.8	n.a.	Eleazer *et al.*, 1997
	58.2	n.a.	n.a.	14.0	Brummer *et al.*, 2014
	63.0	15.0	11.5	9.1	Kinnarinen *et al.*, 2012b
	49.6	15.7	14.9	13.3	Wang *et al.*, 2012a

It has been argued that although waste paper requires less pretreatment than woody or herbaceous materials, a treatment is needed in order to alleviate the hindering effects of inks and certain additives on cellulose accessibility (Kim and Moon, 2003). Moon and Kim (2001) found that ink had a significant negative effect

on the enzymatic hydrolysis of newsprint, whereas the ash content and particle size had a negligible effect. On the other hand, Duff *et al.* (1995) found that ink did not inhibit cellulase enzymes in the hydrolysis of newsprint, but that milling improved the yield. Treating newsprint with ammonia-hydrogen peroxide mixture at 40°C was shown to remove ink from the material, swell the fibres and increase its hydrolysis yield from 68% to 85% (Kim and Moon, 2003). Kuhad *et al.* (2010) reported 60% hydrolysis yield on deinked newsprint. By contrast, it has also been claimed that printing inks have no effect on hydrolysis or fermentation of print products (Rivers and Emert, 1988). Subcritical and supercritical carbon dioxide explosion were shown to improve the digestibility of recycled paper (Zheng *et al.*, 1998). Phosphoric acid pretreatment did not improve the enzymatic digestibility of newspaper but improved the hydrolysis yield of office paper (Chu *et al.*, 2013). Biological treatment with *Sphingomonas paucimobilis* and *Bacillus circulans* strains improved the enzymatic digestibility of office paper from a 30% to a 73% hydrolysis yield (Kurakake *et al.*, 2007). Chen *et al.* (2012) claimed that the high content of ash in office paper is detrimental to its enzymatic hydrolysis due to non-productive adsorption of enzymes on the ash components. Cellulase adsorption on calcium carbonate (17% of total enzyme protein) and clay (38% of total enzyme protein) was detected. The removal of inorganic fillers from office paper by washing and mechanical refining was shown to increase the hydrolysis yield.

Fibre sludge collected from the primary clarifier of a pulp or paper mill is a source of wood-derived lignocellulose, which is currently combusted or disposed to landfills. The composition of fibre sludge varies greatly between different mills. Lynd *et al.* (2001) analysed 44 sludges from different mills and found the mean glucan content to be 39% and mean ash content to be 26%. The composition of the sludge varied over time but the variation was greater between sludges from different mills. Kaolin, $CaCO_3$ and TiO_2 are the main ash components in fibre sludge (Lynd *et al.*, 2001). Fibre sludge has been claimed to be much more susceptible to enzymatic hydrolysis than traditional lignocellulose feedstocks (Lark *et al.*, 1997). As expected, sludge from a thermomechanical plant is less susceptible to enzymatic hydrolysis than kraft or sulphite mill fibre sludge (Duff *et al.*, 1994). Fibre sludges from deinking facilities contain higher amounts of inks and other impurities and are rather recalcitrant towards hydrolysis (Duff *et al.*, 1995). Glucan conversion varied from 38% to 98% in SSF of 39 fibre sludge samples with average conversion of 80% and median conversion of 88% (Lynd *et al.*, 2001). Enzymatic hydrolysis reduces the water holding capacity of the sludge and facilitates the dewatering of the material (Lark *et al.*, 1997). Paper mill fibre sludges are typically neutral or alkaline and the pH must be adjusted with acid prior to enzymatic hydrolysis. According to Lynd *et al.* (2001) <10 g sulphuric acid per kg dry sludge was required in pH adjustment for 80% of the sludges tested representing a relatively small cost factor in the process. Fan and Lynd (2007) assessed the economics for conversion of fibre sludge of a bleached kraft mill to ethanol and concluded that the cash flow is positive with or without xylose fermentation and mineral recovery, and that internal rates of return above 15% can be expected for larger plants. However, the total annual mass of fibre sludge available is typically

rather low and if any development is foreseen, it is rather to minimise the leakage of fibre to the primary clarifier.

1.7 Biofuels and chemicals as biorefinery products

The chemical components in lignocellulosic biomass offer a number of product options for a biorefinery concept. Extensive research and development work has been directed towards converting lignocellulosic carbohydrates to fuels or chemicals via microbial conversion. Ethanol is the first large-volume product on the market produced via this route. Fermentable sugars could also be converted for example to organic acids which function as polymer precursors, and to single cell protein for food and feed purposes. The potential of different polymeric carbohydrates as fibres is vast ranging from web-based and non-woven material solutions to food and pharmacological additives. Lignin can be considered from a material point of view and processed into carbon fibres, composites and resins, or from a chemical point of view as a precursor to aromatic chemicals. Extractives in lignocellulose may turn out to be the component with the highest commercial value. Examples range from the currently valorised tall oil and turpentine to vegetable extracts with pharmaceutical activity. In the next sections, selected biorefinery products from spruce bark and recovered fibres, *i.e.* fermentable sugars, ethanol and tannin, are briefly presented along with their current market situation, production capacity and future potential.

1.7.1 Fermentable sugars

Fermentable sugars are an intermediary product, which could be sold and transported elsewhere for further conversion. Different types of starch and sucrose-based sugar syrups are currently sold and used by the fermenting industry including the producers of industrial enzymes, antibiotics, vitamins, amino acids and organic acids, and by the food industry in products such as beverages, sweeteners and processed foods. Lignocellulosic sugars could in theory replace these starch- and sucrose-based sugars if their producers wish to source their raw material from outside the food chain. However, the main focus is the replacement of starch-based sugars with lignocellulosic sugars in the bioethanol industry and their use in the production of chemicals currently produced from fossil sources. Fermentable sugars may be a product option for a biorefinery if its capacity is not sufficient to sustain the high investment cost of the separation process of the microbial product (e.g. ethanol distillation).

1.7.2 Ethanol

Ethanol is the first high-volume commercial product from lignocellulosic carbohydrates via the fermentation route. The most common microbe used today for the

fermentation step of ethanol production is *Saccharomyces cerevisiae* which ferments hexoses to ethanol with a theoretical yield of 0.51 g/g; the rest of the carbon is converted to CO_2. In practise the ethanol yield is 80-95% from theoretical due to the production of side products and slow growth of the microbe in anaerobic conditions. It is a robust microbe and well-suited for fermenting lignocellulosic hydrolysates but it cannot ferment pentoses (Olsson and Hahn-Hägerdahl, 1996). Some native and engineered bacterial, yeast and fungal strains are able to co-ferment both glucose and xylose, which is a key advantage when processing feedstocks with a high pentose content. The so-called co-fermentation, commonly employed as a simultaneous saccharification and co-fermentation (SSCF) process configuration, uses recombinant strains of *Saccharomyces cerevisiae*, *Escherichia coli* and other microbes to increase the total ethanol yield from biomass (Lindsay *et al.*, 1995; Ho *et al.*, 1998).

The first industrial scale production plant using straw and *Arundo donax*, a bamboo-like energy crop, as the lignocellulosic feedstocks was opened in Italy in 2013 by Beta Renewables (Balan *et al.*, 2013). The POET-DSM plant, which converts corn residues to ethanol, opened in the USA in September 2014 and more openings are planned in the USA, Italy and Brazil for the upcoming years. Lignocellulosic ethanol competes with starch and sucrose-based ethanol in the fuel markets. In 2013, global fuel ethanol production was 90 billion litres, of which 84% came from USA and Brazil (Renewable Fuels Association, n.d.). The market is large but dependent on many factors including policies and governmental incentives, as well as the market of the by-product of corn ethanol production, distillers' grains, as animal food (Sarkar *et al.*, 2012). The demand of diesel versus gasoline also affects the demands for ethanol, as it can only be added to gasoline fuels. Overall, the market potential for lignocellulosic ethanol is huge but it faces tough competition from the existing first generation ethanol industry and from renewable diesel. Being more costly to produce, lignocellulosic ethanol is dependent on government-level decisions to shift from oil to renewable raw materials and from food-based raw materials to non-food sources of carbohydrates.

1.7.3 Tannin

Tannin is a biochemical with established markets and many end uses. We all consume some tannin in our daily diet, as it is present in many fruits, berries, legumes, nuts and beverages such as wine, cider, fruit juices and tea (reviewed by Serrano *et al.*, 2009). Tannins have been shown to possess e.g. bactericidal, molluscicidal, antihepatoxic and antitumor activities (reviewed by Haslam, 1996). Industrially produced tannins of plant origin are called vegetable tannins. Hydrolysable tannin is produced from the galls of Chinese nutgall tree (*Rhus semialata*) and Aleppo oak (*Quercus infectoria*), the leaves and bark of Sicilian sumac (*Rhus coriaria*), tara fruit pods (*Caesalpina spinosa*), myrobalan nuts (*Terminalia chebula*) and the wood of the chestnut tree (*Castanea sativa*) (Bhat *et al.*, 1998). Condensed tannin is produced from the bark of wattle (*Acasia mollissima* and *A.*

mearnsii) and the wood of quebracho (*Schinopsis lorentzii* and *S. balansae*) (Bhat *et al.*, 1998). These two species produce extracts with a naturally high tannin and a low non-tannin content (Roffael *et al.*, 2000). The total production of tannins is in the range of 160 000 – 200 000 tons per year (Vieira *et al.*, 2011, Pizzi, 2006).

Quebracho tannin is produced by extracting chipped quebracho wood with 130°C water in a stainless steel reactor system so that the solvent circulates in a counter-current fashion from tank to tank (Unitan, 2014). The obtained extract is concentrated from 10% to 55% solids content by evaporation and then spray-dried. The solubility and the colour of the product may be adjusted chemically prior to spray-drying. Wattle tannin is extracted very similarly to quebracho tannin (UCL, 2014). There are some reports of softwood bark being used for tannin production. Surminsky (2007) and Grassmann *et al.* (1956) reported the use of spruce bark for e.g. leather tannin in Poland and Germany in the past. Tannin was commercially produced from Radiata pine bark in Chile in the 1980s and 1990s (Valenzuela *et al.*, 2012; Charbonneau, 1988), but later it was considered not economically feasible due to problems associated with achieving both high extraction yields and sufficient quality of the extract for wood adhesives (Li and Maplesden, 1998). Böle Tannery in Northern Sweden currently tans leather for luxury briefcases and bags using spruce bark (Böle Tannery, n.d.).

The main application for vegetable tannins is the production of high quality leather from raw hides. The polyphenolic tannin molecules bind to collagen proteins in the hide making it resistant and flexible and giving it some colour. Vegetable tannin products can be tailored in terms of the colour and also the molecular weight which affects the tanning time of leather (UCL, 2014). The highest quality tannins are used in oenological applications. Their suppliers claim that tannins have a clarifying and colour stabilizing effect, antioxidant, antiradical and bacteriostatic activity, and that they chelate metals, capture thiols and improve the organoleptic properties of wines (Silvateam, 2014). Moderate concentrations of proanthocyanidins slow the degradation of dietary protein to ammonia in the rumen and increase its uptake in the small intestine of ruminant herbivores (Aerts *et al.*, 1999) which is why they are added to animal feed. Tannins can be used as dispersants or coagulants in mineral flotation, oil drilling and sewage treatment and for boiler water treatment, rust conversion, and the cleaning of reverse osmosis systems (Silvateam, 2014). The reactivity of condensed tannins with formaldehyde makes it possible to substitute fossil-based phenol with tannin in phenol-formaldehyde resins. Phenol-formaldehyde adhesives have one of the largest volumes and lowest costs among synthetic resins and they dominate especially in the field of wood adhesives (Ebnesajjad, 2010). The formulation of a formaldehyde free tannin adhesive is also possible (Pizzi, 2006). More recently the use of tannins in rigid insulating foams has been demonstrated (Tondi *et al.*, 2009). Some reports are available on the use of spruce bark tannin extracts in wood adhesives. For example Roffael *et al.* (2000) could replace 20% of quebracho extract with spruce bark extract in the production of particleboards, and even 60% in the production of medium density fibreboard without negatively affecting their properties.

Tannin is a biochemical with existing markets and future potential to replace fossil-based chemicals in a large market segment. Key questions for producing tannin from e.g. spruce bark are the production costs and the suitability of the tannin for existing and new applications. Tannin has to compete with phenol which is currently valued at ca. 1500 €/t in Europe (ICIS, 2014), since investors cannot rely on a green premium paid by the customer for renewable products. It has been claimed that the technology for formaldehyde-free tannin adhesives from current tannin sources is ready for industrial use (Pizzi, 2006), but technical success with spruce bark tannins has not yet been demonstrated.

2. Aims

The overall aims of the thesis were to study the possibilities to produce tannin, fermentable sugars and ethanol from two forest industry-related and underutilized streams, namely spruce bark and recovered fibres, and to understand the future potential of the streams in a biorefinery context. More specifically, the aims were to:

1. Characterize the selected raw materials in respect to their utilization in biorefineries.
2. Develop an extraction procedure for spruce bark tannins.
3. Analyse the enzymatic digestibility of the streams and determine whether there is a need for an additional pretreatment process to improve hydrolysis yield.
4. Study the factors affecting the enzymatic hydrolysis of these streams including the enzyme cocktail composition, solids loading and surfactants.
5. Demonstrate parts of the two forest biorefinery concepts in pilot-scale.

3. Materials and methods

A summary of the materials and methods used in this thesis is presented in this section. Detailed descriptions can be found in the original Publications I-IV.

3.1 Materials

The main lignocellulosic raw materials used in the thesis are listed in Table 5. In addition, Sections 4.3.3 and 4.3.4 present results obtained with two additional reference materials, pretreated wheat straw and pretreated spruce. Details of their origin and composition are given in the paper by Rahikainen *et al.* (2013b).

Table 5. Main lignocellulosic raw materials used in the thesis.

Description	Source	Publication
Solid recovered fuel	Lassila & Tikanoja, Finland	I, IV
Solid recovered fuel	Three commercial suppliers, United Kingdom	IV
Fibre sludge	Kaukas mill, UPM-Kymmene, Finland	I, IV
Wet debarked spruce (*Picea abies*) bark	Jämsänkoski mill, UPM-Kymmene, Finland	II
Dry debarked spruce (*Picea abies*) bark	Lohja Kerto-plant, Metsä Wood, Finland	III

Four different commercial enzymes were used in the hydrolysis of recovered fibres and spruce bark. This dissertation also includes unpublished results on enzymatic hydrolysis of spruce bark water extracts, in which three commercial enzyme products were used. In addition, nine pre-commercial unpurified monocomponent enzyme products were used in the hydrolysis of recovered fibres (Table 6). Surfactants PEG 4000, Lutensol AT 50 and Softanol 90 were used in selected hydrolysis experiments.

Table 6. Commercial and pre-commercial enzymes used in the thesis.

Type	Product name or source organism	Main activity	Publication
Commercial	Econase CEP (AB Enzymes)	Cellulases	I
	Celluclast 1.5 L (Novozymes)	Cellulases	II
	Novozym 188 (Novozymes)	β-glucosidase	II, unpublished
	Pectinex Ultra SP-L (Novozymes)	Pectinase and hemicellulases	II, unpublished
	Viscozym L (Novozymes)	Hemicellulases	unpublished
Pre-commercial	*Acremonium thermophilum* (Roal)	Cellobiohydrolase I	I, IV
	Acremonium thermophilum (Roal)	Cellobiohydrolase II	I, IV
	Chaetomium thermophilum (Roal)	Cellobiohydrolase II	IV
	Thermoascus aurantiacus (Roal)	Endoglucanase	I, IV
	Trichoderma reesei (Roal)	Endoglucanase	IV
	Acremonium thermophilum (Roal)	β-glucosidase	I, IV
	Thermoascus aurantiacus (Roal)	β-glucosidase	IV
	Nonomuraea flexuosa (Roal)	Xylanase	I, IV
	Thermoascus aurantiacus (Roal)	Xylanase	IV
	Trichoderma reesei (Roal)	Mannanase	I, IV

3.2 Analytical procedures

Analytical procedures and enzyme activity assays used in the experimental work are listed in Table 7. The literature references to the analytical procedures and assays can be found in the original publications.

Table 7. Analytical methods and enzyme activity assays used in the thesis.

Analyte	Method	Publication
Solid samples		
Neutral carbohydrates (amount and monosaccharide profile)	Acid hydrolysis, high pressure anionic exchange chromatography with pulsed amperometric detection (HPAEC-PAD)	I, II, III, IV
Acidic carbohydrates (amount and monosaccharide profile)	Methanolysis, gas chromatography	II, III
Non-cellulosic poly- and oligosaccharides	Sequential extraction, glycome profiling using enzyme-linked immunosorbent assay (ELISA)	III
Acid insoluble material (Klason lignin)	Gravimetric quantification after acid hydrolysis	I, II, III, IV
Acid soluble lignin	Spectroscopic quantification after acid hydrolysis	I, II, III, IV
Ash	Gravimetric quantification after combustion at 550°C	I, II, III, IV
Elemental composition of ash	Inductively coupled plasma mass spectrometry	IV
Lipophilic extractives	Heptane or hexane extraction	I, II, III, IV
Tannin	Hide powder method	III
Water soluble compounds		
Reducing sugars	3,5-dinitrosalicylic acid (DNS) assay, glucose standard	I, II, IV
Neutral and acidic mono- and oligosaccharides	Optional mild acid hydrolysis, HPAEC-PAD	I, II, III, IV
Non-cellulosic poly- and oligosaccharides	Glycome profiling using ELISA	III
Fermentation products (ethanol, glycerol, lactic acid)	Liquid chromatography	I, II
Condensed tannin	Acid butanol method	III
Tannin	Hide powder method	III
Total phenolic content	Folin-Ciocalteu method	III
Hydroxyl groups	^{31}P NMR	III
Molecular weight distribution	Size exclusion chromatography (SEC)	III
Enzyme activity assays		
FPU-activity	Hydrolysis of filter paper	I, II
β-glucosidase activity	Hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside	I, II, IV
Pectinase activity	Hydrolysis of polygalacturonic acid	II
MUL-activity	Hydrolysis of 4-methylumbelliferyl-β-D-lactoside	IV

3.3 Fractionation and pretreatment of recovered fibres

In Publications I and IV recovered fibres were fractionated from SRF in small pilot scale (7.5 kg SRF in a 200 L reactor) or large pilot scale (ca. 300 kg SRF in an 8 m^3 reactor) by repulping the material, separating the fibres by filtration, and dewatering the fibres in a board machine, on a belt press or in a decanter centrifuge. For one batch studied in Publications I and IV, 25% fibre sludge was added to the fibres fractionated from SRF prior to dewatering. A 60 min sanitation period at 95°C was carried out prior to or after dewatering to decrease the level of microbial contamination in the material.

In Publication I recovered fibres were steam pretreated as wet pulp (1-6% consistency) in a 400 L reactor at 120-200°C for 5-10 min followed by an explosion step. The pH of the pulp was adjusted to 5 or to 2 with H_2SO_4 prior to treatment.

3.4 Hot water extraction and pretreatment of spruce bark

Hot water extraction of spruce bark was carried out in a 15 L rotating cooking reactor (Publication III), in a 250 L horizontal reactor with ploughshare mixing (Publication II) and in a 250 L vertical reactor in which the bark was placed in filter bags which were under forced extract circulation (Publication III). Additional data is given in Section 4.4.1; crushed bark was extracted in a 1800 L vertical reactor. Solids were removed by filtration and/or centrifugation and the pilot-scale extracts were concentrated by evaporation and spray-dried to yield a dry powder. The solids content of bark during extraction was 5-15% in the rotating reactor and ca. 5-9% in the larger reactors. Native and hot water extracted bark was steam pretreated in a 10 L pressurized vessel at 190°C or 205°C for 5 min followed by an explosion step (Publication II). Acid impregnation with 0.5% H_2SO_4 prior to pretreatment was used for selected samples of native bark.

3.5 Enzymatic hydrolysis

Laboratory-scale enzymatic hydrolysis experiments were carried out under various conditions in Publications I, II and IV as presented in Table 8.

Table 8. Hydrolysis parameters selected for laboratory-scale hydrolysis studies.

Publication	I	II	IV, additional data on high solids hydrolysis	Additional data on spruce bark extract hydrolysis
Solids loading	10 or 30%	1%	1-25%	3%
Working volume	50-1200 ml	3 ml	3-50 ml	3 ml
Buffer	50 or 100 mM sodium acetate buffer pH 5	50 mM sodim acetate buffer pH 5	50 or 100 mM sodium acetate buffer pH 5	No buffer
Enzyme dosing	Based on activity	Based on activity	Based on protein content	Based on protein content
Temperature	45-50°C	45°C	50°C	45°C

Enzymes were dosed per gram of substrate dry matter, either based on activity or based on protein content of the enzyme or enzyme mixture. A 6 h prehydrolysis stage was performed for spruce bark at 15% solids loading prior to ethanol fermentation in Publication II. Pilot-scale enzymatic prehydrolysis of recovered fibres was carried out at 20-30% solids content in a continuously operating stirred tank reactor in Publication I. Hydrolysis yields are always presented as a percentage of theoretical of the carbohydrates in the substrate under hydrolysis.

3.6 Ethanol fermentation

In Publications I and II, spruce bark and recovered fibres were fermented with 3 g/l *Saccharomyces cerevisiae* (strain VTT-B-08014 or Red Star) employing the SSF concept with a prehydrolysis stage. Spruce bark was fermented in batch mode in 1.5 L working volume, and recovered fibres were fermented in pilot scale in continuous mode with a residence time of at least 21 h in ca. 300 L working volume. Yeast slurry and yeast extract were continuously added to the fermentation of recovered fibres. No additional nutrients were used in the fermentation of spruce bark.

4. Results and discussion

4.1 Characterization of spruce bark and recovered fibres as biorefinery feedstocks

The feedstock structure and composition determine the products that a biorefinery can produce and limits the range of technologies that are suitable for the biorefinery concept. Spruce bark and recovered fibres fractionated from SRF (Figure 10) are both relatively unknown and underutilized potential biorefinery feedstocks. In addition, the fractionation of recovered fibres from SRF has not been described in the literature for the purpose of producing fermentable sugars. The composition of both streams is presented and discussed in this work on the basis of the results in Publications III and IV.

Figure 10. Spruce bark and recovered fibres fractionated from solid recovered fuel.

4.1.1 Composition of spruce bark (Publication III)

The composition analysis results of two spruce bark samples are summarized in Table 9. Carbohydrates comprised ca. half (47.6-51.3%) of the bark dry matter containing mainly glucose, and smaller amounts of galacturonic acid, arabinose, xylose, mannose and galactose. Cellulose content calculated by subtracting glucose released in methanolysis from glucose released in acid hydrolysis was 19.0-20.0%. Bark collected from logs felled in the winter contained more non-cellulosic

glucose (11.5%) than bark collected from logs felled in the summer (7.7%). Bark was extracted with six increasingly harsh solvents, which generated extracts enriched in glycans according to the tightness with which they were integrated to bark cell walls. Glycome profiling of the extracts confirmed the presence of the pectic polysaccharides homogalacturonan and rhamnogalacturonan in bark by measuring the binding of their distinctive epitopes on a set of glycan-directed monoclonal antibodies. These polysaccharides were rich in the oxalate extract, the first of the sequential bark extracts, showing that they are among the most easily extractable polysaccharides in bark (Figure 4, Publication III). Xyloglucans, xylans, pectin and arabinogalactans were found in the chlorite extract, indicating that these polysaccharides were at least partially strongly associated with lignin in the cell wall. The abundance of xylan in the 4 M KOH extract produced after chlorite extraction shows that at least a partial delignification of bark is needed for its efficient removal.

The tannin content of bark was measured using the ISO standardized hide powder method in use in the tannin industry. It is not specific to condensed tannins, but it gives an overview of the concentration of compounds behaving like tannin, *i.e.* compounds that precipitate hide proteins. Tannin content measured for wood-free dry-debarked summer bark in Publication III (10.7%) is in line with the rather wide range given in literature (4-15%) as reviewed in Section 1.5.4. The tannin contents of two other spruce bark samples were also analysed (unpublished data). A second sample from the same source containing 10% wood contained 10.5% tannin and a sample of saw mill spruce bark from another Finnish source contained 12.4% tannin.

Lignin analysis from bark is more difficult than from wood because high molecular weight tannins may precipitate in acidic conditions and show up as Klason lignin, which represents the acid insoluble portion of the sample. The analysis of acid soluble lignin is based on absorbance at 215 nm and 280 nm, and is also not fully specific to lignin. The content of acid insoluble material in bark after the removal of lipophilic extractives was 31-34% according to this work. It can be estimated that the actual lignin content of bark was in the range of 25-28%, assuming that approximately 60% of tannins precipitated as acid insoluble material in the Klason lignin analysis. Miranda *et al.* (2012) reported a similar amount of Klason lignin (24-27%) in spruce bark after exhaustive removal of extractives with dichloromethane, methanol, ethanol and water, and the removal of suberin by methanolysis. The characterization of the exact amount and type of lignin in spruce bark remains a topic for further research.

Considering the results presented by Krogell *et al.* (2012) on the carbohydrate composition of inner and outer spruce bark (Table 3), it appears that there was relatively more inner bark than outer bark in the samples studied in this work. The high content of pectin and non-cellulosic glucose differentiates spruce bark from spruce wood, straw, bagasse and many other lignocellulosic feedstocks. The composition analysis results pose a question on the nature and role of the non-cellulosic glucose in spruce bark. Non-cellulosic glucose may be present in bark as a part of hemicellulosic polysaccharides, as free glucose and sucrose, or as a

part of glycosidic compounds. The glycome profiling results indicated that at least a part of the non-cellulosic glucose was present in bark as xyloglucan. Painter and Purves (1960) detected chemically linked glucose and xylose in the bark of *Picea glauca*, but xyloglucan is not reported to be present in spruce bark in the recent literature. Starch is also a potential source of non-cellulosic glucose. Le Normand *et al.* (2012) detected starch in spruce bark based on the iodine test, but did not quantify it further. Based on the enzymatic analysis performed in Publication III, bark contained only 0.7% or less starch, which could not explain the presence of the high amount of non-cellulosic glucose. A part of the non-cellulosic glucose is present in the bark as free glucose, as analysed from water extracts (see Section 4.2). Free glucose was more abundant in extracts produced from trees felled in the winter compared to trees felled in the summer. Thus it appears that the amount of free glucose in bark could be related to the growth cycle of the tree. Weissman (1984) detected the highest amount of free glucose in spruce bark after the growing season and the lowest amount in May and June. Glucose is also present in glycosides, at least in stilbene glycosides (Kylliäinen and Holmbom, 2004; Zhang and Gellerstedt, 2008; Mannila and Talvitie, 1992; Krogell *et al.*, 2012). There is some evidence that glucose could also be bound to condensed tannin units (Pan and Lundgren, 1995; Zhang and Gellerstedt, 2008). Grassmann *et al.* (1956) concluded already decades ago that spruce bark tannin is a glucosidic mixture. If condensed tannins are indeed glycosylated, it would be interesting to determine whether there is a correlation between the amount of glucosidic tannin and free glucose in bark and their dependence on the season.

Table 9. Composition (% of dry matter) of spruce bark and recovered fibres summarized from Publications III and IV. n.a = not analysed

Description of the sample	Dry debarked winter bark	Dry debarked summer bark	SRF from UK, fractionated in small pilot-scale (Sample A)	SRF from UK, fractionated in small pilot-scale (Sample B)	SRF from UK, fractionated in small pilot-scale (Sample C)	SRF from UK, fractionated in large pilot-scale	SRF from Fin-land, fractionated in large pilot-scale, 25% fibre sludge
Publication	III	III	IV	IV	IV	IV	IV
Acid insoluble material	31.1	33.5	19.7	22.8	24.2	24	23.4
Acid soluble lignin	2.7	3.3	0.6	0.6	0.8	0.6	0.5
Lipophilic extractives	2.9	2.4	8.2	3.6	5.3	6.3	6.4
Ash	3.1	3.1	11.9	17.0	15.0	12.5	12.7
Carbohydrates (anhydrous)	51.3	47.6	53.6	53.8	52.4	52.4	53.8
Glucose	30.5	27.7	43.1	43.4	42.8	42.2	40.9
Xylose	2.8	3.5	6.7	6.4	5.5	6.6	7.8
Arabinose	3.7	4.5	0.4	0.5	0.4	0.3	0.4
Mannose	3.1	2.2	2.7	3.1	3.2	2.9	3.9
Galactose	2.2	2.1	0.4	0.5	0.5	0.4	0.7
Rhamnose	0.5	0.6	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Galacturonic acid	7.4	6.0	n.a.	n.a.	n.a.	n.a.	n.a.
Glucuronic acid	0.3	0.4	n.a.	n.a.	n.a.	n.a.	n.a.
O-4-methylglucuronic acid	0.9	0.5	n.a.	n.a.	n.a.	n.a.	n.a.

4.1.2 Fractionation and composition of recovered fibres (Publication IV)

Recovered fibres are not an existing stream ready for a biorefinery process like spruce bark. Instead, they need to be fractionated from solid recovered fuel (SRF). Recovered fibres are more heterogeneous compared to fibres in different grades of recovered paper and board such as newsprint, office paper and old corrugated cardboard. They also contain more impurities, which are not present in the paper recycling system. Although large pieces of plastic are removed during the fractionation process, the presence of residual plastics is probably the most notable difference between recovered fibres fractionated from SRF and other recycled fibre streams.

SRF composition may vary between different producers and over time, because the raw waste is continuously collected from various sources. It is important to determine how the variation affects the yield, composition and the enzymatic digestibility of the fibre stream. Table 1 in Publication IV presents the fractionation yield, the dewatering yield and the resulting fibre yield for three samples of SRF fractionated in the same small pilot-scale equipment (Batches A-C). The fractionation yield describes the separation efficiency of the fibre stream from plastics and other rejects during pulping and sieving. The fractionation yield of the three samples re-pulped in the same experimental set-up was between 35% and 44%. The variation may reflect the fibre content of the sample or the type of fibre products in the sample. For example products prepared using wet-strength agents would be more resistant against disintegration. On the other hand, a very efficient repulping might reduce the particle size of the plastics, thus complicating their separation from the fibres. The dewatering yield describes the yield of dry matter during dewatering of the fibre stream by centrifugal forces or filtration. The dewatering yield is affected by the dewatering technology, the extent of the repulping step and the composition of SRF. The dewatering yields obtained in this work (65-76%) probably reflect the different product spectra in the SRF samples, which result in different particle sizes during repulping. Some material losses during the dewatering step may be beneficial. Dewatering step may be used for example to wash excess ash from the fibres. The total fibre yield (the product of fractionation and dewatering yield) varied between 25% and 33%. The fractionation and dewatering yields were higher in larger pilot-scale equipment resembling industrial systems (51% and 87% respectively) compared to the small-scale pilot system. It is thus likely that industrial equipment would outperform experimental smaller scale set-ups and produce an estimated 50-55% total fibre yield for separating recovered fibres from high quality SRF.

The composition of different samples of recovered fibres is presented in Table 9. Their hexose content (45.5-47.0%, calculated as polysaccharides) was significantly high compared to their pentose content (5.9-8.2%), which indicates that recovered fibres could also be used in those biorefinery concepts which do not include a pentose fermentation step. Glucan accounted for 76-82% of the carbohydrates in recovered fibres. The acid insoluble content of the samples was between 19.7% and 24.2% and the ash content was between 11.9% and 17.0%. The

main inorganic elements in the ash were silicon, calcium, iron and aluminium originating most probably from filler and coating materials such as kaolin, clay and calcium carbonate. The amount of lipophilic extractives varied from 3.6% to 8.2% between the fibre samples. This fraction most probably contains some printing inks and adhesives, which can be expected to be soluble in lipophilic solvents.

The amount of glucan out of total carbohydrates in newspaper, magazine, cardboard and office paper is 71%, 72%, 74% and 80% respectively, as calculated from data in Table 4. Chemical pulp is the main fibre component in office paper, whereas mechanical pulp is the main component in newspaper. The observed relatively high content of glucan in recovered fibres (76-82%) suggests that a significant part of the material has gone through the chemical pulping process in which glucose is enriched in relation to other sugars. The carbohydrate profile of the samples indicates that recovered fibres also contained galactoglucomannan originating mainly from softwood, and xylan originating mainly from hardwood. Unlike bark, recovered fibres are not expected to contain glycosidic sugars or free monosaccharides unless they are introduced in small amounts for example from residual food waste.

The majority of ash and extractives in recovered fibres does not originate from wood but represents other materials used in the fibre products that compose the SRF fibre fraction. The average ash content of the recovered fibre samples in this work was lower than the average ash content of office paper (22%) and magazines (31%), but higher than the average ash content of newspaper (6%) and cardboard (12%) as reviewed in Table 4. This again shows that the fibres in SRF originate from many different sources.

The clarification of the lignin content of recovered fibres would require a more specific analysis method than those in use for wood analysis. In this study the acid insoluble material clearly contained some residual plastics, which could be visually detected on the filter membrane after acid hydrolysis. Some ash components can also be acid insoluble, thus contributing to this gravimetric measurement. The ratio of acid insoluble material to total carbohydrates was 0.37-0.46 in recovered fibres, whereas the literature values in Table 4 give significantly lower ratios: 0.30 for magazines, 0.28 for newspaper, 0.23 for cardboard and 0.08 for office paper. Thus it appears that the acid insoluble material in recovered fibres was not comprised only of material originating in print products. Based on this finding, it can be estimated that lignin comprised 70-80% of the acid insoluble material in recovered fibres, representing 14-19% of the dry matter. The rest of the acid insoluble material (20-30%) was probably composed of plastics and ash.

The enzymatic digestibility of a biomass sample provides information about its structural and compositional aspects. In the case of recovered fibres, it may especially correlate with the amount and ratio of chemical and mechanical pulp in the material. The recovered fibre samples from three different suppliers could be hydrolysed to a varying extent producing final hydrolysis yields from 82% to 100% (Figure 11). At least 70% of the final hydrolysis yield was reached in 6 h, which indicates that the majority of the carbohydrates in recovered fibres are easily digestible. In this study the sample with the highest content of acid insoluble materi-

al (Sample C) produced the lowest hydrolysis yield. A high amount of acid insoluble material could indicate a high lignin content in the sample, probably coming from a large portion of mechanical pulp. Lignin is known to inhibit cellulases by different mechanisms including limiting enzyme accessibility to its substrate and by non-productive binding of enzymes as discussed in Section 1.6.7. Therefore the observed lower hydrolysis yield could be related to the role of lignin and the amount of mechanical pulp in the material. Hydrolysis yields did not correlate with the content of lipophilic extractives indicating that these compounds do not have an effect on the enzymatic hydrolysis of carbohydrates in recovered fibres.

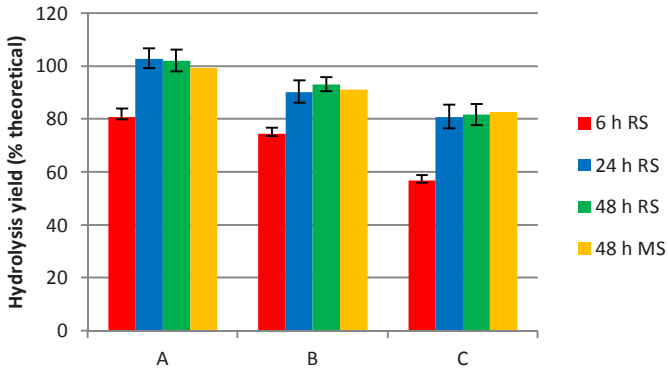

Figure 11. Enzymatic digestibility of three samples of recovered fibres (A, B and C, composition presented in Table 9) fractionated from SRF from different suppliers in the same experimental set-up. Hydrolysis was performed at 1% solids loading at 50°C using 8 mg/g d.m. dosage of a mixture of hydrolytic enzymes. RS = reducing sugar analysis, MS = monosaccharides analysed by HPAEC-PAD. Publication IV.

4.2 Extraction of tannins from spruce bark

Based on the composition of spruce bark and the knowledge of available applications, tannin is suggested to be the most potential new product from spruce bark. Tannin extraction from spruce bark was studied using hot water as the solvent, because it is safe, environmentally friendly and currently used in industrial tannin extraction. The high amount of pectic polysaccharides, hemicelluloses and non-cellulosic glucose in bark indicates that water extracts may contain carbohydrates in addition to phenolic matter. Thus, the effect of extraction parameters on the extraction yield and the extract composition were studied in order to determine whether there is a way to extract tannins selectively and efficiently using water as the solvent. The composition of several extracts was characterized in more detail to preliminarily assess their applicability in different tannin markets. Finally the release of bound sugars in bark extracts was demonstrated using hydrolytic enzymes, which may offer a new method for tannin purification.

4.2.1 Effect of extraction parameters on extraction yield and extract composition (Publication III)

Batch-wise extraction experiments were conducted in ca. 8 L volume at a 60-90°C temperature range and at 5-15% solids content in rotating reactors. Maximum solubilisation of 20.9% bark dry matter was obtained within 120 min (Figure 12). A high extraction temperature of 90°C correlated positively with a high total extraction yield at all levels of solids loading. The increase in solids content from 5% to 15% had little effect on the total extraction yield or carbohydrate yield, but a negative effect (25-35% reduction) on the tannin extraction yield. The temperature increase from 60°C to 90°C increased the tannin yield by at least 77%. Bound carbohydrates, which can be hemicelluloses, oligosaccharides or glycosidic carbohydrates, responded slightly to changes in temperature, but the extraction yield of carbohydrates in the form of free monosaccharides remained constant (1.8-2.3% of bark d.m.) in the experiments (Figure 2, Publication III). The resulting portion of sugars present as monosaccharides was 32.1% out of total carbohydrates in the winter bark extract produced at 75°C in 10% solids content with extraction chemicals, but only 7.8% for the summer bark extract produced in the same conditions (Table 2, Publication III). The bulk of the extracted carbohydrates was composed of glucose followed by galactose and galacturonic acid. Only traces of mannose and xylose were released in the extraction, indicating similarly to the glycome profiling results, that the polysaccharides containing mannose and xylose were tightly bound in the bark cell wall. The use of additional extraction chemicals sodium bisulphite and sodium carbonate (2% and 0.5% of bark d.m.) did not increase the total extraction yield but increased the tannin extraction yield and reduced the relative amount of carbohydrates in the resulting extract (Figure 3, Publication III).

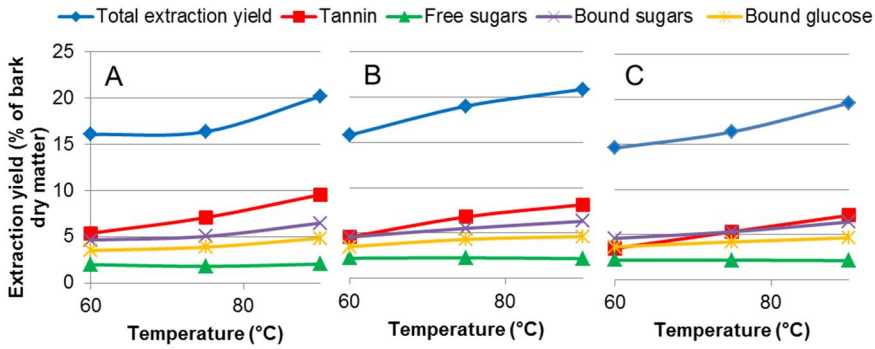

Figure 12. The effect of temperature on hot water extraction yield of spruce bark components at 5% (A), 10% (B) and 15% (C) solids content. Publication III.

Increasing the extraction temperature above 90°C would probably have increased the extraction yield, as reported by Roffael *et al.* (2000). Sequential acetone-water

and pressurized hot water extractions at 100-160˚C can extract up to 42% of spruce bark dry matter according to Le Normand *et al.* (2012). However, Kylliäinen and Holmbom (2004) reported that a substantial part of hydrophilic bark components (12% of d.m.) could be solubilized already at 30°C. The fraction of free sugars in bark and their resulting presence in the hot water extracts is apparently not affected by the extraction parameters but rather the felling time of the tree. Weissman *et al.* (1984) also suggested that the amount of free monosaccharides in bark extracts varies according to the felling time of the year being the highest in the end of the growing season. The positive effect of extraction chemicals on the tannin extraction yield is probably related to their ability to solubilize insoluble phlobaphenes at high temperatures as reported by Grassmann *et al.* (1956).

Despite the slight differences in the response of carbohydrates and tannin to extraction parameters, the results showed that a fully selective extraction of carbohydrates and/or tannin from spruce bark is not possible under these conditions. As reviewed in Section 1.5.4, a few publications indicate that spruce bark tannins may be covalently bound to glucose. Rather than targeting at a selective extraction, it may be beneficial to aim at a high overall extraction yield and develop purification methods for the crude extract, or to aim at developing applications in which crude tannin can be used without purification.

4.2.2 Chemical characterization of bark extracts (Publication III)

Bark extracts were chemically characterized to assess the potential of the crude extract as a source of phenolic compounds. The extracts produced in pilot-scale (see Section 4.4.1) were spray-dried and studied in more detail than extracts produced in bench-scale (Section 4.2.1). Table 10 presents an overview of the composition analysis of three spruce bark extracts. The two comparable extracts produced with extraction chemicals (Batches I and II) were completely water-soluble, whereas the Batch III extract was not fully water-soluble after concentration and spray-drying. pH of the first two extracts was 5.9-6.1 whereas pH of Batch III extract was 4.5. The extract dry matter contained 49.7-57.6% tannin as analysed by the hide powder method, and 22.2-35.3% carbohydrates. Ash content was high (26.5%) when extraction chemicals were used, but low without them (4.2%). The hide powder and acid butanol methods gave very similar results regarding the tannin content of Batch II extract (49.7% vs. 50.5%) but very different results for the Batch III extract (57.6% vs. 23.5%). Batch II extract contained more phenolic groups (35.8%) than Batch I extract (30.2%) according to the Folin-Ciocalteu assay using gallic acid as the standard compound. The method is not specific to phenolic groups in tannin and may thus also detect stilbenes and other phenolic compounds. The content of acid insoluble material and lipophilic extractives was higher in Batch III extract than in Batch II extract.

Table 10. Chemical composition of three spruce bark extracts. The carbohydrate content is expressed as monosaccharide equivalents.

Batch number	**I (Publication III)**	**II (Publication III)**	**III (Unpublished data)**
Extraction parameters			
Tree felling time	Winter	Summer	Summer
Wood content in the raw material	0%	21%	10%
Scale of extraction	Bench-scale	Pilot-scale	Pilot-scale
Extraction chemicals	2% sodium bisulphite and 0.5% sodium carbonate of bark d.m.	2% sodium bisulphite and 0.5% sodium carbonate of bark d.m.	None
Temperature	75°C	75°C	90°C
Solids content	10%	9%	5%
Form	Water extract	Spray-dried powder	Spray-dried powder
Extract composition (% d.m.)			
Water-soluble solids	100	100	80
Tannin (hide powder method)	n.a.	49.7	57.6
Tannin (acid butanol method)	51.0	50.5	23.5
Carbohydrates	35.3	22.2	30.0
Ash	n.a.	26.5	4.2
Acid insoluble material	n.a.	34.5	46.8
Acid soluble lignin	n.a.	12.3	9.4
Lipophilic extractives	n.a.	0.9	2.6

Batch III extract was stored up to 10 days in a concentrated form prior to spray-drying, whereas Batch II extract was dried within a few days of extraction. The storage of the more acidic Batch III extract may have led to condensation reactions between tannin molecules reducing their water-solubility. Water-insoluble phlobaphenes are known to be formed from proanthocyanidins at low pH (Serrano *et al.*, 2009). The higher content of acid insoluble material in Batch III extract could also indicate that condensation reactions occurred between the compounds during storage. Roffael *et al.* (2000) showed that whereas a high extraction temperature improves the extraction yield of spruce bark, it decreases the reactivity of the extract towards formaldehyde. The higher extraction temperature of Batch III ex-

tract and the probable condensation reactions may also have reduced its reactivity in addition to water-solubility. The formation of phlobaphenes may have contributed to the observed difference between the acid butanol assay and the hide powder method results for Batch III extract. The acid butanol assay cannot oxidatively cleave anthocyanidin from phlobaphenes, but phlobaphenes may still bind to hide proteins in the hide powder method. If sugars were covalently bound to tannin, they would also increase the amount of tannin measured by the hide powder method. Batch III extract contained more carbohydrates (30.0%) than Batch II extract (22.2%), which could indicate that more carbohydrates were covalently bound to tannin. This in turn could also explain the relatively higher tannin content analysed by the hide powder method in Batch III.

The weight average molar masses of the ultraviolet light absorbing compounds in Batch I and II extracts were determined to be 3.4 kDa and 3.2 kDa by size exclusion chromatography (SEC). Phenolic groups were also analysed by ^{31}P NMR, which showed that purified quebracho tannin contained almost seven-fold more phenolic groups than Batch II extract (14.6 compared to 2.2 mmol/g). The SEC analysis results indicated that there could be on average 10 catechin subunits (290 g/mol) in each condensed tannin molecule. Even if the impurities such carbohydrates and ash in the sample are accounted for, the ^{31}P NMR results suggest that the tannins in the particular extract contained less phenolic groups per mass unit than the purified quebracho tannin.

Batch I extract dry matter contained 35.3% carbohydrates of which 32.1% were free monosaccharides (see Table 2 in Publication III). Batch II extract contained 22.2% carbohydrates of which only 7.6% were free monosaccharides. Batch II extract contained relatively less glucose, galactose, and methyl-galacturonic acid, but more mannose, arabinose, rhamnose, xylose, galacturonic acid and glucuronic acid than Batch I extract. Bark used for the Batch II pilot-scale extraction contained a major amount of wood as impurities (21% of dry matter), whereas the bark for the bench-scale Batch I extraction was manually sorted to be wood-free. The presence of water-soluble galactoglucomannan from spruce wood may have contributed to the higher mannose content in the Batch II extract. However, the different wood contents of the raw materials cannot explain all the differences between them, for example the higher galacturonic acid content of the Batch II extract.

The glycome profiling analysis which measured the binding of the extract molecules on glycan-directed antibodies (Figure 4, Publication III) revealed the presence of xylan, xyloglucan, pectin and arabinogalactan in the hot water extracts. The strong binding of compounds on xylan directed antibodies was rather surprising because xylose was present in the extracts in very low amounts (0.8-1% of total carbohydrates). In general more detectable epitopes, especially xylans, were found from Batch I extract. Since the extracts were loaded on the ELISA plate at equal carbohydrate concentrations, there must have been either less recognizable hemicellulosic epitopes or more glycosidically bound sugars in the Batch II extract to account for all the undetected carbohydrates. Because the extracts were alcohol extracted and dialysed against a 3.5 kDa cut-off membrane prior to analysis,

these undetected carbohydrates must have been contained in structures that were not alcohol soluble and that were above 3.5 kDa in size. It is possible that the Batch II extract contained glycosidic condensed tannin structures, which were alcohol insoluble, above 3.5 kDa in size and responsible for harbouring the carbohydrates not detected by the ELISA assay.

4.2.3 Enzymatic hydrolysis of carbohydrates in spruce bark extracts

Condensed tannins are typically 500-3000 Da in molar mass (Bate-Smith and Swain, 1962), whereas monosaccharides have a molar mass below 200 g/mol. A size-based separation technology could thus be employed to separate tannins and monosaccharides. Besides removing sugars, the separation could reduce the amount of inorganic salts detected as ash in the extract. To enable tannin enrichment by size-based separation, the carbohydrates in the spruce bark extract should be hydrolysed to monosaccharides.

The enzymatic hydrolysis of carbohydrates in spruce bark extracts was studied using commercial enzymes containing mainly β-glucosidase and, different hemicellulase and pectinase activities. Figure 13 presents the amount of free monosaccharides in the untreated and enzyme-treated Batch I and II extracts and shows the theoretical potential available in them. The enzymes were able to almost completely hydrolyse the carbohydrates in the Batch I extract from winter bark, whereas only a 54.9% hydrolysis yield was obtained for Batch II extract from summer bark. The relatively poor hydrolysis yield of glucose (47.2%) was responsible for the reduced overall yield in Batch II extract. Batch III extract from summer bark was hydrolysed to 64% yield using a mixture of 5 enzyme products (data not shown). The results show that spruce bark carbohydrates can be hydrolysed to monosaccharides at least to some extent even in the presence of bark tannins and extraction chemicals. If a sufficient hydrolysis yield is achieved, an ultrafiltration step could possibly be employed to enrich tannin in the extract.

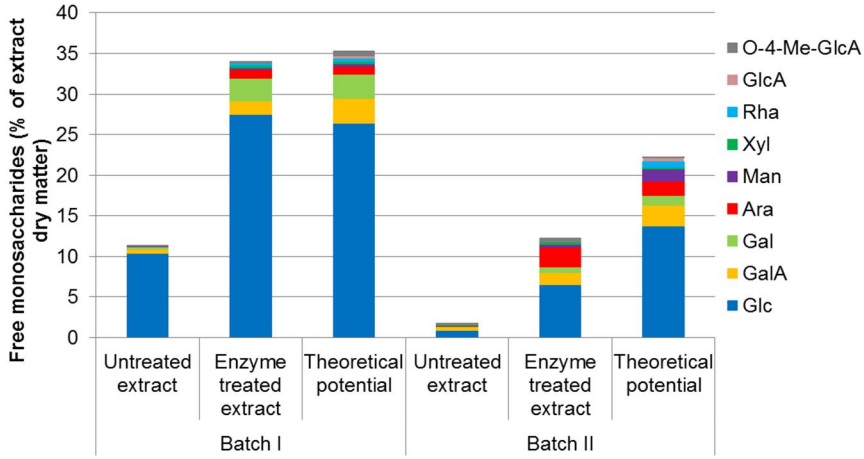

Figure 13. Enzymatic hydrolysis of Batch I (winter bark) and Batch II (summer barl) hot water extracts (30 g/l dry matter content) with an enzyme mixture dosed 10 mg/g d.m. containing equal amounts (based on protein) of Novozym 188, Viscozyme L and Pectinex Ultra SP-L enzyme products. Other parameters: 45°C, 48 h, no pH adjustment. Unpublished data.

4.3 Production of fermentable sugars and ethanol

The effect of pretreatment on the composition and enzymatic digestibility of spruce bark and recovered fibres was studied for the production of fermentable sugars. Selected key questions regarding the composition of the optimal enzyme mixture, the effect of solids loading on enzymatic hydrolysis and the use of surfactants to improve hydrolysis yield were investigated. The fermentability of the bark hydrolysates was confirmed by high consistency ethanol fermentation.

4.3.1 Pretreatment and hydrolysis of spruce bark (Publication II)

The effect of hot water extraction, steam explosion, and a sequential combination of the two on spruce bark composition and hydrolysis was determined. In addition, the effect of temperature and the use of an acid catalyst in steam explosion were investigated. Two temperatures were employed, 190°C or 205°C, for a 5 min treatment time followed by an explosive release of pressure. The corresponding logarithms of the severity factor $R_0 = t \cdot \exp((T-100)/14.75)$ were 3.3 and 3.8 according to the equation by Overend *et al.* (1987). Steam explosion dissolved hemicelluloses from bark, thus enriching glucan in the insoluble solids (Table 1, Publication II). The use of an acid catalyst in the treatment resulted in a more complete hemicellulose removal, as only 14-15% of non-glucan carbohydrates were recovered in the insoluble solids compared to 31-41% without acid. The higher temperature (205°C) and acid catalyst increased the relative amount of compounds analysed as Klason lignin from 35.8% in untreated bark to up to 44.6% in steam ex-

ploded bark. Hot water extraction at 80°C dissolved less non-cellulosic polysaccharides compared to steam explosion: 41% non-glucan neutral polysaccharides and 25% acidic polysaccharides were recovered in the insoluble solids after steam explosion and 74% and 60% respectively after hot water extraction (Table 2, Publication II). Hot water extraction reduced the relative amount of acid insoluble material in the extracted solids (28.2%) compared to untreated bark (32.8%). A sequential hot water extraction and steam explosion resulted in a composition of the solids similar to that obtained after steam explosion.

The removal of hemicellulose during steam explosion enriched lignin in the insoluble solids, but condensation reactions between lignin, carbohydrates and tannin may also have contributed to the increased amount of acid insoluble material in the insoluble fraction. Steam pretreatment may produce so called pseudolignins as a result of condensation reactions between lignin, sugars and sugar degradation products (Heitz *et al.*, 1991). Water-soluble bark tannin may precipitate in dilute acid pretreatment and show up as Klason lignin (Torget *et al.*, 1991). Robinson *et al.* (2002) observed an increase in mass yield when bark was mixed with Douglas fir wood and steam exploded with SO_2. Due to the presence of high tannin content, bark may have a higher tendency to produce new acid insoluble material in pretreatment compared to wood, especially when an acid catalyst is used.

Bark steam exploded at 190°C without acid catalyst performed the best in enzymatic hydrolysis experiments. The treatment improved the digestibility of bark carbohydrates from 36% to 75% (Figure 14). The second best condition was the treatment at 205°C also without the acid catalyst (63% hydrolysis yield). Many articles report that an increase in pretreatment temperature and the use of an acid catalyst improve the enzymatic digestibility of woody biomass (Galbe and Zacchi, 2007; Schutt *et al.*, 2011; Nakagame *et al.*, 2011; Fang *et al.*, 2011). The reason why a lower pretreatment temperature without acid catalyst produced a higher hydrolysis yield could be related to bark tannins and their reactions during the pretreatment in acidic conditions. David and Atarhouch (1987) obtained lower hydrolysis yields after H_2SO_4 treatment for spruce bark compared to the hydrolysis yield after extraction with boiling water only.

Figure 14 shows the enzymatic digestibility of steam exploded bark, hot water extracted bark, and sequentially hot water extracted and steam exploded spruce bark with a cellulase (dosed 10 FPU/g d.m.) and a β-glucosidase product (dosed 200 nkat/g d.m.). Both steam exploded barks could be hydrolysed efficiently, reaching a hydrolysis yield of 68-70%. Hot water extracted bark reached a 54% hydrolysis yield with the same enzyme mix and dosage. The increase of cellulase and β-glucosidase dosages to 25 FPU/g d.m. and 500 nkat/g d.m. respectively improved both the hydrolysis rate and the maximum hydrolysis yield of steam exploded spruce barks (Figure 1, Publication II). The improvement was greater with the samples pretreated using an acid catalyst. A major difference in the hydrolysis of differently pretreated spruce barks was observed when an enzyme product with a high pectinase activity was added in the mixture of hydrolytic enzymes. The performance of hot water extracted spruce bark was significantly

improved when a pectinase product dosed at 5000 nkat/g d.m. was used resulting in yields comparable with those from steam exploded samples (Figure 14). The effect of pectinase was smaller for steam exploded bark, which contained less pectin. Furthermore, the pectinase product Pectinex Ultra SP-L alone was more efficient in hydrolysing hot water extracted bark than steam exploded bark. The pectinase product increased the hydrolysis yield of galacturonic acid most strongly, but also improved the hydrolysis of glucan, xylan, arabinan, mannan and galactan indicating that hemicellulase activities in the product may have contributed to the overall improvement (Table 3, Publication II). The similar hydrolysis yields obtained for steam exploded and sequentially hot water extracted and steam exploded barks reflect the similar chemical composition of the samples.

Steam pretreatment has been shown to increase the tendency of enzymes to bind non-productively on softwood lignin (Rahikainen *et al.*, 2013a). Nakagame *et al.* (2011) reported that lignin isolated from Douglas fir pretreated at higher severity decreased the enzymatic hydrolysis of Avicel more than lignin from a lower severity pretreatment. The results obtained in this work suggest that using an acid catalyst in bark pretreatment affected the properties of bark lignin negatively by increasing its tendency to bind and inactivate hydrolytic enzymes, and that this negative effect could be partially overcome by using a higher enzyme dosage. The reactions that tannin underwent in the pretreatment may have further contributed to this effect. It appears that the pectinase enzyme improved the hydrolysis yield of hot water extracted bark by hydrolysing pectin which increased the enzymatic accessibility of the other polysaccharides. Additional hemicellulase activities present in the pectinase product may also have contributed to the yield improvement. On the basis of the results it can be concluded that steam explosion at 190°C without an acid catalyst is an efficient pretreatment for spruce bark resulting in a 75-79% hydrolysis yield. However, a hot water extraction at 80°C alone could be sufficient as a bark pretreatment when a pectinase-containing enzyme mixture is used for hydrolysis.

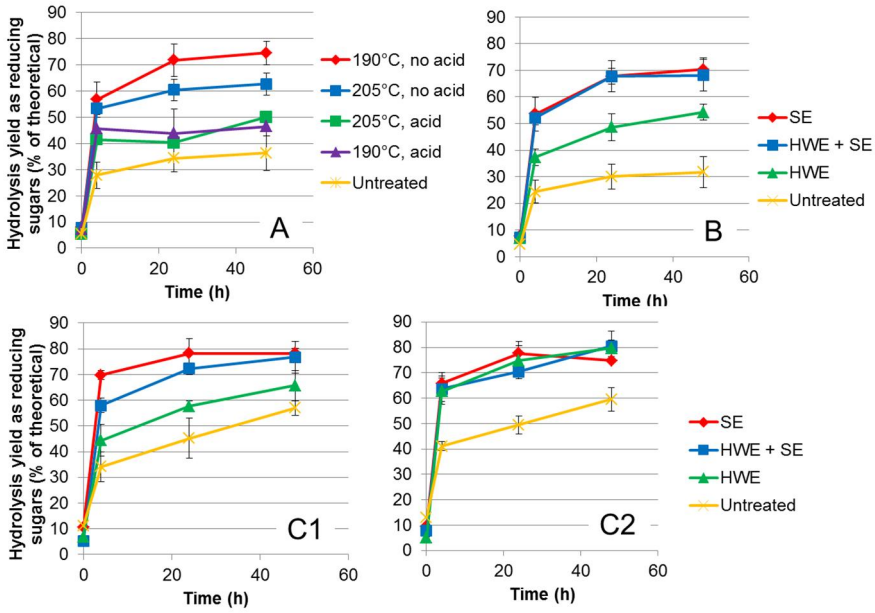

Figure 14. The effect of pretreatment on the enzymatic digestibility of spruce bark at 1% consistency, 50 mM acetate buffer pH 5, 45°C. SE: steam explosion at 190°C without acid catalyst. HWE: hot water extraction at 80°C. A) and B) 10 FPU/g d.m. cellulase activity and 200 nkat/g β-glucosidase activity. C1) 25 FPU/g cellulase activity, 500 nkat/g β-glucosidase activity, no pectinase. C2) 25 FPU/g cellulase activity, 500 nkat/g β-glucosidase activity, 5000 nkat/g pectinase activity. Publication II.

4.3.2 Pretreatment and hydrolysis of recovered fibres (Publication I and IV)

Steam explosion in different conditions was also studied with recovered fibres in order to determine the effect of the pretreatment temperature and lowering the pH with H_2SO_4 on the enzymatic digestibility of the material (Figure 15). Steam explosion decreased the apparent hydrolysis rate and reduced the final hydrolysis yield after 115 h hydrolysis by 10-15%. The effect could already be seen with the material pretreated in the mildest conditions (120°C, pH 5). Treatment temperature at 160°C and above decreased the yield significantly, by over 22% after 24 h hydrolysis. Samples treated at 160°C and above were darker in colour and appeared to have an increased rate of sedimentation compared to untreated fibres on the basis of visual analysis.

The results indicate that recovered fibres are easily digestible without any kind of thermochemical pretreatment. In fact, according to the results a pretreatment

may even decrease the enzymatic digestibility of the material. The plastic impurities, stickies and other non-biomass derived components in the material may behave unexpectedly in the pretreatment, and redistribute so that the accessibility of the carbohydrates is reduced. Moon and Kim (2001) reported that a pretreatment at 170°C reduced the enzymatic digestibility of waste newspaper supporting the results obtained in this work. On the basis of these results, steam explosion can reduce the enzymatic digestibility of recovered fibres. However, the origin of the fibres and the type of the products in the SRF stream may affect the result and the topic should be further studied with a larger and a more varied set of SRF samples.

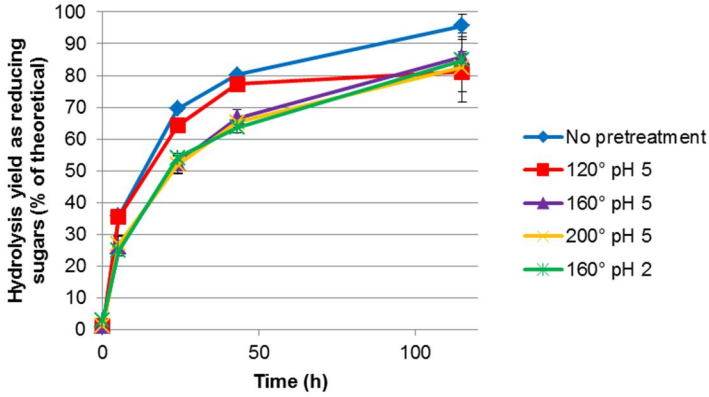

Figure 15. The effect of pretreatment temperature and pH on the enzymatic digestibility of recovered fibres at 10% consistency, 100 mM sodium acetate buffer pH 5, 45°C, with 10 FPU/g d.m. cellulase and 1000 nkat/g β-glucosidase activity. Publication I.

The release of mono- and oligosaccharides during enzymatic hydrolysis of recovered fibres was analysed in order to identify enzyme activities missing from the enzyme mixture in use. Analysis of the hydrolysate revealed the presence of an almost constant concentration (8-9 g/l) of cellobiose from 2 h of treatment time onwards. A higher dosage of β-glucosidase activity or a reduction in solids loading would probably have alleviated the cellobiose build-up. According to Figure 5 in Publication I the mixture lacked enzymes capable of hydrolysing xylose-, mannose- and galactose-containing soluble oligosaccharides, or their hydrolysis rate was significantly slower than that of glucose- and arabinose-containing oligosaccharides during the first 10 h of high consistency hydrolysis. Xylo-oligosaccharides present in the hydrolysate consisted mainly of xylobiose and smaller amounts of xylotetraose, whereas mannose was retained in mannotetraose, mannobiose and mannopentaose. Further experimental work showed that xylose-, mannose-, galactose- and glucose-containing oligosaccharides persisted even after 8 h prehydrolysis and 87 h SSF of recovered fibres. It is apparent that recovered fibres

contain galactoglucomannan that could not be fully hydrolysed to monosaccharides with the enzyme mixture in use, even though the mixture contained a *T. reesei* mannanase enzyme. Similarly, Rättö *et al.* (1993) found that only 3% of solubilized mannose in pine kraft pulp hydrolysates was present in the form of monosaccharides, although the overall hydrolysis yield of mannose was high (85%). The complete hydrolysis of these oligosaccharides should be targeted since they provide hexoses that are fermentable by the common ethanol-producing yeast *S. cerevisiae*.

4.3.3 Effect of solids loading on hydrolysis yield (Publications I and IV)

The effect of solids loading on hydrolysis yield was studied for recovered fibres to assess the enzymatic digestibility of the material in industrially relevant conditions. In Publication I a 40% hydrolysis yield was reached at 30% solids loading in only 10 h with a moderate 8 FPU/g d.m. enzyme loading. Le Costaouec *et al.* (2013) reported <40% hydrolysis yields for pretreated wheat straw at 25% consistency after 24 h hydrolysis, and Cara *et al.* (2007) reported <30% yields for liquid hot water pretreated olive tree prunings at 30% consistency after 12 h hydrolysis. Kristensen *et al.* (2009b) observed <30% hydrolysis yields for filter paper at 20% consistency after ca. 12 h hydrolysis. Zhang *et al.* (2009) reported a 40% hydrolysis yield for organosolv treated hardwood poplar at 20% solids loading after 10 h hydrolysis. Even though the results are not fully comparable due to the use of different enzyme mixtures, dosages and hydrolysis parameters, it appears that recovered fibres can be hydrolysed relatively rapidly at high solids loading compared to other lignocellulosic feedstocks.

The effect of consistency on the hydrolysis yield of recovered fibres was studied in more detail in Publication IV. The results are compared with those obtained for two other raw materials, pretreated wheat straw and pretreated spruce (unpublished data, Figure 16). Recovered fibres could be hydrolysed faster and they reached a higher hydrolysis yield at high solids loading (>15%) compared to pretreated wheat straw and pretreated spruce. Surprisingly, the hydrolysis yield at 1% consistency was low (< 36%) for all substrates in this mixing system in which half-filled bottles rolled freely in a rotating drum. For recovered fibres and pretreated spruce the hydrolysis yield at 48 h increased as a function of the solids loading levelling to a plateau at 15-25% solids loading. The hydrolysis yield of recovered fibres at 6 h decreased slightly above 15% consistency. Wheat straw performed the best at 5% consistency above which the hydrolysis yield decreased almost linearly as the solids loading increased.

The linear decrease of final hydrolysis yield at high solids loading, called the “solids effect” by Kristensen *et al.* (2009b) was not observed for recovered fibres or pretreated spruce in the range investigated. However, the solids effect was clear on pretreated wheat straw in the range of 5-25% solids loading. The effect was very similar to the results presented for pretreated wheat straw by Jorgensen *et al.* (2007). The reason for this difference between the substrates could be related to the content and type of lignin in them. Pretreated spruce contained the high-

est amount of Klason lignin (32.5%) followed by pretreated wheat straw (26.4%) and recovered fibres (14-19% as estimated in Section 4.1.2). However, the botanical source of recovered fibres and spruce is similar and different from wheat straw. Kristensen *et al.* (2009b) concluded that lignin content is not related to the extent of the solids effect, since they observed the same yield reduction on pretreated wheat straw and filter paper. Several studies have concluded that loss of enzyme activity by end-product inhibition or inhibition by other soluble compounds is not the main cause of the decrease in hydrolysis yield at high solids loading; instead the main reason is suggested to be the decline in adsorption of the enzymes on the substrate (Kristensen *et al.*, 2009b; Wang *et al.*, 2011a). The effect of solids loading on the amount of free enzyme activity in the liquid phase after 48 h hydrolysis was studied for recovered fibres. It was observed that the amount of free cellulase and β-glucosidase activity did not correlate with solids loading (Figure 5, Publication IV). The results could indicate that an increase in solids loading does not change the adsorption/desorption tendency of enzymes on recovered fibres and thus have no effect on hydrolysis yield in the studied range. However, reliable experimental proof of this is still lacking.

The significant difference in hydrolysis yield between 1% and 5% solids loading observed for recovered fibres and pretreated wheat straw indicates that enzymes became inactive or otherwise unable to operate at 1% solids loading in the applied conditions. It appears that the enzymes were negatively affected by the combination of the low solids loading and the tumbling type of mixing. The results suggest that a higher substrate and enzyme concentration in the reaction medium helped to retain the enzyme activity in the experiment. Le Costaouec *et al.* (2013) presented similar results showing a clearly lower hydrolysis yield at 2% compared to 10% consistency for pretreated spruce, but a higher hydrolysis yield at 2% compared to 10% for pretreated wheat straw. Significant inactivation of cellulases has been reported when they were exposed to air-liquid interfaces and subjected to shear without a substrate (Kim *et al.*, 1982). The water retention capacity, particle size and particle shape of the substrate would affect the shear forces experienced by the enzyme in the system. Thus the extent of enzyme inactivation could depend on the substrate as was presented above. On the basis of the results it can be concluded that the effect of solids loading on hydrolysis yield is highly substrate specific, and requires studies in various mixing conditions and over a wide range of consistencies to uncover all factors related to it.

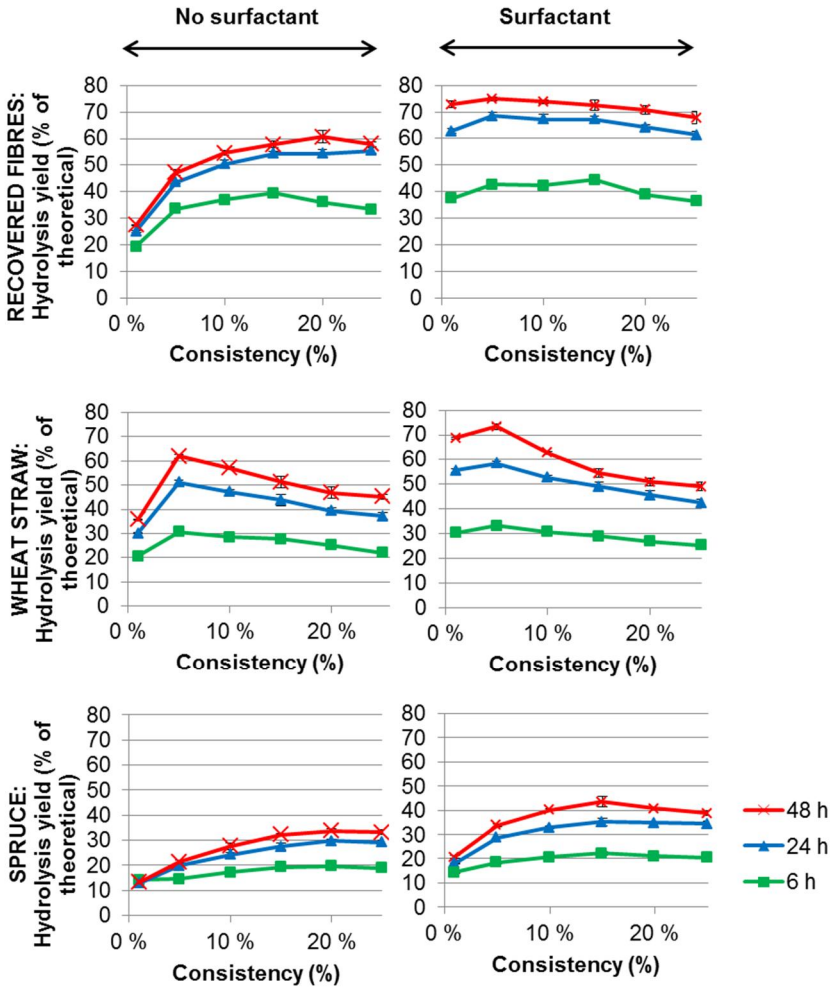

Figure 16. Effect of consistency and the use of surfactant on the hydrolysis yield of recovered fibres, pretreated wheat straw and pretreated spruce measured as reducing sugars. Experiments were carried out with 6 mg/g d.m. enzyme dosage at 50°C, in 100 mM sodium acetate buffer pH 5 with 0 or 1% w/w dosage of non-ionic surfactant Lutensol AT50. Publication IV and unpublished data.

4.3.4 Effects of surfactants on hydrolysis yield (Publication IV)

Surfactants were studied to assess whether they could be used to improve the hydrolysis yield of recovered fibres. In addition, the effects of surfactants were studied over a wide consistency range (1-25%) on three substrates to investigate how the effects of surfactants change in different conditions and between different types of feedstocks.

The effects of two non-ionic surfactants, Lutensol and Softanol, and PEG 4000, a polyethylene glycol product, were studied on recovered fibres at low solids loading (Figure 3, Publication IV). Lutensol and Softanol, which are composed of a polymerized ethylene or propylene oxide chain bound to a hydrophobic fatty acid, improved the final hydrolysis yield of recovered fibres by 8-10%. PEG had a negligible effect on the hydrolysis yield of recovered fibres in these conditions. Although less effective for recovered fibres, PEG improved the hydrolysis yield of a model substrate composed of spruce kraft pulp, birch kraft pulp and spruce TMP by 20%. The effect of PEG was minor on pure TMP, which hydrolysed very poorly overall.

The results indicate that the fatty acid component of Lutensol and Softanol, lacking from PEG, may have played a role in the hydrolysis improvement. However, Börjesson *et al.* (2007a) concluded that the polyethylene oxide moiety of the non-ionic surfactant is more important than the alkyl chain when considering their effects in enzymatic hydrolysis. PEG adsorbs on biomass lignin through hydrophobic interactions, and does not adsorb on crystalline cellulose such as Avicel (Börjesson *et al.*, 2007b). According to the literature, PEG appears to improve the hydrolysis yield most efficiently on substrates with good enzyme accessibility and a relatively high content of lignin, especially softwood lignin. This description matches the model substrate used in this work. It is possible that the lignin content of recovered fibres is too low to benefit from the preventive action of PEG on non-productive adsorption of enzymes on lignin. Jensen *et al.* (2011) discovered a lack of effect of PEG on the hydrolysis yield of thermally treated municipal waste, which contains fibre components similar to those in recovered fibres. Since PEG was efficient on the model substrate but not on recovered fibres, the effects of the non-ionic surfactants Lutensol and Softanol may have at least partially derived from some other mechanism than preventing non-productive binding of enzymes on lignin.

Figure 16 presents the effects of surfactants dosed at 1% of substrate d.m. on the hydrolysis yield of different substrates as a function of solids loading. The effect of surfactant was clearly positive (6-166% improvement at 48 h) for all substrates at all levels of solids loading. However, the effect was smaller at high consistency conditions compared to low consistency conditions. For recovered fibres and wheat straw the significant reduction in yield observed without the surfactant between 5% and 1% solids loading was no longer present when a surfactant was used. For pretreated spruce the final hydrolysis yield at 1% solids loading remained significantly lower (21%) compared to the yield at 5% when surfactant was used (34%). The increase in hydrolysis yield of recovered fibres at 1% solids loading correlated with a significant increase in the amount of free cellulase activity in the liquid phase after hydrolysis when surfactant was used (Figure 5, Publication IV). Overall, the use of surfactants increased the amount of free enzyme activity in the liquid phase. The amount of free cellulase was inversely correlated with the solids loading, whereas the amount of free β-glucosidase remained relatively constant, although higher, with surfactant.

The effect of surfactants may be very enzyme-specific. Börjesson *et al.* (2007b) reported that PEG decreased the adsorption of Cel7B (EGI) in the liquid phase more than the adsorption of Cel7A (CBHI) in the hydrolysis of pretreated spruce. However, on the basis of the results presented in this work the effect of surfactants is also very substrate-specific and dependent on the solids loading. The behaviour of recovered fibres was similar to that of pretreated spruce in terms of the response to solids loading, but similar to wheat straw in terms of the response to surfactants. In addition, the mixing regime affects the system. The improvement obtained with Lutensol in the hydrolysis yield of recovered fibres at 1% solids loading was over 16 times higher in the tumbling type of mixing (166% increase) compared to magnetic mixing in test tubes (10% increase). The results clearly demonstrate that surfactants can be used to improve the hydrolysis yield of recovered fibres and that they may thus enable the reduction of enzyme dosage in the process. The results also show how the complex effect of surfactants is dependent on the plant origin, pretreatment and lignin content of the raw material, on the solids loading, and on the mixing regime.

4.3.5 Fermentation of bark hydrolysates (Publication II)

Ethanol production from pretreated spruce bark was studied in Publication II in batch mode at 15% solids loading in order to confirm the fermentability of bark sugars. The presence of natural inhibitors and pretreatment-derived inhibitors could make spruce bark difficult to use as a feedstock for a biorefinery targeting at ethanol production. Under serious inhibition, yeasts cannot consume the available hexoses, which then accumulate in the reactor in an SSF set-up (Bollok *et al.*, 2000; Hoyer *et al.*, 2009). However, the glucose released from pretreated spruce bark in a 6 h prehydrolysis was fully consumed in less than 4 h after inoculation. Ethanol production continued as more fermentable sugars were released and reached its maximum in ca. 50 h. The hydrolysis yield of glucan after 6 h prehydrolysis was 68.2% for hot water extracted and steam exploded bark and 66.0% for hot water extracted bark with the enzyme mixture containing cellulase, β-glucosidase and pectinase products. Assuming a final hydrolysis yield of 70-75%, the ethanol yield from released hexoses was 83-95% of the theoretical maximum for both materials, which can be considered as efficient ethanol production. The total ethanol yield from theoretically available hexoses was 66.4% for hot water extracted and steam exploded bark and 62.3% for hot water extracted bark. The difference indicates that there may be a slight additional improving effect of steam explosion on the enzymatic digestibility of hot water extracted bark.

Overall, it appears that hot water extraction and steam explosion followed by enzymatic hydrolysis produced a fermentable hydrolysate, which was not toxic to fermenting yeast. By comparison, spruce wood hydrolysates may be severely inhibitory after steam pretreatment (Alriksson *et al.*, 2011). The low content of inhibitors in bark hydrolysates is supported by the results of Robinson *et al.* (2002), who found that Douglas fir bark produced less hydroxymethylfurfural and furfural than Douglas fir wood in SO_2-catalysed steam explosion, and that the

addition of 30% bark in the feedstock did not impact the ethanol yield of the process. Boussaid *et al.* (2001) also detected a lower amount of most fermentation inhibitors in the steam explosion hydrolysate of softwood feedstock containing 9% bark compared to bark-free feedstock, although this hydrolysate did not ferment efficiently. They concluded that high severity pretreatment conditions followed by acid hydrolysis are beneficial for the fermentability of feedstocks containing bark. The results presented in this thesis indicate that the removal of water soluble carbohydrates and phenolic compounds during hot water extraction may have reduced the amount of possibly inhibitory compounds or their precursors making hot water extraction favourable from the point of view of ethanol production. The results show that at least under some conditions, bark can be considered as a suitable feedstock for fuel ethanol production.

4.4 Scale-up of process steps

The extraction of tannin from spruce bark and the production of ethanol from recovered fibres were demonstrated in small pilot scale in order to verify the yields of the critical steps in conditions closer to those of industrial production. The results provided valuable information on the technical feasibility of the processes and data that can be used for investment calculations.

4.4.1 Scale-up of bark extraction (Publication III)

Spruce bark was extracted in two different pilot-scale set-ups. The results for Batch II are published in Publication III and the results for Batch III are hitherto unpublished. Batch II extract was produced by placing the bark in filter bags in a reactor and circulating the solvent in the system using an external pump. The bark for Batch III extract was crushed to enable mixing in a vertical stirred tank reactor at 1800 L volume. The insoluble solids were removed by filtration and centrifugation. Batch II extract was produced by combining the extracts from six extractions treating a total of 114 kg of spruce bark on a dry basis. Batch III extract was produced by combining the extracts from three extractions treating a total of 243 kg of bark on a dry basis. Table 10 presents more details of the extraction parameters.

The average extraction yield in the pilot-scale extractions was 11.8% for Batch II and 14.8% for Batch III. The extraction yield in bench-scale experiments in conditions similar to Batch II (75°C, 10% consistency, extraction chemicals used) was 18.6%. The solids separation and drying steps resulted in material losses at the pilot plant. The final yields of Batch II and Batch III crude tannins from dry spruce bark were thus lower than the extraction yields, 8.3% and 9.1% respectively producing 9.4 kg and 22.1 kg of crude tannin powder.

The reduction in the extraction yield after scale-up could be caused by reduced efficiency of mass transfer, the higher amount of wood in the raw material, or some other unknown factors operating in the pilot-scale system. The difference in the yield between the two pilot-scale extractions was probably caused by a higher

extraction temperature, better mixing system, and a lower content of wood in the raw material for Batch III extract. The produced amounts of spray-dried crude tannin demonstrate that spruce bark extraction is technically feasible and crude tannin can be produced in these pilot-scale set-ups for application studies. The final product yield can be expected to reach 12-13% in industrial systems, which are designed especially for the extraction of spruce bark and in which product losses can be better avoided.

4.4.2 Continuous ethanol production from recovered fibres in pilot-scale (Publication I)

Hydrolysis and fermentation of recovered fibres were demonstrated and further developed during seven pilot-scale experiments that lasted between 5 and 12 days. A process concept consisting of prehydrolysis and SSF operated as a continuous process was selected for the experiments. One of the main reasons for choosing a continuous operation strategy was that the viscosity of the material would remain constant at different stages of the process, which helps to choose the most efficient and cost-competitive reactors for each step in the process design. In addition, continuous fermentation increases the volumetric productivity, reduces cleaning and filling times and reduces labour costs in industrial production (Brethauer and Wyman, 2010). Selected aspects of these pilot-scale experiments were presented in Publication I, with emphasis on the results of the fifth pilot experiment. In addition to the published data, some unpublished data is also presented here.

Figure 17 shows the general process scheme as a simplified diagram and a photograph of the equipment used in the actual pilot-scale experiments. After the fractionation of recovered fibres from SRF (see Section 4.1.2), the material was fed to the prehydrolysis reactor at 40-50% dry matter content using a screw lift. The material was sanitized by heat during the fractionation process (60 min at 95°) and occasionally also in the screw lift using direct steam. The prehydrolysis reactor was a stirred tank reactor with a 4-impeller counter-current mixing element, and it provided a residence time of 6-10 h for the fibres at 20-30% consistency depending on the experiment. The enzymes, pH adjustment chemicals and sodium bisulphite used as a biocide were added to the prehydrolysis reactor continuously or semi-continuously. Sodium bisulphite dosage was selected based on the results of preliminary experiments studying its effect on hydrolytic enzymes and the viability of lactic acid bacteria and yeast. During the prehydrolysis stage the material liquefied forming a thick slurry, which could be transferred to the fermentation vessel by a heavy-duty hose pump. The fermentation stage, where further hydrolysis took place as well, was carried out in one reactor or divided into several reactors. The residence time in fermentation was between 21 h and 44 h. The pilot-scale system processed 2-4 kg/h recovered fibres (d.m.) and the maximum amount of raw material processed in one experiment was ca. 600 kg (d.m). The experiments were started by filling up the reactors one by one occasionally using a

higher flow rate, which was then reduced for the steady-state to provide the residence times planned.

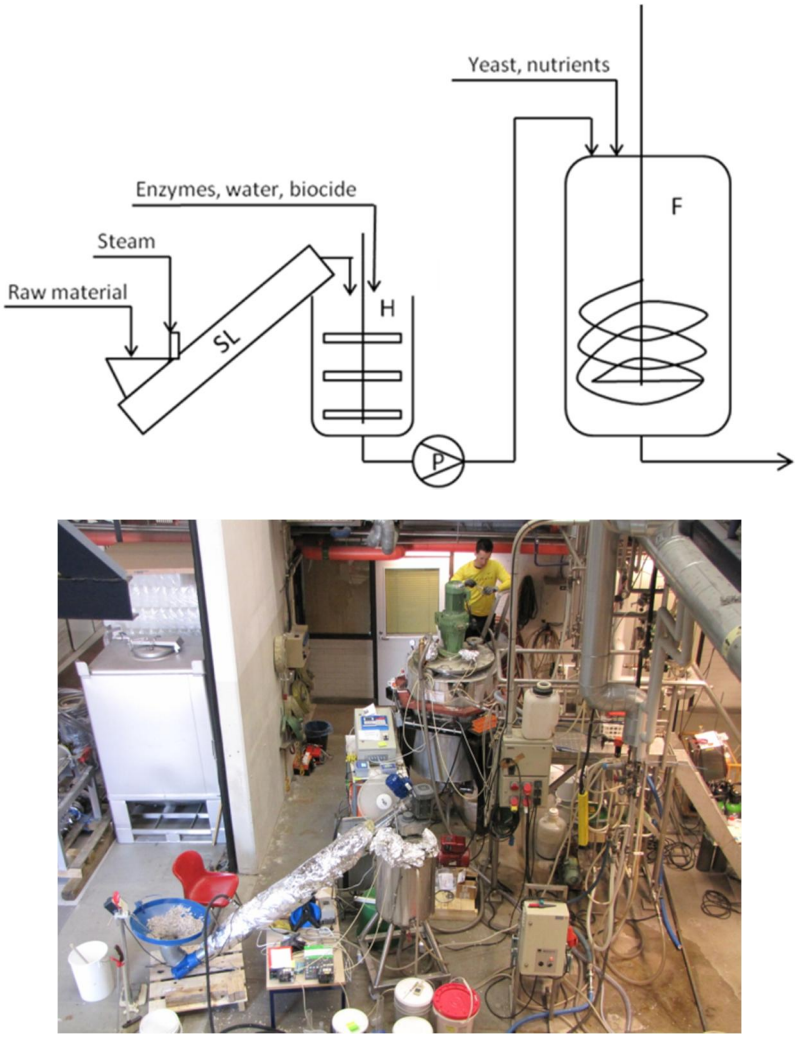

Figure 17. Schematic view of the pilot process and a photograph of the same set-up. SL) screw lift, H) prehydrolysis tank, F) fermenter.

Figure 18 presents the concentration of sugars and metabolites in the fermenter during the fifth pilot experiment, in which only one fermenter was used. The continuous state of the experiment started after 21 h when the reactor used for fermentation was filled. The hydrolysis yield of recovered fibres entering the reactor at 30% consistency was 43-49% measured as reducing sugars. After the 21 h residence time in fermentation was stabilized, a constant ethanol concentration of

ca. 35 g/kg was reached. Due to the short residence time, the average final hydrolysis yield of fermentable hexoses was only 57% and some unused glucose leaked out of the reactor. In later experiments the leakage of unused glucose was prevented by increasing fermentation time and using several fermenters in series. Fermentation yield was 84% as calculated from the hydrolysed hexoses. Xylose was released by the enzymes but not consumed and its concentration stabilized at ca. 13 g/kg. Towards the end of the experiment some xylitol production from xylose was observed. Lactic acid was not detected during the campaign indicating that the microbial contamination could be controlled by heat sanitation of the raw material and by using sodium bisulphite as a biocide in the process. Glycerol production was noticeable (<4.5 g/kg) but acetic acid was not detected in the fermenter.

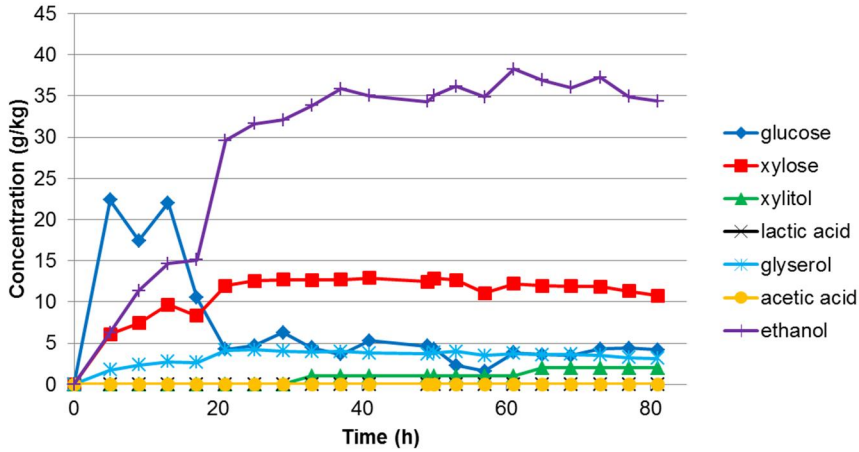

Figure 18. Sugar and metabolite concentrations in the fermenter with 21 h residence time during a continuous SSF process with recovered fibres at 30% solids loading after 6 h prehydrolysis.

Table 11 presents selected parameters and results from three pilot experiments. The experiments varied in terms of their length, total flow rate and consistency, but also in terms of the composition of the enzyme mixture, enzyme dosage, residence times and reactor configurations. Due to the nature of the project where the pilot work was carried out, not all parameters and results can be disclosed in this thesis. The hexose hydrolysis yield was dependent on the enzyme dosage and residence time. The yield reached in the final pilot experiment (73%) can be considered to be sufficient for the process concept, because in the overall concept the idea was to ferment the residual hexoses and pentoses to biogas after ethanol production. Ethanol concentration depended on the hydrolysis yield and contamination problems, and varied during the experiments. However, an ethanol concen-

tration above 4 vol-% was demonstrated which indicates that the distillation of ethanol could be carried out with an acceptable energy consumption.

Table 11. Selected experimental parameters and results obtained in continuous pilot-scale production of ethanol from recovered fibres.

Pilot experiment no.	**5**	**6**	**7**
Duration of the experiment (days)	5	12	12
Raw material	Recovered fibres fractionated from SRF	75% recovered fibres fractionated from SRF, 25% fibre sludge from a pulp and paper mill	Recovered fibres fractionated from SRF
Total flow rate at steady state (kg/h)	14	10	11
Consistency in hydrolysis (%)	30	25	20
Hexose hydrolysis yield (%)	57	59	73
Maximum obtained ethanol concentration (g/kg)	35	40	27
Ethanol yield (L/ton d.m)	156	171	166

Published pilot-scale results on high consistency SSF of lignocellulose remain scarce. Maas *et al.* (2008) reached 48% glucan-to-ethanol yield in a pilot-scale batch hydrolysis and fermentation (53 h) of lime-pretreated wheat straw at 35% dry matter content. Schell *et al.* (2004) reported results of continuous high-consistency SSF of corn fibres at 25% consistency. Their process was hampered by heavy microbial contamination resulting in high concentrations of lactic and acetic acid in the reactors. South *et al.* (1993), Fan *et al.* (2003) and Jin *et al.* (2013) reported results of continuous fermentation of lignocellulosic substrates but not at high solids loadings. The pilot-scale experiments of continuous hydrolysis and fermentation carried out in this work demonstrated that recovered fibres are well suited for ethanol production in a continuous high solids process producing at least 156 L ethanol per ton of recovered fibres.

4.5 Biorefinery concepts based on spruce bark and recovered fibres

The results presented in this thesis help to outline two biorefinery concepts producing fuels and chemicals from feedstocks from the forest industry. Next the concepts based on spruce bark and recovered fibres are described and their capacities and product yields are estimated.

4.5.1 Bark biorefinery concept

The high tannin and carbohydrate content of spruce bark, its residual nature, and its abundant availability in co-location with existing industry makes it a promising feedstock for a future biorefinery. Tannin is a medium value biochemical with existing markets and new opportunities in sight, as reviewed in Section 1.7.3. Carbohydrates are the raw material for all sugar platform biorefinery products, and could contribute to the concept as a secondary value component. Figure 20 presents a scheme of a bark biorefinery co-located at a saw mill or a pulp and paper mill site. It produces tannin and ethanol, extending the traditional product portfolio of the site from wood products, pulp and paper to liquid transport fuels and chemicals. Similar concepts based on spruce bark have been presented by Hytönen and Aaltonen (2008) and Kaijaluoto *et al.* (2011).

The debarking line present at the mill site provides raw material for the process. First the bark enters an extraction process in which tannin is solubilized and the extract is separated from the solids. An existing bark press at a site that carries out wet debarking could be used for the solid-liquid separation, which would decrease the investment costs. According to the results of this thesis, a simultaneously selective and efficient extraction of spruce bark tannin without carbohydrates is not possible using water as the solvent. However, the carbohydrates could possibly be removed and tannin enriched in the extract using hydrolytic enzymes and filtration technology. Solvent-based purification method could also be considered. The results show that the carbohydrates in the insoluble solids from the extraction can be hydrolysed efficiently to fermentable sugars when a pectinase-containing enzyme mixture is used. Fermentation experiments confirmed that ethanol can be produced from bark sugars by microbial conversion. Sugars separated from the extract during purification could be directed to the fermentation step in the process concept. The residual solids are combusted in a unit that is likely already to exist on-site, and the ethanol is concentrated by distillation. The final absolution of ethanol to fuel grade could take place on-site, or at another site combining streams from multiple fermentation sites. The bark biorefinery concept would also produce an effluent stream containing non-fermentable soluble compounds, which would need to be treated in the effluent system of the plant. Biogas production is an option for the effluent stream.

Several modifications to this concept could be envisioned. A very similar concept could also be functional for pine bark or the bark of some other softwood species. Even a mixed bark stream could be considered. Tannin could be formulated directly into a resin on-site and used for gluing the wood products produced on-site. The fermentation product could also be something other than ethanol, for example lactic acid or succinic acid. If the profitable capacity of the tannin plant could not sustain a profitable ethanol unit, the fermentable sugars could be concentrated into a sugar syrup and transported elsewhere for fermentation. In the most limited version of the concept only extraction and extract drying units would be installed and the remaining solids would be combusted directly afterwards.

The realization of this concept requires close cooperation of the supply chain from the bark producer to the user of the tannin-containing product. The investor of the extraction unit could be the company operating the main process on the site, or an external player, for example a company already manufacturing tannin. It might be beneficial if a large part of the produced tannin were to be used in wood products by the mill itself. The mill could give direct feedback on the extract quality to the extraction process, and on the other hand, more easily adapt to for example seasonal changes in the adhesive formulation and behaviour. The freshness of bark and the dry debarking method employed at sawmills would probably make them more suitable locations for the bark biorefinery concept than pulp mills. On the other hand, some valuable components already exist in the debarking effluents of pulp mills (Kylliäinen and Holmbom, 2004).

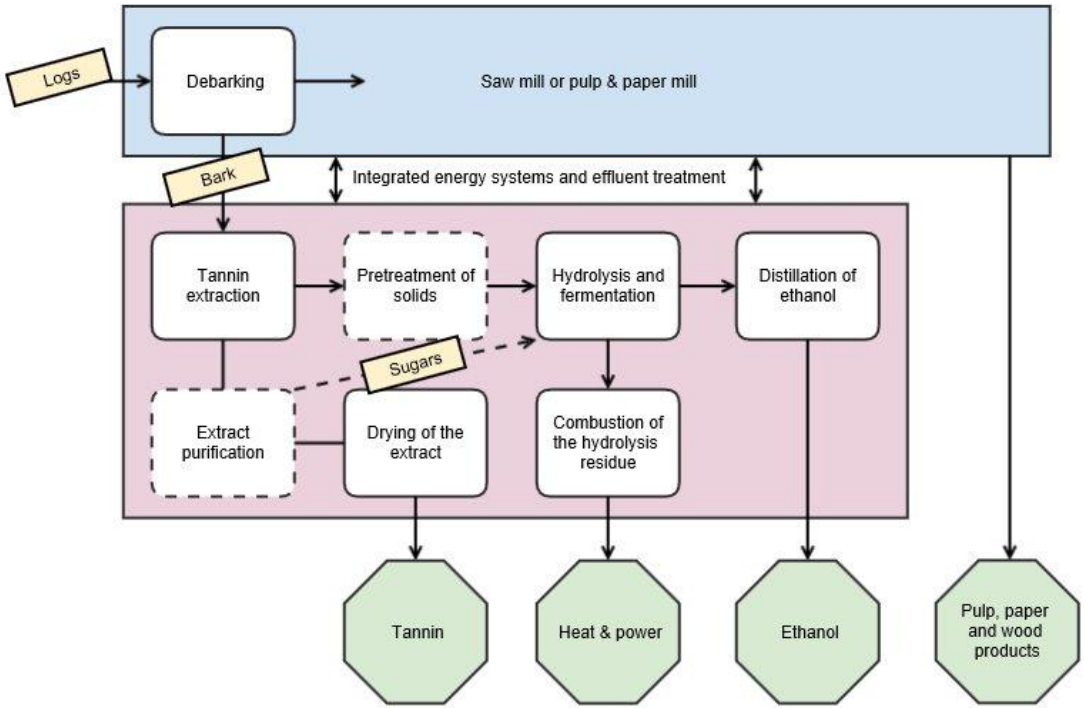

Figure 19. Multiproduct bark biorefinery concept.

4.5.2 FibreEtOH concept

The concept of producing ethanol and biogas from SRF was named “the FibreEtOH concept”. The concept was developed in cooperation between VTT, Pöyry Management Consulting Oy and Skandinavisk Kemiinformation in several projects since 2006. The concept was described in Publication I and some versions of it have been presented in the thesis of Petri Ristola (2012), and conference presentations by Ranta (2010), Sipilä (2012) and the author (Kemppainen *et al.*, 2012). Figure 21 presents an overview of the process concept at a pulp and paper mill site. In the concept SRF is sourced from waste processing facilities located in an urban environment, making the preferred location for the plant near heavily populated areas. The integration of the FibreEtOH concept to a pulp and paper mill was suggested as the preferred solution, but integration of the concept to waste recycling and combustion sites is also an option. A pulp and paper mill using different grades of recycled fibres as a raw material could possibly benefit from co-sourcing of the raw material to both processes, and the fibre sludge produced at the mill could be used as a raw material in the process. In the concept the fibres fractionated from SRF are enzymatically hydrolysed and fermented to ethanol. The results of this thesis showed that a pretreatment step is not necessary in the FibreEtOH concept. An SSF approach with a liquefaction stage is employed directly, and the process from fractionation until fermentation and possibly even to distillation is operated in a continuous mode. Yeast is propagated separately or as a part of the continuous process. Relatively short residence times are chosen rather than targeting over 90% hydrolysis yields. Another key element of the FibreEtOH concept is the use of high consistency conditions during hydrolysis and fermentation. High solids concentration reduces distillation costs and vessel sizes, thus decreasing the capital and operation costs of the plant.

The residual plastic fraction formed in the first fractionation step is combusted in a CHP unit together with other fuels from the pulp and paper processes and possibly with some additional fuels from outside the plant. The plastics could in theory be recycled into new polymer products if a sufficiently pure stream of plastics could be obtained. Biogas production from primarily the C5 sugars in the biomass and excess yeast produced in the process is an integral part of the concept. The biogas production alleviates the additional stress for the effluent treatment of the plant, and produces green electricity that could be sold to the grid. The profitability of bioethanol versus biogas depends significantly on the governmental support for different energy forms through incentives and taxation. In an optimal situation the output of the plant could be shifted between these products depending on the political and market conditions, as is the case in Brazil considering ethanol and sugar production from sugarcane.

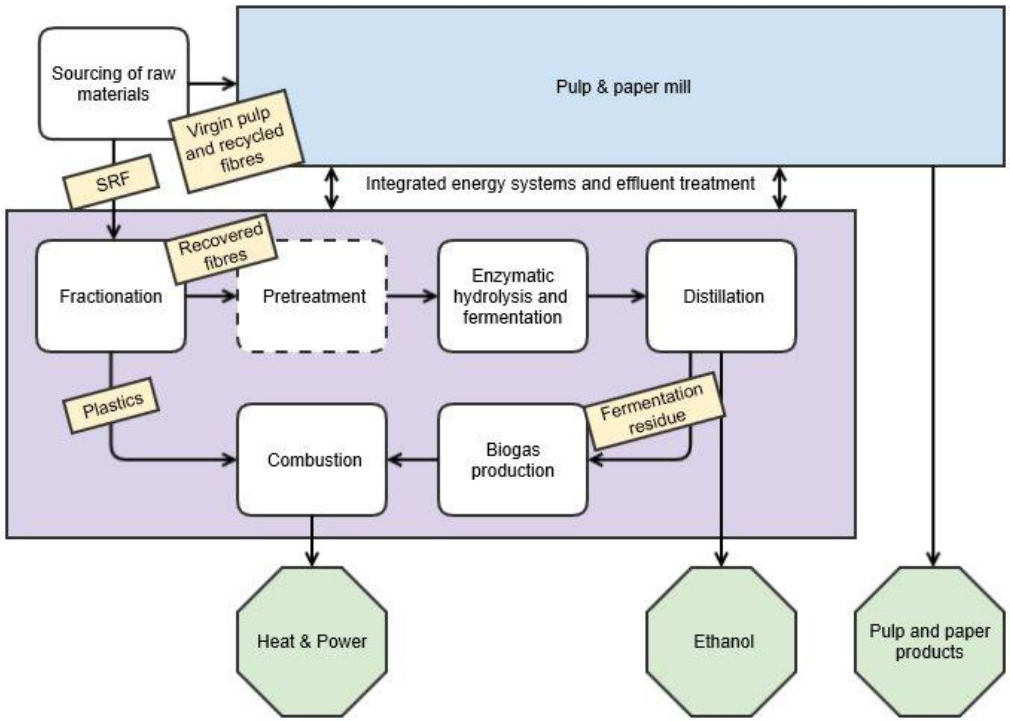

Figure 20. The FibreEtOH concept.

4.5.3 Plant capacity and product yields

A typical large saw mill in Finland consumes 400 000 m^3 logs per year producing ca. 20 000 ton of bark as reviewed in Section 1.4.1. This amount could in theory produce 2100 t/a tannin, which would be about 1% of the current global tannin production. Large quebracho tannin production plants such as the Indunor plant in Argentina have a capacity around 25 000 t/a (Silvateam, 2014), making the envisioned spruce bark tannin production units at sawmills relatively small. Using bark from a large pulp mill could in theory increase the capacity ten-fold, but the raw material would probably be mixed softwood bark from younger trees. It remains to be seen whether this envisioned production capacity at a sawmill can sustain economically feasible production of tannin, but in any case it would be beneficial if the tannin users were on the same site or located nearby.

The theoretical ethanol yield for the spruce bark biorefinery concept is 192 kg from a ton of bark considering 100% hydrolysis and fermentation yields and fermentation of only C6 sugars. As annual capacity this would mean 5 Ml/a ethanol from 20 000 t/a bark, which is about 20 times smaller than the large lignocellulosic ethanol plants being constructed in the US (75-114 Ml/a) and about the same size as the largest first generation ethanol plants operated by St1 Biofuels in Finland (1-7 Ml/a) (Balan *et al.*, 2013; St1 Biofuels, n.d.). It is likely that this capacity would not sustain an economically feasible absolution plant, possibly not even a distillation plant, on a sawmill site. Transportation of the fermentable sugars to a nearby location, or co-location with an ethanol plant also using other raw material sources could be considered as an alternative.

The FibreEtOH concept could in theory produce 261 kg ethanol from the C6 sugars in a ton of recovered fibres. The FibreEtOH project planning capacity was 15 Ml/a ethanol from 170 000 t/a SRF (Sipilä, 2012) making it significantly larger than St1 Biofuels' existing plants in Finland, but several times smaller than the commercial plants currently under construction for the refining of agricultural residues. Biogas in the described FibreEtOH concept would produce ca. 60 GWh/a electricity (Sipilä, 2012). The capacity is ultimately limited by the size and waste generation of the population in the urban area.

5. Conclusions

Two abundant and underutilized biomass streams, namely spruce bark and recovered fibres, were examined in this thesis as biorefinery feedstocks for the production of sugars, ethanol and tannin. The results presented in this work provide new knowledge regarding their composition, response to hot water extraction and steam pretreatment, enzymatic digestibility and fermentation of the enzymatic hydrolysates. Based on the results, two biorefinery concepts were outlined.

Spruce bark was found to contain 11-12% tannin, a valuable biochemical with existing markets and a range of new potential uses. Bark carbohydrates, comprising 48-51% of bark dry matter, were found to be mainly cellulose, pectin, xyloglucan, xylan and arabinogalactan on the basis of the monosaccharide profile of the material and glycome profiling results. The amount of non-cellulosic glucose, present in bark in hemicelluloses, as a part of glycosylated compounds and as free monosaccharides, was distinctively high (8-12% d.m.), and appeared to have seasonal variation.

In hot unpressurized water extraction, up to 21% of bark dry matter could be solubilized in 120 min extraction time. An increase in extraction temperature from 60°C to 90°C increased the extraction yield of tannin and carbohydrates, whereas an increase in solids loading from 5% to 15% of total mass decreased only the yield of tannin. The use of sodium bisulphite and sodium carbonate increased the extraction yield of tannin but not the total solubilisation of bark. The extraction yield of carbohydrates as monosaccharides remained constant in different extraction conditions, but varied between barks collected at different times of the year. The resulting spruce bark extracts were found to contain up to 58% tannin and 22-32% carbohydrates. Different methods employed for analysing the phenolic content of the extract suggested that the extract is a mixture of different tannins and other phenolic compounds with a varying molecular weight and reactivity. It appeared that a selective extraction of only tannins or carbohydrates is not possible using water as the solvent. Instead it is recommended to target at a high overall extraction yield, and to design a purification method that provides an extract of sufficient quality for its proposed end use. On the basis of the results, pectinase, β-glucosidase and hemicellulase enzymes can be used to hydrolyse at least 55% of the carbohydrates in the extract to monosaccharides, which could enable their size-based separation by ultrafiltration from larger tannin molecules.

Steam explosion at 190°C solubilized hemicellulose and pectin and improved the hydrolysis yield of spruce bark carbohydrates from 36% to 75%. Tannin reactions at high temperature and low pH were suggested at least partially to explain why a higher temperature (205°C) or using an acid catalyst during the treatment had a smaller positive effect on hydrolysis yield than pretreatment at 190°C without acid. Hot water extraction removed less pectin and hemicellulose from bark than steam explosion, which hampered the enzymatic digestibility of the material. Hot water extracted bark was found to be less susceptible to enzymatic hydrolysis than steam exploded bark unless a pectinase enzyme was added to the enzyme mixture. The use of a pectinase enzyme increased the hydrolysis yield of hot water extracted bark from 66% to 80%, indicating that hot water extraction alone could be a sufficient bark pretreatment if appropriate enzymes are used in hydrolysis. Furthermore, enzymatic hydrolysates from hot water extracted and steam exploded bark could be fermented to ethanol even at 15% solids loading.

Recovered fibres were fractionated from SRF by repulping, sieving and dewatering. According to the results a steam pretreatment is not recommended for this material as it reduced the hydrolysis yield of the fibre fraction by 10-15%. The fractionation and dewatering yields and the composition of the resulting fibre fraction apparently depended on the type of paper and board products in the SRF, the amount of plastics and other impurities in it, and the scale and efficiency of the fractionation equipment. On the basis of the results, it can be estimated that fibre yields up to 50-55% could be attained from high quality SRF with industrial scale equipment specifically designed for the purpose. Recovered fibres were found to have a high content of carbohydrates composed of C6 sugars (≥ 46%) and a 12-17% ash content. Recovered fibres differ from most other prominent biorefinery feedstocks due to their substantial content of plastics and ash originating from non-biomass sources. The enzymatic digestibility of recovered fibres was found to be high without pretreatment (>82% hydrolysis yield in 24 h) indicating that the non-native ash and plastic impurities did not negatively affect the hydrolytic enzymes. The results indicated that recovered fibres contained galactoglucomannan, which could be solubilized efficiently but was not completely hydrolysed to monosaccharides with the used enzyme mixture containing mannanase activity.

Recovered fibres were found to be efficiently digested to sugars even at high substrate consistency (15-30% dry matter content). Comparison to pretreated straw and pretreated spruce showed that the effect of solids loading on hydrolysis yield was very much substrate specific. The hydrolysis yield of recovered fibres and pretreated spruce at 48 h did not decrease as a function of solids loading, whereas the hydrolysis yield of pretreated straw decreased significantly when solids loading was increased from 5% to 25%. Surprisingly, the hydrolysis yield at 1% consistency was low (< 36%) for all substrates in the mixing system in which half-filled bottles rolled freely in a rotating drum. Surfactants improved the hydrolysis yield of recovered fibres, and the relative hydrolysis improvement was higher at low solids loading. Surfactants interestingly restored the reduced hydrolysis yield observed at low solids loading in the tumbling-type of mixing for recovered fibres and pretreated wheat straw, and the positive effect correlated with an increased

amount of free cellulase activity in the liquid phase in the end of the hydrolysis. The mechanism of the effect of surfactants on hydrolysis was found to be very complex, being clearly dependent on the botanical source of the raw material, pretreatment and lignin content of the raw material, on the solids loading and on the mixing strategy.

Selected steps for processing spruce bark and recovered fibres were scaled up from laboratory scale to a small pilot scale. Up to 22 kg of crude tannin powder was produced from spruce bark representing a 9% yield from dry bark. The obtained lower yield compared to the laboratory-scale experiments was probably due to a lower efficiency of mass transfer during extraction, a higher amount of wood in the raw material, and vessel losses and spills occurring during the pilot experiments. It is estimated that the final product yield could be 12-13% in industrial systems, designed specifically for the extraction of spruce bark. The liquefaction and SSF of recovered fibres were scaled-up and continuous pilot-scale trials were carried out in order to demonstrate the technical feasibility of the process. Promising results were obtained at up to 30% dry matter content showing fast hydrolysis during a 6-8 h liquefaction stage (43-49% hydrolysis yield) and efficient fermentation (84% of the theoretical yield from released hexoses) while keeping microbial contamination under control. The highest ethanol concentration reached in steady state was 40 g/kg, corresponding to 171 L ethanol yield per ton of recovered fibres.

5.1 Common denominators and future prospects

Two biorefinery concepts were outlined on the basis of experimental results: a bark biorefinery concept and “the FibreEtOH concept”. The work carried out in this thesis indicates that both concepts have technical potential for commercialization in industrial scale. In addition, it appears that both concepts may have financial potential to succeed due to several supporting factors. Both raw material streams are available year-round and their collection is already organized, unlike that of many agricultural side-streams. Both concepts have synergy with the traditional forest industry in the areas of raw material supply, process design and product selection. Examples include using the pulp mill surplus energy for ethanol distillation, using an existing bark press for dewatering the barks after extraction, and using the tannin in wood adhesives needed on-site for manufacturing wood products. According to the results the pretreatment step can be omitted from both process concepts, which simplifies the processes and gives the concepts a major economical advantage. Both concepts can be started as streamlined versions with one or two fuel and chemical products, but could develop into multiproduct biorefineries given the time and a suitable market situation.

Several crucial further research targets can be identified for the process concepts. The bark biorefinery concept is dependent on finding a valuable use for spruce tannin. Application tests with crude tannin and, if necessary, the development of sustainable and economically feasible purification methods should be in

the focus of future research. Better understanding of seasonal variation in bark composition and its effect on the extract composition, clarification of the interaction of phenolic compounds with carbohydrates, and the characterization and valorisation of spruce bark lignin could be additional research targets. Using hot water extracted bark or bark hydrolysates as a secondary feedstock in a lignocellulosic ethanol process should be investigated since this is a possible option for the utilization of bark sugars. The FibreEtOH concept, on the other hand, would benefit from a wider comparison of the composition of recovered fibres fractionated from SRF obtained from several countries. The potential of producing chemicals instead of ethanol should be investigated and the effects of the impurities on different types of product recovery processes assessed. Improving the enzyme mixtures by finding new beneficial enzyme activities and optimizing the recipe composition would benefit both process concepts. The effects of solids loading and surfactants on the progress of enzymatic hydrolysis should be investigated in more detail in order to understand the dependence of their effect on substrate composition, pretreatment and mixing type, and their influence on enzyme adsorption and desorption.

The forest biorefinery concepts presented in this thesis pave the way for forest industry renewal through better valorisation of side and waste streams and by opening routes to new market segments. The concepts help to reduce global dependence on fossil oil and point society towards a bioeconomy in which renewable resources are used to produce food, energy, products and services.

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PUBLICATION I

Ethanol and biogas production from waste fibre and fibre sludge – The FibreEtOH concept

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Ethanol and biogas production from waste fibre and fibre sludge – The FibreEtOH concept

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ABSTRACT

The FibreEtOH concept was developed to tackle major challenges in the production of ethanol from lignocellulosics. The two feedstocks, waste fibre fractionated from solid recovered fuel, and pulp and paper mill fibre sludge, provide all-year-round supply of biomass with high hexose content (44–56%) and acceptable ash content (13–14%). They can be liquefied and hydrolysed by enzymes rapidly without a thermal or acidic pre-treatment, although they contain some recalcitrant mannose- and galactose-containing polysaccharides that require additional helper enzymes for complete hydrolysis to monosaccharides. Fractionation of solid recovered fuel, continuous liquefaction, and simultaneous saccharification and fermentation to ethanol, as well as biogas production from the fermented residue were demonstrated in pilot-scale with good results. Total yield consisting of C6 sugar hydrolysis yield (57%) and fermentation yield (84%) was 48% after only 6 h continuous liquefaction and 21 h fermentation. Average biogas production rate was 655 $dm^3\ kg^{-1}$ for fermentation residue from waste fibre and 400 $dm^3\ kg^{-1}$ from fibre sludge with methane content of 69–75%. Based on other results a hydrolysis yield of 75% is reachable within the process concept if the residence time in fermentation is extended. In this scenario 1000 kg of dry feedstock would produce 170 kg ethanol, 310 kg biogas, 360 kg waste sludge and 170 kg CO_2.

1. Introduction

The demand for road transport biofuels is growing rapidly [1]. The current global market is dominated by ethanol, which is predominantly produced from either sucrose or starch – both key raw materials in several food industry value chains. The R&D community, on the other hand, is vigorously trying to develop improved biofuels or more sustainable ways to produce the biofuels already on the market. In respect to ethanol, various non-edible, cellulose-rich raw materials,

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so-called lignocellulosics, have been the main focus of several new concepts. The lignocellulosic raw materials most interesting in terms of economic and environmental sustainability include residues of sugar cane, sugar beet, corn, wood and grain processing, or alternatively energy crops as well as industrial and municipal wastes. In Europe, lignocellulosic ethanol is already today produced commercially using the side-stream of sulphite pulp mills as the raw material (e.g. Domsjö Fabriker in Sweden and Borregaard in Norway).

In the current European biofuels policy framework, new biofuels products should meet the minimum target of greenhouse gas reduction in the production value chain. A threshold value of 35% is valid for current units and for new production plants the threshold is 60% reduction starting in 2017. The lignocellulosic materials from waste streams are attractive sources; for business opportunities the key question will remain on raw material availability and the price of the raw material.

Full commercial deployment of lignocellulosic ethanol is, however, still facing several major constraints [2]. The challenges relate both to the raw material logistics in general as to the actual conversion process. The FibreEtOH concept was developed to tackle most of these challenges (Fig. 1). The concept is based on the utilisation of a side-stream of a pulp and paper integrate (fibre sludge) and waste fibre separated from solid recovered fuel (SRF) and thus, all-year-round supply of inexpensive raw material would be ensured. Moreover, all technologies involved in the production chain except enzymatic hydrolysis, are available on commercial scale. On the other hand, process microbiology, in general, is an additional challenge when processing waste materials.

FibreEtOH is one of the four consortium projects that received funding under the 7th Framework Programme of the European Commission, when the large demonstration projects were selected in 2009. The FibreEtOH project is coordinated by UPM-Kymmene Oyj, Finland, a major global player in the pulp and paper industry. The other partners include VTT Technical Research Centre of Finland (R&D), Pöyry Management Consulting Oy, Finland (process design), Skandinavisk Kemiinformation AB, Sweden (process design), Roal Oy, Finland and AB Enzymes GmbH, Germany (industrial enzyme production and sales), Lassila & Tikanoja Oyj, Finland (supplier of SRF), and St1 Oy, Finland (distributor of fuel ethanol).

The FibreEtOH process consists of raw material handling including the fractionation of SRF, liquefaction of the feedstock, simultaneous saccharification and fermentation (SSF), distillation and absolution of ethanol, and biogas production from the fermented residue. It can be highly integrated to other commercial processes resulting in sound end-uses of all main fractions of the raw material. The process takes advantage of the easy hydrolysability of waste fibres without a thermal or chemical pre-treatment, which is a major capital cost factor in most lignocellulosic ethanol production processes [3]. The advantage of using thermophilic enzymes is also employed. Prehydrolysis with thermostable enzymes can be carried out at higher temperatures, which can increase enzymatic activity, decrease the viscosity of the slurry enabling easier mixing, pumping and working in higher consistency, i.e. lower water to dry matter ratio [4]. Instead of having a residence time of four to six days in fermentation, the process takes a 'quick & dirty' approach hydrolysing and fermenting the easy part of the polysaccharides, approximately 75%, in only two to three days. Complete conversion is not a necessity because the rest of carbohydrates are utilized efficiently in biogas production, which is an important part of the concept from an economical point of view.

In the Helsinki metropolitan area there is a population of 1 million inhabitants. The amount of commercial and industrial waste after source separation to material recovery is about 450 000 t a^{-1}. In the project, the amount of SRF of 200–300 000 t a^{-1} has been estimated to be available for advanced energy applications. Additional raw fibre may be

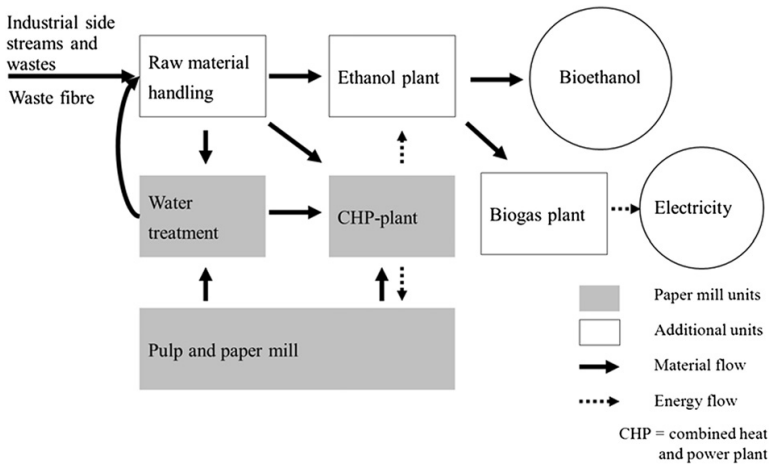

Fig. 1 – The FibreEtOH concept consists of raw material handling including the fractionation of SRF, liquefaction and fermentation of the feedstocks, distillation and absolution of ethanol, biogas and electricity production from the fermented residue, and a shared water treatment and a combined heat and power (CHP) plant with a pulp and paper mill.

available when the ethanol production facility is integrated to pulp and paper industry operations. In addition to fibre sludge, waste fibre fractions from pulp or paper recycling could also be utilized.

Extensive research has been carried out from laboratory scale to pilot-scale and from enzyme development to process design optimization during the past three years. In this paper, R&D results from the FibreEtOH project from all the main areas of the successful bioethanol production concept are presented. The composition of the feedstocks is described in detail and the lack of need for thermal or acidic pre-treatment is demonstrated. High consistency liquefaction is discussed separately before reporting results from a continuous pilot-scale bioethanol production campaign. Finally biogas production from the fermented residue is demonstrated.

2. Material and methods

2.1. Materials

2.1.1. Feedstocks

Two lignocellulosic feedstocks were used in the process: waste fibre and fibre sludge. Waste fibre was fractionated from solid recovered fuel (SRF) provided by Lassila & Tikanoja from their Turku and Kerava facilities. Most plastics, sand, and other contaminants were removed from SRF by screening after repulping the material with water. Material for the pre-treatment experiments was dried on a board machine to 95% mass fraction of dry matter (referred to as dry matter content in this paper). Material for high consistency liquefaction studies and pilot-scale continuous campaigns was dried with a filter press to ca. 50% dry matter content after a 60 min sanitation period at 95 °C. This dried product is called waste fibre. Fibre sludge was obtained from UPM Kaukas pulp and paper mill in 2–3% dry matter content and dried to 40–50% dry matter content using a filter press. Material for pilot campaigns was also produced by mixing 75% waste fibre and 25% fibre sludge (dry matter basis) before drying.

2.1.2. Enzymes

Enzymes used in the experiments have been provided by Roal Oy. The effect of pre-treatment to the hydrolysability of waste fibre was evaluated with commercial Econase CEP enzyme product (10 filter paper units (FPU) g^{-1} dry matter) and a pre-commercial β-glucosidase product from *Acremonium thermophilum* (1000 nkat g^{-1}). A pre-commercial thermostable enzyme mixture was used in the high consistency liquefaction study and pilot-scale bioethanol production with cellobiohydrolase I (CBH I) from *A. thermophilum*, cellobiohydrolase II (CBH II) from *A. thermophilum*, an endoglucanase component from *Thermoascus aurantiacus*, xylanase from *Nonomuraea flexuosa*, mannanase from *Trichoderma reesei* and β-glucosidase from *A. thermophilum*. Enzymes were dosed based on the FPU activity of the mixture. FPU activity was analysed according to Ghose [5] and β-glucosidase activity according to Bailey and Nevalainen [6].

2.1.3. Yeast

The yeast strain used in fermentation was commercial Red Star yeast (Le Saffre). Yeast inoculum was cultivated before every piloting campaign in the previous week in aerobic conditions to maximise the growth of yeast. The yeast was cultivated on a glucose-based medium (70 g dm^{-3}) with peptone (20 g dm^{-3}) and yeast extract (10 g dm^{-3}), from shake flask scale up to 1200 dm^3 via 28 dm^3 and if necessary 200 dm^3 seed fermenter stages. The yeast culture was separated by continuous centrifugation (BTPX-205 SGD, Alfa-Laval) and the concentrated yeast cream was stored in 4 °C until needed during process operation in the following week.

2.2. Feedstock composition analysis

Carbohydrate compositions of the dried feedstocks were analysed by high performance liquid chromatography (HPLC) after air drying, grinding and acid hydrolysis by 70% sulphuric acid to monosaccharides [7,8]. Ash content was analysed from wet samples in a muffle oven by heating samples stepwise first into temperature 103 °C for 7 h and then to 550 °C for 16 h to ash the samples. Ashed samples were cooled and the residues weighed. To analyse the content of extractives and lignin, air dried samples (1 g) were extracted with heptane in a soxhlet extraction system. The heptane extract was dried and the weight of the residue was measured as the gravimetric extractive content. The lignin content was analysed from extracted samples after acid hydrolysis with 70% sulphuric acid. Klason lignin was obtained by weighing the acid-insoluble residue after drying, and soluble lignin was obtained by analysing the UV absorbance of the liquid with wavelength of 203 nm and using absorptivity constant of 128 $dm^3\ g^{-1}\ cm^{-1}$ to estimate lignin concentration. It must be noted that other acid-insoluble substances in the materials such as plastics showed up as Klason lignin in the analysis.

2.3. Pretreatment experiments

The pilot-scale pre-treatment experiments were carried out for waste fibre in a 400 dm^3 multipurpose reactor (Jaro Oy, Finland) at VTT Rajamäki pilot plant. The pH of the pulped and dried waste fibre was adjusted to 5 or to 2 with H_2SO_4, after which the pulps were pretreated in conditions presented in Table 1 with saturated steam. After 5–10 min residence time the pressure was released by "exploding" the pulp to an unpressurized reactor. Batches 4 and 6 were continued from batches 3 and 5 by heating the material again after cooling and sampling.

Table 1 – Batch parameters in the pre-treatment experiments with waste fibre.

Batch	pH	T (°C)	Waste fibre concentration (g dm^{-3})
1	5	120	10–30
2	5	160	10–30
3	5	200	10–30
4	5	200 + 200	10–30
5	2	160	60
6	2	160 + 180	60

2.4. Laboratory scale hydrolysis experiments

Hydrolysis experiments were performed in Schott bottles in 50 ml volume in 100 g kg^{-1} substrate concentration with magnetic mixing. Temperature was adjusted to 45 °C with a water bath, and pH was adjusted to 5 with 100 mol m^{-3} sodium acetate buffer containing 0.02% sodium azide to prevent microbial activity during hydrolysis. Hydrolysis yield was analysed by stopping the reaction by boiling, removing solids by centrifuging, and analysing the reducing sugars in the supernatant by 3,5-dinitrosalicylic acid (DNS) assay [5].

A Hobart pulper was used for liquefaction studies performed in high consistency. It contains a metal vessel with a heat jacket and a lid, and a flat-beater type mixing element. Reaction volume was 1.2 dm^3 and substrate concentration at the start of the reaction was 300 g kg^{-1}. pH was adjusted to 5 with phosphoric acid and temperature was adjusted to 50 °C. Substrate concentration increased from 300 g kg^{-1} to 325 g kg^{-1} during the reaction due to evaporation. Thus dry matter content of the material was routinely measured and taken into account in yield calculations. Due to the high consistency used in the experiments, DNS analysis was performed from 10-fold diluted samples (triplicates) to diminish the solids effect caused by a large share of insoluble solids [9].

Prediluted hydrolysis samples were analysed by HPLC for their total neutral monosaccharide content according to Tenkanen and Siika-aho [8]. A secondary acid hydrolysis with 70% sulphuric acid (1 part acid, 20 parts sample) was carried out for supernatant samples in an autoclave in 120 °C for 1 h. After secondary acid hydrolysis dissolved oligosaccharides could be quantified as monosaccharides from supernatants with HPLC. An oligosaccharide analysis was performed with HPLC for some samples according to Tenkanen et al. [10].

2.5. Pilot-scale continuous experiments

2.5.1. Liquefaction and SSF

Continuous liquefaction and SSF were carried out for 86 h in a pilot-scale system presented in Fig. 2. The dried waste fibre in ca. 50% dry matter content was fed continuously into the process through a screw lift. The material had a high bridging tendency and regular attention was needed in order to ensure continuous feed to the system. In addition to the sanitation conducted before drying, a short steam sanitation was demonstrated by leading steam to the screw lift from a connection above the feed opening. The temperature of the material in the end of the screw was 70–80 °C and the residence time in the screw was approximately 10 min.

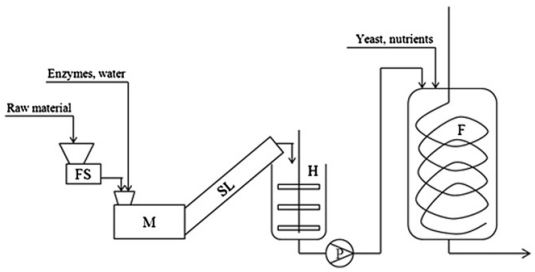

Fig. 2 – The layout of the liquefaction and SSF pilot process. SL = screw lift, H = liquefaction reactor, P = hose pump, F = fermenter.

The liquefaction of the material was carried out in a 100 dm^3 stirred-tank reactor with residence time of 6 h. Counter-current 4-impeller mixing elements were used with a 1.5 kW motor. Enzymes and water were pumped in continuously and biocide sodium bisulfite was dosed to the water feed to give 0.3 g dm^{-3} concentration in the process. Liquefaction pH was adjusted to 5 by adding 80% phosphoric acid to the vessel when needed and temperature was adjusted to 50 °C. Total flow through the system was 14 kg h^{-1} and total solids concentration at start was 300 g kg^{-1}. The liquefied material was pumped to the fermenter with Bredel SPX32 hose pump, which was able to pump very thick paste-like materials without much trouble. Because of the large size of the pumps, the pumping was pulsed with and electric timer (for example 10 s ON, 3 min OFF) to achieve correct average flow.

The fermenter used was a 300 dm^3 vessel with a top-down spiral-type of mixing element with online pH and temperature measurements. Residence time in the fermenter was 21 h, pH was adjusted to 4.5, and temperature to 35 °C. The process was started with 70 dm^3 water and 3 g dm^{-3} yeast in the fermenter. Yeast slurry was pumped in continuously with the feed to obtain 3 g dm^{-3} yeast concentration. Yeast extract (0.5 g dm^{-3}) was added to the fermenter continuously to ensure sufficient trace elements for the yeast. No other additional nitrogen source was used. Antifoam agent (Struktol J633, Schill & Seilacher, Germany) was used when needed. Nitrogen gas was fed to the fermenter headspace to ensure anaerobic conditions.

Samples were taken every 4 h and pre-diluted before analysis because of the high solids content. Metabolite concentrations were determined by HPLC using Aminex HPX-87H column (BioRad Laboratories, USA) with 2.5 mol m^{-3} H_2SO_4 as eluent and flow rate 0.3 ml min^{-1}. The column was maintained at 55 °C. Peaks were detected using a Waters 410 differential refractometer and a Waters 2487 dual wavelength UV (210 nm) detector.

2.5.2. Biogas

Continuous biogas pilot-scale experiments were conducted in a 300 dm^3 reactor at 35 °C. Mixing propeller was timed for sequential low speed mixing. Feeding of the evaporated residue from a waste fibre and fibre sludge pilot-scale fermentations was done manually once or twice a day depending on the amount of the feed. Disposal of the material was done from the bottom of the reactor through overflow principle. Biogas was collected from the upper part of the reactor and routed via gas volume meter.

3. Results & discussion

3.1. Composition of the feedstocks

Feedstock carbohydrate composition sets the limits for the obtainable yield as litres of ethanol per kg of dry feedstock. In the case of feedstocks originating from pulp and paper industry, the ash content and composition is also a significant

factor affecting the hydrolysability and processability of the material as well as acid consumption in the process. Several batches of both feedstocks were prepared over the period of 2 years and their carbohydrate composition was analysed to determine theoretical ethanol yields in the process. The SRF bales arriving from Lassila & Tikanoja were visibly different from each others in terms of their content, and it was clear that the heterogeneity of the materials would have to be accounted for in process design and yield calculations. Table 2 presents the range in the monosaccharide composition analysis results from several measurements. Waste fibre had a higher glucan and xylan content than fibre sludge, while the latter contained more galactomannan type of polysaccharides. The mixture of 25% fibre sludge and 75% waste fibre was evaluated to correspond to a mixture of 35% birch kraft pulp, 34% spruce kraft pulp and 30% spruce thermomechanical pulp based on its monosaccharide profile.

Both feedstocks contained ash originating mostly from paper filling and coating materials as well as extractives and lignin (Table 3). Main components of the ash were determined as $CaCO_3$, kaolin and talc. Ash content in UPM Kaukas mill sludge was lower than in many other reported paper mill sludges [11]. Acid-insoluble material, i.e. Klason lignin, showed all acid-insoluble components in the feedstocks including plastics, which are left as impurities in waste fibre after pulping. Fibre sludge does not contain plastics and so the acid-insoluble material presumably represents Klason lignin.

The glucan content of waste fibre was higher than for example that of corn stover, sugar cane bagasse, wheat straw and switchgrass [12] making it a very promising feedstock for bioethanol production. Because of the high C6 sugar content, the fermentation of C5 sugars is not needed to achieve high ethanol yield per ton of dry matter (theoretical maximum is 224–286 kg t^{-1}). The high ash content has been found problematic in bioethanol production from paper sludge because $CaCO_3$ contained in the ash interferes with pH control demanding high amounts of acids to achieve pH suitable to enzymes [13]. However in these feedstocks, the ash level was rather low compared to waste celluloses in general and although buffering capacity of the material was notable, the acid consumption did not become excessive in the process.

3.2. *The effect of a thermal and acidic pre-treatment to hydrolysis yield*

The target for a pre-treatment of biomass is to improve the rate of hydrolysis and the yield of fermentable sugars by altering or removing structural and compositional impediments to hydrolysis. Liquid hot water treatment with or without pH control is a major class among the pre-treatment technologies reported in literature, often combined with steam explosion [14]. The effect of a thermal and acidic pretreatment to the hydrolysability of waste fibre was studied in pilot-scale. Experiments were run with temperatures from 120 to 200 °C in pH 5 or in pH 2, and the obtained materials were subjected to enzymatic hydrolysis with 10 FPU g^{-1} Econase CEP and 1000 nkat g^{-1} β-glucosidase. Treatments with temperature of 160° and higher visually changed the material making it significantly darker and the rate of sedimentation of the material appeared to increase. According to the results presented in Fig. 3 the thermal and acidic pretreatments did not improve hydrolysis yield but rather decreased the hydrolysis rate especially in the beginning of the hydrolysis. The results were confirmed by HPLC analysis of the released monosaccharides and in another experiment in lower substrate concentration (10 g kg^{-1}, data not shown). According to the results the material appeared to contain a major part of the carbohydrates in easily hydrolysable form (e.g. fibre derived from chemical pulping methods), and a minor part in resistant form (e.g. mechanical pulp, wood residues). The results also suggest that the feedstocks contain components such as lignin that re-distribute within the material as a result of high-temperature pre-treatment and consequently may cover and mask partially the cellulose, make the access of cellulase enzymes to cellulose difficult, and slow down hydrolysis.

Laboratory scale hydrolysis of fibre sludge with commercial cellulases (10 FPU g^{-1}) produced hydrolysis yield over 80% in 48 h without any pre-treatment to the material (data not shown). Thus it can be concluded that both feedstocks are readily available to enzymes as such. This result is in line with results reported in the literature. For example Lin et al. [15], and Lynd et al. [11] have obtained high hydrolysis yields with waste fibre and fibre sludge type of biomasses without any pre-treatment. On the other hand Yamashita et al. [16] found that phosphoric acid treatment alone and combined with ball milling improved the hydrolysability of a batch of paper sludge, which hydrolysed very poorly without any pre-treatment. They concluded that chemical swelling through acidic treatment enhanced the enzymatic saccharification, which supports the conclusion that the high temperature was the detrimental factor reducing the hydrolysability of waste fibre in these experiments.

3.3. *Liquefaction in high consistency*

In order to produce a high ethanol concentration in the broth and thus minimise tank size and the energy consumption in

Table 2 – Composition of carbohydrates from waste fibre and fibre sludge after acid hydrolysis and their combination containing 75% waste fibre and 25% fibre sludge. The values represent the fraction (%) of individual monosaccharides per dry matter released by analytical acid hydrolysis.

Raw material	Glc	Xyl	Man	Gal	Ara	Rha	Monosaccharides	Hexoses
Waste fibre	43.7–50.5	9.6–11.2	2.7–4.3	0.2–0.6	0.2–0.4	0	57.5–65.3	46.1–55.4
Fibre sludge	38.3–40.8	7.1–9.7	4.4–4.7	1.2	0.7	0.2	51.9–57.0	44.1–46.6
Combination	43.5–51.2	7.9–10.1	3.5–4.9	0.5–0.9	0.4–0.5	0	57.8–64.6	47.5–56.1

Table 3 – Ash, extractives, and lignin content of waste fibre and fibre sludge, and their combination containing 75% waste fibre and 25% fibre sludge used in piloting as a fraction (%) of dry matter.

Sample	Ash (%)	Extractives (%)	Acid insoluble material (%)	Acid soluble lignin (%)
Waste fibre	13.3	6.8	19.4	0.5
Fibre sludge	13.7	3.1	30.4	1.4
Combination	13.8	5.7	23.7	0.4

distillation, a high consistency process (>150 g kg^{-1} substrate concentration) is required in the production of ethanol from lignocellulose [17]. Prehydrolysis is an effective way to decrease the viscosity of high consistency biomass to enable easier mixing in further process stages [4].

Liquefaction of waste fibre in high consistency was studied with a table-top Hobart pulper, which produces very efficient mixing already from the beginning of a batch hydrolysis. A mixture of pre-commercial thermostable enzymes was used with 8 FPU g^{-1} dosing and temperature was adjusted to 50 °C. Relatively high hydrolysis yield of 40% from theoretical analysed as reducing sugars was obtained after 8–10 h (Fig. 4). Dissolved monosaccharides were analysed from hydrolysates before and after secondary acid hydrolysis to assess the hydrolysis yield as mono- and dissolved oligosaccharides.

The results show that hydrolysis yield to monosaccharides was lower than hydrolysis yield to mono- and oligosaccharides throughout the reaction time. This indicates that high end-product concentration in the reactor during the reaction (15–50 g dm^{-3} glucose, 17–55 g dm^{-3} total monosaccharides) inhibited the enzymes catalysing the last steps in the chain of reactions needed to hydrolyse polysaccharides to monosaccharides. Literature supports this conclusion as β-glucosidases from *Aspergillus niger* and *T. reesei* have been reported to lose 85% of their activity in 30 g dm^{-3} glucose [18]. The results also suggest that not only does DNS analysis respond to the reducing ends of oligosaccharides in the sample, but it also appears to degrade them to monosaccharides during the analysis. According to this, the analysis of reducing sugars in high consistency hydrolysis is a measure of the conversion of the substrate rather than a measure of the yield of the monosaccharide end-products.

Analysis of the oligosaccharides by HPLC showed concentrations of 5–9 g kg^{-1} cellobiose, 7–11 g kg^{-1} xylobiose, and 1 g kg^{-1} mannobiose in the reactor during the liquefaction. Oligosaccharides corresponding to standards with 5 and 6 xylose units, and 3 and 5 mannose units were also detected by HPLC. Monosaccharide analysis before and after secondary acid hydrolysis showed that after 10 h hydrolysis especially galactose, xylose and mannose remained bound to solubilised oligosaccharides whereas arabinans and glucans were hydrolysed more efficiently to monosaccharides (Fig. 5). Xylan solubilised most easily: as much as 33% of xylan had solubilised already after 30 min hydrolysis (data not shown).

Although there are studies available on the hydrolysis of biomass in high consistency conditions [17,19], very few report anything about the hydrolysis yield during the first 10 h of the reaction. Nevertheless, one very promising process option in ethanol production from lignocellulose is to conduct a short liquefaction prior to SSF to reduce viscosity of the material and to take advantage of the higher enzymatic reaction rate in elevated temperatures. This makes the phenomena during this period industrially relevant. Based on these results it can be concluded that waste fibre liquefies and hydrolyses very quickly in high consistency conditions, but end-product inhibition appears to be a significant factor affecting the hydrolysis rate to monosaccharides. Thus a short liquefaction stage followed by SSF is the preferred process choice as the hydrolysis rate reduces significantly when monosaccharide concentration increases.

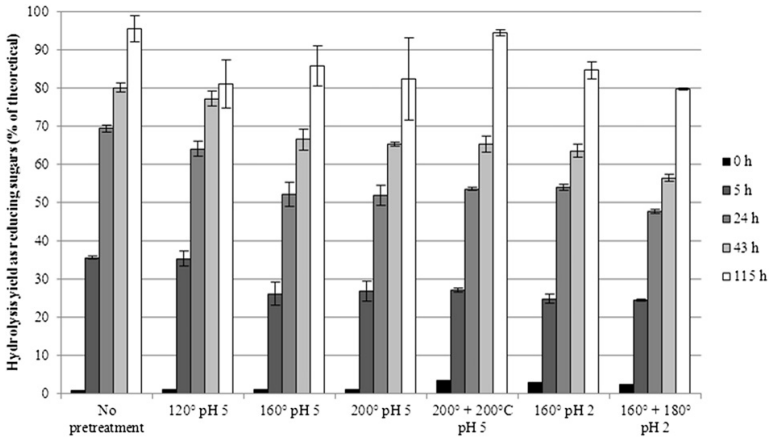

Fig. 3 – The effect of thermal and acidic pre-treatment to hydrolysis yield of waste fibre with 10 FPU g^{-1} cellulase and 1000 nkat g^{-1} β-glucosidase in 100 g kg^{-1} substrate concentration, 100 mol m^{-3} sodium acetate buffer pH 5 at 45 °C. Error bars show the standard deviation of triplicate samples.

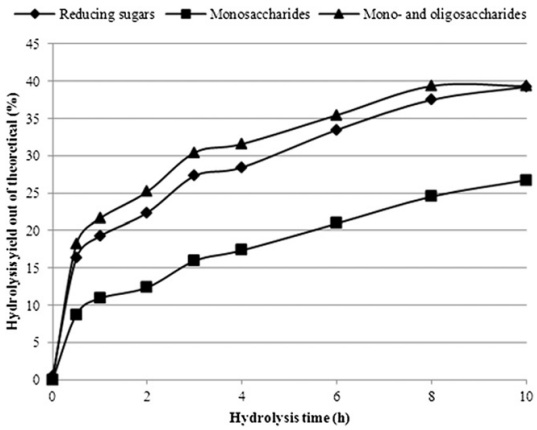

Fig. 4 – Hydrolysis yield as reducing sugars, monosaccharides (HPLC analysis), and mono- and oligosaccharides (HPLC analysis after secondary acid hydrolysis of the supernatant) in liquefaction of waste fibre with 8 FPU g^{-1} in 300 g kg^{-1} substrate concentration, 50 °C and pH 5.

According to the results obtained here, waste fibre contains mannans and galactans, which solubilise with the enzyme mixture containing cellulases, xylanases, and mannanases forming recalcitrant oligosaccharides, which require additional helper enzymes for hydrolysis to fermentable monomers. Rättö et al. [20] hydrolysed isolated galactoglucomannan from pine kraft pulp and found that whereas the hydrolysis yield was high (85%), only 3% of the hydrolysis products were mannose and the rest were mainly mannobiose (35%), mannotriose and larger unidentified oligosaccharides. Adding α-galactose and β-glucosidase only slightly improved hydrolysis to mannose (6%). β-mannosidase could prove beneficial and should be studied to obtain full hydrolysis of these recalcitrant polysaccharides from waste fibre.

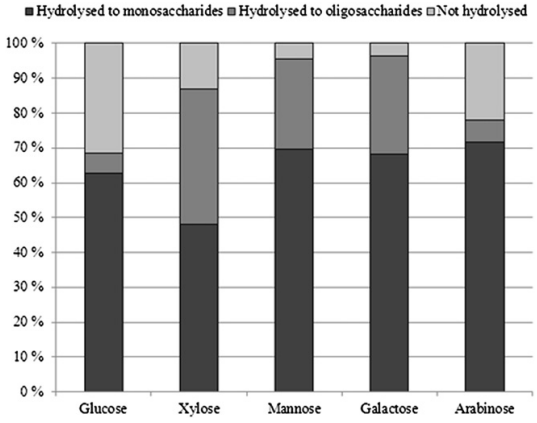

Fig. 5 – Hydrolysis yield of monosaccharides after 10 h liquefaction of waste fibre with 8 FPU g^{-1} in 300 g kg^{-1} substrate concentration, 50 °C and pH 5.

3.4. *Continuous pilot-scale liquefaction and SSF*

Although continuous processing could be an important factor in reducing the costs of producing ethanol from lignocellulose, the studies reporting results from continuous SSF runs are scarce. The advantages of continuous processing in ethanol production include higher volumetric productivity, reduced labour costs, reduced vessel down time for cleaning and filling, and the adaptation of the fermentative organism to inhibitors in the biomass [21]. Continuous processing differs from batch processing in many significant ways but especially high consistency continuous-type of experiments are difficult if not impossible to conduct in laboratory scale. The important hands-on experience on dealing with possible problems for example in raw material handling and microbial contamination can be achieved only by running continuous pilot campaigns. However, the size of the pilot-scale equipment at this stage of the process development does not have to be very large. Smaller volumes and flows ensure easy handling and give flexibility during the campaign but still give good indication of the obtainable yields and functional process parameters.

Several 1–2 week long continuous pilot-scale campaigns have been run over the past two years to verify laboratory results in larger scale experiments. Continuous campaigns were run at VTT Otaniemi bioprocess pilot plant consisting of liquefaction and SSF stages. Total flow through the system varied from 10 to 20 kg h^{-1} containing 250–300 g kg^{-1} solid substrate at the start. The object for the liquefaction stage (also called prehydrolysis) was to achieve rapid initial hydrolysis at a temperature optimal for the enzymes. However, because end-product inhibition reduced the hydrolysis rate significantly in high consistency conditions, the material was transferred to the fermenter almost as soon as it was transformed into pumpable form. In continuous processing mode this meant 6–8 h residence times in the liquefaction reactor. A regular stirred-tank reactor was used because of its simplicity and relatively low cost. Counter-current impellers ensured material flow in three dimensions. Hose pumps were chosen as the pump type because of the high viscosity of the material, and because they are little affected by sand, plastics, bark, or other impurities in the material. Enzyme dosage varied from 8 to 20 FPU g^{-1} in the campaigns.

The main results of one continuous campaign with waste fibre as feedstock are presented and discussed below. Fig. 6 presents the concentration of substrates and metabolites in the fermenter during the campaign. Concentration is presented as g kg^{-1} because of the weight-based predilution before analysis. The material fed to fermentation had a degree of hydrolysis of 43–49% as reducing sugars. Hydrolysis was slightly higher than what Larsen et al. [22] report as the hydrolysis yield of wheat straw in high consistency after 6 h liquefaction in a pilot-scale free-fall mixing system (30–40%). Hydrolysis yield to glucose was lower because of the high end-product inhibition, approximately 28–36%. The campaign was successfully run in continuous mode for 86 h except between 55 and 61 h when the mixer motor in the liquefaction reactor

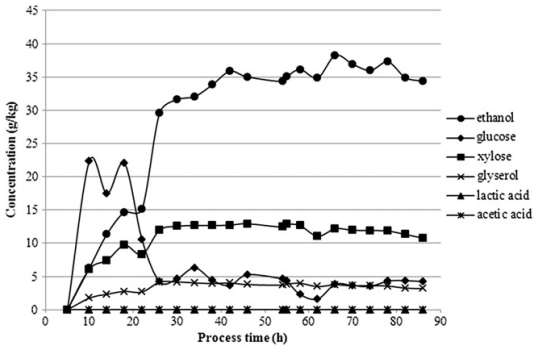

Fig. 6 – The main substrate and metabolite concentrations during a continuous SSF process with waste fibre after continuous 6 h liquefaction in 300 g kg^{-1} substrate concentration.

broke down and had to be changed. During that time no feed went in or came out of the fermenter, which can be seen as a reduction in the glucose concentration in the fermenter.

Fermentation residence time was kept at only 21 h to achieve reliable data from a continuous 5-day campaign and thus the total yield remained rather low. Because of the short residence time, some glucose passed through the reactor unused. This was prevented in later campaigns by dividing the residence time in SSF to two reactors. No lactic acid was produced during the campaign indicating good microbial control throughout the process. Glycerol production was 9–11% of ethanol production while no acetate was detected (detection limit 2 g kg^{-1}). Ethanol productivity was 1.8 g dm^{-3} h^{-1} at its highest. Hydrolysis yield of fermentable hexoses from polysaccharides during the stable face of the campaign was approximately 57% showing that hydrolysis continued further in SSF conditions. Ethanol yield from the hydrolysed hexoses was 84% and thus total yield was 48%. Maas et al. [23] obtained the same glucan-to-ethanol yield (48%) in a pilot-scale batch prehydrolysis and SSF of lime-treated wheat straw in over two times longer fermentation (48 h). This indicates that although final yield here was low, the ethanol productivity rate was rather high, which indicates that a generally shorter fermentation time of 50–70 h is required in this process concept compared to most other proposed process concepts. Good fermentation performance in high dry matter conditions also indicates the low toxicity of the raw materials to the yeast.

Another continuous campaign was carried out using raw material combined from waste fibre (75%) and fibre sludge (25%) (detailed results not shown). The hydrolysis and ethanol yields were on a similar level as with 100% waste fibre. Small pieces of bark and wood sticks originating from the fibre sludge were prone to get stuck in the narrow valves and hoses of the pilot-scale equipment, but otherwise the material was as easily processable as 100% waste fibre.

Schell et al. [24] reported results from the initial runs at the National Renewable Energy Laboratory bioethanol plant with corn fibre feedstock. They encountered significant problems with bacterial contamination in the process during the 10 and 15 day runs and later in longer campaigns [25]. Also Isci et al. [26] report problems with bacterial contamination and lactic acid production in pilot-scale fermentation of pretreated switchgrass. In this campaign reported here, no lactic acid or acetic acid production was observed and the lactic acid bacteria concentration was below 10^4 cfu ml^{-1} in the fermenter throughout the week (data not shown) indicating that sanitation by heat, the use of sodium bisulphite and the short residence time in the fermenter were effective measures for successful bacterial control. In longer campaigns we have also encountered problems with lactic acid bacteria and noted that having high yeast concentration in the fermenter helps to limit their growth. Lactic acid production appeared to start only after the contamination reaches a certain level and thus it is important to take defencive measures (pH shock, cleaning etc.) early on in the process.

3.5. *Biogas production from fermented residue*

Biogas production is an important factor in the FibreEtOH process because it allows the more complete utilization of the feedstock reducing waste and helping to overcome financial challenges related to ethanol production from lignocellulose. It opens up an opportunity to digest additional starch containing materials to biogas when attractive market prices are boosting the economy. The combined ethanol and biogas production will also increase the carbon conversion yield significantly compared to stand-alone ethanol production. Biogas can also be upgraded to biomethane which can also be used in transport applications.

Material from two continuous bioethanol pilot campaigns was used in a biogas production experiment that lasted little over 3 months. First fermentation residue came from a bioethanol pilot campaign using waste fibre and the second from a campaign using fibre sludge as the raw material. The produced ethanol was removed from both residues by evaporation.

During the first 2.5 months when waste fibre based residue was used, total of 655 dm^3 biogas per kg dry matter feed was produced. Loading of material was increased during the experiment from 0.1 to 3.0 kg m^{-3} d^{-1} and best result (990 dm^3 kg^{-1}) was obtained with the lowest loading whereas biogas production with the highest loading was 350 dm^3 kg^{-1}. For fibre sludge residue the total production of biogas during the experiment was 400 dm^3 kg^{-1}. Loadings were varied from 5.9 to 10 kg m^{-3} d^{-1} and the best result (580 dm^3 kg^{-1}) was obtained with the lowest loading. Methane content of the produced biogas was 69–75%. The treated waste fibre based stillage contained 3.6 g dm^{-3} BOD (91% reduction), 48 g dm^{-3} COD (66% reduction) and 51 g dm^{-3} suspended solids. The respective analysis results for fibre sludge based stillage were 12 g dm^{-3} (83% reduction), 104 g dm^{-3} (54% reduction), and 71 g dm^{-3}. Further experiments confirmed that additional nutrients (urea and phosphoric acid) were not needed for effective biogas production.

3.6. *Material balance in the process*

A material balance sheet has been produced based on the results from the pilot campaigns. According to the results

presented in this paper 1000 kg dry combined waste fibre and fibre sludge produces approximately 120 kg ethanol, 350 kg biogas (70% methane), 400 kg (d.m.) waste sludge, and 120 kg CO_2. In the calculations 4% of the feedstock glucan has been appointed for yeast propagation. Based on other pilot-scale experiments a glucan hydrolysis yield of 75% is reachable within the process concept if the residence time in fermentation is extended. For this case the corresponding numbers are 170 kg ethanol, 310 kg biogas, 360 kg waste sludge, and 170 kg CO_2.

4. Conclusions

Waste fibre fractionated from solid recovered fuel and pulp and paper mill fibre sludge are competitive bioethanol feedstocks because of their high hexose content and easy hydrolysability without thermal or chemical pre-treatment. High polysaccharide conversion could be obtained in high consistency liquefaction of the feedstocks in only 6–10 h although hydrolysis to monosaccharides was somewhat slower. Hydrolysis of waste fibre and fibre sludge released recalcitrant mannan- and galactan-containing polysaccharides that require additional helper enzymes to be further hydrolysed to monosaccharides. Continuous pilot-scale campaign consisting of liquefaction and simultaneous saccharification and fermentation was run stably for several days with efficient ethanol production and without problems from bacterial contamination. Biogas was produced with high yields from the evaporated residue of waste fibre and fibre sludge hydrolysis and fermentation. The results presented here demonstrate the feasibility of the FibreEtOH concept as a potential 2nd generation bioethanol process.

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PUBLICATION II

Hot water extraction and steam explosion as pretreatments for ethanol production from spruce bark

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Hot water extraction and steam explosion as pretreatments for ethanol production from spruce bark

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HIGHLIGHTS

- Hot water extraction (80 °C) and steam explosion were studied as pretreatments.
- Steam explosion of spruce bark should be carried out without acid catalyst.
- Hot water extraction is a suitable pretreatment for spruce bark with right enzymes.
- Ethanol production from pretreated enzymatically hydrolysed barks was efficient.
- Spruce bark is a potential feedstock for the production of lignocellulosic ethanol.

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ABSTRACT

Spruce bark is a source of interesting polyphenolic compounds and also a potential but little studied feedstock for sugar route biorefinery processes. Enzymatic hydrolysis and fermentation of spruce bark sugars to ethanol were studied after three different pretreatments: steam explosion (SE), hot water extraction (HWE) at 80 °C, and sequential hot water extraction and steam explosion (HWE + SE), and the recovery of different components was determined during the pretreatments. The best steam explosion conditions were 5 min at 190 °C without acid catalyst based on the efficiency of enzymatic hydrolysis of the material. However, when pectinase was included in the enzyme mixture, the hydrolysis rate and yield of HWE bark was as good as that of SE and HWE + SE barks. Ethanol was produced efficiently with the yeast *Saccharomyces cerevisiae* from the pretreated and hydrolysed materials suggesting the suitability of spruce bark to various lignocellulosic ethanol process concepts.

1. Introduction

The utilization of biomass for the production of transport fuels, chemicals and materials is increasing because of fluctuating price and limited availability of oil, and the need to reduce greenhouse gas emissions. Upgrading of biomass to value-added products may also provide additional profits when compared to its combustion to heat and electricity.

Bark, which constitutes of ca. 12% of the total weight of a tree (Surminski, 2007), is an abundantly available biomass feedstock that is already efficiently collected at pulp, paper, and sawmill sites. With annual industrial wood consumption of 70 Mm^3 (ca. 35 Mt), approximately 3–5 Mt of industrial bark is produced per annum alone in Finland (Finnish Forest Industries Federation, 2011). Most of this bark is combusted for electricity and heat at mill site. In addition to debarking lines producing mixed bark varieties, also pure streams of e.g. spruce and birch bark are available.

Spruce bark and bark in general have been surprisingly little studied recently as a lignocellulosic feedstock for 2nd generation biorefinery processes. The composition of spruce bark is very complex and not well understood as it contains several compounds such as polyphenols and extractives which are not found in wood (Laks, 1991). Bark is not generally considered as a very good source of fermentable sugars because of the high amount of lignin and extractives in the material (Kim et al., 2005; Robinson et al., 2002; Torget et al., 1991; Vazquez et al., 1987). In fact, some non-lignin derived compounds condense and precipitate in sulphuric acid showing up as Klason lignin and making the analysis of true bark lignin difficult (Laks, 1991). Spruce bark contains ca. 19% cellulose and a varying amount of non-cellulosic sugars that are present as free sugars and bound in hemicellulose, pectin and glycosides (Laks, 1991). Most abundant non-cellulosic monosaccharides are glucose, arabinose, galacturonic acid, mannose, xylose and galactose (Le Normand et al., 2011). Additional challenges come from

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high seasonal variation in the bark composition, and the fact that industrial bark may be stored for months and wetted and pressed during debarking, which alters its composition compared to that of fresh bark.

However, some spruce bark extractives, such as lignans, stilbenes, and flavonoids, have been extensively studied because of their antioxidative and biologically active properties (Manners and Swan, 1971; Co et al., 2012). These components are all at least partly extractable with water. The total extraction yield with pressurized or unpressurized water from bark is rather high, 19–35% of bark dry matter (Co et al., 2012; David and Atarhouch, 1987), and unpressurized water extraction can be used to fractionate for example water soluble polyphenols (tannins), stilbenes, stilbene glycosides and hemicellulose from bark (Kylliäinen and Holmblom, 2004). Spruce bark tannins are interesting compounds that could find use for example in adhesives and insulating foam applications (Pizzi, 2006; Tondi and Pizzi, 2009).

In order to design a profitable biorefinery, all components of the feedstock must be efficiently utilized. In this study, the suitability of spruce bark to bioethanol production was studied as such and after extraction of tannins with hot water. Steam explosion was studied as a pretreatment for industrial bark, and the need for further pretreatment after hot water extraction to obtain efficient enzymatic hydrolysis was examined. Finally, the suitability of the materials for ethanol production was studied in industrially relevant conditions.

2. Methods

2.1. Materials

Industrial spruce bark was collected from UPM-Kymmene Oyj Jämsänkoski mill debarking unit (Finland). The dry matter content of the bark was ca. 32%. The material was stored 1–2 weeks outside (subzero temperature) before pretreatment and afterwards below −20 °C.

Enzymes Celluclast 1.5 L, Novozym 188 and Pectinex Ultra SP-L were purchased from Novozymes A/S (Bagsvaerd, Denmark). Filter paper unit (FPU) activity of Celluclast 1.5 L was 49 FPU mL^{-1} measured according to Ghose (1987), β-glucosidase activity of Novozym 188 was 5910 nkat mL^{-1} measured according Bailey and Nevalainen (1981) and pectinase activity of Pectinex Ultra SP-L was 81,900 nkat mL^{-1} measured according to Bailey and Pessa (1989). In addition the xylanase activity (Bailey et al., 1992) and mannanase activity (Stålbrand et al., 1993) of Pectinex Ultra SP-L was measured.

Saccharomyces cerevisiae strain VTT-B-08014 was used in the fermentation experiments. The strain was pre-cultivated aerobically overnight in YP medium supplemented with 2% glucose at 30 °C, and overnight in YP medium supplemented with 5% glucose at 30 °C before inoculation. Yeast cells were washed with 0.1 M sodium phosphate solution pH 7 and diluted to Yeast Nitrogen Base before inoculation.

All used chemicals were of analytical grade.

2.2. Analysis of the substrate composition

Neutral monosaccharide composition of the materials was determined with high-performance anion exchange chromatography with pulsed amperometric detection after a two-step hydrolysis with sulphuric acid according to the NREL Laboratory Analytical Procedure. The analysis was run on Dionex ICS-3000 liquid chromatograph (Dionex Corp., Sunnyvale, CA) according to Tenkanen and Siika-aho (2000) with minor modifications (equilibration with 15 mM NaOH, isocratic elution with water). Acidic monosaccharides galacturonic acid, 4-*O*-methyl glucuronic acid and glucuronic acid were analysed from solid samples after metanolysis and silylation by gas chromatography of the silylated sugar monomers according to Sundberg et al. (1996).

Extractives content was determined gravimetrically after 5 h extraction with boiling heptane in soxhlet. The apparent lignin content was determined after the removal of extractives according to the NREL Laboratory Analytical Procedure with slight modification as previously described by Varnai et al. (2010). Lignin content was calculated as the sum of the Klason- and acid-soluble lignin which was determined at 203 nm with reference absorptivity of 128 g L^{-1} cm^{-1}. The ash content of the samples was measured gravimetrically after combustion in a furnace at 550 °C.

2.3. Pretreatments

Steam explosion (SE) was conducted with and without an acid catalyst. Acid catalyst was added by impregnating the bark prior to steam explosion in 0.5% H_2SO_4 solution for 30 min after which the bark was filtered on a 6 μm wire to ca. 18% dry matter content. Steam explosion of the acid-impregnated and untreated bark was carried out in a 10 L pressurized vessel. The vessel was heated with steam to the desired temperature (190 °C/11.6 bar or 205 °C/16.3 bar) and maintained there for 5 min before releasing the pressure to a collection vessel. In the steam explosion of the hot water extracted material the bark was contained in a 5 L metal wire cage inside the vessel to prevent solid material losses to the collection vessel and to decrease material wetting. The steam condensate was vented out of the reactor during heating. After steam explosion of untreated bark and acid-impregnated bark the dry matter content was from 12% to 17%, and after steam explosion of hot water extracted bark it was 27%. Steam exploded materials were washed four times with hot tap water to remove soluble sugars before hydrolysis experiments.

Hot water extraction (HWE) of bark was carried out in a 250 L horizontal Drais reactor with ploughshare mixing (Dreiswerke, Germany). Consistency during the extraction was 8% and mixing speed 100 rpm. The extraction was carried out in two steps including first a 120 min cold water extraction at 20 °C, after which the extract was let out and replaced with hot water. Hot water extraction was conducted at 80 °C for 120 min. After extraction the bark was pressed to 21% dry matter content. Part of this bark was steam exploded without acid catalyst at 190 °C producing sequentially extracted and steam exploded (HWE + SE) bark.

Another batch of hot water extracted bark was made for fermentation experiments with only 120 min hot water extraction at 80 °C. Experimental data from a large extraction series (data not shown) showed that the amount and composition of the extract as well as the carbohydrate composition of the extracted bark coming from sequential cold and hot water extraction of spruce bark and single 120 min hot water extraction were very similar. Thus in this study both batches of hot water extracted bark can be considered comparable in their composition and behaviour in hydrolysis and fermentation.

2.4. Hydrolysis experiments

Enzymatic hydrolysis experiments were carried out with combinations of different dosages of commercial cellulase, β-glucosidase and pectinase products. Cellulase Celluclast 1.5 L was dosed 10 FPU g^{-1} (low dosage) or 25 FPU g^{-1} (high dosage), β-glucosidase Novozym 188 was dosed 200 nkat g^{-1} (low dosage) or 500 nkat g^{-1} (high dosage) and pectinase Pectinex Ultra SP-L was dosed 0 or 5000 nkat g^{-1} (high dosage). High dosage of Celluclast 1.5 L brought additional 220 nkat g^{-1} and low dosage additional 90 nkat g^{-1} β-glucosidase activity to the mixture on top of the β-glucosidase activity obtained from Novozym 188. The β-glucosi-

dase activity of Pectinex Ultra SP-L and the pectinase activities of Celluclast 1.5 L and Novozym 188 were insignificant (<5%) compared to the dosed activities. High dosage of Pectinex Ultra SP-L brought 115 nkat g^{-1} xylanase activity and 1140 nkat g^{-1} mannanase activity to the enzyme mixture.

Hydrolysis experiments were performed in test tubes in 3 mL reaction volume at 1% dry matter content in 50 mM sodium acetate buffer pH 5, with 0.02% sodium azide to control microbial action. The tubes were placed in a water bath at 45 °C and magnetic mixing was applied at 300 rpm. The materials were not dried after pretreatment but used as such. Hydrolysis was followed by terminating the reaction by boiling the tubes for 10 min after 4, 24 and 48 h from the start. Hydrolysis yield was analysed by measuring reducing sugars with 3,5-dinitrosalicylic acid (DNS) method according to Bernfeld (1955) and by analysing neutral monosaccharides with HPAEC-PAD as described above. Solubilised acidic monosaccharides were analysed similarly as neutral monosaccharides but with a modified gradient (Tenkanen and Siika-aho, 2000). Triplicate tubes were prepared for each sampling point.

2.5. Fermentation

The unwashed pretreated spruce bark was mixed with 0.1 M sodium citrate buffer at pH 5 before moving it to the fermenter. Before starting the fermentations, pH of spruce bark was adjusted to 5 with 10 M NaOH and during the fermentations it was adjusted with 5 M KOH and 5 M H_3PO_4.

The experiments were carried out in a Biostat CT-DCU fermenter by B. Braun (Germany) as a simultaneous saccharification and fermentation (SSF) combined with a short prehydrolysis. Fermenter was equipped with a powerful marine propeller for mixing and a special samples collector device, which enabled sampling during high consistency fermentations. Working volume of 1.5 L was used. Redox balance, dissolved oxygen and pH were measured in situ. Oxygen, carbon dioxide and ethanol were analysed from the exhaust gases with photoacoustic measurement (1309 Multipoint sampler, 1313 Fermentation monitor and LumaSoft Gas Multi Point 7850 software) manufactured by Innova Air Tech Instruments A/S (Denmark). The fermenter was sterilized before the experiment.

A 6 h prehydrolysis in 15% dry matter content was conducted at 45 °C, with 1 L min^{-1} aeration (air) and 100–150 rpm mixing with 10 FPU g^{-1} Celluclast 1.5 L, 500 nkat g^{-1} Novozym 188 and 5000 nkat g^{-1} Pectinex Ultra SP-L.

The consequent simultaneous saccharification and fermentation was carried out at 35 °C under 1 L min^{-1} nitrogen gas flow. Cell density in pre-cultivated yeast was calculated from absorbance at 660 nm (OD_{660}) and the fermenter was inoculated with 3.0 g L^{-1} yeast (OD 10). Samples were withdrawn from the reactor and dry matter content, main metabolic products and monosaccharides were analysed from the supernatant of centrifuged samples using Dionex ICS-3000 liquid chromatograph. Fast Acid Analysis Column (Bio-Rad Laboratories, USA) and Aminex HPX-87H column (Bio-Rad Laboratories, USA) were used with 0.3 mL min^{-1} flow of 5 mM H_2SO_4 as an eluent. Impurities were removed by Cation-H Refill Cartridges (Bio-Rad Laboratories, USA) as a pre-column. Waters 2487 dual λ absorbance detector (wavelength 210 nm) and Waters 2414 refractive index (RI) detector were used.

3. Results and discussion

3.1. Pretreatment of spruce bark

Three types of pretreatments were carried out for spruce bark. The composition of the starting material and the solid fractions after treatments were analysed and component yields compared in order to evaluate how the treatments affected the feedstock composition. First the effects of acid catalyst and temperature conditions during steam explosion were shortly investigated. Mass and component yields in the steam explosion of spruce bark for 5 min at 190 °C (11.6 bar) or 205 °C (16.3 bar) with or without acid catalyst are presented in Table 1. Yields were calculated as total mass of the solid residue or component mass in the solid residue after treatment divided by the original mass or component mass before treatment. Total mass yields are rather low due to material losses during steam explosion and the following washing step.

According to the results, steam explosion dissolved or degraded hemicelluloses and increased the relative share of glucan in the solid residue compared to untreated bark. Steam explosion with acid catalyst resulted in more complete hemicellulose degradation. The relative amount of lipophilic extractives increased slightly during steam explosion whereas some ash was lost especially when using acid catalyst. More severe treatment conditions (impregnation in dilute acid, higher temperature) resulted in an increase of compounds analysed as Klason lignin in the solid residue. It has been suggested that water-soluble bark phenolics such as tannins are rendered insoluble in dilute acid treatment and show up as Klason lignin in the insoluble residue (Torget et al., 1991). High temperature may promote this phenomenon and result in increased Klason lignin content in the more severely pretreated bark samples. Robinson et al. (2002) conducted SO_2 catalysed steam explosions with mixed Douglas fir wood and bark and they observed an increase in mass yield with increasing share of bark in the feedstock mix. This could support the hypothesis that otherwise water-soluble bark components become insoluble by acid pretreatment and remain in the solid residue.

As presented later in Section 3.2, those steam explosion conditions that provided the best hydrolysability (190 °C, no acid catalyst) were used in further experiments where steam explosion and hot water extraction were compared as pretreatment methods. The hypothesis was that hot water extraction at 80 °C would not be a sufficient pretreatment to obtain high enzyme hydrolysis yields. Therefore, a sequential hot water extraction and steam explosion treatment was also carried out for spruce bark. According to the results presented in Table 2 hot water extraction dissolved and degraded less polysaccharides than steam explosion. Especially acidic polysaccharides consisting mostly of galacturonic acid were removed during steam explosion. Apparent lignin content in hot water extracted bark was lower than in untreated and steam exploded bark suggesting partial removal of water soluble phenolics during hot water extraction. Ash was mostly preserved in the treatments, but more extractives were removed in hot water extraction than in steam explosion.

Steam explosion after hot water extraction dissolved and possibly degraded neutral and acidic hemicelluloses resulting in a very similar total yield compared to steam explosion alone except with less extractives and ash. Thus, the composition of the hot water extracted and steam exploded bark was very similar to steam exploded bark with relatively high glucan content and less acidic polysaccharides compared to the starting material (Table 2). Hot water extracted bark on the other hand was similar to the untreated spruce bark with lower glucan and higher hemicellulose content. The neutral hemicellulosic polysaccharides in the untreated bark were composed of 36% arabinan, 20% xylan, 26% mannan, 16% galactan and 3% rhamnan, and acidic hemicelluloses were composed of 89% galacturonic acid, 6% 4-*O*-methyl glucuronic acid and 5% glucuronic acid. Steam exploded bark contained less arabinan (12%) and more xylan (40%) than the hot water extracted and steam exploded material (25% and 29%, respectively). On the basis of the comparison of metanolysis and acid hydrolysis products, 79% of the glucan in the untreated material was cellulose (β-1,4-glucan) and the rest was part of hemicellulose or attached

Table 1
Composition (*C*) of spruce bark as % of dry matter and component and total mass yields (*Y*) as % of theoretical after steam explosion for 5 min at 190 °C or 205 °C with or without acid catalyst.

Component	Untreated bark	Steam exploded samples							
		190 °C, no acid		190 °C, acid		205 °C, no acid		205 °C, acid	
	C	*C*	*Y*	*C*	*Y*	*C*	*Y*	*C*	*Y*
Glucan	28.1	38.8	85	41	81	40.3	91	37.4	83
Other polysaccharides	13.6	9.1	41	3.6	15	6.7	31	3.0	14
Lignin	35.8	33.6	63	37.9	64	38.3	74	44.6	85
Extractives	4.5	6.6	91	6.7	83	6.2	88	6.6	92
Ash	3.6	3.6	62	2.4	37	3.2	56	2.4	41
Mass yield			62		55		63		62

Table 2
Composition (*C*) of spruce bark as % of dry matter, and component and total mass yields (*Y*) after different pretreatments (SE = steam explosion, HWE = hot water extraction, HWE + SE = sequential hot water extraction and steam explosion).

Component	Untreated	SE		HWE		SE after HWE	HWE + SE	
	C	*C*	*Y*	*C*	*Y*	*Y*	*C*	*Y*
Glucan	28.1	38.8	85	33.3	88	99	39.5	87
Other neutral polysaccharides	13.6	9.1	41	13.7	74	57	9.3	42
Acid polysaccharides	7.3	3.0	25	5.9	60	47	3.3	28
Lignin	32.8	33.6	63	28.2	64	98	32.9	62
Extractives	4.5	6.6	91	3.5	59	104	4.4	61
Ash	3.6	3.6	62	3.3	69	72	2.8	49
Total mass yield			62		74	84		62

as glycosides for example to stilbenes. Similar ratios were obtained for the pretreated materials (69–86%).

3.2. Effect of steam explosion conditions on the hydrolysability of spruce bark

Steam explosion was performed on industrial spruce bark at two temperatures (190 or 205 °C) with or without pre-impregnation in 0.5% sulphuric acid, and the hydrolysability of the materials was assayed with commercial hydrolytic enzymes. High or low dosages of cellulase (25 or 10 FPU g^{-1}) and β-glucosidase (500 or 200 nkat g^{-1}) were used. Experiments with and without pectinase (5000 nkat g^{-1}) were also carried out. Fig. 1 presents the results from the hydrolysis experiments. The yields based on reducing sugar assay are calculated as percentage of polysaccharides released as neutral monosaccharides in total acid hydrolysis.

The best conditions for steam explosion of spruce bark can be distinguished from the results. Highest yields and faster hydrolysis was obtained with bark treated at 190 °C without acid catalyst. Hydrolysis yields from 75% to 84% obtained in our studies are comparable to published hydrolysis results obtained for steam pretreated softwood (Monavari et al., 2009; Söderström et al., 2003). The second best steam explosion parameters appeared to be 205 °C without acid catalyst, whereas the acid catalysed materials produced lower hydrolysis rates and yields especially with lower enzyme dosages. Worst hydrolysis results were obtained with untreated material, especially with lower enzyme dosages. However, compared to the hydrolysis of native wood the hydrolysis yield of untreated bark with high enzyme dosages was surprisingly high, 68%.

According to the results, a more severe pretreatment, i.e. the use of acid or higher temperature, decreased the hydrolysis rate and yield. This result is quite the opposite to what is reported for other woody biomasses. Higher pretreatment temperature increases the hydrolysability of for example poplar wood (Schütt et al., 2011), Douglas Fir (Nakagame et al., 2011) and spruce wood (Fang et al., 2011). Possible reason for the finding could be that water-soluble phenolic compounds in the bark, such as tannins, are condensed to insoluble material analysed as Klason lignin by the action of acid and high temperatures so that they cover cellulose surfaces and reduce their accessibility to hydrolytic enzymes. This suggestion is supported by the findings of David and Atarhouch (1987) who obtained lower hydrolysis yields from spruce bark refluxed with dilute H_2SO_4 compared to bark extracted with boiling water.

High cellulase and β-glucosidase dosages improved the hydrolysis rate and final hydrolysis yields, especially when harsh pretreatment conditions were used or the materials were not pretreated at all. The addition of pectinase to the enzyme mixture produced a similar although a smaller effect.

Overall, according to the results presented here steam explosion at 190 °C without additional acid was an effective pretreatment for spruce bark as it more than doubled the hydrolysis yield of the material with low dosages of cellulase and β-glucosidase.

3.3. Comparison of the effect of pretreatments on the hydrolysability of spruce bark

Steam explosion (SE) is not and ideal pretreatment process due to high investment and running costs and possible formation of inhibitors at high temperature during the treatment. It is also not suitable for fractionation and isolation of water soluble polyphenolic components from spruce bark, because the components are not extracted efficiently into the small amount of condensate and the high temperature may cause degradation of the desired compounds (Gaugler and Grigsby, 2009). Unpressurized hot water extraction (HWE) on the other hand allows separation of water-soluble tannins from bark material with less degradation (Kaijaluoto et al., 2010). We investigated whether hot water extraction is a sufficient pretreatment for spruce bark prior to enzyme hydrolysis and fermentation to ethanol or whether additional steam explosion is required. The enzymatic hydrolysis rate and yield of steam exploded (SE), hot water extracted (HWE) and sequentially hot water extracted and steam exploded (HWE + SE) spruce bark were

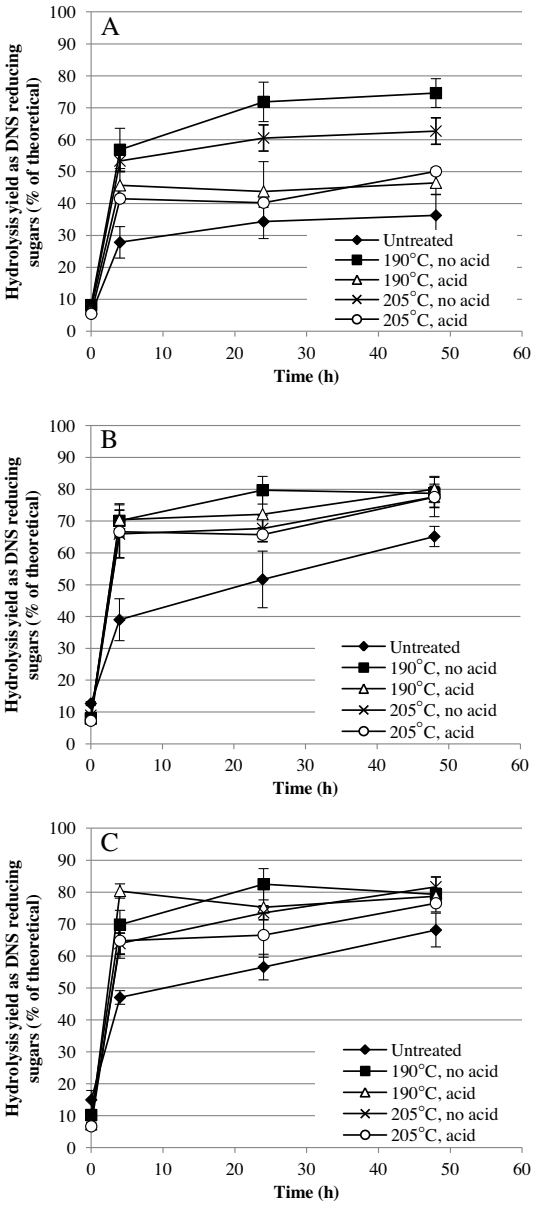

Fig. 1. Hydrolysis yield of untreated and steam exploded spruce bark in 1% consistency in 50 mM acetate buffer, pH 5 at 45 °C. (A) Low dosage of cellulase and β-glucosidase. (B) High dosage of cellulase and β-glucosidase. (C) High dosage of cellulase, β-glucosidase and pectinase.

compared (Fig. 2). The yields based on reducing sugar assay were calculated as percentage of the sum of monosaccharides released in acid hydrolysis (neutral monosaccharides) and methanolysis (acidic monosaccharides) from the solid materials.

SE and HWE + SE barks behaved similarly in hydrolysis although SE bark appeared to hydrolyse somewhat faster with high enzyme dosage. This similar behaviour was expected for these two bark materials as their chemical composition was also similar (Chapter 3.1). HWE bark differed from these two as it hydrolysed slower and the final yield was 22% lower with low enzyme dosages. High cellulase and β-glucosidase dosages improved the hydrolysis of HWE bark but only the addition of pectinase significantly improved its hydrolysis to a comparable level with the two other pretreated materials.

When 500 nkat g^{-1} β-glucosidase and 5000 nkat g^{-1} pectinase but no cellulase was used, the hydrolysis yield was the highest for HWE bark (33% or carbohydrates) and less for SE (28%) and HWE + SE bark (27%) supporting the hydrolysis results with cellulase, β-glucosidase and pectinase (data not shown).

The positive effect of pectinase on the hydrolysis of HWE spruce bark was evident also when low cellulase and high β-glucosidase dosage was used (Fig. 3). Additional pectinase improved the hydrolysis of HWE bark to a comparable level with SE bark. The improvement in the hydrolysis with pectinase was 57% after 4 h and 24% after 48 h. The pectinase activity corresponds only to additional 4% in the protein mixture, which suggests that use of additional pectinase may be inexpensive compared to the total cost of the enzyme treatment. Pectinex Ultra SP-L contains, as well as Novozym 188 to a lesser extent, several accessory activities in addition to pectinase or β-glucosidase activities, which may contribute to the improved hydrolysis of pretreated barks.

Hydrolysates after 48 h enzyme hydrolysis were analysed by HPLC and the specific yield of each monosaccharide was determined (Table 3). The total hydrolysis yields as monosaccharides analysed by HPLC were on average 76% of the hydrolysis yield obtained by reducing sugars assay (standard deviation 5.2). The DNS reagent used in the reducing sugar assay gives response also to the reducing ends of oligosaccharides, and fructose which is found in spruce bark but was not analysed by HPLC. In addition, DNS reagent may degrade some oligosaccharides during the analysis generating more reducing ends. According to the reducing sugar assay, 35% of the soluble dry matter of the hot water extract was reducing compounds and according to HPLC only 1% was monosaccharides (data not shown). This leads to the suggestion that there are some other at least partly soluble components in industrial and also pretreated spruce bark that give response in reducing sugar assay.

Despite the differences in the yield levels, the conclusions from HPLC analysis were similar as those drawn from reducing sugar assay. HPLC analysis showed total hydrolysis yield of 63% for both HWE and HWE + SE bark with low dosage of cellulase and high dosage of β-glucosidase and pectinase. Pectinase improved hydrolysis to monosaccharides, in particular to galacturonic acid, which underlines the importance of the pectinolytic activity for hydrolysis of HWE bark. As spruce bark is reported to contain pectin (Laks, 1991; Le Normand et al., 2011), it can be assumed that at least the majority of the released galacturonic acid came from pectic substances, although it is possible that some activities in Pectinex Ultra SP-L released galacturonic acid from hemicellulose as well. The very low amount of galactose, 4-*O*-methyl glucuronic acid and glucuronic acid in spruce bark caused errors in the calculations and resulted in some cases in yields of over 100%.

A few hydrolysis experiments were carried out with using purified β-glucosidase (Sipos et al., 2009) instead of commercial Novozym 188 enzyme. The yields with purified β-glucosidase were generally slightly lower than with Novozym 188 (results not shown) suggesting that other components in the commercial enzyme preparate play a role in the hydrolysis.

Softwood barks have been found fairly resistant to hydrolytic enzymes in earlier studies (David and Atarhouch, 1987; Vazquez et al., 1987). Tannins contained in the untreated and partly in the pretreated bark have been suggested to bind and precipitate proteins and therefore inactivate cellulase enzymes (Walch et al., 1992). Thus, it was surprising to obtain such high hydrolysis yields (up to 68%) from untreated bark in this study. On the other hand,

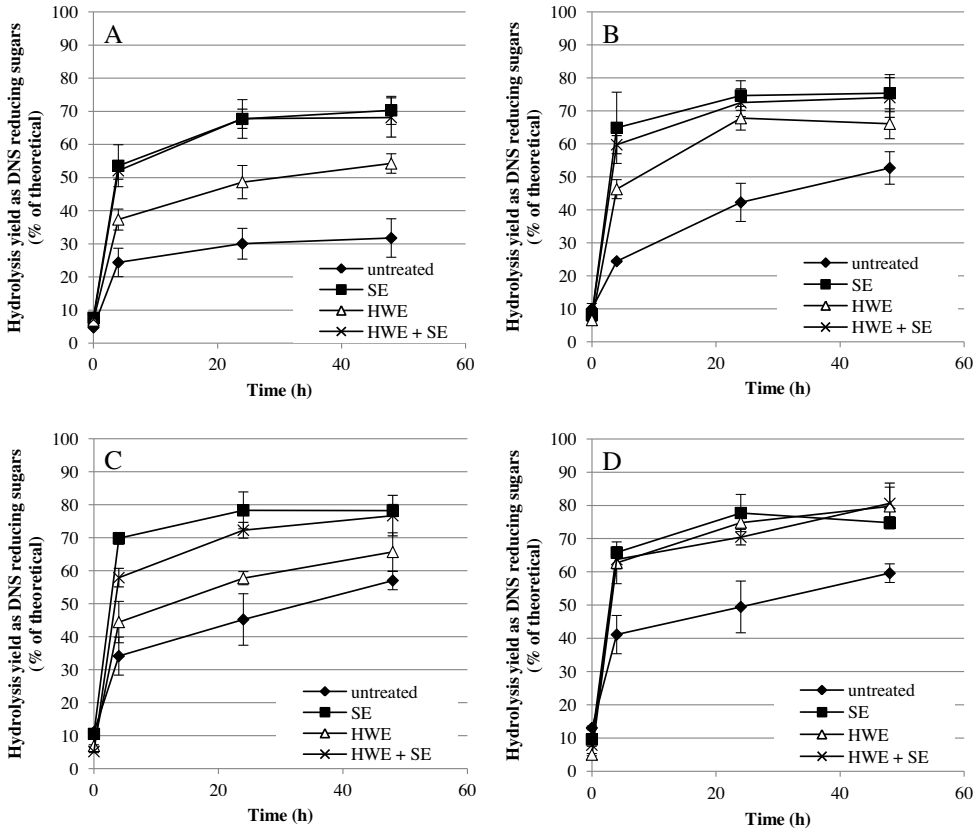

Fig. 2. Hydrolysis of pretreated spruce bark in 1% consistency in 50 mM acetate buffer, pH 5 at 45 °C. Pretreatments used were hot water extraction (HWE), steam explosion (SE) and combination of the two (HWE + SE). Hydrolysis with. (A) Low dosage of cellulase and β-glucosidase, (B) low dosage of cellulase and β-glucosidase, high dosage of pectinase, (C) high dosage of cellulase and β-glucosidase, (D) high dosage of cellulase, β-glucosidase and pectinase.

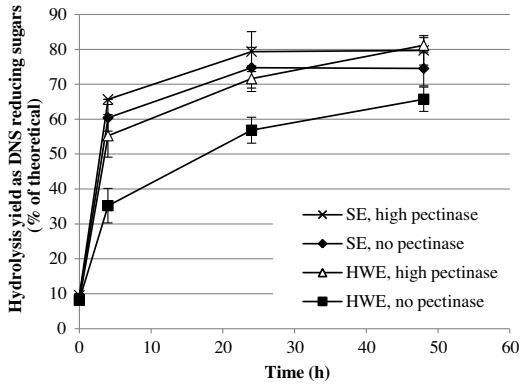

Fig. 3. Hydrolysis of pretreated spruce bark with low cellulase dosage, high β-glucosidase dosage and with or without pectinase in 1% consistency in 50 mM acetate buffer, pH 5 at 45 °C. Pretreatments used were hot water extraction (HWE) and steam explosion (SE).

the specific activity and efficiency of commercial enzymes towards lignocellulosics has greatly developed since 1980's and as mentioned the reducing sugar method used in this study also appears to give response to some other dissolving components in spruce bark in addition to mono- and oligosaccharides.

When comparing our composition and hydrolysis data to literature, it must be noted that industrial bark processed at the debarking unit which we have used in our study is not the same as fresh bark obtained from a recently felled tree, which has been used in many published studied on bark utilisation (Robinson et al., 2002; Torget et al., 1991; David and Atarhouch, 1987). Very large differences have also been found in the hydrolysability of inner and outer bark. David and Atarhouch (1987) obtained 58% hydrolysis yield for spruce inner bark glucan after extraction with boiling water whereas the hydrolysis yield of outer bark was only 18%. Hydrolysability of whole bark was in between (51%), and spruce bark was determined to contain 40% inner bark and 60% outer bark. Altogether, the hydrolysis yields remained lower than those obtained in this study except when spruce bark was delignified with NaOH or NaClO after extraction with boiling water (glucan hydrolysis yields 67–99%). Pretreatment with NaOH has been found to increase the hydrolysability of pine bark only slightly (from 3% to 6%) and delignification with sodium hypochlorite was needed to achieve higher yields (>67%) in 24 h (Vazquez et al., 1987). Eucalyptus bark and hardwood bark in general appear to be easier to hydrolyse enzymatically as 60% cellulose hydrolysis yield has been obtained with eucalyptus bark pretreated hydrothermally with CO_2 (Matsushita et al., 2010). The yields obtained

Table 3
Yields of monosaccharides (% of the amount of the monosaccharides in the starting material) after 48 h enzyme hydrolysis of hot water extracted (HWE) and sequentially hot water extracted and steam exploded (HWE + SE) spruce bark with low (L) or high (H) dosage of cellulase, high dosage of β-glucosidase and with or without pectinase.

Material	Cellulase	Pectinase	Glc	Xyl	Ara	Man	Gal	Rha	GalA	4-*O*-MeGlcA	GlcA
HWE	L	No	64	33	33	20	23	0	7	11	58
	L	Yes	68	58	60	46	58	14	60	8	23
	H	No	58	32	31	25	23	0	5	35	56
	H	Yes	70	54	59	49	56	10	45	23	38
HWE + SE	L	No	57	42	115	28	47	0	19	37	167
	L	Yes	66	62	69	50	118	0	26	63	35
	H	No	68	47	45	36	118	0	6	125	59
	H	Yes	70	62	72	56	120	0	62	30	82

in this study appear to be higher than what has been reported for pretreated bark in the literature.

The results of our experiments point towards a hypothesis that in acid catalysed pretreatment of spruce bark some water-soluble phenolic compounds are condensed and increase the Klason lignin content of the material. This reaction in turn reduces the hydrolysability of the biomass by possibly reducing the accessibility of the enzymes to cellulose. On the other hand, most of these compounds are removed during hot water extraction and further acid catalysed steam explosion may not reduce the hydrolysability of bark as in the case of untreated bark. However, this hypothesis was not verified in this work since acid catalysed steam explosion was not studied on hot water extracted bark.

Altogether, the results suggest that using a right mixture of enzymes including a pectinase product in hydrolysis may render hot water extraction in 80 °C as an adequate pretreatment for industrial spruce bark and remove the need for conventional capital intensive pretreatment technologies such as steam explosion. Steam explosion can also be used to improve the hydrolysability of spruce bark but additional acid catalyst and very high temperature may reduce the efficiency of the pretreatment. It appears that the solid residue from a biorefinery producing tannin from industrial spruce bark by hot water extraction is a good feedstock for 2nd generation bioethanol production or other sugar-based production processes because of its good hydrolysability as such with right enzymes. However, if it is used as an additional feedstock in a process containing a steam explosion unit using acid catalyst, it should be fed to the process after steam explosion. These results suggest that spruce bark can be reconsidered as a feedstock for sugar biorefineries, especially in such concepts in which tannins or other water extractable components are first extracted for use as biochemicals or intermediates.

3.4. High consistency fermentation of pretreated spruce bark

The fermentability of HWE + SE and HWE barks were compared in high consistency (150 g L^{-1} substrate concentration) simultaneous saccharification and fermentation (SSF) experiments to confirm the results from hydrolysis studies and to assess the effect of possible inhibitors to the fermenting yeast *S. cerevisiae*. A new batch of HWE bark was made for this experiment and the materials were used unwashed. HWE + SE and HWE barks contained 49.8% and 48.0% total carbohydrates with 41.4% and 38.4% hexose polysaccharides, expressed as monosaccharides after acid hydrolysis.

During the fermentations it was noticed that a significant amount of ethanol evaporated from the reactor. Evaporation rates were 0.03–0.15 g L^{-1} h^{-1}, and the rate was related to the concentration of ethanol in the reactor. Evaporation rates as high as these have not been observed earlier in the same vessel, and it was concluded that the phenomenon was probably caused by increased gas/liquid surface area in the reactor related to the porous nature of the pretreated bark. Thus the ethanol production was calculated based on CO_2 production assuming that all CO_2 was produced from ethanol fermentation in strictly anaerobic conditions. Ethanol production was also calculated by analysing ethanol from exhaust gas and adding the amount to ethanol concentration analysed by HPLC from samples taken from the reactor. The results supported the CO_2-based calculations.

Fig. 4 presents the release and consumption of glucose and the production of ethanol during the fermentations. Glucose was quickly consumed by the yeast after inoculation at 6 h and 94% of all ethanol was produced during first 50 h in both fermentations. During HWE + SE fermentation 1.7 g L^{-1} acetate and 4.6 g L^{-1} glycerol was produced. The corresponding concentrations for HWE bark were 1.4 g L^{-1} and 4.3 g L^{-1}. The hydrolysis yield of glucan analysed by HPLC after 6 h prehydrolysis was 68.2% for HWE + SE bark and 66.0% for HWE bark showing similar and fast hydrolysability of the materials. Fermentation in 15% consistency produced 21.0 g L^{-1} ethanol from HWE + SE bark and 18.3 g L^{-1} from HWE bark. These concentrations correspond to 66.4% and 62.3% total yields from hexose polysaccharides to ethanol assuming 0.51 g g^{-1} as the theoretical maximum of ethanol from hexoses. Yield of ethanol from 1 kg of pretreated bark was 178 and 155 L correspondingly. Yield of ethanol from untreated bark was 110 and 114 L which could be increased by reducing vessel losses during pretreatment. If a total hydrolysis yield of 70–75% is expected from hexose polysaccharides according to the hydrolysis results, the fermentation yield from hexoses to ethanol was 83–95%, which can be considered as efficient ethanol production.

According to the results, pretreated spruce bark in this consistency did not contain any inhibitors that would have seriously reduced fermentation yield but instead the materials were fermented relatively efficiently. This behaviour is very different compared to spruce wood which is often severely inhibitory after steam pretreatment (Alriksson et al., 2011). Also Robinson et al. (2002) found that up to 30% addition of bark in Douglas fir whitewood pretreated with SO_2-catalyzed steam explosion had a negligible impact on the fermentation of the pretreatment hydrolysate to ethanol by yeast. Hydroxymethylfurfural and furfural production from bark during steam explosion was found to be significantly lower than from wood probably at least partly because of lower carbohydrate content of bark compared to wood. In our case it could also be speculated that hot water extraction removed some water-soluble phenolic compounds that could otherwise be inhibitory to yeast after steam explosion.

Based on this finding it appears that softwood bark produces less inhibitors during steam explosion than softwood in general. Thus it appears that industrial spruce bark either directly after hot water extraction or after pretreatment by steam explosion is a good feedstock for the production of ethanol. Alternatively, it could be used together with other 2nd generation biomass materials as a source of sugars for fermentation. The hydrolysis yield could probably be improved by using the most developed new commercial cellulase products and by optimising the enzyme mixture more carefully, which would lead to higher total ethanol yield.

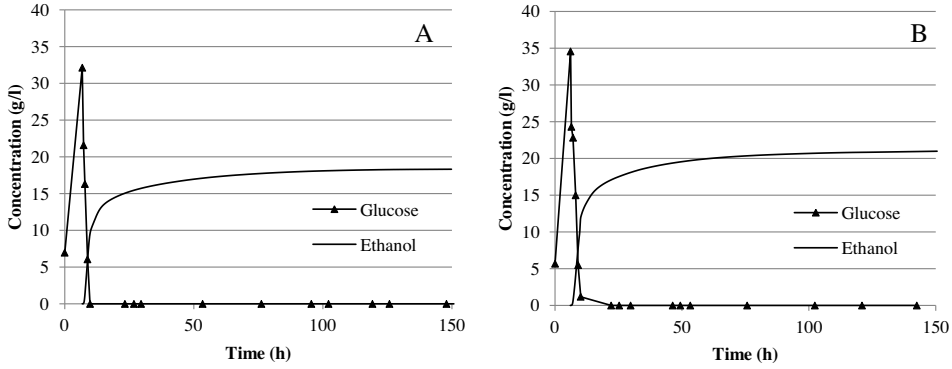

Fig. 4. Glucose release and consumption and ethanol production during the prehydrolysis (6 h) and fermentation of sequentially hot water extracted and steam exploded (A) and hot water extracted (B) spruce bark in 15% consistency.

4. Conclusions

The composition and component yields of industrial spruce bark during two types of pretreatment and their combination were studied and the suitability of the pretreated materials was assessed for 2nd generation bioethanol production. Steam explosion without acid catalyst was found to be an efficient pretreatment improving the hydrolysability of spruce bark. However, hot water extraction was also found to be a sufficient pretreatment for spruce bark when an enzyme mixture containing pectinase is used. Ethanol was efficiently produced from hot water extracted bark suggesting the suitability of hot water extraction as an only pretreatment for spruce bark.

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PUBLICATION III

Spruce bark as an industrial source of condensed tannins and non-cellulosic sugars

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Spruce bark as an industrial source of condensed tannins and non-cellulosic sugars

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ABSTRACT

Norway spruce (*Picea abies*) is an important raw material for the forest industry in Nordic countries. The chemical composition and hot water extraction of spruce bark was studied to find out its potential as an industrial source of condensed tannins. Industrial bark was found to contain a high amount of wood (up to 21%), a sufficient amount of tannin for industrial extraction (10.7% of wood-free bark), and a high amount of non-cellulosic glucose, varying according to the felling season (7.7–11.5% of wood-free bark). Temperature had a major effect on the overall extraction yield. Selective extraction of only tannins or water-extractable carbohydrates was not possible. The extraction was scaled up to pilot-scale and an extract was produced having a promising 50% tannin content. Glycome profiling performed on bark and hot water extracts showed the presence of xyloglucan, pectic polysaccharides and arabinogalactan in bark. In addition the extracts were characterized using size exclusion chromatography and ^{31}P nuclear magnetic resonance spectroscopy. Spruce bark appears to be a promising new source of tannins, however the high content of free, glycosidic, and polymeric sugars in the raw extract may need to be tackled prior to use in applications.

1. Introduction

Increasing energy consumption, depletion of fossil fuel reservoirs, and mitigation of climate change motivate to seek alternatives for petroleum-based chemical industry and transport fuel production. Lignocellulose is the most abundant renewable biomass resource on earth and could offer a sustainable alternative for fuel and chemical production. Softwood bark, especially spruce bark, is regaining interest as a readily available source of valuable precursors and biochemicals. In the past softwood bark was actually used as a tanning agent in e.g. Russia and Poland (Surmiński, 2007). The Finnish forest industry uses on average 23 Mm^3 spruce logs per year producing ca. 0.9–1.3 Mt/a of dry spruce bark calculated with bulk density of 380 kg/m^3 and 10–15% volumetric bark content in logs (Anonymous, 2012). This volume of bark, now being combusted for energy, could provide components suitable for upgrading to higher value products, for instance adhesives, resins and plastics, before being combusted in the end of their life-cycle.

The availability and cost of biomass are of essence when designing feasible biorefinery concepts. The unique composition of the biomass utilized in the particular applications is also important. Interesting opportunities arise when an existing industry is looking for new raw material sources to expand its market portfolio. The tannin industry is currently facing this situation. Tannins are polyphenolic compounds based either on flavan-3-ol monomers (condensed tannins, also called proanthocyanidins), or on gallic or hexahydroxydiphenic acid esters linked to a sugar moiety (hydrolysable tannins). Tannins have molecular weight from 500 to 3000 Da (Bate-Smith and Swain, 1962). Annual production of tannins is ca. 200,000 t out of which condensed tannins cover 90% (Pizzi, 2006). The main use for tannins through the past centuries and still today is leather production, where tannins are used to bind the collagen proteins in animal hides making the leather more flexible and less susceptible to microbial attack (Haslam, 1989). Because of their phenolic nature, tannins can be used to replace fossil-based phenol in many applications such as insulating foams and adhesives (Pizzi, 2006; Tondi et al., 2009). Current main industrial sources of tannin, acacia, quebracho, hemlock, tara, chestnut and sumach species are not abundant enough to provide tannin outside the current markets covering leather tanning, wine industry, animal nutrition, and some industrial uses such as mineral flotation, and oil drilling (Pizzi, 2006). The leather industry is not only looking for collagen binding, but also for the colour of the final tanned leather. On the other hand, the new expanding market of natural polymers is looking for optimal chemical reactivity and physical properties. The tannin industry in general, is looking for new

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inexpensive feedstocks to produce tannin extracts of various qualities for the expanding markets.

The dominant industrial tannin production is based on hot water extraction. The method is also suitable for the extraction of spruce bark tannins, but unlike most current feedstocks, spruce bark has a high content of water soluble extractives other than condensed tannins (Roffael et al., 2000). Up to 33% of spruce bark solids may be extracted with sequential pressurized water extractions (Le Normand et al., 2012). The tannin-rich extract also contains considerable amounts of stilbenes, ash, and sugars in various forms. Optimizing the extraction procedure and understanding the effect of extraction parameters on the component yields is essential when developing applications for tannin. The analysis of the chemical composition of the extract is also highly important since this type of information is lacking in the current literature.

This study describes in detail the composition of spruce bark collected in winter and in summer from an industrially relevant source. We report the effect of the main parameters on the extraction yield, scale up the tannin extraction process from bench-scale to pilot-scale, and report the chemical composition and glycome profile of the barks and the extracts.

2. Materials and methods

2.1. Collection of spruce bark

Two batches of industrial Norway Spruce (*Picea abies*) bark were collected from the debarking unit of Metsä Wood Kertopuu plant (Lohja, Finland). The debarking unit processes fresh moist logs and employs dry debarking technology in the process. The first batch of bark termed as winter bark was collected in February 2012, and larger wood chips were removed manually. The typical felling time is only 1–3 months before debarking, thus the logs were felled between November 2011 and January 2012. Dry matter content of the bark was 39%. The average temperature in January–February 2012 was ca. −6 °C.

The second bark batch termed as summer bark was collected from the same place in August 2012 after a relatively cold summer (ca. 16 °C average temperature in June–August). Dry matter content of the bark was 51%. The trees were felled maximum of three weeks before debarking which was done in the morning of the collection day. No manual separation of wood was carried out except for a small amount (1 kg) for analytical purposes to analyze the composition of the wood-free part of the material.

2.2. Bench-scale extraction

A set of 10 extractions was performed for winter bark in a 15 L pressure cooker rotating 2.3 rpm. The total mass in the extraction was 8 kg out of which 5, 10 or 15% (w/w) was bark (on dry basis). Tap water was heated up to 60, 75 or 90 °C in the reactor, after which warm bark was added into the cooker and timing started. In one experiment 2% of sodium bisulfite and 0.5% sodium carbonate were dosed as percentage of bark dry weight. Extract samples were collected from the reactor after 10, 30, 60, 90 and 120 min and centrifuged for 10 min at 3000 rpm to remove solids. The centrifuged extract samples were stored at −18 °C.

2.3. Pilot-scale extractions

A total of 114 kg of summer bark (on dry basis) was extracted in six separate batches. Extraction was carried out in a 250 L vessel with forced circulation. The bark was placed in 10 μm polypropylene filter bags, which were closed with a cable tie, and the bags were placed in the reactor. The reactor was filled with hot tap water containing the extraction chemicals (2% of sodium bisulfite and 0.5% sodium carbonate), and turbulent circulation with ca. 50 L/min flow rate was started together with heating. Extraction time of 120 min was started when the temperature of the circulation water reached 75 °C. The average solids content of bark in the extractions was 8.8%.

Primary extract was let out of the reactor and the wet extracted bark was washed with ca. 160 L hot (ca. 55 °C) tap water for 5 min. The washing water was let out of the reactor, combined with the primary extract, and concentrated by vacuum evaporation (150 mbar, 50 °C, 200 kg/h evaporation rate). The concentrated extract was spray-dried (210–220 mbar, 175 °C inlet and 75 °C outlet temperature) producing 10.0 kg crude tannin powder with a 6% moisture content. Crude tannin powder was stored in dry conditions in room temperature.

2.4. Yield calculations

Extraction yields related to bench-scale extraction experiments were calculated on mass basis by multiplying the concentration of the compound in the extract sample by the whole mass of water in the system consisting of added water and the moisture contained in the bark. Thus, we assumed that the concentration of dissolved compounds was the same everywhere in the system. The mass of the extracted compound was then divided either by the mass of the original dry bark (yield given as % of bark dry matter) or by the mass of the same compound in the original dry bark (yield given as % of theoretical).

Dry matter and tannin yields for pilot-scale extraction were calculated as above (theoretical extraction yields), but also actual yields were calculated from the amount of the extract that was recovered from the system. The actual yields take into account the losses of solubilized compounds that remain bound to the wet residual bark after extraction.

2.5. Chemical analysis

2.5.1. Extractives and lignin

The content of lipophilic extractives in bark was determined gravimetrically by Soxhlet extraction with heptane from 3 g milled dry bark (extraction time 5 h). The acid insoluble lignin content was determined after the removal of extractives using two-step sulfuric acid hydrolysis (NREL Laboratory Analytical Procedure #003) with minor modifications (60 min incubation in the first step). The acid soluble lignin content was determined according to Goldschmid (1971). The ash content of the samples was measured gravimetrically after combustion in a furnace at 550 °C.

2.5.2. Carbohydrates

The carbohydrate content of bark was analyzed using two methods. Neutral sugars were quantified after the previously described two-step acid hydrolysis using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). HPAEC was run with Dionex ICS-3000 liquid chromatograph (Dionex Corp., Sunnyvale, CA) according to Tenkanen and Siika-aho (2000) with minor modifications (equilibration with 15 mM NaOH, isocratic elution with water). Because acidic sugars degrade in acid hydrolysis, they were quantified by gas chromatography after methanolysis of the sample and silylation of the sugar monomers according to Sundberg et al. (1996). Non-cellulosic glucose content was determined as the glucose released in methanolysis. Cellulosic glucose was determined as the total glucose released in the acid hydrolysis subtracted by the non-cellulosic glucose. Bark carbohydrate content was expressed as anhydrous polymeric carbohydrates.

Soluble free neutral and acidic monosaccharides in the extracts were quantified using the HPAEC-PAD method as described above. The total content of soluble neutral carbohydrates in the extracts

was analyzed by performing a mild acid hydrolysis with 1 part of 72% sulphuric acid and 20 parts of the extract. The mixture was autoclaved at 121 °C for 60 min, and the released neutral monosaccharides were quantified as described above. The total content of soluble acidic carbohydrates was quantified using methanolysis and gas chromatography as described above. The content of bound sugars in the extract was calculated by subtracting the amount of free monosaccharides from the amount of total carbohydrates.

Total starch and β-glucan content of the bark were analyzed using Megazyme's (Ireland) total starch (amyloglucosidase/α-amylase method) and mixed-linkage β-glucan (McCleary method) kits, respectively, according to the manufacturer's instructions.

2.5.3. Tannin and total phenolic content

Tannin content of the summer bark and the dried pilot-scale extract was analyzed according to ISO14088:2012, the so-called hide powder method, which is a quantitative analysis of tanning agents. According to the standard 30 g of dry and ground solid bark sample was extracted 5 times with boiling distilled water collecting in total 1 L of analytical extract. The dried pilot-scale extract containing tannin was dissolved first in water at 4 g/l. A sample (90 mL) of the extracts in water was passed through 7 g of standardized partially chrome-tanned hide powder, which has known reactivity towards polyphenols. Tannin was defined as the dry substance that remained fixed with the hide powder.

Tannin content of the bench- and pilot-scale extracts was analyzed using the acid butanol assay as described by Gessner and Steiner (2005). Commercial solvent purified quebracho tannin product Tannin QS-SOL (Silvateam, Italy) was used to establish the standard curve. Total phenolic content of the extracts was analyzed by using the Folin-Ciocalteu method (Waterhouse, 2003).

2.6. ^{31}P nuclear magnetic resonance (NMR) analysis and molecular size distribution

^{31}P NMR analysis was based on the method developed by Granata and Argyropoulos (1995) and carried out according to Rahikainen et al. (2013). The size exclusion chromatography was carried out according to Rahikainen et al. (2013) using an HPLC system (Waters Corp., MA, USA) equipped with 1000 and 100,000 Å columns (MCX, Polymer Standard Services, Germany) and an UV detector (detection at 280 nm). Results were calculated relative to polystyrene sulphonate sodium salt standard using Waters Empower 2 software.

2.7. Glycome profiling

Glycome profiling of the winter bark was carried out according to Pattathil et al. (2012, 2010). Alcohol insoluble residue (AIR) was prepared from ground dry bark and subsequently extracted with 50 mM ammonium oxalate, 50 mM sodium carbonate (containing 0.5% sodium borohydride), 1 M potassium hydroxide (containing 1% sodium borohydride), 4 M potassium hydroxide (containing 1% sodium borohydride), acidic sodium chlorite at 70 °C and finally again to 4 M potassium hydroxide (containing 1% sodium borohydride). All potassium hydroxide extracts were neutralized with glacial acetic acid. The extracts were dialyzed using 3500 Da molecular weight cut-off tubing and lyophilized prior to subjecting them to enzyme-linked immunosorbent assay (ELISA). The bench- and pilot scale extracts were extracted with 80% ethanol overnight. The insoluble residue was washed, dried, dialyzed and lyophilized similarly to the other extracts according to Pattathil et al. (2012).

Plant cell wall glycan-directed monoclonal antibodies (McAbs) were obtained from laboratory stocks (CCRC, JIM and MAC series) at the Complex Carbohydrate Research Center (available through CarboSource Services; http://www.carbosource.net) or from BioSupplies (Australia) (BG1, LAMP). Details of the McAbs used can be seen in the Supporting Information, Supplement Table 1.

3. Results and discussion

3.1. Composition of spruce bark

Relatively little is published about detailed chemical composition of spruce bark and of its seasonal variation. Here we studied the composition of industrial spruce bark from one Finnish wood products plant collected at two different times of the year: in winter after the growth season and in summer during the growth season.

Winter bark in Table 1 refers to bark collected from the plant in February, out of which the most of residual wood was removed manually. Summer bark collected in August was extracted in pilot-scale without the removal of wood. For analytical purposes a small fraction was manually sorted, and the composition of the bark (79%, w/w) and the wood fractions (21%) were analyzed separately. Based on the gravimetric tannin analysis method used by the tannin industry, the tannin content of summer bark was 10.7%, and the tannin content of the mixture of bark and wood was 8.3%. For comparison the tannin content in current tannin industry raw materials quebracho heartwood and chestnut wood is 25% and 14%, respectively (authors' industrial knowledge). Although the tannin content in spruce bark is not as high as in the currently used tannin raw materials, it may be sufficient for feasible industrial tannin production because of the low price of the bark and its residual nature. Krogell et al. (2012) reported 6% concentration of tannins in inner and 4% in outer fresh spruce bark, whereas Zhang and Gellerstedt (2008) reported 1.7–1.9% of tannins in inner bark and 7.9–8.0% in outer bark. Peltonen, 1981 detected 9.0% of condensed tannins in spruce bark. A wide variety of analysis methods is available for tannin quantification. Contrary to the other recently reported methods, we used a gravimetric analysis in use in the tannin industry.

The carbohydrate profiles of the two wood-free bark fractions were relatively similar, but had a significant difference in the amount of non-cellulosic glucose. The amount of non-cellulosic glucose in general was quite high compared to other hemicellulosic and pectic sugars. In this study more non-cellulosic glucose was found from bark collected after the growth season in winter compared to bark collected during the growth season in summer. It has been reported that some of the non-cellulosic glucose is present in the bark in the form of starch (Painter and Purves, 1960; Le Normand et al., 2012) and callose, which is a type of β-1,3-D-glucan found at least in Scots pine bark (Fu et al., 1972). However, we found that the total starch content was only 0.08% in winter bark and 0.69% in summer bark. The β-glucan contents were 0.41% and 0.05% in winter and summer bark respectively. These two storage polysaccharides could not explain the high non-cellulosic glucose content of spruce bark. It is evident that some glucose is bound as glucosides to stilbenes and other similar bark extractives. Stilbene glucoside content of spruce bark may be as high as 5–10% (Krogell et al., 2012; Kylliäinen and Holmbom, 2004; Mannila and Talvitie, 1992; Zhang and Gellerstedt, 2008). As glucose accounts for 40–45% of the molar mass of stilbene glucosides, the glucose bound to stilbenes may explain a major part of the non-cellulosic glucose found in bark. Non-cellulosic glucose may also be present in the bark as free monosaccharides. Schaberg et al. (2000) found that free monosaccharide content of red spruce stems and needles was the highest in winter, whereas the starch content reached its maximum in late spring. We saw a similar trend here as the non-cellulosic

Table 1
Chemical composition of industrial spruce bark.

	Winter bark, % (w/w)	Summer bark, % (w/w)		
Component	Bark fraction	Bark fraction	Wood fraction	Mixture
Carbohydrates (anhydrous)	51.3	47.6	60.1	50.2
Cellulosic glucose	19.0	20.0	35.3	23.2
Non-cellulosic glucose	11.5	7.7	3.7	6.9
Arabinose	3.7	4.5	0.9	3.7
Galactose	2.2	2.1	1.3	2.0
Xylose	2.8	3.5	3.8	3.5
Mannose	3.1	2.2	11.6	4.2
Rhamnose	0.5	0.6	0.2	0.5
Galacturonic acid	7.4	6.0	1.6	5.1
Glucuronic acid	0.3	0.4	0.8	0.5
O-4-methyl glucuronic acid	0.9	0.5	1.0	0.6
Apparent lignin	33.8	36.8	29.3	35.2
Acid insoluble	31.1	33.5	29.1	32.6
Acid soluble	2.7	3.3	0.3	2.7
Tannin		10.7	0.0	8.3
Ash	3.1	3.1	0.4	2.6
Lipophilic extractives	2.9	2.4	1.0	2.1

carbohydrate content was higher suggesting a higher amount of free monosaccharides, and starch content lower in the winter bark. According to the carbohydrate analysis there were large amounts of pectic polysaccharides containing galacturonic acid in bark as also confirmed by others studies (Krogell et al., 2012; Le Normand et al., 2012). The amount of mannose-containing polysaccharides found abundantly in spruce stem wood was noticeably smaller in bark.

The analysis of lignin content in spruce bark is challenging because water soluble high molecular weight tannins, suberin, and stilbene glucosides precipitate in acidic conditions and show up as acid insoluble material, i.e. Klason lignin (Krogell et al., 2012; Laks, 1991). For example Le Normand et al. (2012) analyzed high amounts of Klason lignin in hot water (100–160 °C) extracts of spruce bark although true lignin is not soluble in water in the extraction conditions. In this study, the bark was pre-extracted only with heptane before lignin analysis. Aqueous acetone and water extractions probably would have decreased the perceived lignin content as described by Krogell et al. (2012). Miranda et al. (2012) analyzed the Klason lignin content of spruce bark after successive extraction with dichloromethane, methanol, ethanol and water. Their analysis result of 26.8% is probably a better estimation of the real lignin content in *Picea abies* bark. The lipophilic extractives content extracted with heptane was higher in our study compared to hexane extractives content of 1.2–2.4% by Krogell et al. (2012). Ash content of spruce bark was considerably higher compared to the ash content of spruce stem wood but on the same level as reported by Miranda et al. (2012). According to them spruce bark ash is composed mainly of nitrogen, calcium and potassium.

3.2. Hot water extraction of spruce bark in bench-scale

A set of 10 bench-scale extractions were conducted to determine the effect of the main parameters (temperature, solids content, the use of chemicals) on the total extraction yield and on the solubilization of the main components in the extract, condensed tannins and sugars. The extractions were carried out for the winter bark. Up to 20.9% of the bark could be solubilized in 120 min of extraction time. Temperature response was evident, as extraction at 90 °C solubilized 19.6–20.9% of bark, whereas extraction at 60 °C solubilized only 14.6–16.1% of bark dry matter. The given yields correspond to the yields obtained in the highest (15%) and the lowest (5%) solids content indicating that the effect of the solids content on the total mass yield was less significant compared to the effect of the temperature. The use of sodium bisulphite and sodium carbonate in the extraction did not increase the total mass yield (18.6% yield with chemicals compared to 19.0% yield without chemicals). Based on the extraction curves showing the time dependency of the extraction yield (data not shown) the total mass yield could have increased a few more percentage points with a prolonged extraction time. Sequential acetone–water and pressurized hot water extractions at 100–160 °C have been reported to dissolve up to 42% of spruce bark dry matter (Le Normand et al., 2012) suggesting that only a part of the hydrophilic compounds in spruce bark were extracted in our experiments. On the other hand Kylliäinen and Holmbom (2004) solubilized 12% of fresh spruce bark at 30 °C within 1 h indicating that a substantial portion of the hydrophilic bark components are already released at low temperatures.

Fig. 1 presents the tannin extraction curves as the amount of solubilized tannin divided by the mass of the bark at the beginning

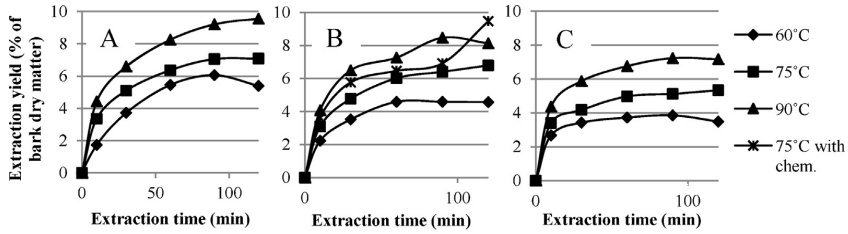

Fig. 1. Extraction yield of tannin from winter bark in bench-scale at varying solids content (A: 5%, B: 10%, C: 15%) and temperature with or without extraction chemicals.

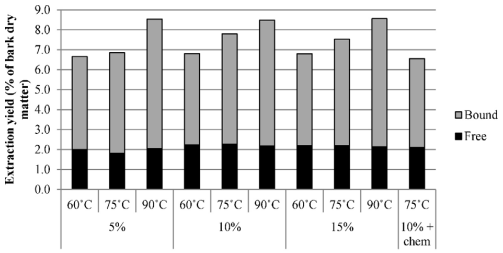

Fig. 2. Extraction yield of sugars from winter bark as free monosaccharides and bound sugars in bench-scale extractions at varying solids content (5–15%) and temperature with or without chemicals.

of the experiment. Considering an approximate 11% tannin content in spruce bark, up to 90% of bark tannins could be extracted without chemicals in 5% solid content at 90 °C (5.0 g/l tannins in the extract) or with chemicals in 10% solids content at 75 °C (10.5 g/l tannins in the extract). However, the tannin yield value reported here is not precise but an estimation, as the tannin analysis method for bark and the extracts were different. Acid butanol method allows fast comparison of the liquid extract samples, whereas the gravimetric method used for dry bark is more time and material consuming and requires drying of the extract. Temperature had a strong effect on tannin extraction yield increasing it 3.5–4.2 percentage points. In addition to temperature, also the solids content affected the tannin yield. Tannin yield reduced 1.8–2.4 percentage points when solids content increased from 5% to 15%. The use of extraction chemicals increased tannin extraction yield up to levels obtained in lower solids content and higher temperature. The highest tannin concentration of 12.6 g/l was analyzed from the extract produced at 90 °C in 15% solids content.

Carbohydrates can be present in the bark extracts in different forms: as monosaccharides, as oligo- and polysaccharides, or bound to stilbenes, or other compounds as glycosides. Here we define monosaccharides as free sugars, and the oligo- and polysaccharides and glycosidic sugars as bound sugars. In our bench-scale extractions the yield of total sugars was 6.6–8.6% of dry bark (Fig. 2). Despite the different extraction conditions the extraction yield of free sugars remained relatively constant at 1.8–2.3% of dry bark, whereas the bound sugars were responsible for the variation in the total sugar yield. Total sugars in the extracts accounted for 18–24% and free sugars 5–6% of the non-cellulosic sugars in the bark. Thus a significant part of non-cellulosic sugars in the bark was not extractable in these conditions. Extraction temperature had a strong positive effect on the extraction yield of bound sugars, whereas the solids content had practically no effect on the yield of bound sugars. Extraction chemicals reduced the solubilization of bound sugars considerably. It appears that there is a certain fraction of free monosaccharides in bark, the release of which was not affected by the changes in the extraction parameters within the tested limits.

Comparison of the extraction yields of individual sugars gives more insight into the type and structure of the bark hemicelluloses and pectins. Table 2 presents the results from the winter bark extraction carried out at 75 °C and 10% solids content using the extraction chemicals. Non-cellulosic glucose was released in the highest amounts from winter bark followed by galactose, glucuronic acid and rhamnose. Only traces of mannose and xylose were released in the extraction suggesting that the polysaccharides containing mannose and xylose are tightly bound in the bark cell wall. Majority of all individual sugars were in bound state in the extract, although a significant part of glucose (39.2%) and galacturonic acid (16.3%) was present as monosaccharides. Some other sugars such as mannose were present as monosaccharides as well, but their total amount in the extract and thus their concentration was very small. Le Normand et al. (2012) studied the extraction of non-cellulosic polysaccharides from bark collected from a sawmill in southern Sweden in May. They were able to extract up to 82% of spruce bark non-cellulosic polysaccharides by pressurized sequential hot water extraction at 100–160 °C, whereas in this study 24–30% of non-cellulosic sugars from the bark were extracted. Mannose and xylose were released least efficiently similarly to our results.

3.3. Scale up of extraction from bench-scale to pilot-scale

Hot water extraction of spruce bark was scaled up to pilot scale to a 250 L volume per batch. In addition to the scale of the extractions, the other differences between the set-ups were the collection time of the bark (winter for bench-scale, summer for pilot-scale) and the amount of wood in the extracted material (higher in the pilot-scale extraction). Extraction parameters (75 °C, 120 min, 10% solids content, 2% sodium sulphite ad 0.5% sodium carbonate) were chosen based on the bench-scale extraction experiments. Although 90 °C extraction temperature would likely have released more tannins, a lower temperature of 75 °C was chosen for practical reasons because the high temperature was feared to cause autocondensation or otherwise negatively modify the bark tannins. On average 11.8% of bark was solubilized during the extraction showing that the efficiency of the extraction was not as high in this set-up as it was in the bench-scale experiments (19.0% yield). At this point on average 70.6% of bark tannin was solubilized. A time series taken from the last batch showed that extraction yield reached close to its maximum in 2 h. A lower extraction yield in pilot-scale was probably due to less efficient mass transfer in the pilot-scale

Table 2
The sugar profile, and the share of free monosaccharides of the total sugars in the corresponding bench- and pilot-scale extracts (75 °C, 10% solids content, 120 min extraction time, extraction chemicals used). For winter bark extract also theoretical extraction yield of individual sugars is presented.

Sugar	Winter bark, bench-scale			Summer bark, pilot-scale	
	Relative amount of sugars, %	Share of free monosaccharides, %	Extraction yield, %	Relative amount of sugars, %	Share of free monosaccharides, %
Non-cellulosic glucose	74.9	39.2	38.5	61.8	6.4
Galactose	8.5	9.9	22.3	5.3	1.9
Mannose	0.8	20.2	1.6	6.7	0.0
Rhamnose	1.1	0.0	12.7	3.1	4.7
Arabinose	2.8	4.7	4.5	7.9	7.9
Xylose	0.8	4.9	1.6	1.0	23.8
Galacturonic acid	8.5	16.3	6.9	11.5	16.9
O-4-methyl glucuronic acid	1.6	3.9	10.1	0.7	100.0
Glucuronic acid	0.9	9.4	20.1	2.0	5.3
Total sugars	100.0	32.1	23.9	100.0	7.8

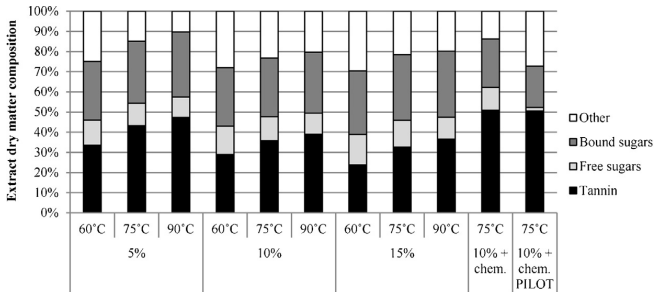

Fig. 3. Composition of bench- and pilot-scale extracts at varying solids content and temperature with or without chemicals.

extraction system. Particle size may have had a larger effect in the extraction using forced circulation and filter bags compared to cooking type of extraction. The higher amount of wood in the summer bark compared to winter bark was expected to slightly decrease the total yield. However, we assume that the extraction efficiency was reduced due to equipment set-up as well.

The extract was drained from the vessel and the bark was washed once with water. Extracts and washing waters were combined. The dry matter analysis showed that 10.2% of bark dry matter could be recovered from the extraction. As a total of 19% of extract dry matter was lost during concentration by evaporation and spray-drying, the final amount of crude tannin powder produced was 10.0 kg (94% dry matter content). Vessel losses in the spray-drying chamber accounted for most of this loss. Tannin yield was 50.3% from the theoretical. Although the high content of wood in the raw material most likely affected the total yields negatively, there remains room for improvement in the pilot-scale extraction set-up to reach yields closer to those obtained in bench-scale experiments. Bocalandro et al. (2012) scaled up 75% ethanol extraction of *Pinus radiata* bark to pilot scale without significant reduction in extraction yields.

3.4. Composition of the bench-scale and pilot-scale extracts

The composition analysis of the spruce bark extract is very important for its industrial exploitation. Many studies present partial results on the composition of spruce bark extracts, however studies covering both phenolic compounds as well as carbohydrates are scarce. Here we present chemical analysis of both phenolics and sugars, molecular weight results as well as glycome profiling data to give complete view on the composition of spruce bark water extracts.

In this study, an extract with over 50% of tannin content could be produced both in bench- (51.0%) and pilot-scale (50.5%) as analyzed by the acid butanol assay. The tannin content of the pilot-scale extract as analyzed by the hide powder method was 49.7%. Weissmann (1981) reports a very similar content of polyphenolic compounds in *Picea abies* hot water extract. The effect of temperature on the tannin content of the extract was positive in our present study, but the most significant difference was observed when extraction chemicals were used. By using the extraction chemicals, which increase the solubility of polyphenolic tannin, the negative effect of solids content could be overcome (Fig. 3). However, based on our results a highly selective extraction of either sugar or tannin is not possible by varying the parameters studied.

Several studies report spruce bark containing condensed tannins, which do not contain a carbohydrate part (Krogell et al., 2012; Navarrete et al., 2011). In contrast, to our knowledge spruce bark has not been reported to contain hydrolysable tannins, in which the subunits are bound to a sugar core. However, there are some speculations that spruce tannins may be bound covalently to sugar moieties (Tišler, 1986). The presence of condensed tannin-stilbene co-polymers has also been reported in spruce bark (Zhang, 2011). These co-polymers might link sugars covalently to tannins, which could partially explain why their selective extraction was not possible.

The total phenolic content of the extracts was measured as gallic acid equivalents using the Folin-Cioucalteau method. The results showed that 23–37% of the bench-scale extract dry matter was phenolic in nature. Total phenolic content of the pilot-scale tannin was 35.8%, which is higher than in the corresponding bench scale extract (30.2%). The structural units of condensed tannins, epicatechin and epigallogatechin, have a higher molar mass per a phenolic hydroxyl group in their structure (61 and 73 g/mol per OH group) than gallic acid (57 g/mol per OH group), which may explain why the tannin content was higher than the total phenolic content.

Phenolic groups were also analyzed using ^{31}P NMR to give additional information on the phenolic nature of tannins (Table 3). Aliphatic hydroxyl groups detected in the analysis are found in monosaccharides but can also be a part of the structure of condensed tannins. According to the ^{31}P NMR analysis the pilot-scale extract had significantly less phenolic hydroxyl groups, slightly more aliphatic hydroxyl groups, and slightly less carboxylic hydroxyl groups compared to solvent purified commercial quebracho tannin used as the reference tannin in the acid butanol assay. Even when the purity of the extracts is accounted for, the phenolic hydroxyl group content of the spruce bark tannin was much lower than that of the reference tannin. Pan et al. (2013) recently reported that there are 3.76 and 2.13 mmol/g phenolic hydroxyl groups in the tannin-containing methanol-water extract of Douglas fir and Loblolly pine respectively. Taking into account the high amount of non-tannin material in our pilot-scale extract, it appears that the

Table 3
The type of hydroxyl groups in the pilot-scale spruce bark extract and a commercial quebracho tannin analyzed by ^{31}P NMR.

Sample	Phenolic	Aliphatic	Carboxylic	Total
Pilot-scale extract (mmol/g)	2.16	4.22	0.18	6.56
Purified commercial quebracho tannin (mmol/g)	14.62	3.99	0.22	18.83

phenolic content of spruce bark tannin is at least on the same level, if not higher, compared to the reported levels in Douglas fir and Loblolly pine barks.

Whereas the tannin contents of the corresponding bench- and pilot-scale extracts were similar, their carbohydrate content and profile differed considerably. Pilot-scale tannin extract contained 20.5% bound sugars and 1.7% free monosaccharides, which is 14% less bound sugars and 85% less free monosaccharides compared to the bench-scale extract suggesting a very different pattern of carbohydrate release. Table 2 allows the comparison of the sugar profiles and the amount of free monosaccharides in the extract between the bench- and pilot-scale extractions. The pilot-scale extract contained less glucose, galactose, and methyl-galacturonic acid, but more mannose, arabinose, rhamnose, xylose, galacturonic acid and glucuronic acid. The presence of wood in the extracted material can be seen clearly in the high content of mannose, but it does not explain all the differences. For example galacturonic acid content of the pilot-scale extract was higher although there is less galacturonic acid in wood compared to bark. Thus it appears that the extractability of the bark polysaccharides also differed between winter and summer barks.

Considerably less glucose, galactose, mannose were present as free monosaccharides whereas more arabinose, xylose and methyl glucuronic acid were present in free form in the pilot-scale extract compared to the bench-scale extract. Le Normand et al. (2012) reported that 8–16% of sugars in their pressurized hot water extracts were present as monosaccharides, which is in between our values for the bench- (32.1%) and pilot-scale extracts (7.7%). Autohydrolysis is not a likely cause for the high concentration of monosaccharides in the bench-scale extract, because extraction temperature was below 100 °C, and pH in the range of 4.5–5.9. We suspect that the variation in the free sugar content of the extracts may be caused by the different felling time of the trees. This conclusion is supported by Weissmann (1984), who showed that the amount of free sugars in spruce bark extracts is the lowest in May/June and highest at the end of the growing season. The lower amount of extracted free sugars from summer bark may have also been partially responsible for the lower total extraction yield obtained in pilot-scale.

The high content of carbohydrates in the extract may reduce its reactivity, increase viscosity and in general reduce suitability for various applications. Therefore it is expected that a fractionation method needs to be developed to lower the sugar content of the extract and enrich the phenolic fraction in it. Interestingly a pine tannin extract containing 30% carbohydrates was found susceptible for thermal degradation at 150 °C, but the reduction of the carbohydrate content to 5% increased the thermal stability of tannin by 50 °C (Gaugler and Grigsby, 2009). Removal of sugars from the extract may not only increase its relative phenolic content but also improve its stability in further processing.

From the technical point of view, it should be noted that the ash content of the pilot-scale extract was found to be fairly high, 26.5%. Based on yield calculations, it can be concluded that the ash came from both the native ash in the bark and also from the inorganic extraction chemicals. The pilot-scale extract contained 0.9% lipophilic extractives accounting for 4% of the lipophilic compounds in the bark.

Molecular size distribution of the bench- and pilot-scale extracts were almost identical having number average molar mass of 1.85 kDa and 1.90 kDa, weight average molar mass of 3.40 kDa and 3.20 kDa and polydispersity of 1.84 and 1.68 respectively. The similar size distribution of the extracts supports the theory that extractable material in the bark samples was similar despite the sugar fraction extracted as monosaccharides, which varied according to the season. Pan et al. (2013) report number averages of 2.15 and 3.28 kDa, weight averages of 5.71 and 11.6 kDa, and polydispersity of 2.66 and 3.54 for Douglas fir and Lobololly pine bark tannins, respectively. The results indicate that spruce bark tannins have a lower molecular weight and narrower size distribution than Douglas fir and Loblolly pines tannins.

3.5. Glycome profiling of spruce bark and the extracts

The glycome profiling analysis was conducted for sequential chemical extracts of winter bark cell walls in order to study overall cell wall glycan compositions in spruce bark (Fig. 4). The increasingly harsh extractions generated a set of six extracts enriched with cell wall glycans based on the tightness, with which they are integrated into the bark cell walls. The extracts were loaded on to the ELISA plate in an equal sugar basis and they were subsequently probed with a comprehensive suite of glycan directed McAbs that can monitor most major plant cell wall polysaccharides excluding cellulose (Supplemental material). The glycome profiling studies conducted here with the winter bark sample was thus instrumental to reveal the overall composition and extractability of major non-cellulosic plant cell wall glycans present in it.

Oxalate extract from winter bark cell walls contained mainly pectin as indicated by the significant binding of McAbs belonging to homogalacturonan backbone 1 and 2 (HG BACKBONE-1 and 2) and rhamnogalacturonan (RG-I) backbone groups. Also, a significant binding of one antibody, JIM137 that belonged to RG-1b group was evident. In carbonate extract, however, most significant binding was exhibited by McAb groups recognizing xyloglucans (XG) especially those belonging to groups XG-1 through 2 and Fucosylated xyloglucans (FUC XG). A marginal abundance of pectic epitopes was noted in this extract as indicated by very low binding of HG BACKBONE-1 and RG-I backbone groups. Both 1 M KOH and 4 M KOH extracts showed largely similar McAb binding patterns with higher abundance of xyloglucan (both fucosylated and non-fucosylated), xylan (indicated by binding of xylan-5 through 7 groups), pectic backbone (indicated by binding of HG BACKBONE-1 and RG-I backbone groups), pectic-arabinogalactan (indicated by binding of RG-I/AG group) and arabinogalactan (indicated by binding of AG-2 through 4 groups) epitopes. Interestingly, in the 4 M KOH extract, the strength in the binding of xylan and pectic backbone antibodies was significantly lesser than that in 1 M KOH extract. Chlorite extract mainly contained xyloglucans, xylan, pectic-backbone and arabinogalactan epitopes hinting that lignin in winter bark cell walls may be associated with these glycans. The 4 M KOHPC (post chlorite 4 M KOH) extract was largely similar to other KOH extracts (1 M and 4 M KOH) in its glycan composition. The main distinction noted in this extract (4 M KOHPC) was the significantly enhanced binding of two McAbs belonging to xylan-6 group (CCRC-M139 and CCRC-M140) in comparison to other base extracts (1 M and 4 M KOH). This could be the result of lignin removal during chlorite extraction causing these epitopes to be released from the wall.

Painter and Purves (1960) fractionated white spruce bark by successive extractions and suggested that the inner bark may contain chemically linked glucose and xylose residues in type of heteropolymeric glucoxylans. According to the glycome profiling results this polysaccharide could actually be xyloglucan. The presence of homogalacturonan, rhamnogalacturonan, and arabinogalactan in bark has now also been shown based on their detection by monoclonal antibodies.

Hot water extracts prepared from winter (bench-scale) and summer barks (pilot-scale) were also ELISA-screened with the entire toolkit of glycan directed antibodies (Fig. 4). Interestingly, extractable glycan epitopes were present in significantly higher levels in the winter bark water extracts compared to summer bark water extract. Xylan epitopes were absent in the summer bark extract while the winter bark extract contained significant

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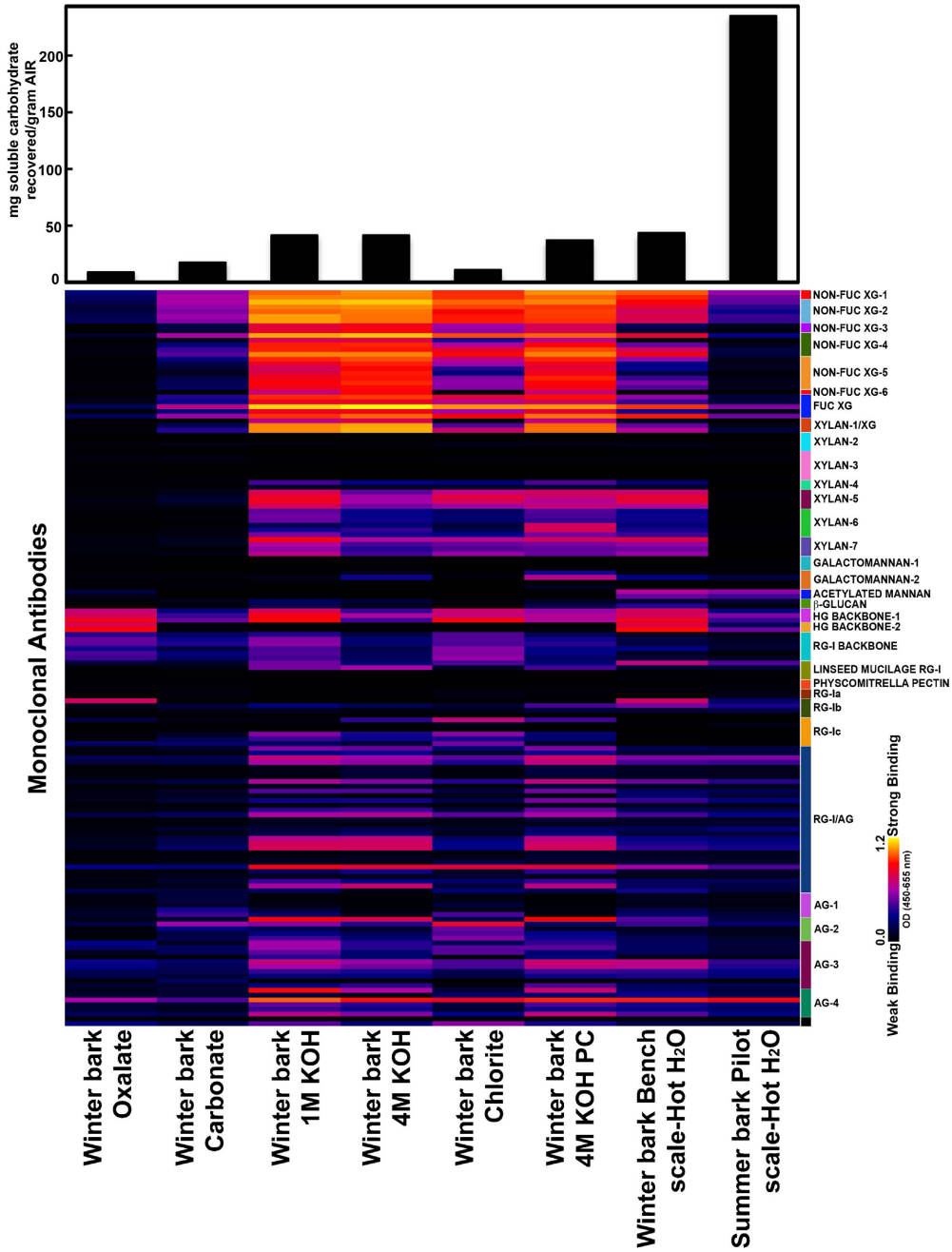

Fig. 4. Glycome profiling of cell wall extracts isolated from spruce winter bark (first 6 columns) and winter and summer bark hot water extracts prepared in bench- and pilot-scale (last 2 columns). The amount of carbohydrate recovered per gram of alcohol insoluble residue (AIR) is depicted as a bar graph on top of the figure. The monoclonal antibodies (McAbs) are grouped on the basis of the glycan classes they recognize and are listed as in the right hand side panel of the figure (a full list of antibodies is given in Supplement material 1). The strength of McAb binding is represented as heatmap with a black–blue–red–yellow–white colour scheme with white colour depicting strongest binding and black no binding. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

abundance of xylan-5 and 7 epitopes. Both winter and summer bark extracts contained epitopes of xyloglucan, pectic-backbone, pectic-arabinogalactans and arabinogalactans. In general hot water appears to have extracted similar compounds as the analytical oxalate, carbonate and KOH extractions. The relative strength of McAbs binding to these epitopes in the summer bark extract was significantly lower compared to winter bark extract. Since the extracts were loaded on to the ELISA plate on equal sugar basis, either there were more oligo- or polysaccharide epitopes present not recognized by the glycan directed antibodies, or more sugars were bound as glycosides to non-carbohydrate structures in the summer extract. Because of the dialysis carried out prior to loading, the sugars must have been bound to structures not passing the 3.5 kDa cut-off membrane. Stilbene glucosides isorhapontin and astringin found in spruce bark (Krogell et al., 2012) have molar mass below 0.5 Da and should have penetrated the dialysis membrane. Thus it appears that these bound sugars not recognized by the glycan directed antibodies in the summer extract could have been bound to larger tannin molecules or some other molecules present in the extract. It is also worth noting, that the sugar yield from the alcohol insoluble part of the summer extract was significantly higher as seen in Fig. 4 bar graph. The high yield was caused by better retention of sugars during the dialysis as the sugar content of the dialyzed extracts was on the same level (479 mg/g in the winter bark extract compared to 475 mg/g in the summer bark extract). The higher content of free monosaccharides in the winter bark extract and their removal during dialysis has at least partially contributed to the lower mass yield observed with the winter bark extract. The results shed light to the nature and extractability of non-cellulosic sugars in bark, but it remains open where and how the sugars not present in these hemicellulosic and pectic epitopes are bound.

4. Relevance of the results for biorefinery concepts using spruce bark

Spruce bark is an abundant but undervalued feedstock for a lignocellulosic biorefinery. The fact that it contains considerable amounts of tannin and water soluble sugars in addition to insoluble cellulose, hemicellulose and lignin makes it different from most lignocellulosic feedstocks. This study provides information on the composition of spruce bark as well as the extraction of tannins from spruce bark and helps to evaluate the potential of spruce bark as a source of precursors and intermediates for the production of chemicals, materials, and fuels.

The relatively high amount of wood in industrial bark streams was discovered in this study. Similarly, also Ngueho Yemele et al. (2013) observed a high content (19.9%) of wood in industrial spruce bark from a sawmill in Quebec, Canada. Even small amounts of bark may disturb pulping and the production of veneers, therefore debarking is conducted at a loss of some stem wood. The composition of wood and bark differ greatly, and thus experimental results obtained with a pure bark stream may not be repeatable in industrial scale. Some adjustments in the debarking depth may be possible to achieve, but sorting the residual wood out of the bark stream prior to extraction is probably not feasible.

The debarking method affects the bark quality and effluent production. Here we worked with bark from a dry debarking process, where the bark is in contact with water only during log de-icing (Bajpai, 2010). In the wet debarking process the bark becomes wet and is pressed prior to combustion releasing water soluble substances into the debarking effluent. Tannins and stilbene glycosides make up the main soluble material in these sometimes very concentrated effluent streams (Kylliäinen and Holmbom, 2004). In case a biorefinery is planned at a location using wet debarking technology, the effluent composition and the possibility to extract valuable components from it should be investigated. Debarking effluents have high biological and chemical oxygen demand and contain pollutants toxic to aquatic life (Bajpai, 2010), which is why their exploitation may also serve another purpose as waste water management.

We used simple unpressurized hot water extraction for spruce bark, but several studies report use of solvents, pressure, or other types of extraction systems. For example a laboratory scale pressurized fluid extraction was found more effective than supercritical carbon dioxide, or conventional solvent extraction in extracting antioxidants from spruce bark (Co et al., 2012). Although analytical extraction procedures use various solvents and their aqueous mixtures, the yield obtained with pure water was so high, that it is probably not worth replacing water with less sustainable organic solvents requiring expensive recycling. The reduction of particle size and size classification may lead into rough fractionation of bark components. Miranda et al. (2012) reported the fines fraction of spruce bark to contain more ash (5.3%) and extractives (25.3%) than the medium and coarse fractions (1.2–3.2% ash, 20.6–21.5% extractives). Nevertheless, the fractionation does not appear efficient enough to justify applying it in a larger scale. Extraction chemicals such as urea and sodium metabisulphite can be used in the extraction to prevent autopolymerisation and the formation of phlobaphenes (Sealy-Fisher and Pizzi, 1992). In this study the chemicals were found to improve the extraction yield of tannin and increase the relative tannin content of the extract. It depends on the application whether the partial sulphonation of the hydroxyl groups in the condensed tannin structure is accepted.

Tannin is the main water soluble component in spruce bark, but also other interesting possibly high-value compounds are extracted in a hot water treatment. Stilbene glucosides isorhapontin, astringin, and piceid may account for up to 7.2% of *Picea abies* bark dry weight (Krogell et al., 2012). These compounds and their stilbene counterparts have been shown to possess antileukaemic and other bioactive properties in many studies (Mannila and Talvitie, 1992; Shen et al., 2009). However, the separation of these compounds from tannin may be difficult in industrial scale.

As described by this study, various types of sugars were extracted from bark alongside tannin. It appears that highly selective extraction of tannins or sugars is not possible using hot water as the solvent. Among the extracted sugars are at least pectic and hemicellulosic poly- and oligosaccharides, sugar attached to other compounds as glycosides, and free monosaccharides. The amount and type of sugars extracted appear to be affected by the seasons, as higher amount of non-cellulosic glucose and free monosaccharides were found in the bark and the water extracts from trees felled in the winter. The amount and type of sugars in the crude bark extract affects its behaviour in various applications. The presence of a high amount of free sugars may reduce the microbial stability of the product, and a high amount of sugars and other non-phenolic compounds reduce the overall reactivity of the extract. It remains to be seen what level of reactivity is needed for possible applications, and whether a sugar removal step is needed for the crude extract prior to use as a source of renewable phenolic compounds. Further understanding on the nature and binding of the carbohydrate fraction in the extracts is needed to be able to design an efficient and inexpensive method for their removal from the extract. Glycome profiling could possibly be used to further study and predict the behaviour of bark in extraction at various times of the year.

The water-soluble bark sugars may not only be a nuisance but instead provide additional value as raw material for future biorefineries. The extracts contain mainly C6 sugars, which are natural substrates for many microbes used in bioprocesses. Another abundant sugar, galacturonic acid may also be fermented to e.g. ethanol by some strains (Grohmann et al., 1998) or used as an acidic agent

in food and cosmetic industry. The water soluble xyloglucan in the extract could possibly be interesting for drug-delivery technologies, food technology and in textile industry applications (Mishra and Malhotra, 2009). The insoluble sugars in the residue may be hydrolysed to monosaccharides with enzymes even without a separate thermal or chemical pretreatment (Kemppainen et al., 2012). The residual lignin has the highest calorific value out of all the compounds in bark (White, 1987) and could provide good quality fuel for a possible combustion plant on site. Soil improvement and landscaping are other options for the lignin-rich residue in addition to the currently developed lignin applications in adhesives, resins and dispersants. Spruce bark contains a combination of high and low value precursors, the exploitation of which should be clarified. Tannin, however, is one of, if not the most promising compound in spruce bark being easily extractable, reactive and constituting over 50% of the crude bark extract dry matter.

5. Conclusions

Spruce bark, having a sufficiently high content of tannin and good availability, could be a promising new feedstock for industrial tannin production. Extracts with up to 50% tannin content could be produced in pilot-scale in unpressurized conditions using water as the solvent. Temperature had a major effect on the extraction yield, but the composition of the extract and especially its sugar fraction was also affected by the felling season of the logs. The forms in which the sugars exist in the extract, partially identified by glycome profiling in this study, should be well understood to be able to develop a method for their removal to increase the phenolic content of the extract.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2013.10.009.

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PUBLICATION IV

Hydrolysis and composition of recovered fibres fractionated from solid recovered fuel

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Hydrolysis and composition of recovered fibres fractionated from solid recovered fuel

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HIGHLIGHTS

- Recovered fibres were fractionated from solid recovered fuel and characterised.
- Recovered fibres have a high content of carbohydrates and ash.
- The carbohydrates in recovered fibres can be hydrolysed to sugars with enzymes.
- The effect of solids loading and surfactants on enzymatic hydrolysis was studied.

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ABSTRACT

Fibres fractionated from solid recovered fuel (SRF), a standardised market combustion fuel produced from sorted waste, were considered as a source of lignocellulosic fermentable sugars. The fibre yield from four samples of SRF was 25–45%, and the separated material consisted of 52–54% carbohydrates, mainly glucan, with a high content of ash (12–17%). The enzymatic digestibility of recovered fibres was studied at low and high solids loading and compared with model substrates containing only chemical and mechanical pulps. Above 80% hydrolysis yield was reached at 20% solids loading in 48 h, but variation was observed between different samples of recovered fibres. Surfactants were found to improve the hydrolysis yield of recovered fibres especially in tumbling-type of mixing at low solids loading, where hydrolysis was found to stagnate without surfactants. The results suggest that SRF is a potential source of easily digestible lignocellulosic carbohydrates for use in biorefineries.

1. Introduction

Sugars are raw material for microbial and chemical processes producing biofuels such as ethanol and butanol, polymer precursors like lactic and succinic acid, and other chemicals including xylitol and furfural (Menon and Rao, 2012). In addition a vast fermentation industry producing e.g. amino acids, antibiotics and industrial enzymes is using starch- or sucrose based glucose as a raw material. As the sustainability of using sugars from the food chain for the production of non-food products has been disputed, there is a need to find alternative, non-food sources of sugar for the non-food industry. Various lignocellulosic feedstocks could, in this respect, serve as an interesting alternative. The first commercial-scale lignocellulosic ethanol plants are currently under construction or have recently started operations (Balan et al., 2013). Corn stover, sugar cane bagasse, wheat straw and the energy crop *Arundo donax* are favoured as feedstocks in these first industrial plants. Other potential biomass sources besides agricultural residues are forest industry related biomass streams. Native woody streams like logs, harvest residues, saw dust and bark, and some more processed streams like pulp and paper mill sludges are rich in carbohydrates, but may be expensive or challenging to process. Recovered pulp and paper industry products are an alternative non-food source of sugars, which could be used for

Abbreviations: CBH, cellobiohydrolase; CHP, combined heat and power; EG, endoglucanase; GMO, genetically modified organism; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; MUL, 4-methyl-umbelliferyl-β-D-lactoside; PEG, polyethylene glycol; SRF, solid recovered fuel; TMP, thermomechanical pulp.

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the production of transport fuels and chemicals instead of being directly combusted to energy.

Solid recovered fuel (SRF) is defined as a solid fuel prepared from non-hazardous waste meeting the classification and the specification requirements laid down in the standard EN15359 (ERFO, 2013). SRF is typically municipal solid, industrial, or commercial waste, which is homogenised and upgraded to a quality that can be traded amongst producers and users. SRF is a heterogeneous fuel with a net calorific value from 3 to above 25 MJ/kg depending on the source. The current production of SRF is circa 12 Mt/a in the EU, however, the potential is much higher, estimated at circa 70 Mt/a (Straetmans, 2010). The production of refuse-derived fuel (RDF), which is shredded and sorted but not standardised, is even larger (Rotter et al., 2011). SRF is utilised for energy production in cement kilns, coal-fired power plants, lime kilns, industrial boilers and combined heat and power (CHP) plants reducing the amount of waste going to landfill. The largest European CHP and power plant capacities for SRF are currently in Germany, Finland and Sweden (ERFO, 2013).

The lignocellulose content of SRF is a key attribute as it is the climate neutral part of the fuel (Flamme and Geiping, 2012). Astrup et al. (2009) estimated the paper content of SRF to be 54% of dry content, and reviewed the biogenic carbon content to be from 45% to 85% of the total carbon, which covers 45% of SRF dry weight. This biogenic carbon present mostly in the form of cellulosic and non-cellulosic carbohydrates offers a waste-based low-cost source of fermentable sugars alternative to agricultural residues, which are seasonal and more recalcitrant. The fractionation of carbohydrates from SRF in the form of recovered fibres, and their recalcitrance towards enzymatic hydrolysis are the key factors in producing fermentable sugars from SRF. We have recently published pilot-scale trial data on an interesting biorefinery concept consisting of the fractionation of SRF, the hydrolysis of recovered fibres, ethanol fermentation and biogas production (Kemppainen et al., 2012). What is yet to be reported are the fractionation yields of recovered fibres from SRF, their composition, and the detailed behaviour of the material in enzymatic hydrolysis compared to its pure main constituents, chemical and thermomechanical pulp. The present literature on similar concepts is typically based on experiments at low processing consistency although a relatively high processing consistency appears to be necessary for feasible conversion processes of lignocellulosics (Modenbach and Nokes, 2013). It is thus important to understand the effect of solids loading on the hydrolysis yield of a particular feedstock. Surfactants are widely used chemicals that have been shown to improve the enzymatic hydrolysis of several different types of biomass (Eriksson et al., 2002). Assessing the effect of surfactants on a new type of biomass can bring down enzyme costs in an envisioned industrial plant. This paper analyses the suitability of recovered fibres fractionated from solid recovered fuel as a source of sustainable fermentable sugars.

2. Methods

2.1. Raw materials, enzymes and chemicals

SRF samples were received from three suppliers in the United Kingdom and one supplier in Finland. A sample of pulp and paper mill fibre sludge was received from an integrated pulp and paper mill in Finland. Never-dried birch and spruce kraft pulps, received as samples from Finnish pulp mills, were mixed in ratio of 51/49 w-% for use in hydrolysis experiments. A model substrate was composed by mixing birch kraft pulp, spruce kraft pulp and Finnish spruce thermomechanical pulp in ratio of 35/34/31 w-%.

Several different enzyme mixtures were constituted from nonpurified monocomponent enzymes provided by Roal Oy, Finland. The basic enzyme mixture contained Cel7A from *Acremonium thermophilum* (cellobiohydrolase I) and Cel5A from *Thermoascus aurantiacus* (endoglucanase), and was supported by the addition of Cel6A (cellobiohydrolase II) from *A. thermophilum* or *Chaetomium thermophilum,* Cel7B (endoglucanase) from *Trichoderma reesei*, xylanase from *Nonomuraea flexuosa* or *T. aurantiacus,* mannanase from *T. reesei* and β-glucosidase from *T. aurantiacus* or *A. thermophilum*. Protein content of the enzymes used in Figs. 1–3 was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA), which is based on the Lowry assay using bovine gamma globulin as standard. Protein content of the enzymes used in Figs. 4 and 5 was measured using Bovine Serum Albumin as standard.

The nonionic surfactant products used in the experiments were PEG 4000 (Merck, Germany), Lutensol AT 50 Flakes (BASF, Germany), and Softanol 90 (Ineos, Switzerland). Other chemicals were analytical or technical grade depending on their purpose of use.

2.2. Fractionation of SRF

Fibres were fractionated from SRF in small (7.5 kg dry weight SRF treated per batch) and large pilot-scale (300 kg SRF treated per batch). Fibre batches A, B and C were produced by re-pulping the SRF samples from the United Kingdom in small pilot-scale for 20 min at 5% consistency (150 kg total mass) in a 200 L and a 11 kW batch pulper (Tampulping, Finland). The disintegrated fibre sludge was let out of the reactor through a sieve plate with 3 mm width holes. The reject was washed 3 times with a total of 130 L water. The sludges and washing waters from two pulping batches were combined, and pH was adjusted to 5 using strong sulphuric acid. The fibre sludge was dewatered in a decanter centrifuge producing a stream of recovered fibres at 34–38% dry matter content, and heat treated at 95 °C for 60 min to reduce microbial load.

Batch D fibres were fractionated from SRF from the same supplier as batch A fibres, but in large pilot-scale using a 8 m^3 pulping tank with a 110 kW motor. Sample E was fractionated similarly to sample D but before pH adjustment a volume of dilute pulp and paper mill fibre sludge was added to make up 25% of the dry matter of the total sludge. Heat treatment was conducted after adjustment of pH with strong phosphoric acid. The dewatering of the recovered fibres was carried out on a belt press to produce circa 320 kg (on dry) recovered fibres per batch at 43% average dry matter content.

2.3. Hydrolysis experiments

Hydrolysis experiments were carried out as triplicates in two experimental setups: in test tubes in a water bath under magnetic mixing, and in round plastic bottles in a rotating drum placed in a heat cabinet. Conditions for test tube hydrolysis were: 1% solids content, 3 ml total volume, 400 rpm magnetic mixing. Conditions for bottle hydrolysis were: 1–25% solids content, 50 ml total volume (40% bottle fill volume), 24 rpm of the rotating drum. The bottles were moving freely in the rotating drum. Temperature was controlled at 50 °C and sodium acetate buffer with 0.02% sodium azide was used in all experiments. Buffer strength of 50 mM was used in the test tube experiments, whereas in the bottle experiment the final buffer strength in the reaction was adjusted to 100 mM. Enzyme mixtures were dosed as mg protein per g of substrate using dosages in the range of 4–16 mg/g. Surfactants were dosed 1% w/w of the substrate dry weight and added to hydrolysis by dissolving them to the buffer. Hydrolysis was stopped by boiling

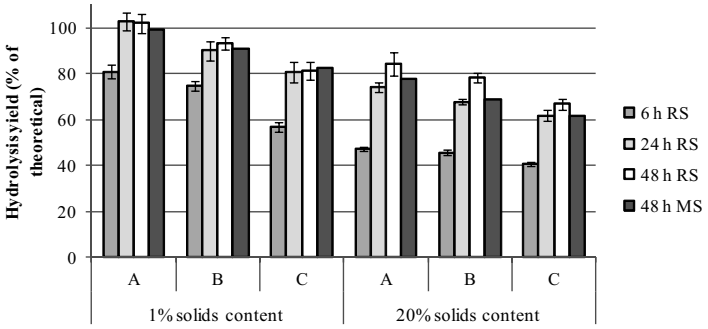

Fig. 1. Enzymatic hydrolysis of recovered fibres from SRF batches A–C at low (1%) and high (20%) initial solids content, 1% w/w Lutensol AT 50. RS = reducing sugars, MS = monosaccharides. The basic enzyme mixture was supplemented with *Acremonium thermophilum* Cel6A, *Trichoderma reesei* Cel7B, *Thermoascus aurantiacus* xylanase, *Thermoascus aurantiacus* β-glucosidase, and *Trichoderma reesei* mannanase (total enzyme dosage 8 mg/g).

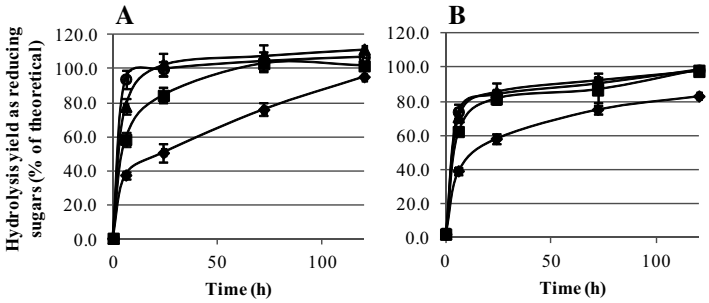

Fig. 2. Dosage response curves for chemical pulp (A) and recovered fibres from batch E (B) at low (1%) initial solids content. Enzyme dosages: Open circles 16 mg/g, triangles 12 mg/g, squares 8 mg/g, diamonds 4 mg/g. The basic enzyme mixture was supplemented with *Acremonium thermophilum* Cel6A, *Trichoderma reesei* Cel7B, *Nonomuraea flexuosa* xylanase, *Acremonium thermophilum* β-glucosidase, and *Trichoderma reesei* mannanase.

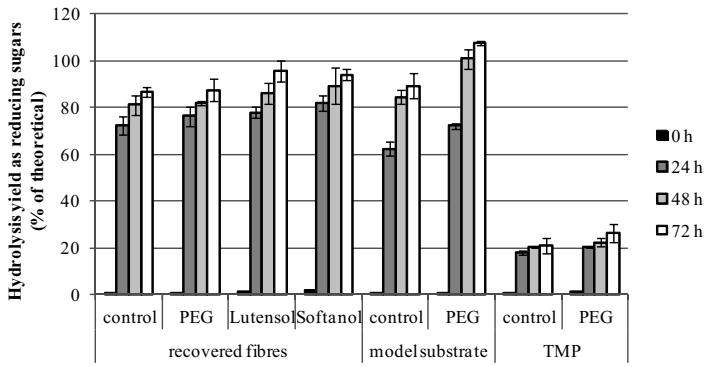

Fig. 3. Hydrolysis of recovered fibres (batch E), model substrates, and TMP in low initial solids content (1%) with different surfactants (1% w/w). The basic enzyme mixture was supplemented with *Acremonium thermophilum* Cel6A, *Trichoderma reesei* Cel7B, *Nonomuraea flexuosa* xylanase, *Acremonium thermophilum* β-glucosidase, and *Trichoderma reesei* mannanase (total enzyme dosage 4 mg/g).

a sample for 15 min, and insoluble solids were removed by centrifuging the sample. All samples taken from the bottle set-up were pre-diluted by a factor of ten before solids removal to eliminate the effect of high content of water insoluble solids on yield calculations (Kristensen et al., 2009). Hydrolysis yields were expressed as percentage of the theoretical maximum hydrolysis yield.

2.4. Analysis of chemical composition and hydrolysis products

The composition analysis of the fibre samples and the analysis of hydrolysis products were carried out as described by Kemppainen et al. (2012) except the acid soluble lignin content of fibre samples A–D was analysed according to Goldschmid (1971). As the acid soluble lignin content was very small, the

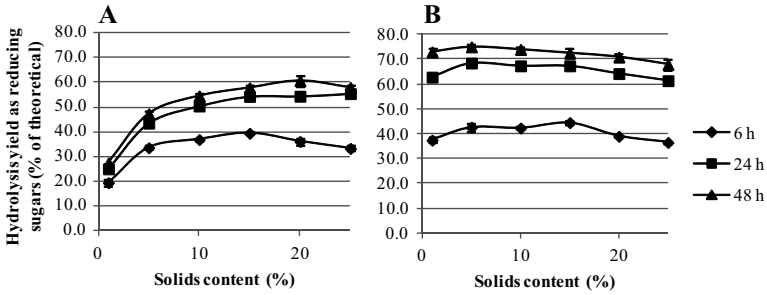

Fig. 4. Enzymatic hydrolysis of recovered fibres (batch E) under gravitational mixing at varying solids loading without (A) or with (B) surfactant (1% w/w Lutensol AT 50). The basic enzyme mixture was supplemented with *Chaetomium thermophilum* Cel6A, *Thermoascus aurantiacus* xylanase and *Thermoascus aurantiacus* β-glucosidase (total enzyme dosage 6 mg/g).

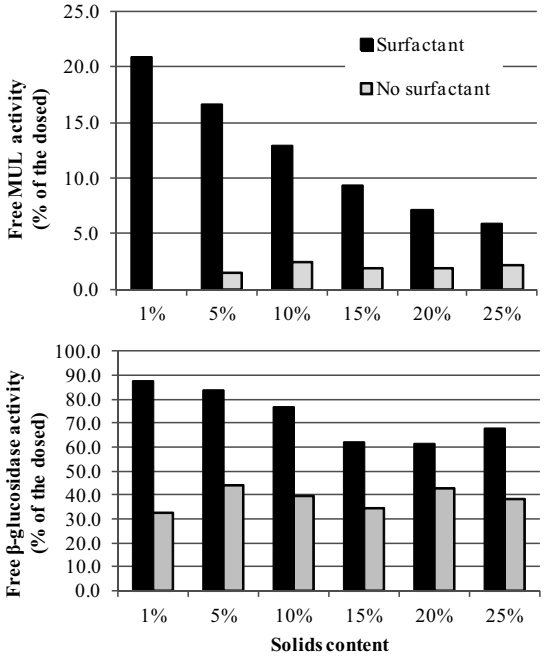

Fig. 5. Free MUL- and β-glucosidase activity in the solution after 48 h hydrolysis with or without surfactant at 1–25% solids loading. The basic enzyme mixture was the same as in Fig. 4.

change of method is not expected to have a significant effect on the reliability of the results. The elemental composition of ash was analysed by inductively coupled plasma mass spectrometry after microwave assisted hydrofluoric acid–nitric acid–hydrochloric acid dissolution.

2.5. Enzyme activity assays

The cellulase activity present in the hydrolysis supernatants was assayed using 4-methyl-umbelliferyl-β-D-lactoside (MUL) according to Bailey and Tähtiharju (2003) except that the only inhibitor compound used in the experiment was glucose. β-glucosidase activity was assayed using 4-nitrophenyl-β-D-glucopyranoside as described by Bailey and Nevalainen (1981).

3. Results and discussion

3.1. Separation of recovered fibres from SRF

Prior to using the fibre components from SRF as a source of lignocellulosic sugars, they have to be separated from the plastics and other main components in the feedstock, and chemically characterised. This paper, to the authors' knowledge is the first description of separation yields and the chemical composition of the fractions. The first target was to study the yield and composition of recovered fibres separated from SRF obtained from three different suppliers. Visually the fuel samples looked very different from each other. The SRF used to produce the fibre sample A had the smallest particle size (circa <5 cm) and most homogenous appearance being composed only of soft plastics, paper, and board. Sample C SRF had the largest particle size and contained hard objects like metal cans, pieces of wood, hard plastics, and rocks, that were removed manually prior to pulping. The particle size of the SRF for sample B was between the previous two, and the material contained long flexible pieces of hard plastic that were removed prior to pulping. The separation of recovered fibre batches A–C was carried out by high force repulping in small pilot scale to disintegrate the paper and board components. The fibre sludge was let out of the reactor through a sieve plate and the fractionation yield was increased by washing the reject 3 times with water. The dry matter content of the combined sludge and washing water was between 0.8% and 1.1%. Depending on the sample 35–44% of the dry mass of the SRF was recovered in the combined sludge and washing waters forming the accept (Table 1). The accept contained some small pieces of plastics but the majority of plastics was retained in the reject. The differences in yields may reflect the fibre content

Table 1
Separation yields and chemical composition of recovered fibres, batches A–E.

Batch	A	B	C	D	E
Fractionation yield, %	44.1	41.1	34.7	51.2	n.a.
Dewatering yield, %	75.7	64.4	72.3	87.1	n.a.
Total yield, %	33.4	26.5	25.1	44.7	n.a.
Carbohydrates, % of dry matter	53.6	53.8	52.4	52.4	53.8
Glucan	43.1	43.4	42.8	42.2	40.9
Xylan	6.7	6.4	5.5	6.6	7.8
Mannan	2.9	3.1	3.2	2.9	3.9
Arabinan	0.4	0.5	0.4	0.3	0.4
Galactan	0.4	0.5	0.5	0.4	0.7
Lipophilic extractives, % of dry matter	8.2	3.6	5.3	6.3	6.4
Acid insoluble material, % of dry matter	19.7	22.8	24.2	24.0	23.4
Acid soluble lignin, % of dry matter	0.6	0.6	0.8	0.6	0.5
Ash, % of dry matter	11.9	17.0	15.0	12.5	12.7

of the SRF sample but also the type and origin of the fibre components. Products prepared using wet-strength chemicals like liquid packaging boards, paper bags and some tissues disintegrate less efficiently and may remain in the reject. In efficient industrial pulping equipment a slightly increased particle size may be beneficial for the process limiting the size reduction of the plastics during pulping and thus reducing the amount of impurities in the fibre fraction. In this study the SRF sample with the smallest particle size and most homogenous appearance produced the highest fractionation yield, but as observed later, also had a high content of carbohydrates.

The separation efficiency of dewatering carried out using a decanter centrifuge is affected by the pumping rate and the particle size of the material. The dry matter content of the filtrate was 0.3–0.4%, whereas the dry matter content of the dewatered fibre fraction was 34–38%. The dewatering yields achieved ranging from 64% to 76% probably reflect the content of fines in the fibre sludge as some insoluble material was lost during dewatering despite of the slow pumping rate of the slurry to the decanter centrifuge. Ash and other material solubilised during pH adjustment and very fine insoluble ash may also constitute a part of the dry matter lost during dewatering.

Fractionation of SRF for batch D fibres in large pilot scale reached higher fractionation and dewatering yields compared to small pilot-scale. It is suspected that the disintegration was more efficient in the large pilot scale leaving fewer fibres in the reject. In the belt press the fibres may function as a filter aid improving the retention of fines. Overall the reported total yields in this study are expected to present the minimum, which can be reached, when fractionating fibres from SRF in a suboptimal pilot scale equipment. A process designed especially for these materials should be able to increase the total yield up to 50–55% and produce a relatively clean reject stream that could be either combusted or even recycled for plastics. In this case, assuming 54% paper-based content in SRF (Astrup et al., 2009) and 90% yield in fractionation, the plastic and other impurities would account for 3–12% of the recovered fibre fraction. However, it must be noted that also the paper-based fraction contains ash, lignin and other non-carbohydrate components.

3.2. Composition of recovered fibres

The composition of the recovered fibres was analysed (Table 1). The results indicated large variations between different suppliers and fractionation scales. Batches A–D comprised only fibres from SRF, but the batch E also contained 25% pulp and paper mill fibre sludge. The largest variation between the samples was observed in the content of ash and lipophilic extractives. Lipophilic extractives extracted with heptane may contain native wood extractives but also chemicals used in inks, glues and coatings. Ash in the recovered fibres is composed of mainly inorganic coating and filler materials, such as kaolin, calcium carbonate, and talc. Main inorganic elements found from the batch E materials were Si (2.0% of dry weight), Ca (1.7%), Fe (1.2%), Al (0.6%), Mg (0.3%), P (0.3%), S (0.2%) and K (0.1%). Si, Ca, Al and Mg originate from the abovementioned filler materials, but the source of iron can only be speculated. For comparison, Kang et al. (2010) concluded that the ash in a sample of kraft paper mill primary sludge (36%) was composed mainly of calcium carbonate, clay and titanium oxide. Both the ash and the extractives content of the recovered material is greatly affected by the type of the fibre product since the majority of these compounds do not originate from wood. The same applies at least partially to the acid insoluble material termed as Klason lignin for wood, because it covers also acid insoluble plastic impurities in the material. In addition it contains the acid insoluble portion of the ash. Chemical pulp contains 3–5% lignin, and the lignin content of mechanical pulp is close to that of native softwood, 25–35% (Biermann, 1996). Chemical pulp is used in sack papers, the lining of particle boards, some wrapping papers, liner boards, and on the top ply of packaging boards as well as in printing papers. Mechanical pulp often forms the centre ply of packaging board and is used in newspapers and other print products. Recycled fibre pulp is commonly used in making packaging materials and it may be composed of varying combinations of chemical and mechanical pulp. Wang et al. (2013) report 17.1% acid insoluble lignin in newspaper, 4.7% in office paper, 13.9% in magazines and 14.2% in cardboard. The determination of the actual lignin content of our materials would require additional separation methods, but it can be estimated that Klason lignin comprised less than 70–80% of the acid insoluble material.

There was little variation in the carbohydrate content or the monosaccharide profile of the recovered fibres. Having a high content of C6 sugars (50.6–52.2% as monosaccharides) the material is very suitable for concepts carrying out only C6 fermentation with common non-GMO yeasts. The content of xylan and mannan reflects the fact that the material is a combination of hardwood and softwood pulps. For example the xylan contents of bleached kraft hardwood and softwood pulps are 25% and 7.4% respectively, whereas their mannan contents are 0.5% and 6.6% (Sjöholm et al., 2000). High content of xylan could suggest a higher content of chemical pulp since mechanical pulp is made solely from softwood. A material similar to these fibres, paper pulp derived from MSW, was found to contain 55% glucan, 12% xylan and 6% arabinan/galactan/mannan (Puri et al., 2013). The higher total carbohydrate content (73%) was probably reached by removing ash by washing during the production of the paper pulp, as the ash content of the material was only 3%. Negative side to ash removal by washing is the loss of fines, which decreases the yield of easily hydrolysable material. For comparison, Kinnarinen et al. (2012) report 78% carbohydrate content, 11.5% lignin content and 9% ash content in cardboard waste.

The variation between the batches in the most important component regarding the production of sugars, the carbohydrates, was small. However, a similar carbohydrate content does not guarantee that the hydrolysability of these carbohydrates is also similar, as they may originate from many paper and board grades. The non-native extractives, ash and acid insoluble materials may likewise have a large impact on the behaviour of the material in enzymatic hydrolysis. In this work the scale-up of fractionation (comparison between batches A and D) produced a higher fractionation yield and increased the amount of acid insoluble material in the fibre fraction. It is possible that the fibre products containing more mechanical pulp, and thus lignin, have been more resistant to disintegration. In a more efficient pulping also they disintegrate increasing the fractionation yield and the relative amount of acid insoluble material in the fibres. Fibre sludge from a mill producing chemical and mechanical pulp and paper contains fillers and coating chemicals as well, which may contribute to the fraction of acid insoluble material in addition to lignin. However, the plastic residues present in fibres recovered from SRF are lacking from pulp and paper mill fibre sludge. Thus, although the content of acid insoluble material is similar in batches D and E, it is possible that the batch E contains more lignin, because it contains 25% fibre sludge. Based on the results, the composition of the recovered fibres is influenced by the source material and the fractionation efficiency.

3.3. Enzymatic hydrolysis of recovered fibres

The recovered fibres from batches A–C fractionated in the same set-up were hydrolysed with low and high solids loading with an enzyme mixture containing cellulases and hemicellulases (Fig. 1).

According to reducing sugar analysis the hydrolysis yields and rates were affected by both the source of the raw material and the solids loading. Batch A fibres hydrolysed the fastest and reached the highest hydrolysis yield resulting in practically a complete hydrolysis of the carbohydrates in 24 h. Batch C fibres hydrolysed slower and reached a lower yield at low solids loading (82%) even after 48 h. The lower hydrolysis yield obtained with batch C fibres could be caused by limited accessibility of the matrix, increased non-productive binding of the enzymes, or inhibition of the enzymes by some solubilised compounds. Batch C contained more acid insoluble material, which could correlate with a higher lignin content and explain the reduced hydrolysis yield due to limited accessibility and non-productive binding. Based on the results a high content of lipophilic extractives does not affect negatively the hydrolysis yield of the material.

Hydrolysis with high solids loading was in general slower and the final yield was decreased. However, relatively good hydrolysis yield was achieved already in 6 h showing promise for commercial concepts employing high consistency conditions in the process. HPAEC-PAD analysis of the released monosaccharides supported the conclusions. The monosaccharide concentration was close to the amount of reducing sugars at 1% solids content, but 8–12% less at 20% solids content. One explanation for this finding could be the higher relative share of oligosaccharides present in the high consistency hydrolysate due to stronger end-product inhibition. These oligosaccharides give response in the reducing sugar assay in addition to monosaccharides, and they may also be further hydrolysed in the assay conditions. For all materials glucan and xylan hydrolysed to monosaccharides most efficiently ($\geqslant$89% hydrolysis yield) at 1% solids content compared to mannan ($\leqslant$10%), arabinan ($\leqslant$46%), and galactan ($\leqslant$7%). Glucan and xylan hydrolysed more efficiently compared to other carbohydrates at 20% solids loading as well, but their hydrolysis rate was clearly reduced in 20% solids concentration as compared to 1% solids concentration. The poor hydrolysability of galactoglucomannans in recovered fibres was previously reported by our group (Kemppainen et al., 2012). Additional accessory enzymes such as mannosidases and galactosidases could help to increase to overall hydrolysis yield.

According to the results the hydrolysability of the carbohydrate fraction of SRF varies from supplier to supplier and could also vary from batch to batch. This fact needs to be taken into account when designing process concepts. The hydrolysis is at least partially limited by the recalcitrance of the material, especially the mechanical pulp portion. Thus adaptation is needed to accommodate the process for changes in the feedstock.

3.4. Comparison of recovered fibres to chemical and thermomechanical pulp

The hydrolysis of recovered fibres and chemical pulp was compared to evaluate the effect of mechanical pulp and impurities in the material compared to almost pure cellulose and hemicellulose (Fig. 2). Chemical pulp used in the experiment was a mixture of spruce and birch kraft pulps with 70.3% glucan content, 15.2% xylan and 3.2% mannan content. Its behaviour in the hydrolysis suggested that the hydrolysis rate was limited only by the enzyme dosage. On the other hand, the hydrolysis rate and yield of recovered fibres appears to be limited by the structure and accessibility of the material and possibly also inhibition of enzymes. Hydrolysis rate was on the same level with dosages from 8 to 16 mg/g indicating that the hydrolysis was mainly limited by accessibility.

To better determine the effect of thermomechanical pulp (TMP) in the material, a model substrate was created comprising of birch kraft pulp, spruce kraft pulp and spruce thermomechanical pulp in ratio of 35/34/31. The ratio of the components was chosen so that the monosaccharide profile of the model substrate was as close as possible to the profile of batch E fibres. Thermomechanical pulping of wood retains the chemical structure of wood fibres close to their native form, as only small amounts of water soluble polysaccharides are removed during pulping. TMP is resistant to enzymatic hydrolysis because its high content of softwood lignin is masking the carbohydrates and preventing the swelling of the fibre (Mooney et al., 1998) and causing non-productive adsorption and possible denaturation of the enzymes (Eriksson et al., 2002). Fig. 3 shows the results from the hydrolysis of recovered fibres, model substrate and TMP. Recovered fibres hydrolysed the fastest suggesting that they in fact contain perhaps less TMP than the model substrate. As the enzyme mixture was dosed as protein per gram of dry matter, it must be noted also that the enzyme dosage per gram of carbohydrate was higher for recovered fibres. Comparison to the model substrate also suggests that the impurities in the recovered fibres do not cause enzyme inhibition at least in low consistency conditions. The hydrolysis yield of the model substrate shows that TMP fibres can be hydrolysed to some extent, since the material contained 31% TMP but reached a hydrolysis yield higher than 69%. The hydrolysis yield of TMP alone was very low compared to the other substrates. It appears that the enzymes became soon non-productively bound and possibly inactivated halting the hydrolysis reaction before it was limited by the recalcitrance of the material. Probably the addition of fresh enzyme to TMP hydrolysis would have increased the hydrolysis yield further. The results are supported by Mooney et al. (1998) who observed a large difference in the hydrolysability of kraft pulp and mechanical pulp, as well as Li et al. (2012) who reached only 11% hydrolysis yield with TMP. However, Mooney et al. (1998) do not speculate on the possibility of non-productive binding of enzymes on lignin which was later discovered as one of the major factors limiting the hydrolysis.

3.5. High consistency hydrolysis of recovered fibres

To evaluate the suitability of a feedstock to industrial production of lignocellulosic sugars, the hydrolysis experiments have to be conducted at high solids concentration. Without high solids loading in the hydrolysis, the resulting low end product concentrations challenge the feasibility of the product separation and the overall concept. Increasing the solids concentration in enzymatic hydrolysis affects the system in many ways including effects on end-product and other inhibition mechanisms, mass transfer issues, and challenges with mixing (Modenbach and Nokes, 2013). Increasing solids loading has been reported to cause the so called 'solids effect', where at increasing substrate concentration the corresponding hydrolysis yield decreases (Kristensen et al., 2009).

The hydrolysis reaction for recovered fibres was carried out in round plastic bottles, which were freely tumbling inside a rotating drum. Very little yield decrease was observed in the high consistency hydrolysis of recovered fibres (Fig. 4). It appears that the aforementioned 'solids effect' is weak on recovered fibres making them a promising feedstock for biorefinery concepts where high end-product concentration is critical. However, an interesting difference was observed between this experimental set-up and the test-tube set-up, as hydrolysis yield in 1% solids content was dramatically reduced in the rotating drum. Only 27.4% hydrolysis yield was reached, which indicates that the enzymes were not working optimally in the reaction conditions. Hydrolysis yield increased as a function of the solids content up to 15% solids content, after which the yield at 6 h started to decrease even though the final yield remained at 58–61%. It appears that the enzymes suffered from the free-fall mixing at low solids loading resulting in most likely enzyme inactivation and thus stagnation of the hydrolysis. Magnetic mixing in test tubes is apparently gentler

for the enzymes in low substrate loading, as the hydrolysis yield of this material at 1% loading in test tubes resulted constantly in yields above 90% (data not shown). Le Costaouec et al. (2013) show similar results for pretreated spruce and wheat, where 2% consistency produced lower yields compared to 10% consistency under combined gravity and vortex mixing. However, the effect was less dramatic and was not discussed in detail in the paper. In our work the hydrolysis yields calculated from HPAEC-PAD analysis were constantly slightly lower but otherwise aligned with the reducing sugar yields. The yields based on HPAEC-PAD analysis of monosaccharides and post-hydrolysed oligosaccharides varied between 88% and 91% of the corresponding reducing sugar yield after 48 h hydrolysis (data not shown). Post-hydrolysis of the samples with mild acid hydrolyses oligosaccharides to monosaccharides. Therefore the difference between reducing sugar assay and HPAEC-PAD analysis results is caused by something else than different the reducing ends of oligosaccharides, for example the different response of the reducing sugar assay on different monosaccharides, or the presence of other reducing compounds in the substrate.

Cellulase and β-glucosidase activities were measured after the hydrolysis from the supernatants to see whether the amount of residual enzyme activity in the supernatant correlates with the hydrolysis yields or solids loadings. MUL is a substrate for certain classes of endoglucanases, cellobiohydrolases and β-glucosidases. MUL activity was analysed using glucose to inhibit β-glucosidase activity, and it thus shows the cellobiohydrolase and endoglucanase activity in the solution. As CBHII and EGII cannot hydrolyse MUL, the measured activity correlates with CBHI and EGI enzyme activities (Reinikainen, 1994). It needs to be noted that whereas the results show the residual activity in the solution after the hydrolysis, they do not show whether the rest of the enzyme is adsorbed on solids or inactivated in the solution. The analysis of MUL activity from the supernatants showed very low amounts of free activity in the liquid phase independent of solids loading. The activity loaded at 1% solids concentration was low and close to the detection limit of the analysis, thus no conclusions should be drawn on the zero activity measured at 1% solids loading. The free β-glucosidase activity was significantly higher compared to MUL activity and did not show correlation with the solids loading. Similarly Varnai et al. (2011) have shown that *Trichoderma reesei* Cel7A (CBHI) and Cel5A (EGII) remain highly adsorbed on steam pretreated spruce and Avicel at low substrate concentration, whereas β-glucosidase remains mostly free in the solution. This is natural as cellobiose is the substrate for β-glucosidase and it is soluble. In a later study it was found that the adsorption of intact Cel7A is even stronger at high solids loading compared to low solids loading (Varnai et al., 2013). According to these results, the free enzyme activity in solution did not correlate with solids loading in the hydrolysis of recovered fibres.

3.6. Effect of surfactants on enzymatic hydrolysis of recovered fibres

In an attempt to increase the hydrolysis yield of recovered fibres with low enzyme dosages the effect of surfactants was studied, as they have been shown to improve enzymatic hydrolysis (Ooshima et al., 1986; Helle et al., 1993; Eriksson et al., 2002). Two non-ionic surfactants Lutensol and Softanol, and polyethylene glycol (PEG) were dosed as 1% w/w of the substrate together with buffer to the reaction medium, and the effect of PEG was measured also on the model substrate and TMP (Fig. 3). Small positive effect could be detected with Lutensol and Softanol on recovered fibres. PEG notably increased the hydrolysis yield of the model substrate both in the beginning and the end of the hydrolysis. PEG addition did not significantly improve the hydrolysis yield of TMP.

PEG is composed of a polymerised ethylene oxide which is a carbon chain where every third atom is oxygen. Thus the hydrophobic carbon–carbon areas and more hydrophilic surroundings of the oxygen atom alter rapidly. Lutensol and Softanol products are composed of a polymerised ethylene or propylene oxide chain bound to a strongly hydrophobic saturated fatty alcohol. Softanol has 9 and Lutensol has on average 50 ethyl oxide groups whereas PEG 4000 is composed of 91 ethyl oxide groups. A PEG product and a non-ionic surfactant having an ethylene oxide chain of similar length have been shown to produce a positive effect of similar magnitude on steam pretreated spruce (Börjesson et al., 2007a) suggesting that the poly(ethylene oxide) part of the non-ionic surfactant is more important than the alkyl chain. Most effective surfactants have been shown to contain more than 75 ethylene oxide units in their structure (Börjesson et al., 2007a). Nevertheless, the alkyl chain appears to have contributed to the positive effect of Softanol and Lutensol on recovered fibres because of the lack of effect of PEG on recovered fibres in this experiment.

PEG adsorbs on lignin through hydrophobic interaction and prevents the deactivation of enzymes by exclusion of enzymes from lignin surfaces (Börjesson et al., 2007b). Improvements in hydrolysis yield and rate have been shown on steam pretreated spruce but not on delignified spruce (Börjesson et al., 2007a). PEG does not adsorb on Avicel (microcrystalline cellulose), but nevertheless improves its hydrolysis rate slightly (Börjesson et al., 2007b). PEG appears to improve hydrolysis yield most efficiently on materials with good enzyme accessibility and a relatively high content of lignin, such as the model substrate in this experiment. Jensen et al. (2011) found no positive effect of PEG on thermally treated municipal solid waste, which contains same components as the materials in this paper. The positive effect of surfactants containing the fatty acid part could be related to some non-fibre constituents in the material and their interactions together. The lack of effect on TMP may be related to the dosage of the surfactant. Non-ionic surfactants may improve the hydrolysis of crystalline substrates by varying the adsorption balance of endo- and exoglucanases. Surfactants may prevent the non-productive attachment of endoglucanases to the cellulose surface after reaction, which prevents the access of the saccharifying exoglucanase enzymes to the newly formed cellulose chain ends (Ooshima et al., 1986).

The effect of surfactant Lutensol AT50 was studied at varying solids loading to evaluate its effect in conditions required in industrial processes (Fig. 4). Interestingly, applying 1% of non-ionic surfactant (0.1 g/kg concentration in the reaction medium at 1% solids content) restored the hydrolysis yield at low solids loading in the rotating drum. Surfactant addition improved hydrolysis yield 166% at 1% solids content, 58% at 5% solids content, and 17–35% at solids content of 10–25%. There was a slight reduction in the final hydrolysis yield towards increasing solids content when surfactant was used. For comparison, Ma et al. (2011) obtained 5–30% improvement with surfactant Tween 80 in the hydrolysis of pretreated cassava bagasse at 10–25% solids loading. Kim et al. (2007) report the increase of hydrolysis yield from 49.5% to 70.6% on newspapers when using surfactant Tween-80 in low consistency hydrolysis.

The effect of surfactants on the free MUL activity after hydrolysis was clear and now correlated negatively with the solids loading (Fig. 5). The most dramatic difference was observed at 1% solids loading where now a significant portion of the dosed MUL activity was free in the liquid phase. Surfactant also increased the free β-glucosidase activity in the liquid phase. Also Börjesson et al. (2007b) reported a decrease in enzyme adsorption when using surfactants on pretreated spruce. They found 10.0% of the original Cel7A (CBHI) and 13.0% of Cel7B (EGI) activity (measured by ρ-nitrophenyl-β-D-cellobioside) in the solution after 6 h adsorption

to steam pretreated spruce on a well plate. The addition of PEG increased the amount of free activity correspondingly to 13.0% and 21.1% showing a larger effect on Cel7B. The positive effect of PEG on free enzymatic activity on isolated pure lignin was even larger compared to pretreated spruce. Park et al. (1991) reported significant increases in hydrolysis yields when using non-ionic surfactants, and correlate the improvement to decreased enzyme adsorption, suggesting that the effect comes from the surfactant's ability to improve enzyme desorption.

Results reported here show that an increase in free MUL activity positively correlates with improvement on the hydrolysis yield on this substrate. It appears that the surfactant was able to prevent the enzyme inactivation that took place in the rotating drum at low solids loading. The positive effect of surfactants could be related to their suggested capability to shelter enzymes from inactivation by shear forces, thermal inactivation, and non-productive adsorption resulting in denaturation of the enzyme on the lignin surface. The positive effect at high solids loading could also originate from the viscosity reducing effect of the surfactants (Modenbach and Nokes, 2013). It appears more probable that the surfactant in these experiments prevented thermal and/or shear force induced inactivation of the desorbed enzymes at low solids loading. High substrate concentration increases the possibility of enzyme-substrate interaction (Varnai et al., 2013), which would suggest that if the main effect was the prevention of non-productive adsorption, the improvement would be the largest at high solids loading. As a conclusion it appears that surfactants could be used to protect enzymes from inactivation in the hydrolysis of recovered fibres in high consistency conditions relevant to industrial concepts.

4. Conclusions

Fibres fractionated from SRF are a distinct source of fermentable sugars, unique in their origin and high carbohydrate and ash content. Although variation was detected between fibres from different SRF suppliers, SRF-derived fibres can in general be hydrolysed rapidly to soluble sugars even at high solids loading. The TMP present in the material slows down the hydrolysis compared to chemical pulp, but does not fully stop it. Non-ionic surfactants can be used to improve hydrolysis yield and rate, and they appear to shield enzymes from inactivation caused by tumbling type of mixing at low solids loading.

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Title	**Production of sugars, ethanol and tannin from spruce bark and recovered fibres**
Author(s)	Katariina Kemppainen
Abstract	Valorisation of forest industry-related side- and waste streams in a biorefinery context could help to reduce dependence on fossil resources and introduce new value chains and sources of income for the forest industry. This thesis examined two abundant and underutilized biomass streams spruce bark and recovered fibres as biorefinery feedstocks for the production of sugars, ethanol and tannin. Spruce bark was found to contain 11-12% tannin and 48-51% carbohydrates, mainly cellulose, pectin and non-cellulosic glucose. Up to 21% of spruce bark dry matter could be solubilised by hot water extraction at 60-90°C and the results indicated that a selective extraction of only tannins or carbohydrates is not possible in these conditions. The resulting spruce bark extracts were found to contain up to 58% tannin and 22-32% carbohydrates, out of which a minimum of 55% could be enzymatically hydrolysed to monosaccharides. Steam explosion solubilised pectin and hemicellulose, and increased the enzymatic digestibility of spruce bark carbohydrates from 36% to 75%. Hot water extracted bark could be hydrolysed efficiently (80% hydrolysis yield) without steam explosion when an enzyme mixture containing pectinase activity was used, indicating that an additional pretreatment step is not needed. Recovered fibres were fractionated in pilot scale from solid recovered fuel (SRF), a standardised combustion fuel composed mainly of packaging waste, and the composition and enzymatic digestibility of the material were determined. A fibre yield of 25-45% was obtained in the pilot-scale fractionation of three different SRF samples, and produced material contained at least 46% hexose polysaccharides and 12-17% ash. The enzymatic digestibility of recovered fibres was found to be high without pretreatment and the hydrolysis yield of recovered fibres in high consistency conditions was found to be higher than the hydrolysis yield of steam pretreated wheat straw and spruce. Non-ionic surfactants improved the hydrolysis yield of recovered fibres and the results indicated that their effect is dependent on the botanical source, pretreatment and lignin content of the feedstock as well as the mixing regime. Selected steps for processing spruce bark and recovered fibres were scaled up from laboratory- to small pilot scale. Up to 22 kg of crude tannin powder was produced from spruce bark representing a 9% yield from dry bark. Ethanol production was demonstrated from recovered fibres in seven up to 12-day long continuous pilot experiments according to the FibreEtOH concept. The results of the work carried out in this thesis indicate that the biorefinery concepts presented for spruce bark and recovered fibres have technical potential for industrial application.
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Nimeke	**Sokerien, etanolin ja tanniinin tuotto kuusen kuoresta ja jätekuidusta**
Tekijä(t)	Katariina Kemppainen
Tiivistelmä	Metsäteollisuuden sivu- ja jätevirtojen hyötykäyttö biojalostamossa voisi vähentää riippuvuutta öljystä ja luoda teollisuuden alalle uusia arvoketjuja ja tulonlähteitä. Tässä väitöstyössä selvitettiin kahden mittavan biomassavirran, kuusen kuoren ja jätekuidun käyttöä biojalostamossa sokerien, etanolin ja tanniinin tuotannon raaka-aineena. Kuusen kuori sisälsi 11–12 % tanniinia ja 48–51 % hiilihydraatteja, pääasiassa selluloosaa, pektiiniä ja ei-selluloosaperäistä glukoosia. Jopa 21 % kuoresta liukeni kuumavesiuutossa (60–90 °C), ja lämpötilan nosto vaikutti positiivisesti uuttosaantoon. Tulosten perusteella voitiin kuitenkin todeta, ettei tanniinien tai hiilihydraattien selektiivinen uutto ole mahdollista tällä tekniikalla. Uutteet sisälsivät jopa 58 % tanniinia ja 22–32 % hiilihydraatteja, ja vähintään 55 % uutteen hiilihydraateista pystyttiin entsymaattisesti hydrolysoimaan monosakkarideiksi. Höyryräjäytys liuotti pektiiniä ja hemiselluloosaa ja paransi kuoren hydrolysoitavuutta 36 %:sta 75 %:iin. Kuumavesiuutettu kuori hydrolysoitui tehokkaasti (80 % saanto), kun käytettiin pektinaasia sisältävää entsyymiseosta. Tämän perusteella voitiin todeta, ettei erillistä esikäsittelyvaihetta tarvita. Jätekuitu fraktioitiin pilot-mittakaavassa erilleen standardoidusta kierrätyspolttoaineesta (SRF, solid recovered fuel), joka sisältää pääasiassa pakkausjätettä. Kuitusaanto kolmesta kierrätyspolttoainenäytteestä oli 25–45%, ja tuotetut materiaalit sisälsivät vähintään 46 % heksoosipolysakkarideja ja 12–17 % tuhkaa. Jätekuidun havaittiin hydrolysoituvan tehokkaasti ilman erillistä esikäsittelyä, ja jätekuidun hydrolyysisaanto korkeassa sakeudessa oli korkeampi kuin esikäsitellyn oljen ja kuusen. Varauksettomat surfaktantit paransivat jätekuidun hydrolyysisaantoa ja tulosten perusteella voitiin todeta, että ilmiöön vaikuttavat myös materiaalin alkuperä, esikäsittely, ligniinipitoisuus ja sekoitustapa hydrolyysin aikana. Valikoidut vaiheet kuusen kuoren ja jätekuidun prosessoinneista vietiin laboratoriosta pilot-mittakaavaan. Kuusen kuoresta tuotettiin enimmillään 22 kg raakatanniinijauhetta, joka vastasi 9 % saantoa kuivasta kuoresta. Etanolin tuotanto jätekuidusta osoitettiin viidessä jatkuvatoimisessa 5–12 päivän pituisessa pilot-kokeessa Fibre-EtOH-konseptin mukaisesti. Työn tulosten perusteella kuusen kuorella ja jätekuidulla on teknistä potentiaalia teollisen mittakaavan biojalostamon raaka-aineiksi.
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Production of sugars, ethanol and tannin from spruce bark and recovered fibres

Valorisation of forest industry-related side- and waste streams in a biorefinery context could help to reduce dependence on fossil resources and introduce new value chains and sources of income for the forest industry. This thesis examined two abundant and underutilized biomass streams spruce bark and recovered fibres as biorefinery feedstocks for the production of sugars, ethanol and tannin.

Hot water extraction of tannins from spruce bark, steam explosion to reduce the recalcitrance of the feedstock towards hydrolytic enzymes, enzymatic hydrolysis of bark carbohydrates and fermentation of released sugars to ethanol were demonstrated and the effect of main process parameters studied. Recovered fibres were fractionated from solid recovered fuel, a standardised combustion fuel composed mainly of packaging waste, and the composition and enzymatic digestibility of the material were determined. The effect of pretreatment, solids loading and the use of surfactants on hydrolysis yield was studied. Selected steps for processing spruce bark and recovered fibres were scaled up from laboratory- to small pilot scale.The results of the work carried out in this thesis indicate that the biorefinery concepts presented for spruce bark and recovered fibres have technical potential for industrial application.

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