

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Process development for platform chemical production from
agricultural and forestry residues

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CHALMERS UNIVERSITY OF TECHNOLOGY
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Cover: Illustration of a typical biorefinery system from feedstock production to the main product leaving the biorefinery

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“The best way of predicting the
future is shaping it.”
Willy Brandt

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ABSTRACT

As part of a bio-based economy, biorefineries are envisaged to sustainably produce platform chemicals via biochemical conversion of agricultural and forestry residues. However, supply risks, the recalcitrance of lignocellulosic biomass, and inhibitor formation during pretreatment impair the economic feasibility of such biorefineries. In this thesis, process design and assessment were developed with the aim of addressing these hurdles and improving the cost-effectiveness of lignocellulose-derived platform chemicals.

To expand the feedstock base and reduce operational costs, logging residues served as underutilised and inexpensive raw material. The major impediment in converting logging residues was their high recalcitrance and low cellulose content, which resulted in low attainable ethanol titres during simultaneous saccharification and co-fermentation (SSCF). Pretreatment optimisation reduced inhibitor formation and recalcitrance, and led to enzymatic hydrolysis yields at par with those obtained for stem wood, despite the less favourable chemical composition. Upgrading logging residues with carbohydrate-rich oat hulls increased ethanol titres to $>50 \text{ g}\cdot\text{L}^{-1}$ using batch SSCF at 20% WIS loadings, demonstrating the potential to further decrease downstream processing costs.

To alleviate the toxicity of inhibitors generated during pretreatment, preadaptation was applied to *Saccharomyces cerevisiae*. Exposure to the inhibitors in the pretreated liquid fraction improved ethanol production during subsequent fermentation. Transferring the concept of preadaptation to lactic acid production by *Bacillus coagulans* cut the process times by half and more than doubled the average specific lactic acid productivity, showcasing how preadaptation could decrease operational costs.

To assess the performance and robustness of process designs against process input variations, a multi-scale variability analysis framework was developed. The framework included models for bioprocess, flowsheet, techno-economic, and life cycle assessment. In a case study, multi-feed processes, in which solids and cells are fed to the process using model-based predictions, were more robust against variable cellulolytic activities than batch SSCFs in a wheat straw-based ethanol biorefinery. The developed framework can be used to identify robust biorefinery process designs, which simultaneously meet technological, economic, and environmental goals.

Keywords: Biorefinery, lignocellulose, logging residues, pretreatment, multi-feed SSCF, preadaptation, multi-scale variability analysis, mixed feedstocks, ethanol, lactic acid platform chemicals

Preface

This doctoral thesis partly fulfils the requirements for a PhD degree at the Department of Biology and Biological Engineering, Division of Industrial Biotechnology, Chalmers University of Technology, Sweden. The work presented in this thesis was performed between 2016 and 2021 and was funded by the Swedish Energy Agency under the application title “Bioethanol from spruce and oat shells via High Gravity Multi-Feed SSF” (Grant no. 41272-1) as a collaborative project between the Division of Industrial Biotechnology, Chalmers University of Technology, RISE Processum AB, Örnköldsvik, Sweden, and RISE Research Institutes of Sweden, Department of Energy and Resources, Division of Built Environment, Gothenburg, Sweden. The work on multi-scale variability analysis also involved collaboration with the Department of Technology Management and Economics, Division of Environmental Systems Analysis, Chalmers University of Technology, and was funded by the Chalmers Area of Advance Energy.

The main part of the work was carried out at the Division of Industrial Biotechnology at Chalmers University of Technology under the supervision of Ass. Prof. Carl Johan Franzén, Dr. Rickard Fornell, and Dr. Fábio Luis Da Silva Faria Oliveira. Pretreatment of raw materials was performed by Dr. Emma Johansson, Carolina Jogner, Dr. Andreas Hörnberg and Dr. Björn Alriksson at RISE Processum AB, Örnköldsvik, Sweden. Techno-economic analysis for the multi-scale model was performed by Dr. Rickard Fornell, RISE Research Institutes of Sweden, Department of Energy and Resources, Division of Built Environment, Gothenburg, Sweden. Life cycle assessment for the multi-scale model was performed by Dr. Matty Janssen, Chalmers University of Technology, Department of Technology Management and Economics, Division of Environmental Systems Analysis, Gothenburg, Sweden.

David Benjamin Nickel
January, 2021

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I **Nickel D. B.**, Nielsen F., Franzén C. J. (2021). Response surface modelling identifies dilute acid-catalysed steam pretreatment conditions with high sugar yields and minimal inhibitor formation for spruce logging residues. *Submitted manuscript*

- II **Nickel D. B.**, Nielsen F., Franzén C. J. (2021). Upgrading low-value logging residues with oat hulls in integrated ethanol production: Effects on the design of batch and model-based multi-feed SSCFs. *Manuscript*

- III Aulitto M., Fusco S., **Nickel D. B.**, Bartolucci S., Contursi P., Franzén C. J. (2019). Seed culture pre-adaptation of *Bacillus coagulans* MA-13 improves lactic acid production in simultaneous saccharification and fermentation. *Biotechnology for Biofuels*, 12(1), 45.
DOI: 10.1186/s13068-019-1382-2

- IV **Nickel D. B.**, Fornell R., Janssen M., Franzén C. J. (2020). Multi-scale variability analysis of wheat straw-based ethanol biorefineries identifies bioprocess designs robust against process input variations. *Frontiers in Energy Research*, 8, 55.
DOI: 10.3389/fenrg.2020.00055

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Author's contributions

- Paper I:** First author. I designed the study, planned and performed the experiments, analysed, and interpreted the results, and wrote the manuscript.
- Paper II:** First author (shared). I contributed in conceiving and designing the study, planned and performed most of the experiments, analysed and interpreted the results, and wrote the manuscript together with my co-authors.
- Paper III:** Third author. I participated in designing and performing the fermentation experiments and subsequent analytics. Together with my co-authors, I analysed the data, interpreted the results, drafted and critically commented on the manuscript.
- Paper IV:** First author. I initiated and designed the study, evaluated published data, evaluated the bioprocess model, programmed the interface for techno-economic analysis, and collected the data from all system scales. I analysed and interpreted the results with my co-authors and wrote the manuscript.

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Abbreviations

| | |
|-----------------|---|
| 3-HP | 3-Hydroxypropionic acid |
| 5-HMF | 5-Hydroxymethylfurfural |
| CBH | Cellobiohydrolase |
| CO ₂ | Carbon dioxide |
| DoE | Design of Experiments |
| FDCA | 2,5-Furandicarboxylic acid |
| LCA | Life cycle assessment |
| RSM | Response surface model |
| SHCF | Separate hydrolysis and co-fermentation |
| SHF | Separate hydrolysis and fermentation |
| SSCF | Simultaneous saccharification and co-fermentation |
| SSF | Simultaneous saccharification and fermentation |
| TEA | Techno-economic analysis |
| WIS | Water-insoluble solids |
| XDH | Xylitol dehydrogenase |
| XR | Xylose reductase |

1. Introduction

The effect of climate change, hastened by the unsustainable use of fossil resources, calls for a paradigm shift from a linear towards a circular economy. A combination of bio-based and circular economy is envisaged to mitigate climate change by reducing and reutilising waste streams, as well as by supporting a more sustainable production of energy, fuels, and chemicals from renewable resources. The work presented in this thesis contributes to the shift by developing processes to sustainably produce platform chemicals from agricultural and forestry biomass.

1.1 Background

Today's society relies heavily on fossil resources, such as crude oil, natural gas, peat, and coal. They are utilised to generate heat and electricity, transport fuels, and building blocks for petrochemical production of olefins, aromatics, and thermoplastics (Levi & Cullen, 2018). Due to expected demographic (United Nations, 2019) and economic growth (Christensen et al., 2018), global energy demand is predicted to soar (British Petroleum, 2019). As a result, the demand for fossil resources will rise. The increase will be mainly driven by petrochemical industry (International Energy Agency, 2018). However, the burning of fossil resources releases sequestered carbon from its geological reservoirs into the atmosphere and terrestrial biosphere in the form of climate-active gases, such as carbon dioxide (CO₂) and methane. Increased emissions of these gases have resulted in anthropogenic climate change, with multiple negative environmental impacts at local (Abatzoglou & Williams, 2016; Williams et al., 2019) and global scale (Hansen & Stone, 2016; Rosenzweig et al., 2008). The increase in average global temperatures and the occurrence of droughts have already sounded the alarm bell. In a panoply of international agreements spearheaded by the Paris Agreement (United Nations FCCC, 2015) and followed by other conferences (United Nations FCCC, 2017), most countries have agreed to implement political measures to mitigate climate change and limit the increase in average

global temperature to 2 °C compared to pre-industrial levels of 1850–1900. The European Union, for example, aims to reduce its greenhouse gas emissions by at least 40% until 2030 and by 80% until 2050, compared to 1990 levels (European Commission, 2018). To reach these objectives, policies need to be implemented to decarbonise industries, increase the efficiency of energy transformation processes, implement a circular economy based on recycling resources, and find renewable, environmentally friendly alternatives to fossil resources.

Renewable resources, such as plant and algal biomass, will play an important role in this industrial and economic transformation process. As part of terrestrial and marine biological carbon cycles, plants and algae assimilate atmospheric CO₂ into carbohydrates via photosynthesis. When biomass is used instead of fossil fuels, assimilated atmospheric carbon replaces the geologically sequestered one. This approach prevents an additional increase in net CO₂ emissions. Although decarbonisation is one of the primary goals in mitigating climate change, current manufacturing is largely hydrocarbon-based. Most petrochemical products cannot be replaced by non-carbon sources. The carbohydrates stored in biomass might not be directly used in the hydrocarbon-based chemical industry but can be converted to platform chemicals for further processing.

The European Union identified 25 biorefinery-produced compounds with strong potential to serve as platform chemicals (Taylor et al., 2015). These platform chemicals, which include ethanol and lactic acid (Figure 1), can serve as bulk intermediates for more sustainable production of higher-value chemicals, such as acrylic acid (Dagle et al., 2020), butadiene (Angelici et al., 2013), and polylactic acid (Datta & Henry, 2006). Accordingly, biomass can replace finite fossil resources as renewable feedstock for value chains in the chemical industry.

1.2 The concept of biorefineries

Biorefineries enable economically feasible conversion into fuels and chemicals via an integral, efficient use of biomass. Like oil refineries, biorefineries are envisaged to convert biomass feedstocks into building blocks suitable for the generation of food, feed, chemicals, fuels, and energy in the form of heat or power (de Jong et al., 2012). Biorefinery conversion processes are generally classified as either thermo-chemical or biochemical. Thermo-chemical conversion includes the combustion of biomass for heat and power generation (IRENA, 2019), biomass gasification to syngas (Göransson et al., 2011), pyrolysis (Wang et al., 2017), hydrothermal liquefaction (Gollakota et al., 2018), and pulping processes. The biochemical conversion pathway is based on the microbial conversion of fermentable sugars into fuels, chemicals, and biogas (McKendry, 2002).



Figure 1: The concept of a biorefinery for the biochemical conversion of lignocellulose into potential platform chemicals. The listed platform chemicals were identified by the US Department of Energy (Bozell & Petersen, 2010). Ethanol and lactic acid (highlighted in bold) were selected as products in my thesis to demonstrate the application of biorefineries for platform chemical production and to discuss the development of process designs to improve process performance. 5-HMF, 5-Hydroxymethylfurfural; FDCA, 2,5-Furandicarboxylic acid; 3-HP, 3-Hydroxypropionic acid.

For biorefineries based on biochemical conversion, various feedstocks have been identified, including starch- or lipid-containing food crops, lignocellulose-based biomass, municipal and industrial waste, and algae. The first and still most utilised feedstock is represented by food crops specifically grown for biorefinery purposes, such as corn, sugarcane or sugar beet (Balan, 2014). However, the utilisation of food crops for chemical production received wide public criticism due to concerns about food security and changes in land use, referred to as the food versus fuel debate (Rulli et al., 2016). Therefore, research has shifted towards second-generation biorefineries that can use lignocellulosic feedstocks, such as residues from agricultural or forestry practice, wood, and energy crops. Examples include straw, stover, switchgrass, wood, logging residues, as well as by-products from industrial biomass operations (*e.g.* saw dust or hulls from milling processes). However, the sugars in these feedstocks are embedded in a recalcitrant lignocellulosic structure, which requires additional processing steps. These steps decrease fermentable sugar yields compared to first-generation feedstocks, yet economic competitiveness relies on second-generation feedstocks remaining inexpensive sources of biomass.

Lignocellulose-based biorefineries that employ fermentative conversion adhere to a generalised process scheme (Figure 2). After harvest, the feedstock is collected, stored, and transported to the biorefinery. Particle size reduction is followed by pretreatment, intended to decrease the inherent recalcitrance of lignocellulose and to fractionate the feedstock into a cellulose-rich, easily hydrolysable solid fraction plus a liquid fraction rich in hemicellulose, lignin or combinations thereof. After pretreatment, the material is subjected to enzymatic hydrolysis to release fermentable sugars stored in the lignocellulosic structure. During fermentation, these sugars can be metabolised by microorganisms to produce platform chemicals as intermediates for further valorisation or for direct utilisation as commodity chemicals. After hydrolysis and fermentation, the product is separated from the fermentation broth. To increase economic viability and sustainability, biorefineries are typically designed to maximise resource utilisation through process integration.

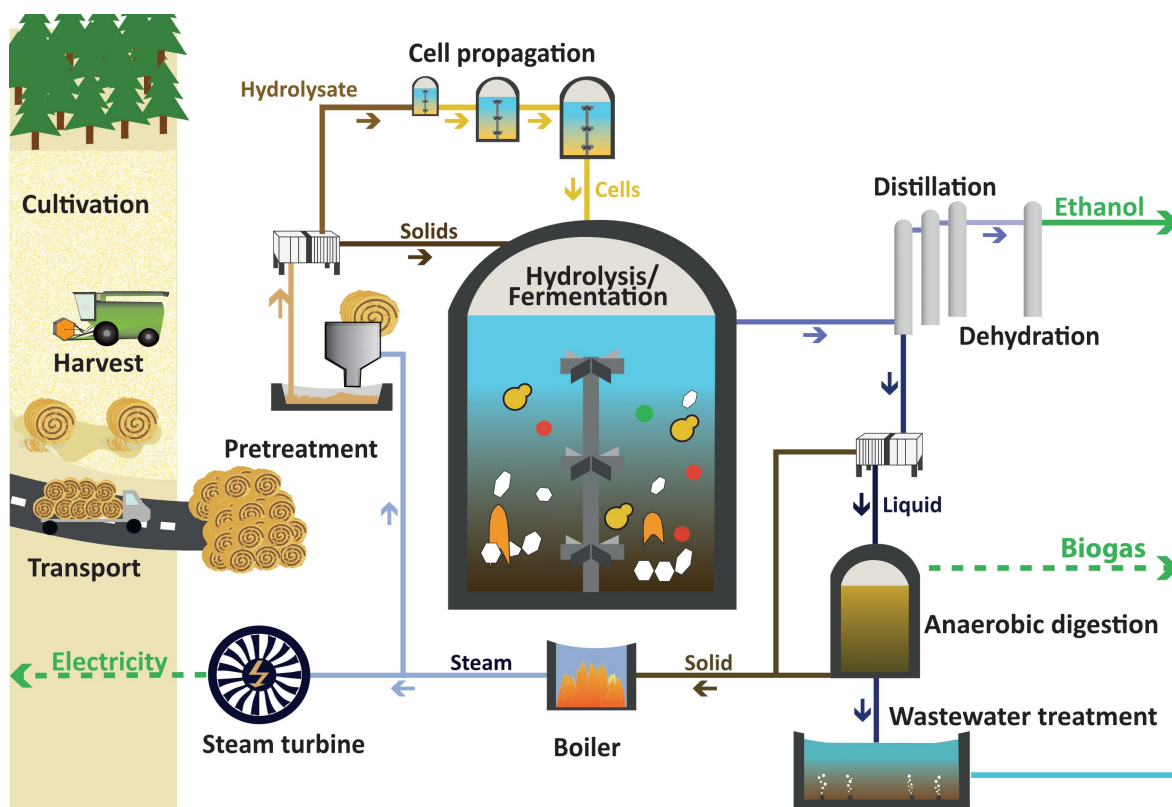


Figure 2: Simplified process scheme of a lignocellulose-based biorefinery. The products of the biorefinery are ethanol, biogas, and electricity. The main unit processes in lignocellulose-based biorefineries are pretreatment, cell propagation, hydrolysis and fermentation, product separation and purification (in the case of ethanol this is achieved by distillation and dehydration), co-product generation (here represented by anaerobic digestion and heat and power generation), and wastewater treatment. Adopted from **Paper IV**.

Nonetheless, reaching cost-competitiveness with fossil fuel-based platform chemical production remains a challenge for lignocellulose-based biorefineries. The main hurdles are represented by ensuring a constant, year-round supply of inexpensive feedstock, the inherent recalcitrance of lignocellulosic biomass to enzymatic hydrolysis, and the toxicity of pretreatment by-products towards industrial microorganisms. Overcoming these drawbacks is necessary to achieve the capital and operational cost savings required to reach cost-competitiveness.

1.3 Aim and scope

The overall goal of this work was to develop and assess process designs that could improve the performance of biorefineries based on fermentation of agricultural and forestry residues for platform chemical production. The goal was approached in the following ways:

Expanding the feedstock base to novel, inexpensive feedstocks was the first approach. In my thesis, spruce logging residues (**Paper I**), combined spruce and pine logging residues (**Paper II**), and oat hulls (**Paper II**) were assessed as feedstocks for ethanol production. To efficiently convert these feedstocks to platform chemicals, *pretreatments tailored to novel feedstocks* had to be devised. In **Paper I**, the acid-catalysed steam pretreatment conditions for spruce logging residues were optimised with the help of response surface models (RSMs). This allowed concurrent high sugar recoveries after pretreatment and enzymatic hydrolysis, and low concentrations of inhibitors.

Using mixed feedstocks to upgrade the low carbohydrate contents in logging residues was investigated in **Paper II**. Logging residues contain comparably low amounts of carbohydrates and abundant lignin. The low content of hydrolysable solids in enzymatic hydrolysis limits fermentations with high solid loadings. High solid loadings, though, are required to reach ethanol titres suitable for an economically feasible distillation process. Therefore, the mixing of carbohydrate-rich oat hulls with logging residues during simultaneous saccharification and co-fermentation (SSCF) was investigated for batch and model-based multi-feed SSCFs. In multi-feed SSCFs, solids and cells were added throughout the process according to hydrolysis models to ensure mixability at high solids loadings. To identify potential bottlenecks to SSCF and process integration caused by the respective feedstock, the biochemical composition, hydrolysability, and toxicity of the pretreated liquid phase towards cellular growth were assessed.

One way to improve the cost-competitiveness of biorefineries is to increase volumetric and specific productivities. For *Saccharomyces cerevisiae*, preadaptation has been proven effective at increasing ethanol productivity through improved inhibitor tolerance (Nielsen et al., 2015). Such process design was applied in **Paper II**. During propagation, cells were exposed to hydrolysate containing toxic lignocellulose-derived inhibitors to preadapt them to the subsequent fermentation step. In **Paper III**, *transfer of the preadaptation method from ethanol production by S. cerevisiae KE6-12A to lactic acid production from wheat straw by Bacillus coagulans MA-13* was assessed. To find the optimal trade-off between preadaptation and biomass formation during propagation, cells were propagated at different hydrolysate concentrations to screen for the impact of inhibitors on maximal growth rates. Lactic acid production was compared at different inoculum sizes between batch SSCFs with non-adapted cells and cells preadapted at the various hydrolysate concentrations.

Biorefinery processes are typically assessed with regards to their technical design, economic potential, and environmental sustainability. Therefore, quantitative assessments at different system scales, ranging from bioprocess models to global-scale life cycle evaluations, are required. In **Paper IV**, a multi-scale model was developed, which enabled a holistic assessment of biorefineries from bioprocess scale to techno-economic analysis (TEA) and life cycle assessment (LCA). A *multi-scale variability analysis framework* was developed, which could quantify the impacts of input variations on the overall biorefinery performance and the robustness of different unit processes, including pretreatment (**Paper I**), fermentation (**Paper II**), and propagation (**Paper III**). Using a wheat straw-based ethanol

biorefinery as a case study, the impact of variations in enzymatic activity on batch and multi-feed SSCFs was analysed from a bioprocess, techno-economic, and life cycle perspective.

In the first part of this thesis, the characteristics of lignocellulose-based biorefineries employing fermentative conversion are presented. In **Chapter 2**, a short overview of the chemical composition of lignocellulosic feedstocks is provided, followed by a description of the criteria for feedstock selection and the application of these criteria to the feedstocks utilised in this thesis. In **Chapter 3**, a broad overview of pretreatment techniques for lignocellulosic biomass is given, before focusing on steam pretreatment to compare the findings presented in **Paper I** with published data. The overall bioprocess, including preadaptation, enzymatic hydrolysis and fermentation, is presented in **Chapter 4**, as well as in **Papers II** and **III**. In **Chapter 5**, an overview of assessing biorefinery performance at different system scales by modelling is provided and related to the findings in **Paper IV**.

2. Lignocellulosic feedstocks for biorefineries

Over the past decades a range of feedstocks have been investigated for biochemical biorefinery applications. Potential feedstocks encompass food crops, lignocellulosic biomass, algae, as well as industrial and household waste. Lignocellulosic biomass includes agricultural and forestry residues, wood, and energy crops, and is categorized based on phylogeny and taxonomy into softwoods, hardwoods, and herbaceous biomass. Herbaceous biomass is represented by agricultural residues, such as cereal straw and hulls, stover, and energy crops (*e.g. Miscanthus*). Hardwoods include, for example, aspen and poplar, whereas spruce, pine, and Douglas fir are examples of softwoods. The suitability of lignocellulosic feedstocks as substrates for fermentative processes is determined by their biochemical composition, mostly by the relative abundance of cellulose, hemicellulose, and lignin. The biochemical composition, as measured per National Renewable Energy Laboratory protocols, varies within species and biomass categories, as shown in Table 1.

Table 1: Chemical composition of lignocellulosic feedstocks, as measured per National Renewable Energy Laboratory protocols, in % of dry matter. The measured chemical composition of feedstocks used in this thesis is highlighted in italics.

| Biomass type | Glucan | | Hemicellulose | | | Lignin | Extractives | Ash | References |
|----------------------------------|--------|----------|---------------|----------|----------|--------|-------------|-----|---|
| | Mannan | Galactan | Xylan | Arabinan | Arabinan | | | | |
| <i>Herbaceous biomass</i> | | | | | | | | | |
| Wheat straw | 30–32 | 0–2 | 1 | 19–22 | 3–4 | 17–21 | 13 | 2 | (Erdei et al., 2013; Tomás-Pejó et al., 2008) |
| Oat hulls | 32.8 | 0.1 | 1.2 | 33.2 | 3.4 | 17.2 | 5 | 2.9 | Paper II |
| Corn stover | 36–48 | 1–2 | 2–3 | 22–23 | 4 | 17–20 | --- | 2–7 | (Bondesson et al., 2015; Öhgren et al., 2007) |
| <i>Hardwood</i> | | | | | | | | | |
| Poplar | 42–49 | 1 | 0 | 16 | 0 | 24–28 | 4 | 1–2 | (Cantarella et al., 2004; Stoklosa & Hodge, 2012) |
| <i>Softwood</i> | | | | | | | | | |
| Spruce stem wood | 40–54 | 12–18 | 3 | 5 | 1 | 32–34 | 7 | <1 | (Monavari et al., 2010; Pielhop et al., 2016a) |
| Spruce bark | 23–27 | 3 | 1–2 | 4–5 | 4–5 | 27–34 | 20–28 | 2–4 | (Frankó et al., 2015; Neiva et al., 2018) |
| Spruce needles | 25–31 | 9–12 | 2–3 | 2–4 | 3–5 | 28–35 | 10–26 | | (Johansson, 1995) |
| Logging residues | 29.1 | 7 | 4.3 | 5.8 | 2.2 | 34.7 | 12.7 | 2.6 | Paper I |
| | 25.0 | 6.3 | 4.0 | 6.0 | 2.1 | 43.9 | 7.6 | 3.1 | Paper II |
| Pine stem wood | 38–43 | 11–14 | 2–4 | 5–7 | 2 | 27–32 | 3–9 | 0 | (Hanhikoski et al., 2019; Normark et al., 2014) |

2.1 Lignocellulose and its characteristics

Lignocellulosic feedstocks are dominated by the major constituents of plant cell walls: Cellulose, hemicellulose and lignin. Most plants have a multi-layered cell wall structure, consisting of the outermost middle lamella, the primary cell wall, and the secondary cell wall surrounding the cell membrane. The primary cell wall is the site of cell growth. The middle lamella connects the primary cell walls of different cells. Secondary plant cell walls define the structure of plants (Martin et al., 2001), provide rigidity (Burton et al., 2010) and, depending on the site, protection against microbial degradation (Cantu et al., 2008; Vorwerk et al., 2004). The chemical composition of plant cell walls changes according to species variety (Benjamin et al., 2014; Welch et al., 1983), age (Rencoret et al., 2011), cell type (Burton et al., 2010), and cell localization (Collins et al., 2014). In the following sections, the characteristics of the various components of lignocellulosic biomass, such as cellulose, hemicellulose, lignin, extractives, pectin, and inorganic compounds are detailed.

2.1.1 Cellulose

Cellulose is a linear polysaccharide consisting of glucopyranosyl units linked via β -1,4-glycosidic bonds. The degree of polymerisation depends on the biomass and pretreatment method used. For native biomass, degree of polymerisation ranges of 1045–2660 for herbaceous biomass, 3063–5000 for softwoods, and 3500–5000 for hardwoods have been reported (Hallac & Ragauskas, 2011). Hydrogen bonds between the hydroxyl groups and oxygen atoms, and Van-der-Waals interactions cause cellulose chains to align in flat, parallel structures. Stacked together, cellulose chains form crystalline microfibrils, which themselves aggregate in a highly ordered structure, interrupted by amorphous cellulose. The topology of amorphous and crystalline cellulose, and their interaction, result in a hydrophobic, recalcitrant cell wall component aimed at providing tensile strength (Sjöström, 1993).

2.1.2 Hemicellulose

Hemicellulose provides rigidity to the plant cell wall by binding to cellulose (Morris et al., 2004), lignin (Balakshin et al., 2011), and pectin (Tan et al., 2013). In contrast to cellulose, hemicellulose consists of various hexose and pentose sugars, including arabinose, galactose, glucose, mannose, rhamnose, and xylose. Substitutions by acetyl groups (Bååth et al., 2018; Jaafar et al., 2019), glucuronic acid (Urbanowicz et al., 2012), and ferulic acid esters (Harris & Trethewey, 2010) further increase hemicellulose diversity. These substitutions vary between species. In softwoods, xylans are not acetylated, whereas acetylation is abundant in herbaceous biomasses. Softwood hemicellulose is dominated by galactoglucomannans (Ek et al., 2009); while glucuronoxylans dominate hardwood (Ek et al., 2009). Furthermore, arabinoglucuronoxylans predominate in herbaceous hemicellulose, with xylan being the major sugar building block in hardwood and herbaceous hemicellulose. Owing to its

amorphous structure, hemicellulose is less recalcitrant to enzymatic hydrolysis than cellulose.

2.1.3 Lignin

Cellulose and hemicellulose are embedded in a complex lignin matrix. Lignin is a variable aromatic polymer generated through radical polymerisation of substituted phenyl propylene units (Ragauskas et al., 2014). Although there is considerable variability, lignin is primarily formed by the monolignols *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, which give rise to hydroxyphenyl, guaiacyl, and syringyl subunits, which further polymerise. The distribution and abundance of lignin subunits vary greatly between plant species. Softwoods lack syringyl units, but contain abundant guaiacyl units (Dellon et al., 2017), resulting in a branched lignin (Ragauskas et al., 2014). Hardwood lignins are guaiacyl- and syringyl-rich (Sjöström, 1993), whereas in herbaceous biomass all three monomer units are present (Dellon et al., 2017). Together with hemicellulose, lignin forms lignin-carbohydrate complexes (Du et al., 2013), which reinforce the recalcitrance of plant cell walls to enzymatic degradation. Breaking the bonds between hemicellulose and lignin is essential to achieve efficient enzymatic hydrolysis.

2.1.4 Pectin, extractives, and inorganic compounds

Plant cell walls also contain pectin, extractives, proteins, and inorganics. Pectin is a polysaccharide with α -1,4-linked galacturonic acid as the main building block, which is found mostly in the middle lamella and in the primary cell wall (Mohnen, 2008). Pectin binds to cellulose in primary cell walls (Wang et al., 2012b) and its abundance varies between species.

Extractives encompass a highly diverse group of compounds soluble in water and neutral organic solvents. It comprises non-structural sugars, waxes, sterols, fatty acids, glycerides, terpenoids, steroids, and phenolics (Prinsen et al., 2012; Sjöström, 1993). Extractives' content varies considerably between species and even within plants. For example, in the logging residues used for **Paper II**, extractives accounted for 5.0% of the wood fraction, 15.5% of bark, and 16.4% of needles.

Biomass contains inorganic compounds, such as phosphate, silicates, and potassium salts, which are converted to ash upon combustion. Typically, wood contains less ash than herbaceous biomass (Tao et al., 2012). Ash content is influenced by the harvesting method, with biomass harvested near the ground usually displaying significantly more ash because more soil is taken up during collection.

2.2 Selection of feedstocks for biorefineries

The suitability of feedstocks for biochemical biorefineries is assessed based on the planned conversion process and product in mind. Generally, the assessment is based on several selection criteria: Biochemical composition, potential supply, and environmental impact of growing and harvesting the feedstock.

The biochemical composition of candidate feedstocks determines the design of all processing steps in a biorefinery. For biorefineries based on the fermentative conversion of sugars, a high carbohydrate content is of prime interest. Elevated carbohydrate levels increase the expected concentration of fermentable sugar concentrations after pretreatment and enzymatic hydrolysis, and thereby potential product titres. Higher product titres are often associated with lower energy demands during subsequent product purification, in turn lowering overall operational costs. Both cellulose and hemicellulose are important sources of fermentable sugars, whose release depends not only on the carbohydrate content, but also on the inherent recalcitrance of the raw material to enzymatic hydrolysis. The recalcitrance of lignocellulosic feedstocks is determined by lignin and hemicellulose shielding the underlying cellulose, cellulose crystallinity and degree of polymerisation, accessible surface area, particle volume, pore size, and interactions thereof (Zhao et al., 2012). It has been identified as one of the main impediments to economic competitiveness of lignocellulosic biorefineries (Himmel et al., 2007).

In addition, suitable feedstocks should contain low amounts of ash. Soil ash can buffer acid catalysts during pretreatment, thereby decreasing effective catalyst loads (Weiss et al., 2010). Solid fermentation residues rich in lignin and ash are usually combusted to generate heat and power in integrated biorefinery systems. Silicate-rich ashes of herbaceous biomass such as rice straw (Liu et al., 2013) can lead to slagging, fouling, and sintering during combustion (Lindström et al., 2007; Wang et al., 2012a). To overcome these problems, additives are introduced, thus increasing operational costs (Wang et al., 2012a).

The potential supply is an important criterion for feedstock selection. A constant, year-round feedstock supply has been identified as a major challenge for the operation of biorefineries. Biomass availability is subjected to natural variations. Seasonal and geographic variations in biomass yields and composition (Lewandowski & Heinz, 2003; Ray et al., 2020), harvest seasons, and weather conditions influence biomass availability and, therefore, feedstock prices. As the latter contribute heavily to operational costs (Humbird et al., 2011), fluctuations in feedstock prices have direct consequences on the economic feasibility of lignocellulose-based biorefineries. Therefore, suitable feedstocks should be available all year round and, preferably, have a constant chemical composition. However, agricultural feedstocks are particularly prone to variations in biomass availability because of harvest seasons. Storage solutions such as baling (Argo et al., 2013) or ensiling (Wendt et al., 2018) can help avoid supply chain disruptions, but add to process costs. Furthermore, advanced supply chains are necessary to ensure a steady supply of biomass. Bulk transport capacity is required to handle the supply of low-density lignocellulosic biomass such as logging



residues, which should ideally occur at low moisture contents. To minimise transport costs and CO₂ emissions, suitable feedstocks should be harvested within a short distance from biorefineries or depots with bulk transport capacity (Noon et al., 2002).

Another important criterion guiding feedstock selection is the environmental impact of biomass growth and harvest for biorefinery applications. This is determined through LCA, as in the case of wheat straw in **Paper IV**. As the political and public support for biorefineries is to a large extent based on the expectation that biorefinery products significantly reduce CO₂ emissions compared to their fossil fuel-based counterparts, a low climate impact of growth and harvest is paramount. In particular, the need for fertilisers should be minimised as they contribute to eutrophication and soil acidification. Repurposing of land use for feedstock should also be minimised to avoid elevated prices for arable land devoted to food production.

2.3 Residual biomass for biorefinery applications

Currently, biorefineries rely mainly on first-generation feedstocks, including corn, sugarcane or sugar beet (Balan, 2014). Due to the food versus fuel debate (Rulli et al., 2016) and an increased use of land for biofuel production (Havlík et al., 2011), the utilisation of food crops for biorefinery applications has been placed under scrutiny. The European Union has mandated restrictions on land-use change for biofuel production (European Union, 2018), promoting instead the utilisation of lignocellulosic biomass.

Table 2: Suitability of feedstocks investigated in this thesis with respect to the selection criteria defined in Chapter 2.2.

| |  |  | |
|-------------------------|---|--|-------------------------|
| | Wheat straw | Oat hulls | Logging residues |
| Potential supply | +++ | + | +++ |
| Biomass availability | +++ | + | +++ |
| Feedstock price | ++ | unknown | +++ |
| Biochemical composition | ++ | +++ | + |
| Environmental impact | ++ | ++ | +++ |

+ low performance ++ moderate performance +++ high performance

Lignocellulosic feedstocks exert a lower environmental impact than first-generation feedstocks (Parajuli et al., 2015). Thus, lignocellulosic biomass has a strong potential to meet the policy-driven goals on climate impact and land-use change. However, the economics of lignocellulose-based biorefineries suffer from the lower carbohydrate content per dry metric ton and higher recalcitrance of lignocellulosic biomass. To retrieve fermentable sugars, pretreatment and enzymatic hydrolysis are required. These unit processes are associated with the highest costs within the biorefinery process chain (Yang & Wyman, 2008). To reach cost-competitiveness with first-generation feedstocks, further savings in capital and operational costs are required.

In lignocellulose-based biorefineries, feedstock is the largest contributor to operational costs, amounting to 30–40% depending on feedstock and process design (Chovau et al., 2013; Humbird et al., 2011; Klein-Marcuschamer et al., 2010). One way to lower the cost is to use inexpensive feedstocks such as residual biomass from agricultural and forestry operations. Wheat straw is one of the most abundant agricultural residues; annual global estimates place it at 134–192 TWh (Bentsen et al., 2014; Jørgensen et al., 2019)¹. However, not all wheat straw can be utilised for biorefinery applications. To ensure sufficient soil organic matter, typically 25% of the harvested wheat straw is left on the field (Nemecek et al., 2007). Furthermore, wheat straw is used for heat and power production (Bentsen et al., 2018), as bedding material (Tuytens, 2005), and as soil cover in mushroom and vegetable production (Kretschmer et al., 2012). Still, the bioeconomic potential for wheat straw is high, and has been estimated at 11 TWh for the European Union alone (Thorenz et al., 2018)¹.

Wheat straw is a locally or regionally traded commodity with significant price differences among regions. While wheat straw prices range between €10.8–19.4 MWh⁻¹ in southern Sweden (Bentsen et al., 2016) and between €18.0–19.8 MWh⁻¹ in Denmark (Bang, 2013), in other countries no market for wheat straw exists yet as it is burned on the field without further valorisation (Talebna et al., 2010). High biomass availability in combination with elevated carbohydrate content (Table 1) and locally low prices make wheat straw a promising feedstock for biorefinery applications (Table 2). Thus, in **Paper III** wheat straw was used as feedstock for lactic acid production. Several attempts to commercialise wheat straw-based biorefineries have been made. As of 2021, Clariant is constructing a plant in Podari, Romania, with a planned annual capacity to convert 300.000 t of wheat straw into 50.000 t of ethanol based on its proprietary sunliquid[®] process (Clariant AG, 2020).

Attempts to establish viable biorefineries have not been restricted to agricultural residues. In countries with a strong forestry sector, the utilisation of wood as feedstock has been broadly investigated. As wood is already used in the pulp and paper industry, as construction material, manufacturing board, and furniture, interest has been directed towards coniferous logging residues obtained as by-products from thinning and logging operations.

¹ Converted from Mt assuming a conversion factor of 4166 Wh·kg⁻¹ according to the higher heating value of wheat straw (Montero et al., 2016)

In this thesis, the utilisation of logging residues for bioethanol production was investigated in **Papers I** and **II**. In Sweden, logging residues cost on average €14.4 MWh⁻¹ (Grahn, 2019)². Usually, a portion of these residues is used for combined heat and power generation (Ericsson & Werner, 2016), the rest is left on the ground to provide nutrients to the soil, especially carbon and nitrogen (Hyvönen et al., 2000). However, logging residues have been associated with an increased risk of wildfires (Evans & Finkral, 2009) and pests in productive forests (Bernhold et al., 2006; Hanssen et al., 2018), which can be prevented by their removal. In Sweden, the annual bioeconomic potential of extracting logging residues has been estimated to be 33 TWh and for Canada 175 TWh (IRENA, 2019), and is in line with environmental standards. In comparison to wheat straw, logging residues can be available all year round and do not require large capital investment into storage facilities.

Utilisation of inexpensive feedstocks such as logging residues typically comes at the price of an inferior feedstock quality. As softwoods, coniferous logging residues are more recalcitrant to enzymatic hydrolysis than herbaceous biomass (Galbe & Wallberg, 2019), requiring more severe pretreatments (Galbe & Zacchi, 2012) and higher enzyme loadings (Arantes & Saddler, 2011). The heterogeneity of logging residues and their low carbohydrate content (Table 1) add to the challenges posed by softwood biomass to biorefinery processes. The heterogeneity stems from the bark, needle, and wood fractions having different physical and chemical characteristics. The bark fraction of logging residues contained half as much glucan (15.8%) as the wood fraction (33.4%), but significantly more lignin (Table 1, **Paper II**). The higher lignin content contributes to the higher recalcitrance of spruce bark compared to wood chips (Frankó et al., 2015) and, consequently, would require more severe pretreatment conditions to ensure efficient enzymatic hydrolysis (Frankó et al., 2015). Unit processes for heterogeneous feedstock mixtures such as logging residues cannot be optimised for individual feedstock fractions (*e.g.* bark). Instead, process conditions are the result of trade-offs between the process optima of each fraction, which typically leads to yield losses. Moreover, the low glucan content of logging residues (Table 1) restricts attainable fermentable sugar concentrations and, in turn, product titres. As discussed in Chapter 2.2, low product titres often translate into high energy demands for downstream processing, lowering the profitability of biorefineries. Thus, the biochemical composition is the main limitation to the utilisation of logging residues for biorefinery applications (Table 2).

One way to overcome the unfavourable biochemical composition of carbohydrate-poor lignocellulosic biomass is to upgrade the substrate via feedstock mixing. This practice has been discussed primarily in the context of reduced supply chain risks (Baral et al., 2019; Oke et al., 2016). Improving the biochemical composition and specifically carbohydrate content could potentially reduce operational costs by increasing product titres. In most studies, the integration of lignocellulosic feedstocks into existing first-generation ethanol plants has been discussed (Erdei et al., 2013; Persson et al., 2020). This choice seems natural from two perspectives. First, integration of first- and second-generation feedstocks into one

² Exchange rate for SEK to € in 2019: 0.095, according to (European Central Bank, 2020)

biorefinery can reduce costs and, thereby, pave the way for lignocellulose-based biorefineries. Second, processing of first-generation feedstocks results in sugar solutions too concentrated for fermentation; hence, they could be diluted with carbohydrate-poor lignocellulose-derived hydrolysate. As biofuel and biorefinery policies in the European Union (European Union, 2018) and United States of America (Congressional Research Service, 2020) mandate an increased share of lignocellulose-based biorefinery products, carbohydrate-rich lignocellulosic raw materials are preferred over first-generation feedstocks.

Oat hulls represent a carbohydrate-rich lignocellulosic raw material. The total carbohydrate content of oat hulls utilised in **Paper II** was 71%, and the lignin content 17% (Table 1). Because of their high cellulose and hemicellulose content, oat hulls are attractive for biorefineries (Schmitz et al., 2020). As by-products of the oat milling process, oat hulls form a homogenous feedstock with predictable characteristics, which distinguishes them from other agricultural residues such as wheat straw. However, oat hulls suffer from low bulk density and high flowability, which makes transport inefficient and expensive. Therefore, they are typically considered as waste in oat mills, and are not a traded or priced commodity (Table 2). Recently, the combustion of oat hulls for heat and power generation, previously associated with slagging problems, has been investigated (Zhang et al., 2013). The global annual technical potential of oat hulls is estimated at 5–8 Mt, equivalent to 1.1–1.8 TWh³, considering a global oat production in 2018 of 23 Mt (FAO, 2020) and that oat kernels contain 22–35% oat hulls (Welch et al., 1983). Accordingly, the technical potential of oat hulls is approximately 100 times lower than that of wheat straw. Despite other alternative uses, including as dietary fibre in food (Stevenson et al., 2011) and feed (Hsu et al., 1987), industrial-scale production of furfural from oat hulls (Brownlee & Miner, 1948) implies that supply is sufficiently high enough to establish local oat hull-based biorefineries, possibly integrated with oat mills. An example of such process integration is xylitol production from steam-pretreated oat hulls at Fazer's oat mill in Lahti, Finland, which is currently under construction (Oy Karl Fazer Ab, 2020).

In conclusion, the main benefits of utilising residual biomass sources are their low price and low environmental impact, paired with an elevated local supply. In contrast, common drawbacks include their lower carbohydrate content and higher recalcitrance compared to first-generation feedstocks. Wheat straw is currently one of the most investigated feedstocks for lignocellulose-based biorefineries. The comparably high carbohydrate content and vast supply are the main reasons for its use in biorefineries. Wheat straw utilisation was demonstrated in **Paper III** for lactic acid production. Compared to wheat straw, logging residues have a higher supply potential, which makes them interesting for biorefinery applications; however, as shown in **Papers I and II**, the low carbohydrate and lignin contents contribute to higher recalcitrance to enzymatic hydrolysis. In comparison, oat hulls contain elevated amounts of carbohydrates, making them interesting for biorefinery applications, but

³ Assuming a higher heating value of 16,118 kJ·kg⁻¹ (Zhang et al., 2013)

are limited by low supply potential, which favours the location of oat hull-based biorefineries close to established oat mills.

3. Biomass pretreatment

As explained in Chapter 2, the high recalcitrance of lignocellulosic biomass derives from its complex biochemical composition, determined, among others, by the degree of polymerisation, crystallinity, and shielding of cellulose (Zhao et al., 2012). Therefore, enzymatic hydrolysis of untreated feedstocks typically results in low hydrolysis rates and yields (Mosier et al., 2005), both which need to be improved to enable lignocellulose-based biorefineries.

Pretreatment is essential to reduce the recalcitrance of lignocellulosic materials and, thereby, turn lignocellulosic feedstocks into feasible substrates for enzymatic hydrolysis and fermentation. A broad range of pretreatment methods has been developed (Table 3). In general, they rely on two common mechanisms: Biomass fractionation into a cellulose-rich solid phase plus a liquid phase enriched in lignin, hemicellulose or both, which reduces shielding effects; and/or material fragmentation plus size-reduction to increase the accessible surface area. To achieve efficient fractionation and fragmentation, severe conditions are required, making pretreatment one of the most expensive unit processes in biorefineries (Yang & Wyman, 2008). Suitable pretreatment methods should require low energy inputs or enable efficient process integration, and have low operational and capital costs (Galbe & Zacchi, 2012). So far, only a few methods have been found suitable for commercial application, including steam pretreatment for herbaceous biomass (Larsen et al., 2012) and acid-catalysed steam pretreatment for the more recalcitrant softwoods (Galbe & Zacchi, 2012).

Table 3: Main mechanism and typical feedstocks for selected pretreatment methods.

| Pretreatment | Main mechanism | Typical feedstocks | Reference |
|---|--|--|---|
| Organosolv | Lignin extraction Hemicellulose hydrolysis | Herbaceous biomass Softwood Hardwood | (Zhang et al., 2016) |
| Ammonia fibre expansion | Lignin extraction | Herbaceous biomass | (Jin et al., 2012; Tao et al., 2011) |
| Ionic liquids | Fractionation of macromolecules | Herbaceous biomass Softwood Hardwood | (Brandt et al., 2013) |
| SPORL (Sulfite pretreatment to Overcome Recalcitrance of Lignocellulose) | Hemicellulose hydrolysis, lignin sulfonation | Softwood | (Dien et al., 2016; Zhang et al., 2012; Zhu et al., 2009) |
| Dilute acid pretreatment | Hemicellulose hydrolysis | Herbaceous biomass | (Tao et al., 2011) |
| Biological pretreatment | Fungal-induced degradation | Herbaceous biomass Softwood Hardwood | (Ray et al., 2010) |
| Steam pretreatment | Hemicellulose hydrolysis, fragmentation | Herbaceous biomass Softwood Hardwood | (Ewanick et al., 2007; López- Linares et al., 2015; Tengborg et al., 1998) |

3.1 Steam pretreatment

Steam pretreatment, also referred to as steam explosion, is a well-established technique derived from the Masonite process for fibreboard production (Boehm, 1930). Originally developed for steam-treating wood, this method has been proven suitable for a broad range of feedstocks, from agricultural residues, including corn stover (Liu & Chen, 2016) and wheat straw (Nielsen et al., 2017), to hardwoods such as willow stem wood (Sassner et al., 2006), and softwood biomass such as spruce stem wood (Pielhop et al., 2016a; Söderström et al., 2003). In this work, steam pretreatment was applied to logging residues (**Papers I and II**), oat hulls (**Paper II**), and wheat straw (**Paper III**). Steam pretreatment has been tested in several units at demonstration and pilot scale and is close to commercialisation (Galbe & Zacchi, 2012). During steam pretreatment the feedstock is

rapidly heated up by the application of high-pressure live steam and the temperature is held at 160 °C to 240 °C for a few seconds or up to several minutes (Galbe & Zacchi, 2012), depending on the feedstock. Afterwards, the feedstock is decompressed by a sudden pressure drop, while the material is discharged from the pretreatment reactor.

3.1.1 Mechanism

Steam pretreatment changes the chemical composition and physical characteristics of the feedstock. Hemicellulose is solubilised into the liquid phase, from here on referred to as hydrolysate, whereas cellulose and lignin remain in the solid phase. Hemicellulose hydrolysis is governed by protonation reactions targeting either the glycosidic or ring oxygen. The generated carbonium ions react with water to form monosaccharides (Sella Kapu & Trajano, 2014).

Without an acid catalyst, hemicellulose hydrolysis is mainly dependent on autohydrolysis. Steam pretreatment causes the release of acetyl groups from hemicellulose. Once in solution, they turn into acetic acid, which can serve as proton donor in hydrolysis reactions. Because autohydrolysis reactions are limited by the acetyl content in the hemicellulose fraction, they can result in efficient hemicellulose solubilisation of acetyl-rich herbaceous biomass and hardwoods, but not of acetyl-poor softwood (Galbe & Zacchi, 2012). Acid catalysts, such as H₂SO₄ or SO₂, are added to provide protons for hemicellulose hydrolysis, thereby reducing reaction times and temperatures.

Following pretreatment, lignin and cellulose remain in the solid phase. Lignin is relatively enriched due to hemicellulose solubilisation. Accordingly, in **Paper I**, hemicellulose content declined from 29% to <3% after pretreatment, while lignin content increased from 35% to 64–82%, depending on pretreatment conditions. Steam pretreatment reduces the degree of cellulose polymerisation, freeing up more reducing ends for exoglucanase activity during subsequent enzymatic hydrolysis (Hallac & Ragauskas, 2011). Compared to hemicellulose, cellulose is more stable because of its microfibrillar, crystalline structure (Li et al., 2005).

Lignin is relocated during steam pretreatment, and its structure changed. Above the glass transition temperature, lignin has been reported to cycle between the liquid and solid phase as a result of solubilisation, phase transitions (Trajano et al., 2013), and depolymerising chemical reactions, including the formation of carbonium intermediates under acidic conditions, which cause cleavage of ether bonds in β -O4'-linked lignin structures (Li et al., 2007). At the same time, lignin can repolymerise by building carbon-carbon linkages between the carbonium intermediate and its aromatic ring structures (Li et al., 2007; Pielhop et al., 2016b). Repolymerised lignin differs in its physical properties, presenting an increased specific surface area, which further hinders enzymatic hydrolysis (Pielhop et al., 2016b).

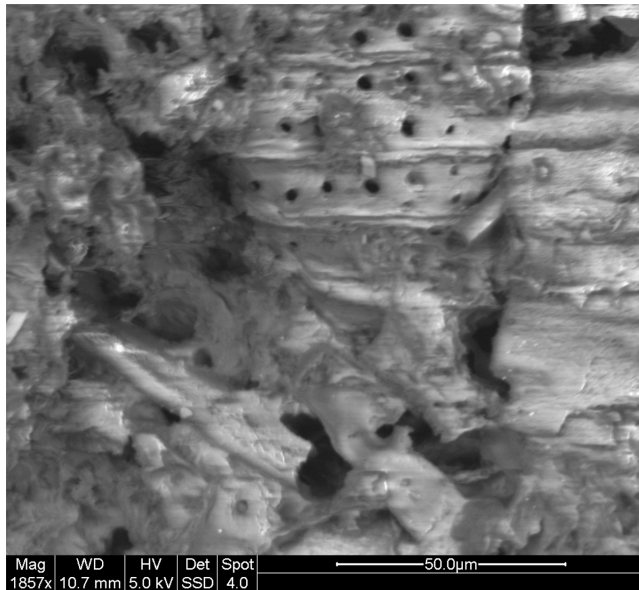


Figure 3: Surface characteristics of pretreated logging residues visualised by environmental scanning electron microscopy. Logging residues were steam-pretreated at 214°C for 20 min as described in **Paper I**. Environmental scanning electron microscopy was performed with the SSD detector of a Quanta 200 ESEM at 5 kV in low-vacuum mode to visualise the presence of condensates (darker colour) on the surface of logging residues and the pores generated during acid-catalysed steam pretreatment (unpublished data). Images were taken at the Chalmers Material Analysis Laboratory.

Extractives and carbohydrates have been reported to form lignin-like condensates, which are measured as Klason lignin and are referred to as pseudo-lignin. Pseudo-lignin is also recalcitrant to enzymatic hydrolysis (Hu et al., 2012). However, the extent of its recalcitrance compared to natural lignin remains unclear (Shinde et al., 2018). For the logging residues used in **Paper I**, the presence of condensation products on the surface of pretreated logging residues could be visualised by environmental scanning electron microscopy (Figure 3).

The physical characteristics of feedstocks vary in response to changes in biochemical composition and pretreatment conditions. Hemicellulose removal and lignin redistribution increase cellulose accessibility. The adiabatic expansion of the material upon decompression further increases cellulose accessibility due to fibre fragmentation and smaller particle sizes (Pielhop et al., 2016a). Furthermore, steam pretreatment generates pores and augments pore size through changes in the chemical composition and expansion of steam in the tracheid (Muzamal et al., 2015), as exemplified in Figure 3 for steam-pretreated logging residues used in **Paper I**.

3.1.2 Optimisation

From a process perspective, pretreatment is arguably the most influential unit process in lignocellulose-based biorefineries. Pretreatment conditions affect size reduction of the raw material, enzymatic hydrolysis, fermentation, the potential for co-product generation, and

wastewater treatment requirements (Yang & Wyman, 2008). Therefore, efficient reduction of feedstock recalcitrance is not the only criterion assessed during pretreatment development. Rather, pretreatment must meet the demands of up- and downstream unit processes. For example, suitable pretreatments should repress the formation of lignocellulose-derived inhibitors, which prevent microbial growth and product formation. By addressing these issues, the inherent recalcitrance of lignocellulosic feedstocks and the decreased fermentation efficiency in response to lignocellulose-derived inhibitors may be alleviated.

Consequently, pretreatments are often subjected to multi-parametric assessments that evaluate enzymatic hydrolysability, fermentability, as well as monosaccharide, solid, and inhibitor concentration in the hydrolysate (Table 4). Low inhibitor concentrations are desirable to reduce potential toxic effects on microbial growth and fermentation. Combined with analysis of the chemical composition of the solid phase, assessing monosaccharide concentration in the hydrolysate can provide information on the solubilisation of hemicellulose and cellulose. In addition, enzymatic hydrolysability and fermentability are evaluated directly, either separately or in SS(C)F processes.

The assessment of enzymatic hydrolysability serves two purposes: Evaluation of the ability of a pretreatment to efficiently reduce recalcitrance (*i.e.* hydrolysis efficiency), and estimation of the potential to release sugars during enzymatic hydrolysis from a given amount of solids (*i.e.* technical hydrolysis potential). Typically, enzymatic hydrolysability is expressed as hydrolysis yield based on glucan. However, for the design of enzymatic hydrolysis and fermentation, the technical hydrolysis potential is also important, as these processes are designed based on solid and not glucan loadings. That is because glucan content in the solid fraction changes with different pretreatment conditions. As a result, the technical potential might be higher for a material with elevated glucan content and inefficient hydrolysis than for a material with low glucan content but high hydrolysis efficiency. Therefore, the technical hydrolysis potential, expressed as hydrolysis yield based on water-insoluble solids (WIS), is an important additional measure in the evaluation of pretreatments.

Table 4: Typical response variables for the development of steam pretreatment. The response variables serve as direct measures and proxies for parameters used to evaluate pretreatment.

| Name | Unit | Measure for |
|--|---|--|
| Sugar concentration in hydrolysate | $[\text{g}\cdot\text{L}^{-1}]$ | <ul style="list-style-type: none"> • Initial sugar concentration in fermentation • Hemicellulose solubilisation (in combination with solid phase assessment) |
| Sugar recovery | $[\%]$ | <ul style="list-style-type: none"> • Overall conversion yields |
| Inhibitor concentration in hydrolysate | $[\text{g}\cdot\text{L}^{-1}]$ | <ul style="list-style-type: none"> • Inhibition of enzymatic hydrolysis • Inhibition of microbial growth • Inhibition of product formation |
| Solid content after pretreatment | $[\%]$ | <ul style="list-style-type: none"> • Potential for high solid loadings in enzymatic hydrolysis and fermentation • Demand for wastewater treatment |
| Microbial growth rate | $[\text{h}^{-1}]$ | <ul style="list-style-type: none"> • Inhibition of microbial growth • Overall hydrolysate toxicity • Nutrient availability |
| Hydrolysis yield | $[\text{g}_{\text{Glc}}\cdot\text{g}_{\text{Glucan}}^{-1}]$ | • Hydrolysis efficiency |
| | $[\text{g}_{\text{Glc}}\cdot\text{g}_{\text{WIS}}^{-1}]$ | • Sugar potential |
| Product titre | $[\text{g}\cdot\text{L}^{-1}]$ | • Product formation potential |
| Product yield | $[\text{g}_{\text{Product}}\cdot\text{g}_{\text{Glucan}}^{-1}]$ | • Product formation efficiency |

Most reactions during steam pretreatment, including hemicellulose solubilisation or lignin relocation and repolymerisation, are influenced by temperature, holdup time, and catalyst load. Consequently, these variables are routinely used to optimise process conditions. Other relevant factors include moisture content of the feedstock and chip size (Olsen et al., 2015). Because the exact reaction mechanisms during pretreatment are not fully understood, design of experiments (DoE) is combined with response surface methodology Black Box modelling to optimise pretreatment conditions. For Black box modelling, an in-depth knowledge of the system under study is not necessary. DoE strategies can minimise the number of experiments required to derive information from which to estimate the effects, interactions, and variations of pre-defined predictor variables on selected response variables. RSMs are typically generated by fitting polynomial models to the data and offer rapid identification of optimum

conditions in a pre-defined design space. In **Paper I**, the conditions previously developed for H₂SO₄-catalysed steam pretreatment of spruce stem wood were adjusted to and optimised for logging residues, accounting for structural and compositional differences. To find optimum pretreatment conditions, a central composite DoE strategy was combined with RSMs, setting temperature and holdup time as predictor variables.

Hemicellulose was efficiently solubilised under all investigated conditions, as indicated by solid phase analysis (**Paper I**). Maximum concentrations of hemicellulose-derived sugars in the hydrolysate were measured under the least severe pretreatment conditions. For example, maximum xylose concentrations, indicated by the lavender-coloured area in Figure 4, were obtained when either one predictor variable was minimal and the other maximal, or when both temperature and holdup time were minimal. Cellulose solubilisation required more severe pretreatment conditions than hemicellulose, which can be explained by the more resistant, microfibrillar, crystalline structure of cellulose (Li et al., 2005). Therefore, maximum glucose concentrations (light blue area, Figure 4) were found at a higher temperature (206–208 °C) than for hemicellulose-derived sugars. A decline in glucose concentration under more severe conditions, *i.e.* at higher temperatures and longer holdup times, was caused by the degradation of released monosaccharides, as shown also for spruce stem wood (Stenberg et al., 1998), and dilution by condensing steam.

In acidic environments and at commonly applied pretreatment temperatures, solubilised monosaccharides undergo degradation reactions to form compounds that are toxic to microorganisms. Furfural and 5-hydroxymethylfurfural (5-HMF) are the ones most commonly used to assess hydrolysate toxicity (Galbe & Wallberg, 2019). Furfural is the degradation product of pentose sugars and 5-HMF of hexoses (Jönsson et al., 2013). The mechanisms of furfural and 5-HMF formation and their toxic effects will be detailed later in this chapter. RSMs showed that the concentrations of 5-HMF (dark red area, Figure 4) and furfural (light red area, Figure 4) were dependent on both temperature and holdup time. Because cellulose solubilised at higher temperatures than hemicellulose, 5-HMF concentration was maximal at higher pretreatment temperatures than for furfural (Figure 4). Notably, the minima for 5-HMF and furfural matched the maxima for hemicellulose-derived sugar concentrations, *i.e.* xylose (lavender-coloured area, Figure 4).

The optimum conditions for an efficient hydrolysis, expressed as enzymatic hydrolysis yields based on glucan (light yellow area, Figure 4), coincided with the optimum conditions for glucose concentrations in the hydrolysate. However, the maximal technical potential to release glucose from solids, expressed as hydrolysis yield based on WIS (dark blue area, Figure 4), was found under the least severe pretreatment conditions. These conditions coincided with the highest ethanol concentrations in SSCF experiments (green dots, Figure 4).

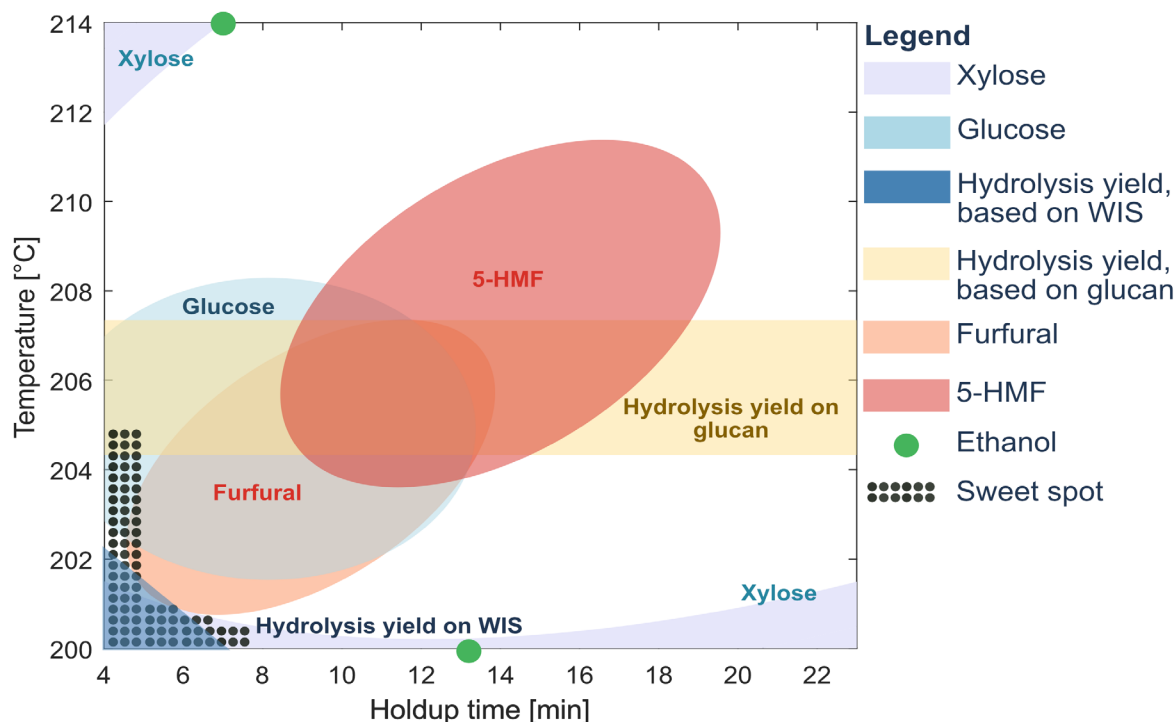


Figure 4: Sweet spot analysis for the optimisation of H₂SO₄-catalysed steam pretreatment conditions of logging residues. The plot shows the maximum concentrations of xylose (lavender), glucose (light blue), furfural (light red), and 5-HMF (dark red) in the hydrolysate after pretreatment; the maximum hydrolysis yields, based on glucan (light yellow) and WIS (dark blue); the maximum ethanol concentrations after a 72 h SSCF in shake-flasks (green dots); and the joint sweet spot (black dots). The sweet spot was a trade-off between high fermentability and high hydrolysis yields, based on glucan (**Paper I**).

Thus, the optimisation procedure in **Paper I** resulted in a trade-off between high enzymatic hydrolysability, based on glucan, and improved fermentability (Figure 4). The identified sweet spot (4.4–8 min, 200–205 °C; black dotted area Figure 4) ensured hydrolysis yields, based on glucan, at par with reported ones for spruce stem wood (Wang et al., 2018), high glucose concentrations in the hydrolysate, as well as limited 5-HMF and furfural formation and thereby reduced inhibition of microbial growth or product formation.

The influence of the acid catalyst, which was not tested in **Paper I**, remains under debate. The most common acid catalysts are sulphuric acid and sulphur dioxide, although other catalysts, *e.g.* acetic acid (Bondesson & Galbe, 2016), have been tested. Contradictory results have been published on the impact of catalyst loads. In acid-catalysed steam pretreatment of spruce wood chips, the SO₂ load had no significant impact on pretreatment results (Stenberg et al., 1998), whereas a significant effect has been reported for SO₂-catalysed steam pretreatment, without explosive decompression, of spruce logging residues (Janzon et al., 2014). In contrast, the type of acid catalyst has a more discernible effect. Comparison between H₂SO₄ and SO₂ has revealed superior hydrolysability by the latter (Tengborg et al., 1998; Wang et al., 2018), which has been associated with smaller particle sizes caused by SO₂ pretreatment (Wang et al., 2018). The impact of the catalyst on ethanol

fermentation has been characterised by contradicting results, leading to both lower (Martín et al., 2002; Tengborg et al., 1998) and higher ethanol yields (Wang et al., 2018) with H₂SO₄ compared to SO₂ as catalyst.

Optimal pretreatment conditions strongly depend on the feedstock and vary not only between biomass species, but also between different anatomical parts of the same species. The optimal pretreatment temperatures found in **Paper I** for spruce logging residues (200–205 °C) were significantly lower than those reported for spruce stem wood (~210 °C) (Stenberg et al., 1998). A contributing factor could be the lower particle size of logging residues, which comprise significant amounts of fines and needles, in comparison to stem wood chips. Feedstocks less recalcitrant than softwoods such as oat hulls (**Paper II**) require less severe pretreatment conditions to achieve high enzymatic hydrolysis yields.

3.1.3 Lignocellulose-derived inhibitors

The severe conditions of steam pretreatments induce the formation of a panoply of by-products, some of which are toxic to microorganisms. These inhibitory compounds can decrease biomass and product formation in terms of both yields and productivity. Thus, inhibitors constitute a major obstacle to achieving cost-competitive platform chemical production in lignocellulose-based biorefineries. Inhibitor formation depends on pretreatment conditions, mainly temperature, holdup time, and pH, as well as on feedstock type. Typically, inhibitors are grouped according to their chemical properties. The most important inhibitor groups are furaldehydes, weak organic acids, and phenolics.

The furaldehydes 5-HMF and furfural are the degradation products of hexoses and pentoses, respectively (Figure 5). Pretreatment of pentose-rich oat hulls resulted in more furfural than 5-HMF, whereas in logging residue hydrolysate the opposite was true (**Paper II**). Furaldehydes are formed by temperature-dependent dehydration reactions (Ramos, 2003). However, as shown in **Paper I**, both temperature and holdup time significantly influenced furaldehyde concentrations (Figure 4). Longer holdup times increase the dilution of pretreated material by condensing steam, providing an explanation for the effect of holdup time on furaldehyde concentrations.

In *S. cerevisiae*, furfural and 5-HMF inhibit several essential enzymes, including alcohol, acetaldehyde, and pyruvate dehydrogenases (Modig et al., 2002). Furfural has been reported to inhibit two key glycolytic enzymes, hexokinase and glyceraldehyde-3-phosphate dehydrogenase (Banerjee et al., 1981), as well as damage the mitochondria, vacuoles, actin cytoskeleton, and nuclear chromatin by inducing reactive oxygen species (Allen et al., 2010).

Several microorganisms, among them *S. cerevisiae* (Almeida et al., 2009) and some but not all *Bacillus coagulans* strains (van der Pol et al., 2016; Ye et al., 2014), can convert furfural into less toxic compounds. Under respiratory conditions, both *B. coagulans* and *S. cerevisiae* can oxidise furfural to furoic acid (Horváth et al., 2003; Ye et al., 2014). Under respiro-

fermentative conditions, *S. cerevisiae* can convert furfural to both furoic acid via oxidation and furfuryl alcohol via reduction (Horváth et al., 2003); whereas under anaerobic conditions, furfuryl alcohol is generated (Almeida et al., 2009). *B. coagulans* MA-13, the strain used in **Paper III**, has also been shown to metabolise furfural under anaerobic conditions (Aulitto et al., 2017) and express genes for *in situ* detoxification (Aulitto et al., 2019). Furthermore, *S. cerevisiae* can convert 5-HMF to the less toxic 2,5-bis-hydroxy-methylfuran (Liu et al., 2004).

In *S. cerevisiae*, furfural conversion is NAD(P)H-dependent and, therefore, lowers the intracellular NAD(P)H level. To cope with the increased NAD(P)H demand under aerobic conditions, *S. cerevisiae* augments the flux through the pentose phosphate pathway to regenerate NADPH (Pornkamol & Franzén, 2015). Under anaerobic conditions, NADH-dependent furfural detoxification compensates for the re-oxidation of NADH by glycerol formation and thus results in a net decrease in glycerol (Ylittervo et al., 2013). At the same time, furfural reduction causes competition for alcohol dehydrogenase, which reduces both furfural to furfuryl alcohol and acetaldehyde to ethanol. Under anaerobic conditions, this competition results in an intracellular accumulation of acetaldehyde, which blocks cell replication (Palmqvist et al., 1999a). Overall, the combination of stress induced by reactive oxygen species, reduced intracellular NAD(P)H and ATP levels, and inactivation of and competition for enzymes decrease the specific growth rate, biomass yield, ethanol productivity and yield, and cause a lag phase (Almeida et al., 2007). For *S. cerevisiae*, the duration of the lag phase depends on the concentrations of furfural and 5-HMF prior to their detoxification. In **Paper I**, furfural concentrations could be used as a proxy for the lag phase during aerobic growth of *S. cerevisiae* KE6-12A due to a strong correlation between the two ($R^2=0.85$). Low furfural concentrations can favour xylose consumption in *S. cerevisiae*. Under anaerobic conditions, furfural can serve as an electron acceptor to produce NAD^+ , which can be utilised in recombinant xylose-fermenting yeast strains expressing the xylose reductase (XR) / xylitol dehydrogenase (XDH) pathway (Wahlbom & Hahn-Hägerdal, 2002).

Increasing the severity of pretreatment conditions promotes further conversion of 5-HMF and furfural. 5-HMF is transformed into levulinic acid (Girisuta et al., 2007), whereas furfural and 5-HMF can be degraded to formic acid (Jönsson et al., 2013) (Figure 5). Furthermore, the labile acetyl groups in hemicellulose solubilise into the liquid phase, forming acetic acid (Figure 5). This is particularly obvious during pretreatment of herbaceous biomass and hardwoods due to their abundant hemicellulose acetylation. As shown in **Paper II**, the hydrolysate fraction of steam-pretreated oat hulls contained more acetic acid ($6.3 \text{ g}\cdot\text{L}^{-1}$) than logging residue hydrolysate ($3.8 \text{ g}\cdot\text{L}^{-1}$) did. Acetic, levulinic, and formic acids reduce microbial growth and final product yields (Almeida et al., 2007) in a variety of microorganisms, ranging from *B. coagulans* (Cubas-Cano et al., 2020) to *S. cerevisiae* (Ullah et al., 2012). The primary toxicity mechanism of weak organic acids is common to all microorganisms. They enter predominantly via passive diffusion in their undissociated form (Trček et al., 2015). Inside cells, the local pH causes the acids to dissociate, driving an acid influx until the concentrations of undissociated organic acid inside

and outside the cells are in equilibrium (Lindahl et al., 2018). Consequently, as protons and organic anions accumulate, an active transport of protons and, for some microorganisms, of the acids themselves is required to maintain the typically higher intracellular pH. The ATP-dependent active proton transport decreases ATP availability for growth. The cells can only grow while proton influx and efflux rates are equal. Influx in the undissociated form depends largely on the cultivation pH and pK_a of the acid. Because of its low pK_a of 3.75, formic acid can be more toxic than levulinic (pK_a : 4.64) or acetic (pK_a : 4.76) acids (Guo & Olsson, 2014).

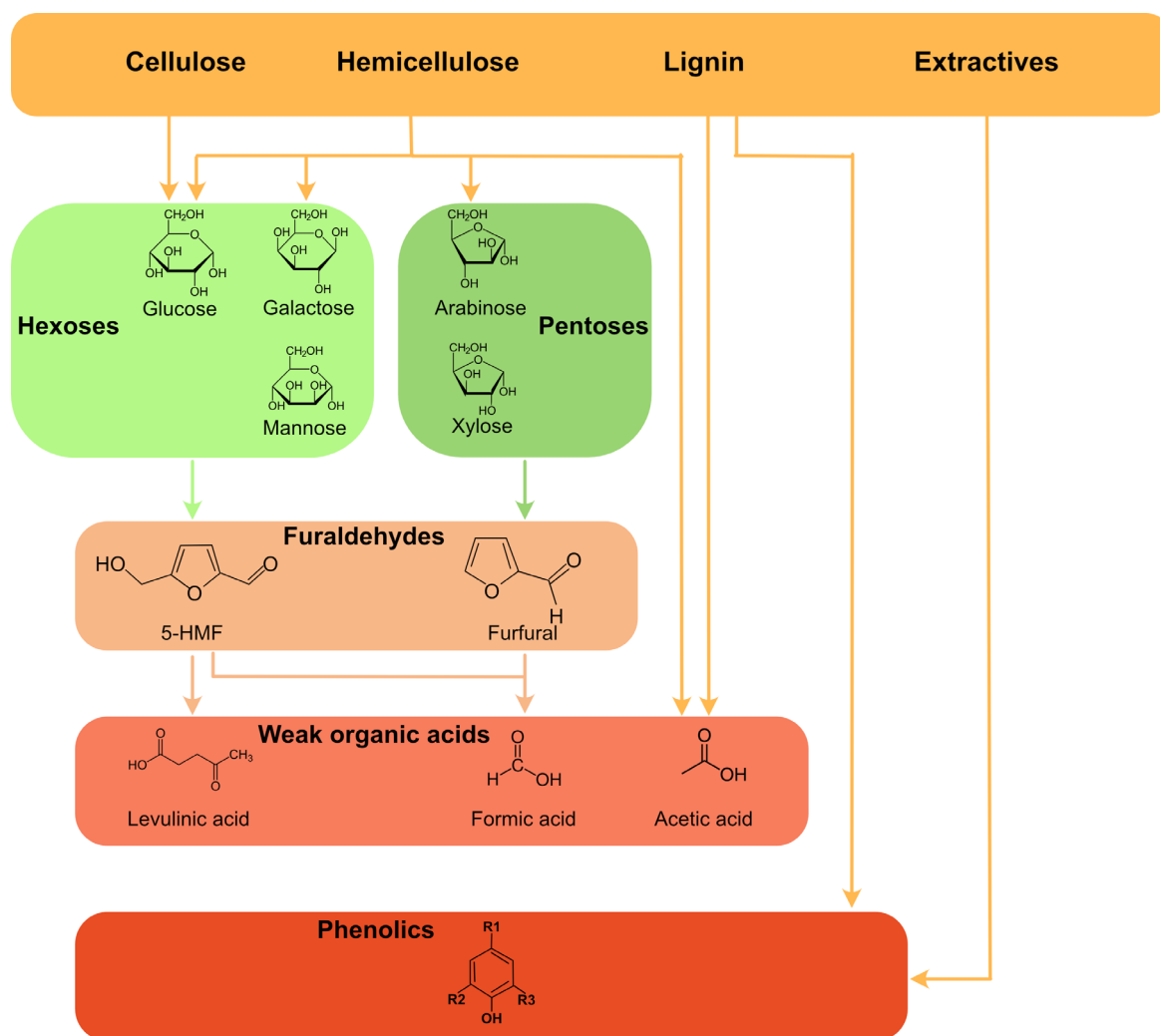


Figure 5: The most important fermentable monosaccharides and lignocellulose-derived inhibitors in pretreated hydrolysates. Hexose (light green) and pentose (dark green) sugars are carbon sources for fermentative microorganisms. Furaldehydes (light red), weak organic acids (red), and phenolics (dark red) inhibit microbial growth and product formation. Furaldehydes are sugar degradation products, the weak organic acids levulinic acid and formic acid are furaldehyde degradation products, acetic acid is mainly a product of acetyl groups released during hemicellulose solubilisation, and phenolics result from degradation of lignin and extractives.

Additionally, weak organic acids can elicit various microorganism-specific secondary toxicity mechanisms, ranging from the generation of apoptosis-inducing reactive oxygen species in *S. cerevisiae* (Ludovico et al., 2001) to exhaustion of the overall glutathione pool (Guo & Olsson, 2014). Nevertheless, under anaerobic conditions, low concentrations of weak organic acids can favour the production of ethanol in *S. cerevisiae* (Palmqvist & Hahn-Hägerdal, 2000) and lactic acid in *B. coagulans* (**Paper III**), while generating the ATP required for active proton transport.

Phenolic compounds, such as vanillin and coniferyl aldehyde, are also generated from lignin or the extractive fraction during steam pretreatment (Figure 5). Due to their elevated structural heterogeneity and the large variety of different toxicity mechanisms, only few phenolic compounds have been studied in detail. The inhibitory effects of phenolics on microbial activity are determined by their functional groups (Jönsson et al., 2013) and the substitution position (Almeida et al., 2007). *S. cerevisiae* can detoxify some phenolics, including coniferyl aldehyde, ferulic acid, and *p*-coumaric acid (Adeboye et al., 2015). Phenolic compounds not only inhibit microbial activity, but also cellulolytic enzymes by forming hydrogen bonds with proteins (Ximenes et al., 2011).

The toxicity of inhibitors in the hydrolysate fraction should not be assessed separately. Several studies have shown synergies between inhibitors (Ding et al., 2011; Palmqvist et al., 1999b), or between fermentation products at high concentrations and inhibitors (Lindahl et al., 2018; Westman et al., 2017). In **Papers I, II, and III**, the effects of logging residue, oat hull, and wheat straw hydrolysates on growth of *S. cerevisiae* KE6-12A and *B. coagulans* MA-13 were assessed. Both microorganisms were grown in the presence of increasing hydrolysate concentrations to identify the thresholds at which microbial growth was completely inhibited. Increasing hydrolysate concentrations decreased the maximal specific growth rates of *B. coagulans* MA-13 (**Paper III**) and *S. cerevisiae* KE6-12A (**Paper II**); maximal specific growth rates in the negative controls without oat hull and logging residue hydrolysate did not differ significantly. Direct comparison revealed that oat hull hydrolysate had a higher inhibitory effect on the maximal specific growth rate of *S. cerevisiae* KE6-12A than logging residue hydrolysate. Specifically, the toxicity threshold was reached at an oat hull hydrolysate concentration of 80% (Figure 6). At the same logging residue hydrolysate concentration, cells still showed significant growth as indicated by a relative maximum specific growth rate of 56% compared to the negative control (Figure 6).

The higher inhibitory effect of oat hull hydrolysate can be attributed to the higher acetic acid and furfural concentrations ($6.3 \text{ g}\cdot\text{L}^{-1}$ and $4.3 \text{ g}\cdot\text{L}^{-1}$, respectively) compared to logging residue hydrolysate ($3.8 \text{ g}\cdot\text{L}^{-1}$ and $1.2 \text{ g}\cdot\text{L}^{-1}$, respectively), a result of the higher acetyl and xylan content of oat hulls (Table 1). Furthermore, under all investigated conditions, oat hull hydrolysate led to a lag phase, which is in agreement with reported lag phases caused by furfural detoxification (Almeida et al., 2009).

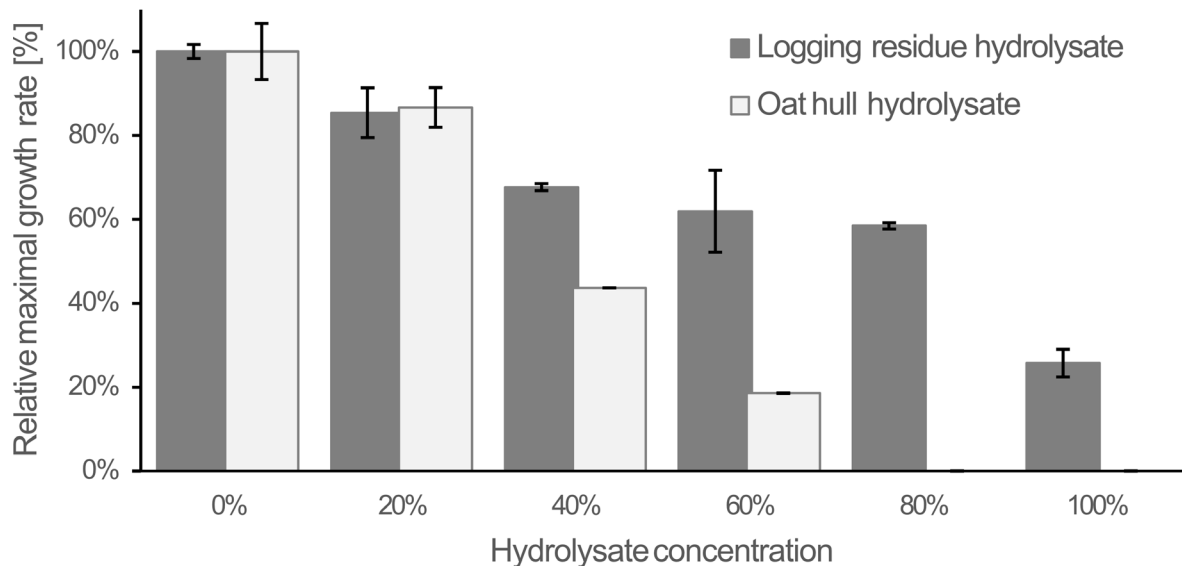


Figure 6: Effect of logging residue and oat hull hydrolysate on the relative maximal specific growth rate of *S. cerevisiae* KE6-12A. Cells were cultured in flowerplates in the BioLector platform (m2p-labs GmbH, Baesweiler, Germany) in $4 \text{ g}\cdot\text{L}^{-1}$ peptone and $2 \text{ g}\cdot\text{L}^{-1}$ yeast extract, and supplemented with different hydrolysate concentrations. To ensure comparability, the concentrations of glucose, galactose, mannose, and xylose were adjusted to those measured in respective the undiluted hydrolysates. The difference between the maximum specific growth rates of the negative controls without oat hull or logging residue hydrolysate was within the measurement error. Error bars indicate the standard deviation of at least technical duplicates (**Paper II**).

The screening experiments on logging residue and oat hull in **Paper II** did not only reveal the inhibitory effects of pretreated hydrolysates, but they also confirmed the advantage of utilising oat hull hydrolysate at low concentrations. While the final biomass concentration decreased with increasing logging residue hydrolysate concentrations, oat hull hydrolysate concentrations below 40% had the opposite effect. While cells in the negative controls entered a linear growth phase without depleting xylose after exponential growth on glucose, cells grown on low oat hull hydrolysate concentrations benefitted from xylose consumption, which resulted in extended exponential growth. The results suggest that in the absence of hydrolysate, the cells became nutrient-limited under the tested conditions and that low oat hull hydrolysate concentrations could overcome this nutrient limitation.

In conclusion, the steam pretreatment conditions developed for spruce stem wood were adapted to logging residues in **Paper I**, resulting in hydrolysis yields at par with stem wood despite the unfavourable biochemical composition of the logging residues. Optimal pretreatment conditions for logging residues reflected a trade-off between enzymatic hydrolysability and fermentability. Inhibitors formed during steam pretreatment impeded microbial growth and fermentability (**Papers I, II, and III**) and, thus, represent an additional obstacle that needs to be overcome in lignocellulose-based biorefineries. As shown in **Paper II**, despite favourable sugar concentrations, oat hull hydrolysate was more toxic to *S. cerevisiae* than logging residue hydrolysate, but could, at low concentrations, provide necessary nutrients to facilitate growth.

4 Enzymatic hydrolysis and fermentation

Pretreatment lays the foundation for enzymatic hydrolysis and fermentation. As outlined in Chapter 3, the main targets of pretreatment are to efficiently reduce recalcitrance, ensure high sugar recovery after pretreatment *and* enzymatic hydrolysis, and avoid inhibitor generation. Cellulose remains in the solid phase in most pretreatment methods, which prevents solubilised cellulose from excessive degradation under severe pretreatment conditions. One way to increase the accessible surface area of cellulose is to solubilise hemicellulose and/or lignin and, thus, reduce their shielding effect on cellulose. Overall, the solids generated during pretreatment are typically enriched in cellulose and can contain varying levels of lignin and hemicellulose depending on the pretreatment method used. Enzymatic hydrolysis is necessary to release the carbohydrates that remain in the solids into the liquid phase to provide fermentable monosaccharides which serve as substrates to fermentation.

4.1 Enzymatic hydrolysis

Even after pretreatment, lignocellulosic material is characterised by high structural complexity, which necessitates the application of several enzymes, including cellulolytic, hemicellulolytic and accessory enzymes, to efficiently hydrolyse cellulose and hemicellulose (Van Dyk & Pletschke, 2012). Cellulolytic enzymes are classified into endoglucanases, β -glucosidases, and cellobiohydrolases (CBH). CBHs hydrolyse either the reducing (CBH I) or non-reducing ends (CBH II) of crystalline cellulose, endoglucanases cleave within cellulose chains, and β -glucosidases release glucose by hydrolysing the glucooligomers released by CBHs and endoglucanases (*e.g.* cellobiose or cellodextrins) to glucose (Bornscheuer et al., 2014). Endoglucanases are produced also by *B. coagulans* MA-13, which was used in **Paper III** (Aulitto et al., 2017). They attack the amorphous regions of cellulose (Bubner et al., 2013) and reduce the degree of polymerisation.

Cellulolytic enzymes act synergistically, meaning that the enzymatic activity of the enzyme mixture is higher than the sum of the individual enzyme activities (Van Dyk & Pletschke, 2012). Cooperation between cellulolytic enzymes has been suggested, whereby endoglucanases rapidly degrade amorphous cellulose to uncover CBH-binding sites in the crystalline cellulose region and enable subsequent CBH hydrolysis (Ganner et al., 2012). Furthermore, cellulolytic enzymes act synergistically with lytic polysaccharide monooxygenases (Tokin et al., 2020). The latter are copper-dependent enzymes that cleave glycosidic bonds in crystalline cellulose in the presence of oxygen and an electron source (Beeson et al., 2015; Johansen, 2016). As a result, they provide new binding sites for CBHs. Hydrolysis of crystalline cellulose is slower than that of the amorphous region (Ganner et al., 2012), which is reflected by overall hydrolysis kinetics. An initial phase of rapid hydrolysis of amorphous cellulose entities is followed by a rate-retardation phase towards linear hydrolysis behaviour (Arantes & Saddler, 2011).

Various hemicellulolytic enzymes are required to efficiently saccharify lignocellulosic biomass, accounting for the structural heterogeneity of hemicellulose. Depending on biomass type, different activities are needed. While the hydrolysis of hardwood hemicellulose is predominantly governed by xylanases, mannanases dictate the saccharification of softwood hemicellulose (Álvarez et al., 2016). In addition, accessory enzymes remove substituents such as acetyl groups from hemicellulose backbones to increase accessibility to depolymerising enzymes. Hemicellulolytic enzymes act synergistically with cellulolytic enzymes on lignocellulosic biomass by increasing the accessibility of cellulose and are essential for an efficient hydrolysis even in raw material containing little hemicellulose after pretreatment (García-Aparicio et al., 2007).

Cellulolytic, hemicellulolytic, and accessory enzymes are typically combined in enzyme cocktails to obtain high enzymatic hydrolysis yields (Van Dyk & Pletschke, 2012). In **Papers I, II, and III**, the commercial enzyme cocktail Cellic CTec2 was applied. The performance of these cocktails is dictated by their constituents, the pretreated material they are applied to, and reaction conditions. The accessibility of binding sites on the cellulose surface of pretreated material is one of the limiting factors for efficient enzymatic hydrolysis. This is especially important for feedstocks with a low glucan content such as logging residues, because the accessible cellulose surface area is small and hydrolysis yields are thereby low (Arantes & Saddler, 2011).

Accordingly, at low enzyme loadings ($5 \text{ FPU} \cdot \text{g}_{\text{WIS}}^{-1}$), hydrolysis yields based on glucan, were substantially lower with logging residues (27%) than with oat hulls (96%) (**Paper II**). Given that the hydrolysis yield on oat hulls was already high at the lowest enzyme dosage, a ceiling was reached regardless of further increases in enzyme dosage (Figure 7). The ceiling indicates that hydrolysis proceeded until only the highly crystalline, undegradable cellulose was left (Arantes & Saddler, 2011). In contrast, hydrolysis yields on logging residues increased with greater enzyme dosages, but even at the highest enzyme dosage ($20 \text{ FPU} \cdot \text{g}_{\text{WIS}}^{-1}$), they remained 41% lower than with oat hulls (Figure 7). Because of the lower recalcitrance of oat hulls in comparison with logging residues, as indicated by higher

hydrolysis yields and ~200% higher hydrolysis rate (**Paper II**), oat hull solids were rapidly liquefied by enzymatic hydrolysis. In contrast, a combination of low glucan and high lignin content resulted in strong recalcitrance of logging residues, which was a major hurdle to efficient saccharification and liquefaction of the medium.

The high lignin content in logging residues (Table 1) presents an additional challenge to enzymatic saccharification. Besides decreasing the accessible surface area of cellulose through shielding effects (Kumar et al., 2012) as discussed in Chapter 3, lignin and pseudo-lignin diminish hydrolysis rates through non-specific binding of cellulolytic enzymes on their surface (Rahikainen et al., 2013). As shown in **Paper II**, the addition of bovine serum albumin was more successful at increasing hydrolysis yields on logging residues than on oat hulls. Bovine serum albumin binds to hydrophobic surfaces and prevents cellulolytic enzymes from non-specific binding to lignin (Eriksson et al., 2002). The effect of non-specific binding was larger in lignin-rich logging residues than lignin-poor oat hulls.

The extractive fraction of pretreated solids can play an important role in enzymatic hydrolysis. Extractives have been associated with impaired saccharification (Nitsos et al., 2019). This was confirmed upon the removal of extractives with ethanol, whereby hydrolysis yield on oat hulls showed a minor, yet significant increase from 97% to 100%. Increased hydrolysis yields after ethanol extraction have been attributed to the removal of surface lignin and pseudo-lignin (Nitsos et al., 2019). In contrast, ethanol extraction decreased enzymatic hydrolysis yield on pretreated logging residues by 72%. This may be explained by amphiphilic wood extractives preventing cellulolytic enzymes from irreversible, non-specific binding to the cellulose surface (Leskinen et al., 2015). By removing these extractives with ethanol extraction, irreversible, non-specific binding on the cellulose surface increases and hydrolysis yields decrease.

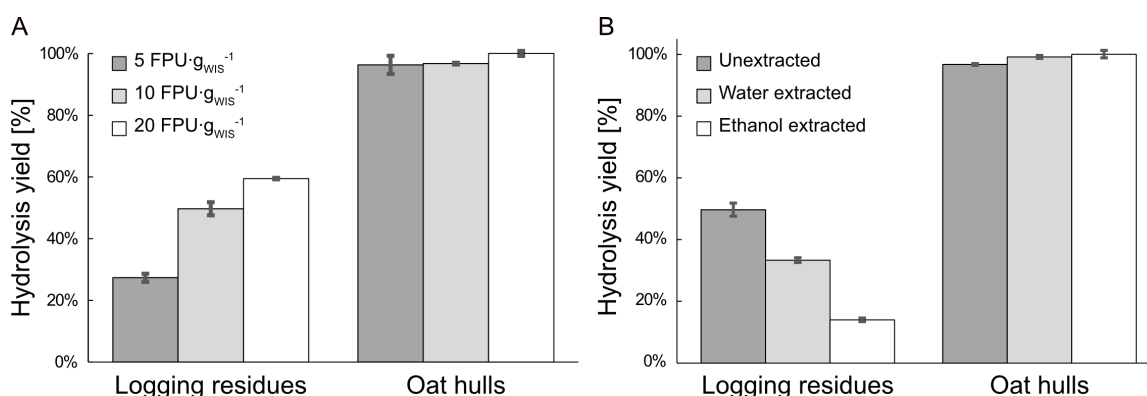


Figure 7: Influence of enzyme dosage and removal of extractive fractions on enzymatic hydrolysis of logging residues and oat hulls. (A) Hydrolysis yields, based on glucan, of logging residues and oat hulls at Cellic CTec2 loadings of 5 FPU·g_{wis}⁻¹ (dark grey), 10 FPU·g_{wis}⁻¹ (light grey), and 20 FPU·g_{wis}⁻¹ (white). (B) Hydrolysis yields, based on glucan, of unextracted (dark grey), water-extracted (light grey), and ethanol-extracted (white) logging residues and oat hulls at 10 FPU·g_{wis}⁻¹ of Cellic CTec2. All reactions were performed at 1.5% WIS and 50 °C for 72 h (**Paper II**).

Enzymatic hydrolysis performance is influenced also by reaction conditions. Most cellulolytic and hemicellulolytic enzymes have temperature optima of approximately 50 °C and pH optima between 4 and 6 (Singhania et al., 2010). Hence, enzymatic hydrolysis is a milder process than pretreatment. The mild conditions favour high glucose recovery as sugar degradation only occurs at the high temperatures at which pretreatments are conducted. Another important factor is solids loading. As shown in **Paper II**, hydrolysis yields on logging residues decreased with increasing solid loadings, which is in line with previous findings (Humbird et al., 2010; Weiss et al., 2019). The solids effect has been attributed to increased end-product inhibition (especially for older enzyme cocktails), mass transfer limitations, water availability at the reaction site (expressed as water constraint) and the effect of biochemical composition, especially lignin inhibition (da Silva et al., 2020). Compared to other reports, whereby the solids effect was detected at 15% WIS loadings (Weiss et al., 2019), here, it was already apparent at 4% with logging residues, probably due to elevated lignin content in logging residues and consequent increased non-specific binding of cellulolytic enzymes.

4.2 Fermentation in lignocellulosic medium

Fermentation processes in lignocellulose-based biorefineries rely on the release of fermentable sugars during enzymatic hydrolysis and the ability of microorganisms to convert these sugars at high rates and yields in the presence of lignocellulose-derived inhibitors. To ensure high yields, primary metabolites should be produced anaerobically, thus avoiding carbon losses to biomass growth and to complete substrate oxidation to CO₂ (Weusthuis et al., 2011). This is of particular importance for bulk products, such as ethanol and lactic acid, which must be produced at high yields to achieve economic feasibility. Hence, microorganisms suitable for the production of primary metabolites should be anaerobes or facultative anaerobes, such as *B. coagulans* and *S. cerevisiae*. In lignocellulose-derived medium, these microorganisms face two challenges: The variety of monosaccharides derived from pretreated biomass, all of which should be utilised to obtain high product yields, and the inhibitors generated during pretreatment.

Besides glucose, lignocellulosic media contain also hemicellulose-derived sugars. As discussed in Chapter 2, their abundance depends on the lignocellulosic feedstock. Xylose and arabinose are the most abundant hemicellulose-derived monosaccharides in agricultural feedstocks; whereas mannose and galactose are the most common in softwoods. After pretreatment, oat hull hydrolysate contained more than double the concentration of xylose than glucose (58.8 g·L⁻¹ and 25.1 g·L⁻¹, respectively) (**Paper II**).

However, most industrially relevant microorganisms are unable to convert all the different monosaccharides present in lignocellulosic media. *B. coagulans* MA-13 can metabolise glucose, cellobiose, and mannose but is unable to convert other hemicellulose-derived sugars (Aulitto et al., 2017). Further metabolic engineering would be necessary to achieve high technical yields on lignocellulosic substrates by introducing xylose and arabinose utilisation

pathways. The natural ability of several *B. coagulans* strains to convert arabinose and/or xylose (Pleissner et al., 2016; Ye et al., 2013) should simplify these metabolic engineering efforts. *S. cerevisiae* can naturally convert glucose, mannose, and galactose (Dynesen et al., 1998; Ostergaard et al., 2000), but is unable to efficiently metabolise xylose and arabinose. Although *S. cerevisiae* harbours genes to metabolise xylose, its conversion remains negligible (Toivari et al., 2004). Hence, various attempts to introduce xylose and, to a lesser extent, arabinose conversion pathways into *S. cerevisiae* have been made (Endalur Gopinarayanan & Nair, 2019).

S. cerevisiae KE6-12A, the strain used in **Papers I** and **II**, has been metabolically engineered to utilise xylose following the introduction of the XR gene *xyl1* and the XDH *xyl2* from *Scheffersomyces stipitis*, as well as overexpression of the endogenous xylulokinase gene *XKS1* (Wahlbom et al., 2003). After metabolic engineering, the strain underwent several rounds of evolutionary engineering to improve xylose utilisation and inhibitor tolerance at elevated temperatures (Albers et al., manuscript in preparation; Novy et al., 2017). In the XR/XDH pathway, XR catalyses the NAD(P)H-dependent conversion of xylose to xylitol, and XDH further converts xylitol to xylulose while generating NADH (Eliasson et al., 2001). Under anaerobic conditions, the different co-factor dependence in the XR/XDH pathway causes a co-factor imbalance, which results in increased xylitol production and diversion of xylose away from ethanol production (Kötter & Ciriacy, 1993). Furthermore, xylose uptake in *S. cerevisiae* relies on non-specific hexose transporters with a several-fold higher affinity for glucose than for xylose (Kötter & Ciriacy, 1993). Therefore, an efficient xylose uptake requires low glucose concentrations, which can be achieved by process design.

Lignocellulose-derived inhibitors generated during pretreatment hinder product formation and lower cell viability. To alleviate inhibitory effects, various hydrolysate detoxification methods have been developed (Jönsson et al., 2013); however, they increase costs by introducing an additional unit process. Therefore, research has focused on promoting inhibitor tolerance of microorganisms, using adaptive laboratory evolution (Almario et al., 2013), metabolic engineering (Larsson et al., 2001; Petersson et al., 2006), and preadaptation (Alkasrawi et al., 2006; Nielsen et al., 2015; Van Dijk et al., 2019). In this context, tolerance can be defined as the ability of cells to maintain viability and productivity in the presence of lignocellulose-derived inhibitors.

In preadaptation, also referred to as short-term adaptation, cells are exposed to dilute hydrolysate during propagation prior to fermentation (Alkasrawi et al., 2006; Nielsen et al., 2015; Van Dijk et al., 2019). Exposure to inhibitors activates several cellular responses, such as the upregulation of genes involved in oxidative stress response, thiamine and biotin biosynthesis, and furaldehyde detoxification (Van Dijk et al., manuscript in preparation). During preadaptation, phenotypes with an increased inhibitor tolerance come to dominate the cell population due to, for example, faster *in situ* detoxification. As outlined in Chapter 3.1.2, this represents an essential tool of microorganisms to cope with the toxicity exerted by inhibitors. The detoxification mechanisms help reduce inhibitor concentrations over time,

alleviate inhibitor-induced stress, and form an integral part of preadaptation. Consequently, preadapted cells have been shown to exhibit a shorter lag phase in subsequent fermentations because of improved furfural and 5-HMF reduction (Narayanan et al., 2016).

Improved inhibitor tolerance leads to higher product formation rates during fermentation, especially at higher solid loadings (Alkasrawi et al., 2006). This phenomenon is observed also in xylose-utilising *S. cerevisiae*, as the *in situ* detoxification of low furfural concentrations improves xylose utilisation and ethanol yields (Tomás-Pejó & Olsson, 2015). The improvement is achieved indirectly as reduction of furfural to furfuryl alcohol results in both NADH oxidation to NAD⁺, which can be reutilised in the XR/XDH pathway, and in a decrease in xylitol production, so that more xylose can be co-consumed to form ethanol. Therefore, *in situ* detoxification elicited by preadaptation benefits xylose co-consumption (Nielsen et al., 2015).

Preadaptation in Bacillus coagulans MA-13

In **Paper III**, the concept of preadaptation was applied to lactic acid production by *B. coagulans* MA-13 using steam-pretreated wheat straw as substrate. Unlike *S. cerevisiae*, *B. coagulans* MA-13 was propagated anaerobically, which prevented the accumulation of acetoin and acetic acid. Instead, lactic acid was produced to regenerate ATP required to cope with hydrolysate-induced stress. As outlined in Chapter 3.1.3, anaerobic growth of *B. coagulans* MA-13 was screened at different wheat straw hydrolysate concentrations. While maximal specific growth rates decreased with increasing hydrolysate concentration, specific lactic acid productivity increased until reaching a maximum at 50% hydrolysate concentration. Beyond this point, inhibitor-induced cell stress decreased lactic acid formation. Hence, under anaerobic growth conditions, specific lactic acid productivity could be used as a proxy for cell stress to identify optimal conditions in the trade-off between microbial growth and adaptation in the propagation phase.

In agreement with previous studies on *S. cerevisiae* (Van Dijk et al., 2019), preadaptation in *B. coagulans* MA-13 improved the volumetric and specific productivities during subsequent fermentation. Propagated in the presence of 30% wheat straw hydrolysate, *B. coagulans* produced lactic acid at a 115% higher average specific productivity within 50% shorter process times in subsequent simultaneous saccharification and fermentation (SSF) processes based on wheat straw, compared to non-adapted cells (Table 5). Higher hydrolysate concentrations during preadaptation resulted in reduced cell vitality and, in turn, decreased specific and volumetric lactic acid productivities and increased process times in batch SSF (Table 5). The results point to an optimum hydrolysate concentration for the propagation of *B. coagulans* MA-13.

Table 5: Effect of different hydrolysate concentrations during the propagation of *B. coagulans* MA-13 on lactic acid production in subsequent SSFs. SSFs were conducted anaerobically at 10% WIS, 10 FPU·g_{WIS}⁻¹ of Cellic CTec2, 0.01 g_{Cells}·g_{WIS}⁻¹ of *B. coagulans* MA-13, pH 5.5, and 55 °C on steam-pretreated wheat straw in 3.6-L BioEtOH double-jacket flat-bottom bioreactors. Pre-hydrolysis was conducted for 30 min prior to SSFs (**Paper III**).

| Hydrolysate in propagation [%] | SSF process time [h] | Avg. vol. lactate productivity [g·L ⁻¹ ·h ⁻¹] | Avg. spec. lactate productivity [10 ⁻¹¹ g·CFU ⁻¹ ·h ⁻¹] |
|--------------------------------|----------------------|--|---|
| 0 | 30 | 1.11±0.13 | 0.52±0.02 |
| 30 | 15 | 1.74±0.11 | 1.11±0.07 |
| 40 | 24 | 1.23 | 2.06 |
| 50 | 24 | 1.19±0.01 | 1.15±0.13 |

Another interesting observation was the appearance of a lag phase in batch SSFs when the inoculum size was reduced to 0.005 g_{Cells}·g_{WIS}⁻¹. The lag phase lasted 10 h for cells propagated without hydrolysate and 5 h for cells propagated in 40% hydrolysate. For cells propagated in 30% hydrolysate, no lag phase was observed. The lag phase was likely the result of impaired cell replication, as indicated by the decreased colony-forming unit counts. Lower counts can be attributed to higher inhibitor concentrations per cell, resulting in extended cell stress and increased need for detoxification. Again, the results pointed to a maximum hydrolysate concentration above which the effect of inhibitor toxicity outweighed the inhibitor-induced tolerance.

Paper III shows that the concept of preadaptation can be successfully adapted to lactic acid production by *B. coagulans*. Due to the activation of general stress response mechanisms, it seems reasonable to assume a general transferability of preadaptation to improve inhibitor tolerance – although the extent to which it actually happens might vary from strain to strain as shown in yeast (Van Dijk et al., 2019), and from feedstock to feedstock. The ability of a strain to detoxify inhibitors might be crucial in determining the success of preadaptation.

From a process design perspective, preadaptation combines several benefits compared to other detoxification strategies. First, unlike in most detoxification strategies, there is no need to add water, chemicals or other microorganisms. Second, process integration is less complicated for preadaptation than for other detoxification methods as only one additional filtration step is required to separate the hydrolysate from the pretreated slurry. At the same time, the filtration step enables high solid contents during saccharification and fermentation, which is necessary to reach sufficient product titres for efficient downstream processing. Third, preadaptation increases productivity and yields, which typically results in lower capital costs and unit cost of production. However, preadaptation comes at the expense of lower biomass yields during propagation, which must be balanced by higher sugar inputs. Therefore, preadaptation is a trade-off between high biomass yields and high specific growth rates during propagation on the one hand, and an increased inhibitor tolerance resulting in higher productivities during fermentation on the other hand.

4.3 Process configurations for efficient enzymatic hydrolysis and fermentation

The design of enzymatic hydrolysis and fermentation determines, to a large extent, the product formation potential and profitability of biorefineries. Several process configurations have been developed, the most important ones being separate hydrolysis and fermentation (SHF) and SSF. In case of sugar co-fermentation, SHF and SSF are referred to as separate hydrolysis and co-fermentation (SHCF) and SSCF, respectively.

In SH(C)F, enzymatic hydrolysis and fermentation are conducted in different vessels, which allows them to proceed at both of their optimal conditions. The temperature optimum of most hydrolytic enzymes is around 50 °C (Singhania et al., 2010), whereas that of most industrially relevant microorganisms, such as *S. cerevisiae* and *E. coli*, ranges around 30–37 °C (Olofsson et al., 2008a). Given that enzymatic hydrolysis and fermentation can be operated at optimal conditions, higher enzymatic hydrolysis rates can be generally achieved by SH(C)F. However, the large amounts of glucose and cellobiose released at optimal hydrolysis conditions lead to end-product inhibition of cellulolytic enzymes, which reduces hydrolysis yields (Hsieh et al., 2014). With improved enzyme cocktails, end-product inhibition has been reduced in SH(C)F (Cannella & Jørgensen, 2014).

In SS(C)F, enzymatic hydrolysis and fermentation are performed simultaneously in a single vessel. Monosaccharides released during enzymatic hydrolysis can be directly taken up and metabolised by the microorganism. Hence, glucose concentration in SS(C)F remains fairly low, which reduces end-product inhibition (Olofsson et al., 2008a), lowers the risk of contamination, favours xylose uptake, and promotes glucose and xylose co-consumption (Olofsson et al., 2008a). As a result, xylose conversion yields are typically higher in SSCF than in SHCF. Moreover, the low sugar concentrations in SS(C)Fs can reduce the risk for contaminations. Higher ethanol yields were reached in SSCFs than in SHCFs when xylose-utilising yeast was used (Nielsen et al., 2017; Tomás-Pejó et al., 2008). Furthermore, SS(C)F processes can reduce capital costs by 20% as a single reaction vessel is necessary (Wingren et al., 2003).

The main drawback of SS(C)F is the trade-off between the temperature optima for enzymatic hydrolysis and fermentation (Olofsson et al., 2008a). As shown in **Paper I**, enzymatic hydrolysis conducted at 30°C, which corresponds to the optimum temperature of *S. cerevisiae*, reduced enzymatic hydrolysis yields by approximately 20–25%, depending on pretreatment conditions. For ethanol production by *S. cerevisiae* in SS(C)F, temperatures of 32–35 °C are typically applied as a compromise (Olofsson et al., 2008b; Rudolf et al., 2008). Above 35 °C, *S. cerevisiae* is severely inhibited by heat stress which, in combination with lignocellulose-derived inhibitors, can result in stuck fermentation (Westman et al., 2017). One way to resolve the discrepancy between the temperature optima of enzymatic hydrolysis and fermentation is to apply thermotolerant organisms such as *B. coagulans* MA-13, a thermophilic strain that grows optimally at 55 °C (Aulitto et al., 2017). With the temperature

optimum of the microorganism matching the one of the applied enzyme mixture Cellic CTec2, both enzymatic hydrolysis and fermentation can be performed under optimal conditions. Moreover, running the process at higher temperature reduces the demand for cooling and, thereby, operational costs (Abdel-Banat et al., 2010).

High solid loadings represent an additional challenge to SS(C)F batch processes. For ethanol production, solid loadings of 20%, based on WIS, have been generally regarded high enough to achieve the target titres of >4% (w/w) ethanol (Xiros et al., 2017), which enable cost-efficient distillation and dehydration (Zacchi & Axelsson, 1989). High solid loadings are of special importance for feedstocks with low carbohydrate content such as logging residues. As shown in **Paper II**, solid loadings of 15% WIS were too low to achieve final ethanol titres above 4% (w/w), although ethanol yields of $78\pm 2\%$, based on glucose and xylose theoretically available in the raw material, were reached. Increasing the solid load to 20% improved final ethanol titres above 4% (Figure 8A), while ethanol yields did not change significantly. The ethanol yield⁴ achieved at 20% WIS was only slightly lower compared to published data for batch SSFs which were performed at 10% WIS and $20 \text{ FPU}\cdot\text{g}_{\text{WIS}}^{-1}$ on bark and stem wood mixtures (Frankó et al., 2015) at similar wood-to-bark ratios as found in the logging residues used in **Paper II**. Furthermore, the achieved ethanol yield of $76\pm 2\%$ was not significantly different to published yields for batch SSFs on 10% WIS spruce stem wood (Hoyer et al., 2009). In accordance with previous publications on SSCF using spruce stem wood (Hoyer et al., 2009), in batch SSCF at 20% WIS yeast viability declined over time (Figure 8A), which limited batch SSCF performance at high solid contents.

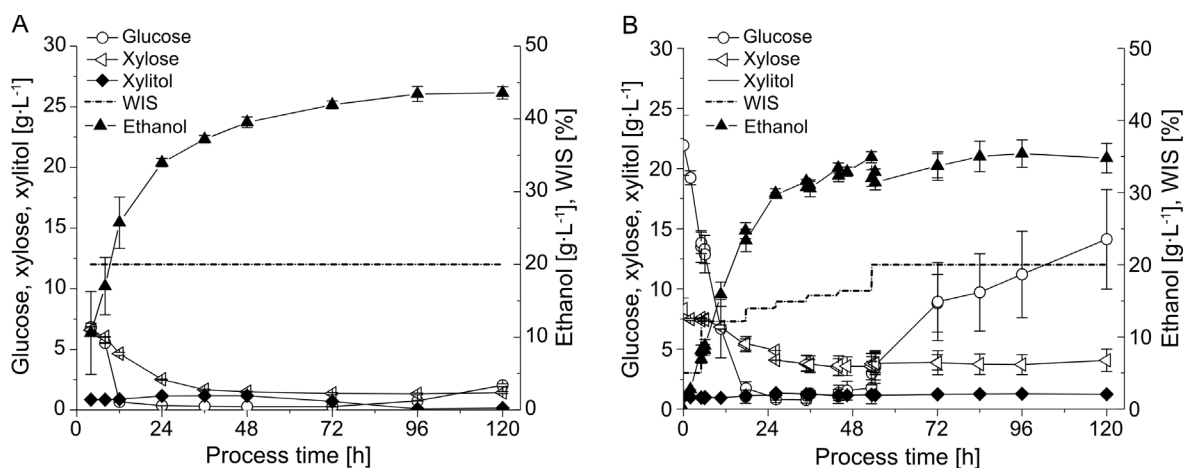


Figure 8: Effect of process configuration on ethanol production by *S. cerevisiae* KE6-12A from steam-pretreated logging residues. (A) Time-courses of duplicate batch SSCFs. (B) Time-courses of duplicate model-based multi-feed SSCFs. Glucose, xylose, xylitol, ethanol, and WIS concentrations are plotted over time. SSCF processes were performed at cumulative WIS loadings of 20% and enzyme loadings of $10 \text{ FPU}\cdot\text{g}_{\text{WIS}}^{-1}$, controlled at $35 \text{ }^\circ\text{C}$ and pH 5.2. The experimental conditions are further detailed in **Paper II**.

⁴ Note: The basis of yield calculation was changed for results in **Paper II** from glucose and xylose theoretically available in the raw material to glucose and mannose to aid comparability between the studies

Batch SS(C)F processes at high solid loadings are prone to inhomogeneities arising from the low solubility of lignocellulosic solids, especially in the initial phase (Koppram et al., 2014). Inhomogeneities, often observed as phase separation between liquids and solids, can hinder heat and mass transfer, with detrimental effects on enzymatic hydrolysis and fermentation. Moreover, inhomogeneities can impair process monitoring and control due to pH and temperature gradients (Koppram et al., 2014). These challenges are typically resolved by the increasing liquefaction in the course of enzymatic hydrolysis, which decreases viscosity. Hence, one of the primary goals in the design of SS(C)F processes is to reach rapid liquefaction of the lignocellulosic medium to obtain full process control and ensure proper and prolonged mixability.

Several fed-batch strategies have been developed with the aim of ensuring efficient mass transfer and coping with the slow liquefaction of lignocellulosic materials. Their unifying element is the addition of solids throughout the process to keep the actual solid loading below a predetermined threshold, while achieving high cumulative solid loadings (Olofsson et al., 2010; Wang et al., 2016). In this way, permanent mixability is ensured. The low glucose concentrations caused by the solid feedings benefit xylose co-consumption. The addition of enzymes, cells or combinations thereof with solids has been realised in multi-feed processes to maintain high hydrolysis rates and cell viability. To rationalise the additions in fed-batch SSCFs, model-based feeding strategies using feed-forward control have been developed and applied to pretreated birch stem wood, wheat straw, and sugarcane bagasse at laboratory and demonstration scale (Unrean et al., 2016; Wang et al., 2014; Wang et al., 2016; Westman et al., 2017). Macro-kinetic models were used to predict viable cell concentrations, cellulose conversion, actual WIS loadings, and ethanol concentrations. The predictions were used to design solid and cell additions.

In **Paper II**, a similar multi-feed SSCF strategy was developed to reach a cumulative solid loading of 20%. Instead of a full kinetic model predicting the interplay between enzymatic hydrolysis and fermentation (Wang et al., 2016), a hydrolysis model was used for solid additions. The model was a hybrid between steady-state statistical Black Box modelling in the form of RSMs and dynamic models to capture the dynamics of the enzymatic hydrolysis process (Figure 9). RSMs predicting the effect of solid and enzyme loadings during the course of hydrolysis were established and linked to second-order exponential decay models. The latter reflect the two phases of enzymatic hydrolysis: initial rapid hydrolysis and rate-retardation towards a linear hydrolysis pattern (Arantes & Saddler, 2011). To account for their predictive uncertainty, RSMs were weighed by their standard error in prediction to fit the exponential decay models. The hydrolysis model was used to predict hydrolysis yields and rates as function of time, enzyme load, and WIS. By implementing the modelling approach with a combination of RSMs and second-order exponential decay models, only a few experiments based on a DoE strategy without prior knowledge of hydrolysis kinetics were necessary to establish a hydrolysis model for a complex, heterogeneous, and uncharted substrate such as logging residues.

Instead of the hydrolysis yield, which was used in previous publications as a criterion for solid feedings (Wang et al., 2016; Westman et al., 2017), here, the hydrolysis rate was used as a criterion to keep liquefaction at high levels. Therefore, solids were fed to a pre-determined maximum solid concentration, when the hydrolysis rate was half-maximal, which coincided with the transition from rapid hydrolysis to the rate-retardation phase. However, the biochemical composition of logging residues hampered a consistent feed design. Due to their low carbohydrate content, non-hydrolysable solids accumulated over time, resulting in more frequent but smaller solid additions over time. Eventually, planned additions would exceed pre-set process time limits. Feeding substrate at later process times would result in an incomplete hydrolysis. Consequently, solids were fed according to model-based feeding trajectories in the first half of the process. The remaining solids were added with the last solid addition within 60 h, thereby exceeding the experimentally determined maximum solid load and risking to temporally decrease mixability (Figure 8B). To increase cell viability, 95% of the total cells were added in proportion to the hydrolysate fraction within the first 60 h, whereas the remaining 5% were added at 72 h to promote continued fermentation and limit the loss in cell viability observed in multi-feed processes over time (Wang et al., 2014).

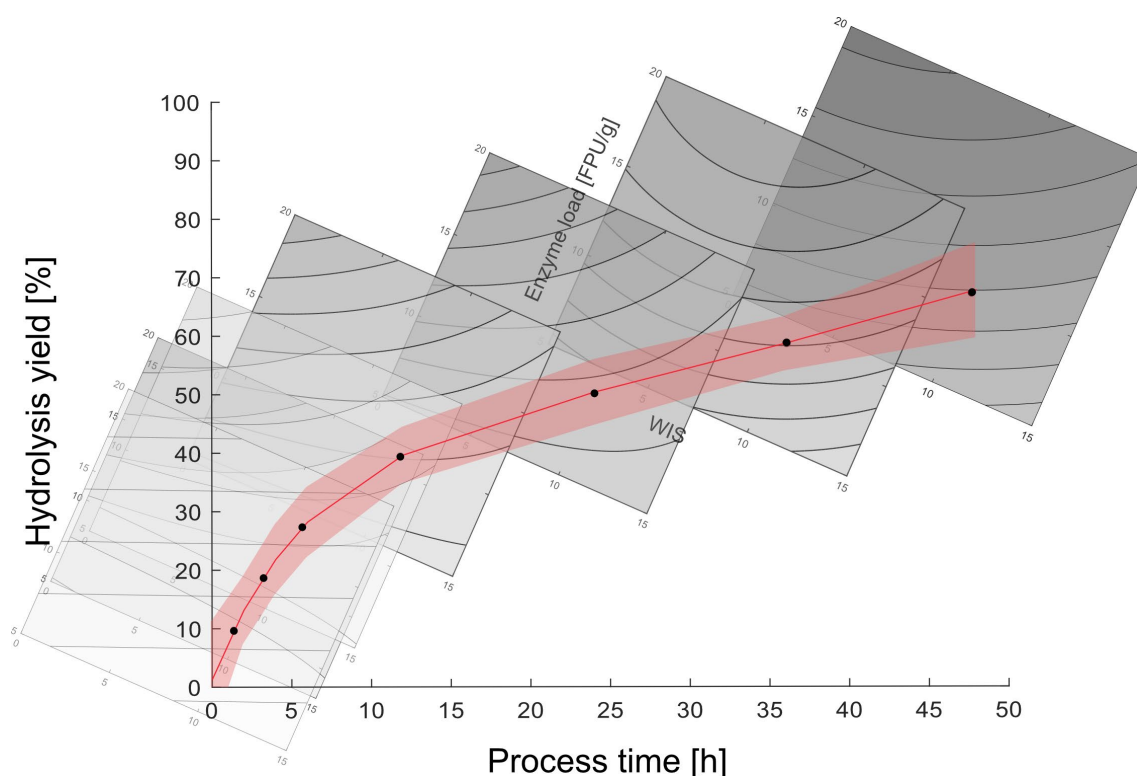


Figure 9: Multi-feed model development. The modelling strategy is exemplified for an enzymatic hydrolysis at $10 \text{ FPU} \cdot \text{g}_{\text{WIS}}^{-1}$ and 10% WIS. The RSMs were established to predict hydrolysis yields based on glucan, indicated by black dots on the response surfaces, as function of enzyme and WIS load. The hydrolysis yields were combined in a second-order exponential decay model (red curve) describing the progression of hydrolysis yields over time. Prediction uncertainties of the RSMs were incorporated in model fits by weighting each RSM by its standard error in prediction. The red area around the red curve shows the 95% confidence interval around the mean for the example (**Paper II**).

Compared to batch processes, the multi-feed strategy resulted in significantly lower ethanol titres and yields (Figure 8B). Final ethanol titres remained below the minimum requirement for efficient distillation. To be competitive with batch processes, the multi-feed design needs improvements in the following areas:

(1) Yeast viability at the later stages of fermentation.

Yeast viability dropped after 54 h following the last, large solid addition after as indicated by immediate glucose and xylose accumulation. The addition of yeast cells at 72 h to sustain viability had only a minor benefit, which is in accordance with previous results on wheat straw (Westman et al., 2017). A possible solution, applied successfully to multi-feed SSCFs on wheat straw, is to lower the temperature to alleviate temperature-induced stress (Westman et al., 2017).

(2) Xylose conversion.

High hydrolysis rates required for rapid liquefaction result in high glucose concentrations, which prevent xylose uptake. Rapid liquefaction is necessary to gain proper process control, especially for logging residues with their high lignin content. Thus, xylose should be utilised ahead of multi-feed SSCF in a pre-fermentation step, previously shown to improve xylose utilisation in fed-batch schemes (Nielsen et al., 2017).

With these improvements, the multi-feed strategy could potentially outperform batch SSCF processes. However, the current design favours batch processes for logging residues despite the challenges they pose.

4.4 Effect of mixed feedstocks on enzymatic hydrolysis and fermentation

The main disadvantage of logging residues is their low carbohydrate content, which creates multiple problems in process design and performance. These include the difficulty to design feeding schemes ensuring proper mixability in multi-feed SSCF, slow liquefaction due to impaired enzymatic hydrolysis, and the need to conduct SSCF at high solid loadings to achieve sufficient product titres. In **Paper II**, upgrading low-carbohydrate logging residues with carbohydrate-rich oat hulls was investigated.

The concept of upgrading the carbohydrate content by feedstock mixing has been applied mainly to mixtures of lignocellulosic material with first-generation feedstocks, in so-called 1.5G bioethanol production (Erdei et al., 2010; Erdei et al., 2013; Persson et al., 2020; Xu & Wang, 2017). Upgrading the carbohydrate content can improve final ethanol titres and, in turn, reduce downstream processing costs (Joelsson et al., 2016). In several cases, positive synergistic effects of mixed feedstocks on ethanol yields have been shown, further improving process economics (Persson et al., 2020; Xu & Wang, 2017). In **Paper II**, a weak

synergistic effect was shown for the enzymatic hydrolysis of oat hulls and logging residues, alleviating their recalcitrance. Oat hull addition at a 1:3 ratio in batch SSCF and at 20% solid loadings resulted in ethanol titres above 50 g·L⁻¹ at technical yields, which did not differ significantly from batch SSCFs on logging residues only (Figure 10A).

The high ethanol titres reached upon substrate upgrading demonstrate the potential of oat hull addition in reducing unit production costs. Furthermore, increased final ethanol titres can lower the requirement to run processes at high solid loadings. The main bottleneck in SSCF containing oat hulls was xylose utilisation. Due to the elevated cellulose content of oat hulls, glucose-limited conditions were reached later than for SSCF on logging residues only. However, in the later process stages xylose utilisation typically declines (Nielsen et al., 2017; Olofsson et al., 2010), possibly due to an overall decreased cell viability caused by prolonged exposure to inhibitors (Nielsen et al., 2017) or the fermentation-dependent formation of compounds specifically inhibiting the XR/XDH pathway (Olofsson et al., 2010).

The high carbohydrate content and low recalcitrance of oat hulls was beneficial for the design of multi-feed SSCFs. In contrast to logging residues, the high amount of degradable solids in oat hulls enabled a feasible solid feeding strategy. However, the high residual glucose and xylose concentrations in multi-feed SSCFs on oat hulls and logging residues (Figure 10B) imply a strong inhibitory effect leading to partial yeast inactivation. The latter is explained by the higher initial concentrations of inhibitors, such as acetic acid and furfural, in the oat hull hydrolysate. Due to partial cell inactivation early in the multi-feed process, the cells were sensitive to further solid additions, resulting in compromised process robustness and, in turn, lower ethanol titres and yields compared to batch processes (Figure 8B).

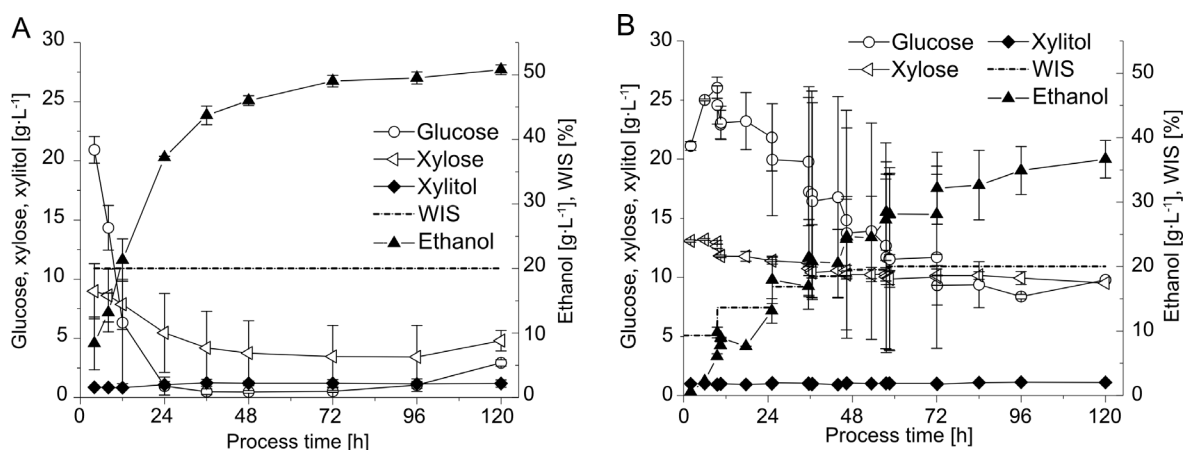


Figure 10: Effect of process configuration on ethanol production by *S. cerevisiae* KE6-12A from steam-pretreated logging residues mixed with oat hulls at a 1:3 ratio. (A) Time-courses of duplicate batch SSCFs. (B) Time-courses of duplicate model-based multi-feed SSCFs. Glucose, xylose, xylitol, ethanol, and WIS concentrations are plotted over time. SSCF processes were performed at cumulative WIS loadings of 20% and enzyme loadings of 10 FPU·g_{WIS}⁻¹, controlled at 35 °C and pH 5.2. The experimental conditions are further detailed in **Paper II**.

One way to improve the multi-feed design is to start the process with logging residues and feed oat hulls gradually together with logging residues. The slower glucose release from logging residues could reduce initial glucose concentrations and, thus, result in improved xylose utilisation. Furthermore, the lower inhibitor concentrations in logging residue hydrolysate could improve yeast viability. Despite a possible higher inhibition effect at later process stages, originating from the synergistic action of ethanol and acetic acid from oat hull hydrolysate, this strategy has the potential to improve ethanol titres and yields in multi-feed SSCF.

In conclusion, upgrading the low carbohydrate content of logging residues with oat hulls in batch SSCF improved ethanol titres and may lower operational costs. Improved xylose fermentation would allow the full carbohydrate potential of oat hulls to be exploited, possibly further increasing both ethanol titres and yields.

The mixing of feedstocks has wide implications on biorefineries. From a supply perspective, it decreases supply risks. Biorefineries operating at industrial scale require large amounts of feedstock to reach an economy of scale. Postulated feedstock demands vary between 2000–5000 metric tons per day (Argo et al., 2013; Humbird et al., 2011). As discussed in Chapter 2.2, seasonal and geographic variability can lead to supply chain risks, which translate into economic risks (Golecha & Gan, 2016a). Feedstock mixing, preferably of similar materials, has been described as a suitable option to ensure a secure feedstock supply and hedge risks arising from feedstock price volatility (Golecha & Gan, 2016b). Furthermore, feedstock mixing can address a dilemma faced by industrial-scale biorefineries. On the one hand, transport distances and costs increase with biorefinery size, resulting in a diseconomy of scale (Richard, 2010); on the other hand, large-scale biorefineries are required to reach economically feasible bioconversion processes for inexpensive bulk products such as platform chemicals. Feedstock mixing can reduce transport distances and, thus, improve the economy of biorefineries.

The benefits of supply chains relying on multiple feedstocks can be increased when integrating processes based on multiple feedstocks into existing infrastructure. In the case of oat hulls this implies integration with oat mills; in the case of mixing first with second-generation feedstocks, it involves biorefineries already using first-generation feedstocks. The choice of mixable feedstocks has implications on pretreatment. Feedstocks with similar characteristics can be pretreated together, which reduces capital costs compared to two separate pretreatment units (Nielsen et al., 2019). Even for feedstocks with very different composition, common pretreatment can be beneficial when synergies between the two become advantageous, *e.g.* acetic acid released from herbaceous biomass aids hemicellulose solubilisation of woody biomass (Vera et al., 2015). Nonetheless, most simultaneous pretreatments will result in a trade-off scenario, which must be carefully evaluated in terms of drawbacks, such as lower product formation rates or yields (Oke et al., 2016).

Another advantage of mixed feedstocks is the possibility to generate value-added co-products. In the case of oat hulls mixed with logging residues (**Paper II**), biogas and

electricity can be produced. The xylose-rich oat hull hydrolysate could be further valorised to produce other value-added compounds, including furfural or xylitol. This type of co-production could increase the profitability of biorefineries. In summary, feedstock mixing can overcome limitations imposed by an unfavourable biochemical composition of a feedstock and benefit product formation. Additional benefits include a safer supply chain and increased ability for co-product generation. Still, mixing different feedstocks necessitates case-to-case evaluation because of potential trade-offs in pretreatment and fermentation strategies, which could lower process yields and increase operational costs.

5 Assessment of process designs for lignocellulose-based biorefineries

5.1 Modelling of simultaneous saccharification and fermentation: From hydrolysis modelling to life cycle assessment

Biorefineries are envisaged to play a key role in the transition from fossil fuels to renewable resources. To fill this role, biorefineries must simultaneously meet technical, economic, and environmental goals. Ideally, they should be capable of rapidly converting biomass into products at high yields, be profitable, and benefit the environment. The performance of SS(C)F processes and their impact on biorefinery systems have been typically assessed along these goals to guide process development. Mathematical models at various system levels have been established to formalise process knowledge, quantitatively assess SS(C)F process performance, and guide their design through rational optimisation (Figure 11).

At bioreactor scale, hydrolysis models have been established to formalise knowledge on hydrolysis kinetics, reveal the mechanisms governing any synergisms and hydrolysis rate reduction, as well as optimise hydrolysis conditions with respect to pH, temperature, and solid loadings (Bansal et al., 2009) (Figure 11). Besides empirical models in the form of RSMs (*e.g.* those developed in **Paper II**), numerous mechanistic models have been developed to predict hydrolysis dynamics and the interaction of hydrolytic enzymes with their substrates. A representative model, which serves as the basis for several other hydrolysis models (Angarita et al., 2015; Hodge et al., 2009; Prunescu & Sin, 2013), was developed by Kadam et al. (2004). Although several model parameters were unidentifiable (Sin et al., 2010), its simplicity made the model, either in its original form or with reduced parameter subsets, useful for SS(C)F process design.

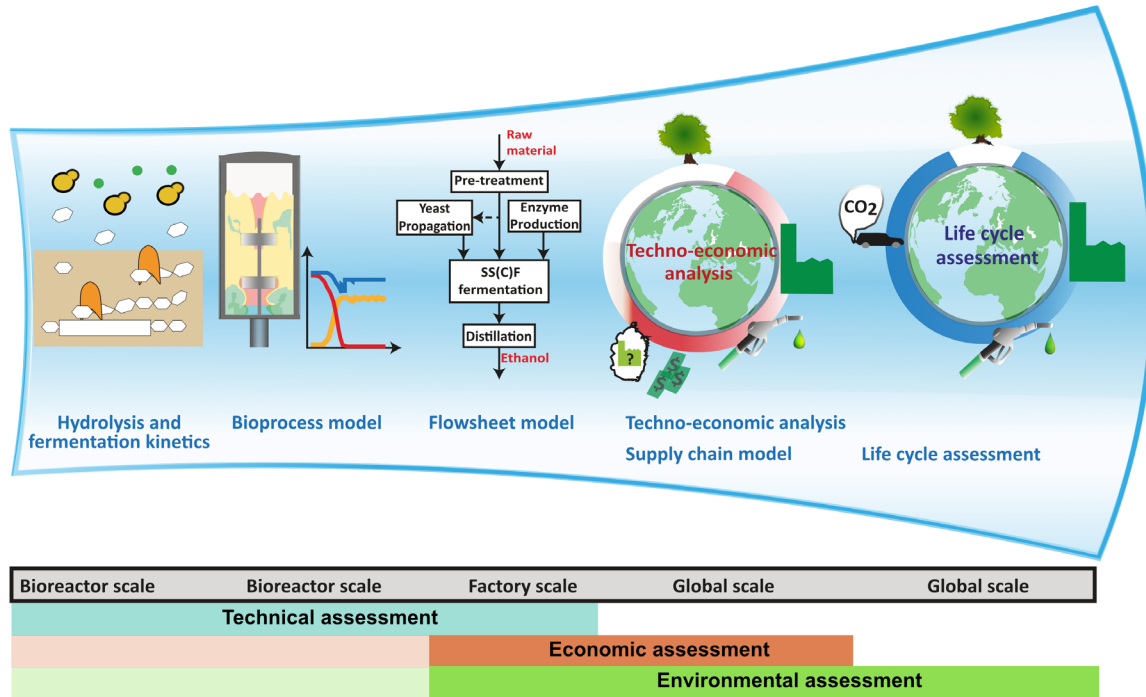


Figure 11: Assessment of SS(C)F performance at different system scales. The system scales range from hydrolysis kinetics at bioreactor scale to LCA at global scale. Technical assessments range from bioreactor to factory scale (blue bar); whereas economic and environmental assessments range from factory to global scale (brown and green bars, respectively). Economic and environmental assessments can be extended to bioreactor scale (light brown and light green bar).

Typical for most mechanistic enzymatic hydrolysis models, Kadam et al. (2004) modelled enzyme adsorption by Langmuir isotherms as shown in **equation 1**:

$$c_{EB} = \frac{E_{max} \cdot k_{ad} \cdot c_{EF} \cdot c_S}{1 + k_{ad} \cdot c_{EF}} \quad (1)$$

where c_{EB} is the bound enzyme concentration, E_{max} is the maximum amount of enzyme that can adsorb to the solids, k_{ad} is the dissociation constant for enzyme adsorption, c_{EF} is the concentration of enzyme free in solution, and c_S is the substrate concentration. Although the underlying assumptions such as homogenous binding sites over time, are not met in reality, Langmuir isotherms are frequently used as mathematical expression providing a good fit to experimental data (Bansal et al., 2009). In **Paper IV**, the enzymatic hydrolysis model described enzyme adsorption by second-order adsorption kinetics, with a dynamic adsorption equilibrium accounting for changes in adsorption and substrate characteristics over time (Wang et al., 2014).

Hydrolysis reaction rates including end-product inhibition are usually modelled by Michaelis-Menten kinetics with competitive monosaccharide inhibition (Kadam et al., 2004) as described in **equation 2**:

$$r_H = \frac{k_R \cdot c_E \cdot c_S}{1 + \frac{c_{SP}}{K_{SP}}} \quad (2)$$

Where r_H is the hydrolysis reaction rate, k_R is the reaction rate constant, c_E is the enzyme concentration, c_S is the substrate concentration, c_{SP} is the concentration of the resulting sugar product, and K_{SP} is the inhibition constant. The temperature-dependency of reaction rate constants is typically modelled using the Arrhenius equation for temperature dependency as presented in **equation 3**:

$$k_R = c \cdot e^{\frac{-E_A}{R \cdot T}} \quad (3)$$

Where k_R is the reaction rate constant, c is a pre-exponential coefficient, E_A is the activation energy, R is the universal gas constant, and T is the temperature. Although the underlying assumptions, especially homogenous catalysis in a single phase, are not met for enzymatic hydrolysis processes, models using the Arrhenius equation generally display a good fit and are widely employed. Hydrolysis models have been applied to predict hydrolysis dynamics, optimise enzyme cocktails (Niu et al., 2016), and predict solid additions in fed-batch hydrolysis processes (Hodge et al., 2009).

Kinetic enzymatic hydrolysis models are typically combined with fermentation kinetics in mechanistic SS(C)F bioprocess models (Figure 11). Mechanistic SS(C)F models describe the dynamic mass balances of SS(C)F processes in a set of ordinary differential equations, and are numerically solved in MATLAB or Python (Zhuang et al., 2013). The general structure of these mechanistic models is presented in **equation 4**:

$$\frac{dx(t)}{dt} = f(x(t), t, \theta); y(t) = g(x(t)); x(t_0) = x_0 \quad (4)$$

Where $x(t)$ are the state variables, t is the process time, θ are the model parameters, and $y(t)$ are the model outputs. Besides hydrolysis and fermentation kinetics, population balance models (Wang et al., 2014) and dynamic flux balance models (Unrean et al., 2016) have also been integrated into SS(C)F process models. The main outputs of SS(C)F process models are cellulose, glucose, ethanol, and biomass concentrations over time.

The integration of hydrolysis and fermentation kinetics into dynamic mass balance models is important to accurately describe the intertwined dynamics between these two processes such as the decreased glucose inhibition caused by simultaneous sugar uptake by fermentative microorganisms (Wang et al., 2014). SS(C)F process models have been used to gain insights into the interplay between enzymatic hydrolysis and fermentation (Westman et al., 2017) and guide the design of SSCF processes, for example, of model-based feeding trajectories in multi-feed SSCFs (Wang et al., 2014; Wang et al., 2016).

For plant-wide modelling of biorefineries, a host of flowsheet models has been established (Chovau et al., 2013; Gnansounou & Dauriat, 2010). Developed in SuperPro Designer, Aspen Plus, or CHEMCAD, these models assess entire biorefineries and solve mass balances for all unit processes and operations (Zhuang et al., 2013) (Figure 11). In contrast to bioprocess models, flowsheet models are evaluated at steady-state conditions. The main outputs of flowsheet models are the overall mass balances, and the demand for heat and energy in each process step. Therefore, flowsheet models are used to identify potential targets for process integration (Fornell & Berntsson, 2012; Joelsson et al., 2015; Ojeda et al., 2011). For example, in **Paper IV**, the steam generated in the boiler from the combustion of solids leftover from fermentation and anaerobic digestion is used for steam pretreatment.

Usually, plant-wide flowsheet models are combined with global-scale economic models in TEAs (Figure 11). The main outputs of such economic analyses are minimal product selling prices, net present values, and internal rates of return (Cheali et al., 2016). TEAs and flowsheet models have been widely applied in lignocellulosic process development to identify bottlenecks in process integration and economics (Gnansounou & Dauriat, 2010), devise economically feasible process configurations (Olofsson et al., 2017), as well as to compare different biorefineries regarding processed feedstocks (Duque et al., 2015; Frankó et al., 2016) and co-products (Joelsson et al., 2016). The latter is particularly important in the context of lignocellulosic biorefineries, as production of platform chemicals alone will likely not result in profitable biorefinery processes (Rosales-Calderon & Arantes, 2019).

The environmental impact of biorefineries and different process configurations are studied at global scale in LCAs (Figure 11). To compare the effects of different process configurations and designs on environmental impact categories, attributional LCAs are typically conducted. They focus on a description of the environmentally relevant physical flows from and to a life cycle system and its subsystems (Finnveden et al., 2009). Therefore, environmental burdens are allocated (*e.g.* between different products) by partitioning the environmental burden based on physical, chemical or economic causes (Finnveden et al., 2009). The impact of different process designs is usually investigated in cradle-to-gate LCAs, in which the product life cycle is assessed from feedstock growth to the product leaving the biorefinery (Borrion et al., 2012). Some LCAs on bioethanol also included the use phase, resulting in cradle-to-grave LCAs (González-García et al., 2012; Rajagopalan et al., 2017). Typical functional units used to compare different process strategies in cradle-to-gate LCAs of biorefineries are kg or L of generated product (Borrion et al., 2012). LCA outputs encompass the assessed impact categories, which include the climate impact, eutrophication and acidification potential, and ecotoxicity, all of which are relative to the functional unit. LCAs have been used to study the environmental impact of lignocellulose-based biorefineries and their different process designs (Janssen et al., 2014; Janssen et al., 2016; Spatari et al., 2010). Accordingly, off-site enzyme production was identified as a major contributor to the climate impact of lignocellulose-based ethanol biorefineries (Olofsson et al., 2017), and ethanol yield as the key variable affecting all impact categories in LCAs for ethylene production from woody biomass (Liptow et al., 2013).

5.2 Multi-scale models as tools for consistent biorefinery assessment across system scales

Biorefineries are highly complex systems, in which unit processes and operations are highly intertwined at several system scales. For example, changes in pretreatment conditions can lead to different inhibitor concentrations, as shown in **Paper I**, which in turn can affect bioprocess dynamics and product formation rates. Ultimately, these may impact the downstream process chain, and, possibly, process economics and environmental impact. Thus, all three dimensions for biorefinery assessment, *i.e.* technical, economic, and environmental, can be affected by changes in a single process step or process input. Consequently, models should assess the effects of changes in unit processes and operations on all three dimensions by integrating multiple models at several system scales.

Multi-scale models fill this role as they connect models from different scales to enable a multi-dimensional process assessment. They have been widely applied in biotechnology, mainly to link dynamic flux balance analysis to bioprocess models (Pornkamol & Franzén, 2015; Zhuang & Herrgård, 2015), population balance models with bioprocess models (Heins et al., 2015), and computational fluid dynamics with bioprocess modelling (Fernandes et al., 2012; Wang et al., 2015). So far, integration of biorefinery models has focused mainly on the combination of TEAs and LCAs, and the integration of bioprocess models into flowsheets. Integrating dynamic bioprocess models into TEAs has been used to study process dynamics in flowsheet models, which are normally evaluated under steady-state conditions (Morales-Rodriguez et al., 2011, 2012). These coupled models have been used to assess and optimise different process configurations with process scheduling (Morales-Rodriguez et al., 2011), as well as to identify uncertain model parameters and quantify their effect on biorefineries using global sensitivity analysis (Morales-Rodriguez et al., 2012). Furthermore, TEA and LCA have been combined to compare both economics and environmental impacts for different process configurations (Kadhun et al., 2018; Olofsson et al., 2017; Tao et al., 2014).

However, only a few multi-scale models spanning many system scales have been implemented. They targeted mostly the integration of dynamic flux balance models into dynamic bioprocess models, which were in turn integrated into flowsheet models (Ploch et al., 2019) or into flowsheet models coupled with economic network models (Zhuang & Herrgård, 2015). Their ultimate goal was to identify targets for metabolic engineering and to understand intracellular dynamics during bioprocesses.

In **Paper IV**, a multi-scale model was developed by connecting a previously existing SS(C)F process model with a flowsheet model, TEA, and LCA. The SS(C)F process model was a refinement of an existing model describing the hydrolysis of steam-pretreated wheat straw and fermentation to ethanol (Wang et al., 2016). The flowsheet model and TEA were developed to describe a wheat straw-based biorefinery with ethanol as main product and biogas and electricity as co-products. The cradle-to-gate attributional LCA described the

biorefinery process from wheat straw production to ethanol leaving the plant. The developed multi-scale model has several benefits.

First, it allows the incorporation of SSCF process dynamics into flowsheet models via time-dependent yields and yearly flow rates. Accordingly, the effects of bioprocess dynamics can be evaluated with respect to all three dimensions of biorefinery assessment, which enables a thorough investigation of different process configurations (Figure 11). Compared to previous integrations of dynamic process models into flowsheets (Morales-Rodriguez et al., 2011, 2012), the approach described in **Paper IV** focuses on the effects of bioprocess dynamics. Introduction of, for example, scheduling and dynamics of other unit processes and operations will further improve the multi-scale model and can highlight important bottlenecks in plant design, especially when operating on multiple feedstocks, as investigated in **Paper II**.

Second, both TEA and LCA benefit from model integration with more detailed datasets. This is especially important for attributional LCAs, whereby allocations between different products could be avoided thanks to the provided flowsheet data. Because the basis for allocation is to some extent subjective, as when determining whether they should be performed based on expected economic value, physical flow or other measures, allocations are typically prone to controversies. Hence, model integration augments the validity of LCA studies by removing causes for subjective interpretation. For example, in **Paper IV**, allocations on product distributions by partitioning the environmental burden based on economic value were avoided thanks to the provided mass flows from flowsheet modelling.

Third, the multi-scale model enables a holistic process assessment and, in turn, multi-objective optimisation procedures and uncertainty or variability assessments. Uncertainty and variability analyses are inevitable in the context of lignocellulose biorefineries. Several publications have reported considerable variability of feedstock composition for corn stover (Kenney et al., 2013; Ray et al., 2020; Templeton et al., 2009), wheat straw (Collins et al., 2014; Kenney et al., 2013), and *Miscanthus* (Kenney et al., 2013). In case of bioethanol production, compositional variability can lead to significant differences in ethanol yields (Kenney et al., 2013; Ruth & Thomas, 2003) and minimum selling prices (Ruth & Thomas, 2003). Hence, variations in feedstock composition, process parameters, amounts of added hydrolytic enzyme or other process inputs can have significant effects on bioprocess outcomes and propagate through system scales.

In **Paper IV**, a multi-scale variability analysis framework was developed, in which published or laboratory data were applied to obtain the variability in one or more process inputs and propagate it through the multi-scale model. The variability, either in the form of raw data or sampling data from a fitted distribution, was directly propagated through the bioprocess model (Figure 12). The application of an automated stop criterion based on product formation rate resulted in the spreading of product yields and process times. The results were analysed in terms mean, median, maximum, minimum, 5th, 25th, 50th, 75th, and 95th percentiles, which were then transferred to the flowsheet scale. The mass balances from

flowsheet modelling, together with information on the process flows (e.g. the distribution between products), were used as inputs for attributional cradle-to-gate LCA. The model outputs at each scale, such as process yields, energy demand, internal rate of return, and climate impact, were assessed via data analysis and used to calculate the variation in the performance indicators across scales (Figure 12).

The multi-scale variability analysis framework was applied in a case study to determine the effect of reported variability in activity of the commercially available hydrolytic enzyme cocktail Cellic CTec2 on the robustness of performance indicators at multiple system scales for batch and multi-feed processes in a wheat straw-based biorefinery. Despite concerns about the irreproducibility of the filter paper assay to assess total cellulolytic activity (Dashtban et al., 2010), filter paper units are still the prevalent approach to report enzyme loads in SS(C)F. Failure to accurately quantify the enzymatic activity can lead to impaired process design or prevent the comparison of process designs among different studies.

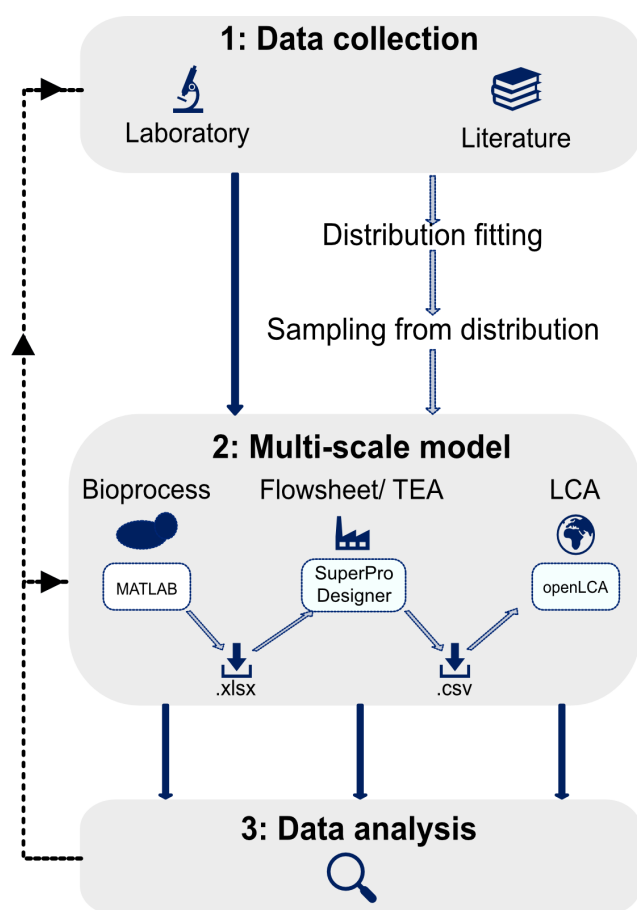


Figure 12: Illustration of the multi-scale variability analysis framework. Literature or laboratory data on the variability of process inputs are either direct input to the multi-scale model or are used for distribution fitting and Monte-Carlo sampling from the distribution. Variability/uncertainty is propagated through the multi-scale model, which consists of a bioprocess model in MATLAB, a flowsheet model in SuperPro Designer, and LCA in openLCA. The model outputs are used for data analysis and can be employed to pinpoint bottlenecks in process design (**Paper IV**).

Therefore, reported enzyme activities, expressed as filter paper units per mL, were used as inputs in the multi-scale variability analysis framework. When assessing median values, the differences between bioprocess, economic, and environmental performance indicators were insignificant except for the internal rate of return and payback time. In both metrics, multi-feed processes performed slightly better than batch processes, which can be attributed to the lower capital costs of the former. Compared to batch processes, multi-feed processes benefit from lower flow rates due to higher ethanol titres, which in turn result from higher solid loadings.

Contrary to the experimental observations on logging residues and oat hulls in **Paper II**, the variation in performance indicators of multi-feed processes was at least 50% lower at all system scales compared to batch processes. Hence, the results showed that under the investigated conditions, multi-feed processes were more robust to varying enzymatic activities than batch processes. The lower spread under multi-feed conditions resulted from the lower spread in ethanol yields and process times. Furthermore, counteracting enzymatic variability by adjustments in enzyme loads reduced the variation in process economics, but significantly increased the variability in climate impact (**Paper IV**). Moreover, the study revealed the potential to meet both economic and environmental goals by choosing the right process design; in the investigated case, this would be using multi-feed processes and the same enzyme loads despite different activities. Hence, multi-scale variability analysis can be applied to investigate the robustness to input variations of different process configurations at multiple system levels. Such approach allows stakeholders to choose process designs, in which technical, economic, and environmental goals align, and performance parameters can be reliably estimated. This is especially important for biorefineries, in which several process inputs, such as feedstock composition and quantity, are subject to natural variation as is the entire process chain.

In conclusion, multi-scale models enable the assessment of biorefinery processes from a holistic perspective by integrating technical, economic, and environmental impact metrics. The detailed information passed from one system scale sub-model to another improves modelling procedures because estimates and assumptions (*e.g.* on allocation) are replaced by calculated variables from lower system scales. The multi-scale variability analysis framework, developed in **Paper IV**, offers an efficient tool to identify process bottlenecks with regard to varying process inputs. As shown by the example of varying enzymatic activities, process design can be used to suppress, at least to a certain extent, the propagation of input variations through the biorefinery system. The holistic approach of multi-scale modelling can help determine the potential to align technical, economic, and environmental goals through process design.

6 Conclusions and outlook

6.1 Conclusions

In this thesis, process designs and assessments were developed to improve the performance of lignocellulose-based biorefineries for platform chemical production from agricultural and forestry residues and, thereby, advance towards cost-competitiveness with platform chemicals produced from first-generation feedstocks or fossil fuels.

In **Papers I and II**, the possibility to utilise logging residues as a presently underutilised and inexpensive source of biomass for bioethanol production was explored to *expand the feedstock base of biorefineries*. However, it was shown that the elevated potential supply of logging residues came at the cost of an unfavourable chemical composition. The inherent heterogeneity of logging residues, combined with their low carbohydrate and high lignin content, resulted in high recalcitrance to enzymatic hydrolysis. Optimisation of H₂SO₄-catalysed steam pretreatment in **Paper I** efficiently reduced such recalcitrance and resulted in hydrolysis yields at par with published ones on spruce stem wood. Optimal conditions included lower pretreatment temperatures than for spruce stem wood, showing the need to *tailor pretreatment* to novel feedstocks. Under these conditions, inhibitor formation was repressed, while high sugar concentrations were recovered in the liquid phase. Thus, the sweet spot was a trade-off between fermentability and hydrolysability.

Low biomass carbohydrate content calls for high solid loadings during SSCF to achieve sufficient product titres and ensure cost-effective downstream processing. *Mixing in carbohydrate-rich oat hulls to upgrade the low carbohydrate content of logging residues*, as was done in **Paper II**, significantly increased final ethanol titres to above 50 g·L⁻¹ in batch cultivations at 20% solid loadings. This strategy offered an opportunity to decrease separation and purification costs. Alternatively, to improve mixability and process control in batch SSCFs, solid loadings could be decreased, as current model-based multi-feed designs were unable to improve ethanol titres. Furthermore, enzymatic hydrolysis benefitted

from a weak synergistic effect between the two substrates. The utilisation of two feedstocks in a biorefinery concept can facilitate co-product generation and decrease supply chain risks.

An imminent challenge of lignocellulose-based conversion processes is represented by the inhibitors generated during pretreatment even under optimal conditions, as shown in **Paper I**. Inhibitors can decrease both product formation rates and titres with detrimental effects on overall process performance. In **Paper III**, the concept of *preadaptation*, in which cells were exposed to lignocellulose-derived inhibitors in the form of hydrolysate during cell propagation, was successfully *transferred from ethanol production by *S. cerevisiae* to lactic acid production by *B. coagulans**, suggesting the general applicability of preadaptation. By preadapting *B. coagulans* cells with 30% hydrolysate, volumetric lactic acid productivity was increased by 50%, while process time was shortened by 50%. Accordingly, preadaptation offers another possibility to decrease operational costs in lignocellulose-based biorefineries.

Lignocellulose-based biorefineries are evaluated not only by their economics. In fact, technical, economic, and environmental goals must be met simultaneously. To allow such a holistic assessment, multi-scale modelling was used in **Paper IV**, combining a bioprocess model with flowsheet modelling, TEA, and LCA. The *multi-scale variability analysis framework* enables the comparison between different process designs in terms of their robustness to variations in process inputs. In a case study, multi-feed SSCF processes were shown to be more robust than batch processes against fluctuations in enzyme activity. With the holistic assessment of biorefineries and their input variations, multi-scale variability analysis provides a tool to identify promising process configurations capable of aligning technical, economic, and environmental goals.

The results of my thesis show that the suitability of novel feedstocks for biorefineries and the identification of bottlenecks associated with these feedstocks requires an assessment on a case-to-case basis. The main bottlenecks for logging residues were their low carbohydrate content and high recalcitrance; whereas the main hurdle for the conversion of oat hulls was toxicity of the pretreated hydrolysates and low potential supply. Knowing the individual bottlenecks, some hurdles can be mitigated through process design by: Optimised pretreatment to reduce recalcitrance, preadaptation to alleviate the effects of lignocellulose-derived inhibitors, or substrate upgrading to compensate for low carbohydrate contents. Still, process design cannot solve all problems associated with lignocellulosic feedstocks. In particular, residual biomass is characterised by a high natural variation originating from feedstock heterogeneity and harvest practices. The developed multi-scale variability analysis framework provides an effective tool to assess the impact of such variations on biorefineries and can thus be used to choose process designs best suited to address these variations, instead of developing a dedicated process strategy.

6.2 Outlook

The concept of lignocellulose-based biorefineries to produce fuels and chemicals has been widely investigated over the past two decades. Still, further improvements are required to ensure a wide application of lignocellulose-based biorefineries, especially for the production of low-value platform chemicals, such as ethanol and lactic acid.

The need to introduce inexpensive, underutilised feedstocks such as logging residues for low-value chemical production by biorefineries will increase as more biomass is used to reach the planned climate objectives, *e.g.*, for the direct production of speciality chemicals, heat and power generation, or the production of novel materials. The developed multi-scale variability analysis could serve as an important tool to holistically assess process robustness and the robustness of performance indicators in response to variability of these novel feedstocks. The framework should be further automated to enable a simultaneous assessment and comparison of various potential feedstocks and process configurations, *e.g.* by automating pinch analysis via mixed-integer linear programming (Celebi et al., 2017).

In forest-rich regions such as the Nordic countries, residues from forestry practice and industry, including logging residues, saw dust, industrial bark, and wood shavings, will become more important as feedstocks for biorefineries to generate additional value for forest owners and the industry, while also meeting climate objectives. Although the potential supply is large, further research will be required to enable stable processing of these heterogeneous feedstocks in a biorefinery context. In case of logging residues, future studies should target the contribution of the different anatomical fractions to enzymatic hydrolysis and fermentation. Accordingly, potential bottlenecks in the process could be related to these fractions and cost-efficient pre-processing of logging residues could be suggested, either within forestry practice or in the biorefinery. Moreover, SSCF processes must be designed to operate on logging residues at solid loadings above 20%.

Multi-feedstock biorefineries are a promising avenue towards aligned economic and environmental goals. The utilisation of several feedstocks can decrease supply chain risks, as well as the risks related to large monocultures and their detrimental effects on biodiversity. Moreover, by relying on several feedstocks, biorefineries may be established in areas, in which a single feedstock cannot be reliably supplied. The growing interest in mixed feedstocks could provide interesting insights into the hydrolysis and fermentation dynamics of such substrates. Details about any synergistic effects could unveil the underlying mechanisms, which can be applied for rational process optimisation. Hydrolysis and fermentation designs must be further developed to optimally utilise mixed feedstocks, while meeting economic and environmental targets in up- and downstream unit processes. In case of oat hull/logging residue mixtures the process designs in **Paper II** should be further improved to stimulate xylose co-consumption through, for example, pre-fermentation. An important question regarding the process design and optimisation is whether pretreatment and bioconversion should be tailored to the different feedstocks to maximise conversion efficiency or be designed to be as robust as possible to maximise process stability. In this

context, the acetic acid formed by oat hulls during pretreatment might be used to reduce the demand for acid catalyst during a combined pretreatment with logging residues. Regarding the inhibitors generated during pretreatment, future studies should be directed to the underlying mechanisms of preadaptation, *e.g.* to find markers for the monitoring of preadaptation or to identify the optimal trade-off between biomass production and preadaptation.

The utilisation of oat hulls in **Paper II** pointed at the importance of the location as another factor in biorefinery development as oat hulls are a by-product of oat mills. The evolving utilisation of industrial biomass by-product streams, such as oat hulls or bark, favours the integration of biorefinery units directly at the source of those by-products. Compared to feedstocks extracted directly from fields and forests, these by-products typically underwent some form of pre-processing and are therefore more homogeneous, and their characteristics are more predictable and controllable. Using by-product streams, which are presently considered waste, could be a strong incentive to develop local biorefineries which, due to their integration with other processes, might not need the size of stand-alone biorefineries to become profitable.

In the context of lignocellulose-based biorefineries for platform chemical (*e.g.* ethanol and lactic acid) production, further processing is of utmost importance. Not all compounds derived from bio-based platform chemicals can be produced in a cost-competitive way when compared to their fossil fuel-based counterparts. The production of ethylene from bioethanol, for example, will likely be unprofitable as the current price of ethylene is lower than the price of the stoichiometric quantity of ethanol required to produce it (Dagle et al., 2020). However, further processing to ethylene carbonate, a major component of the electrolyte in lithium batteries, could be profitable (Dagle et al., 2020). In fact, the CO₂ required for the reaction could be produced during ethanol fermentation itself. This example illustrates how more research needs to be directed towards the integration of biorefineries in the larger context of platform chemical utilisation instead of focusing only on the fermentation products. Ideally, biorefineries should aid further chemical processing by generating the co-products required for later chemical process steps, such as the CO₂ in the example above.

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