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An inquiry into factors affecting conversion performance

JONAS OHLSSON

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Jonas Ohlsson
Faculty of Natural Resources and Agricultural Sciences,
Department of Molecular Sciences,
Uppsala

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Cover: Processing a *Salix* feedstock on multiple scales.
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Salix as a biorefinery feedstock

Abstract

Lignocellulosic biomass is one of the world's greatest natural resources, with potential for replacing a considerable portion of current fossil resource use. Although lignocellulosic biomass can be readily utilized for generating heat and power via combustion, its conversion into storable fuels such as ethanol and biogas presents a greater challenge. Biomass can be converted into such fuels in biorefineries, although the inherent resistance of lignocellulosic biomass to deconstruction, *biomass recalcitrance*, incurs financial penalties. The genus *Salix* contains several woody species known for their high biomass production, low requirements for agricultural inputs, and short harvest rotations. These species could serve as optimal feedstocks for the production of transportation biofuels, if their recalcitrance can be overcome. In this thesis, several aspects related to *Salix* as a biomass feedstock for use in biorefineries are considered, including breeding, conversion methodology, and cultivation.

Biomass recalcitrance and several other traits relevant to the breeding of improved *Salix* varieties were quantified in a population of natural accessions of *S. viminalis*, and narrow-sense heritabilities and genetic and phenotypic correlations were calculated. Narrow-sense heritabilities (h^2) for biomass recalcitrance were 0.23 to 0.29, indicating a certain level of genetic control. Shoot weights and ratios of syringyl to guaiacyl monomers in lignin were negatively correlated with biomass recalcitrance, serving as possible proxy traits for selection of improved variants in breeding programs.

The conversion performance of a subset of clones from the aforementioned population were further evaluated using two different conversion systems, enzymatic hydrolysis (EH) and anaerobic digestion (AD). Despite fundamental differences between these systems, measures of conversion performance correlated well, indicating that recalcitrance improvements are not specific to one conversion system. In AD, biomass recalcitrance more strongly affected conversion rates than final yields, and the AD system was overall less sensitive to variations in recalcitrance.

Several other aspects related to the use of *Salix* as an AD feedstock were evaluated. By using a sequential reactor setup, methane yields of non-pretreated *Salix* were shown to be similar to those reported for steam pretreated material, allowing its use in smaller-scale facilities where pretreatment may not be feasible. Moreover, the choice of clone and rotation length were shown to affect conversion yields under AD, whereas there was no apparent effect of nitrogen fertilization.

Making biofuels cost-competitive with their fossil counterparts will likely require a considerable amount of further work in several scientific and engineering disciplines. The contents of this thesis will hopefully contribute toward that goal.

Keywords: Salix, anaerobic digestion, breeding, biomass recalcitrance, enzymatic hydrolysis

Author's address: Jonas Ohlsson, SLU, Department of Molecular Sciences
P.O. Box 7015, 750 07 Uppsala, Sweden
E-mail: jonas.ohlsson@slu.se

Salix som råvara för bioraffinaderier

Sammanfattning

Oätlig växtbiomassa, lignocellulosa, är en av jordens viktigaste naturresurser, med potential att kunna ersätta en stor del av vår nuvarande fossila resursanvändning. Sådan biomassa kan lätt omvandlas till värme och elektricitet via förbränning, men att framställa biodrivmedel som etanol och biogas från den innebär en större utmaning. Biomassa kan omvandlas till biodrivmedel i bioraffinaderier, men dess inneboende motstånd mot yttre påverkan, dess *strukturella härdighet*, gör denna omvandling kostsam. *Salix* är ett släkte innehållande flera vedartade växter med hög biomassaavkastning, låga krav på gödning och korta skördecykler. Därför kan *Salix* utgöra ett utmärkt råmaterial för framställning av biodrivmedel, givet att dess inneboende härdighet övervinns. I denna avhandling undersöks flera aspekter kring användning av *Salix* som råvara för bioraffinaderier, bland andra förädling, omvandlingsmetodologi och odling.

Strukturell härdighet och flera andra egenskaper med betydelse för växtförädling av salixsorter för användning i bioraffinaderier undersöktes i en population av naturliga *S. viminalis*-kloner, och heritabilitet i snäv bemärkelse samt genotypiska och fenotypiska korrelationer beräknades. Heritabiliteten (h^2) för härdighet var 0.23–0.29, vilket indikerar att denna egenskap står under viss genetisk kontroll. Vikt på största skott och förhållandet mellan syringyl- och guaiacylmonomerer i lignin var negativt korrelerade med strukturell härdighet, vilket skulle kunna underlätta förädling genom förenklad fenotypning.

Omvandlingsutbytet hos ett antal kloner från ovan nämnda population undersöktes ytterligare via både enzymatisk hydrolys (EH) och anaerob degradering (AD). Mått på utbyte korrelerade väl mellan omvandlingssystemen, trots stora skillnader i verkningssätt mellan de två metoderna. Detta antyder starkt att minskad härdighet direkt kan omsättas i förbättrade utbyten, oavsett omvandlingssystem. Vidare kunde ses att där strukturell härdighet under EH manifesterar sig som minskat utbyte, visar den sig inom AD främst som en lägre omvandlingshastighet.

Vidare undersöktes andra aspekter av omvandling av *Salix* via AD. Genom att använda ett system med två seriekopplade reaktorer kunde metanutbytet hos icke-förbehandlad *Salix* uppnå samma nivåer som för förbehandlat material, vilket skulle kunna tillåta användning av *Salix*-råvara i AD-anläggningar utan möjlighet till förbehandling, såsom gårdsskaleanläggningar. Vidare så visade det sig att klon och skördeålder påverkade metanutbytet, medan kvävegödsling inte hade någon effekt på detta.

För att göra biodrivmedel lika kostnadseffektiva som fossila motsvarigheter återstår fortfarande mycket arbete, inom både vetenskapen och ingenjörskonsten. Förhoppningsvis kan innehållet i denna avhandling bidra något till att nå detta mål.

Contents

List of Figures

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Ohlsson, J. A.**, Hallingbäck, H. R., Jebrane, M., Harman-Ware, A. E., Shollenberger, T., Decker, S. R., Sandgren, M., Rönnberg-Wästljung, A.-C. (2019). Genetic variation of recalcitrance in a natural *Salix viminalis* (L.) population. *Biotechnology for Biofuels* 12:135. DOI: 10.1186/s13068-019-1479-7

II **Ohlsson, J. A.**, Harman-Ware, A. E., Sandgren, M., Schnürer, A. (2020). Biomass Recalcitrance in Willow Under Two Biological Conversion Paradigms: Enzymatic Hydrolysis and Anaerobic Digestion. *BioEnergy Research* 13:260–270. DOI: 10.1007/s12155-019-10079-6

III **Ohlsson, J. A.**, Rönnberg-Wästljung, A.-C., Nordh, N.-E., Schnürer, A. (2020). Co-Digestion of *Salix* and Manure for Biogas: Importance of Clone Choice, Coppicing Frequency and Reactor Setup. *Energies* 13(15):3804. DOI: 10.3390/en13153804

IV **Ohlsson, J. A.**, Schnürer, A., Jebrane, M., Wallberg, O., Weih, M., Sandgren, M. Effect of genotype and fertilization on biomethanation potentials and biomass composition of steam pretreated *Salix*. Manuscript.

My contribution to the papers included in this thesis was as follows:

I Participated in planning the study, and performed most of the laboratory work and data analysis. Wrote the bulk of the manuscript.

II Participated in planning the study, and performed most of the laboratory work and data analysis. Wrote the bulk of the manuscript.

III Planned and performed most of the data analysis, and wrote the bulk of the manuscript.

IV Participated in planning the study, and performed biomethane potential assays and data analysis. Wrote the bulk of the manuscript.

During the timeframe of this thesis work, I have also contributed to the following scientific output:

Dauphinee, A. N., Cardoso, C., Dalman, K., **Ohlsson, J.A.**, Fick, S. B., Robert, S., Hicks, G. R., Bozhkov, P. V., Minina, E. A. (2019). Chemical screening pipeline for identification of specific plant autophagy modulators. *Plant Physiology* Nov; 181(3):855-–866. DOI: 10.1104/pp.19.00647

Weih, M., Hansson, P.-A., **Ohlsson, J. A.**, Sandgren, M., Schnürer, A., Rönnberg Wästljung, A.-C. (2020). Sustainable production of willow for biofuel use. In C. Saffron (Ed.), *Achieving carbon-negative bioenergy systems from plant materials*. Cambridge: Burleigh Dodds Science Publishing. DOI: 10.19103/AS.2019.0027.17

Dauphinee, A. N., **Ohlsson, J. A.**, Minina, E. A. (2020). Tandem Tag Assay Optimized for Semi-automated in vivo Autophagic Activity Measurement in *Arabidopsis thaliana* roots. *Bio-Protocol* 10(5). DOI: 10.21769/BioProtoc.3535

Ohlsson, J. A., Olstorpe, M., Passoth, V., Leong, S.-L. L. Yeast single cell protein production from a biogas co-digestion substrate. biorXiv preprint. DOI: 10.1101/766345

Molinder, R., **Ohlsson, J.A.**, Fatma, S., Sandgren, M., Johansson, A.-C. Production of renewable transportation fuels from *Salix* through a biochemical and a thermochemical route. Manuscript.

Ohlsson, J. A., Leong, J. X., Elander, P. H., Dauphinee, A. N., Ballhaus, F., Johansson, J., Lommel, M., Hofmann, G., Betnér, S., Sandgren, M., Schumacher, K., Bozhkov, P. V., Minina, E. A. SPIRO – the automated Petri plate imaging platform designed by biologists, for biologists. bioRxiv preprint. DOI: 10.1101/2021.03.15.435343

Abbreviations

AD	anaerobic digestion
BMP	biomethanation potential
CBP	consolidated bioprocessing
CV	coefficient of variation
EH	enzymatic hydrolysis
G	guaiacyl
GBS	genotyping by sequencing
GC	gas chromatography
GS	genomic selection
GWAS	genome-wide association study
GHG	greenhouse gas
H	hydroxyphenyl
HPLC	high-performance liquid chromatography
HRT	hydraulic retention time
HR-TGA	high resolution thermogravimetric analysis
LCA	life cycle assessment
LC-MS	liquid chromatography–mass spectroscopy
MAB	marker-assisted breeding
NMR	nuclear magnetic resonance
OPLS-DA	orthogonal projections to latent structures–discriminant analysis
py-MBMS	pyrolysis–molecular beam mass spectrometry
S	syringyl
SD	standard deviation
SNP	single-nucleotide polymorphism
SRC	short rotation coppice
SSF	simultaneous saccharification and fermentation

1 Background and aims

Climate change, to a large part caused by the temporal uncoupling of carbon fixation and emission in our current energy system, is seen as one of the most pressing global issues of our time (Steffen et al., 2015). Using plant biomass as an energy source (i.e, *bioenergy*) could allow us to transition away from using ancient carbon stores, thereby recoupling carbon storage and utilization.

Each year, immense amounts of solar energy is captured via photosynthesis and stored as chemical energy by living organisms such as plants. Globally, aboveground plant biomass is estimated to contain 320 Gt (gigatons) carbon (Bar-On et al., 2018), with 123 Gt carbon fixated yearly (Beer et al., 2010). In comparison, anthropogenic carbon emissions from fossil fuel combustion amounted to 9 Gt carbon (33.1 Gt CO_2) in 2018 (IEA, 2019). However, only a small fraction of plant fixated carbon is realistically available for use in current or future energy systems. The amount of biomass available for use as bioenergy, the biomass potential, is dictated by several factors. Common definitions of biomass potential include *theoretical potential*, equalling the total amount of biomass in existence (the previously quoted figure); *technical potential*, the amount which can be harvested and converted; and *economic potential*, the part of the technical potential which can be utilized given current economic constraints (Thrän et al., 2010). The work in this thesis ultimately concerns improving the economic potential of a specific energy crop species, the rapidly-growing shrubby tree *Salix*, when used as a feedstock for the production of liquid or gaseous biofuels (henceforth simply referred to as *biofuels*), by investigating factors related to its biofuel conversion performance.

1.1 Biofuels from lignocellulosic crops

Biomass, in the form of firewood, has been used as a source of energy for humans for at least 6000 years, but the controlled use of fire likely dates back to one million years ago (Guo et al., 2015). The use of biomass for producing storable and transportable biofuels via fermentation is much more recent.

Anaerobic digestion (AD), a process whereby organic matter is converted into a combustible gas mixture consisting primarily of methane (CH_4) and carbon dioxide (CO_2), was used to produce gas for heating from animal manure hundreds or possibly as far back as 2000 years ago (Abbasi et al., 2012; Guo et al., 2015). In the late 1800s, the AD process was employed for sewage treatment, enabling its more widespread modern use (Abbasi et al., 2012). The 1800s also saw the rise of ethanol as a fuel for combustion engines. Although the use of ethanol as vehicle fuel has since then largely been inversely linked with the availability of fossil fuels, such as during wartime or the 1970s oil crisis, ethanol is currently used as an additive in gasoline blends to improve its properties as a fuel (Solomon et al., 2007).

Transportation accounts for a large part of global greenhouse gas (GHG) emissions (Fulton et al., 2015), but shifting this sector to non-fossil energy presents a number of challenges. Fuel requirements differ between sectors; an electric car can easily satisfy most requirements of a personal vehicle, whereas heavy long-haul transport and airplanes require energy-dense fuels. Current battery technology has an energy density of $0.5\,\mathrm{MJ\,kg^{-1}}$ to $1\,\mathrm{MJ\,kg^{-1}}$ (Thackeray et al., 2012), which is considerably lower than the corresponding values for ethanol ($29.7\,\mathrm{MJ\,kg^{-1}}$) or gasoline ($45\,\mathrm{MJ\,kg^{-1}}$; Wolfram|Alpha, 2020). Thus, a large part of transportation may not be electrified for the foreseeable future, and increased biofuel use remains the most viable strategy for reducing the carbon footprint of these vehicle classes (Fulton et al., 2015).

Motivations for using biofuels over fossil fuels have shifted over the years. Perhaps the strongest policy push for increased use of biofuels and bioenergy came during the oil crises of the 1970s, which caused oil price hikes throughout large parts of the world. Fears over energy security gave rise to important biofuel policy changes, such as the Brazilian sugarcane ethanol program (Solomon et al., 2007; Su et al., 2015) and support schemes for renewable energy in the US (Su et al., 2015), Sweden (Ericsson et al., 2004; Perttu, 1998), and Finland (Ericsson et al., 2004). Over time, the stated goals of the bioenergy support programs have largely shifted away from energy security and towards environmental targets such as GHG reductions, and, in some countries, rural development (Tomei and Helliwell, 2016).

The increased production of biofuels prompted the so-called food versus fuel debate of the early 2000s (summarized in Thompson (2012)), triggering a shift towards promoting “second-generation” biofuels, produced from inedible plant matter (lignocellulose) and waste products in *biorefineries* (analogous to fossil-based oil refineries). Ambitions to reduce competition between food and fuel uses of biomass while still claiming GHG emission reductions are reflected in recent support programs and policies in the US and the EU

(European Commission, 2018; Su et al., 2015).

Although lignocellulosic biofuels may provide several advantages over their “first-generation” forebears, the widespread adoption of lignocellulosic materials as feedstocks for certain biofuel processes such as ethanol production is still to be realized (Lynd, 2017). Certain conversion processes, such as AD which has a longer history of utilizing lignocellulosic material, have fared better and are processing these materials at a relatively large scale (Scarlat et al., 2018). At the core of the problem of transitioning from using readily degradable material such as cereal grains into lignocellulosic feedstocks is the inherent resistance of such plant material to deconstruction, a phenomenon which has been referred to as *biomass recalcitrance* (Himmel et al., 2007; Section 2), and which is a key factor determining the financial viability of second generation biofuel production.

1.2 *Salix* as a biofuel feedstock

Salix (willows) is a genus encompassing hundreds of tree species, some of which have a long history of human use. Baskets and ropes made out of willow are probably among the first products produced by humans in areas native to willow, as the flexible, durable quality of its twigs makes it exceptionally well suited for such applications (Going, 1903). Willows were also treasured for their medicinal properties, with the anti-inflammatory properties of its bark having been recognized since ancient times (Jack, 1997). Other traditional uses of willow include dyeing, ornamentation, and flood protection (Going, 1903). The 1980s saw a resurgence in willow growing, motivated by an increased interest in bioenergy (Kuzovkina et al., 2008).

When cultivating *Salix* for bioenergy use, a short rotation coppice (SRC) growing system is used. In this type of cultivation system, the plant is initially cut back to stimulate shoot production, with the resulting shoots being harvested after 3–6 years, depending on the development of the stand (Kuzovkina et al., 2008; Ledin, 1996). A SRC plantation can be harvested in this manner for 20–25 years until reestablishment is necessary (Karp et al., 2011).

In many ways, *Salix* is an ideal feedstock for second-generation biofuel production. Not only does it provide high biomass productivities (Mola-Yudego et al., 2015; Perlack et al., 2011), it does so with low nitrogen requirements, which means that it can be cultivated in poorer soils compared to annual species (Karp et al., 2011), which combined with less frequent harvesting and lower fertilization requirements translates into higher net energy returns in comparison to perennial herbaceous energy crops (Boehmel et al., 2008). The technology for planting and harvesting *Salix* (Figure 1) is mature due to its established use as a bioenergy feedstock (Ledin, 1996), and further

Figure 1: Harvest of mature *Salix* shoots. Note that *Salix* is normally harvested before the start of the growing season. Photo credit: Nils-Erik Nordh.

development is ongoing (Perlack et al., 2011). The fast turnover from planting to harvest means that SRC *Salix* more effectively recouples carbon uptake and emission compared to species with longer harvest cycles. Forest biomass has recently come under scrutiny for its perceived inability to reduce GHG emissions within the timeframes required for meeting agreed-upon carbon emission reduction quotas (Searchinger et al., 2018). Moreover, short harvest cycles, ease of propagation, and facile crossing and hybridization enables efficient breeding, which has been demonstrated by successful breeding programs in Sweden, the UK, the US, and elsewhere (Brown et al., 2019; Kuzovkina et al., 2008; Larsson, 1998).

Salix is closely related to the genus *Populus* (poplars), both belonging to the family Salicaceae. The two species likely diverged around 50 million years ago (Dai et al., 2014). This relatedness greatly benefits *Salix* research, as the species *P. trichocarpa* is used as a hardwood model species in biologic research, and its genome sequence has been available for 15 years (Tuskan et al., 2006). Research performed in *Populus* is often directly or indirectly relevant to *Salix*. Wood anatomy, ultrastructure, and gross chemical composition is similar between the species, and the genomes are sufficiently similar to allow transfer of genomic resources between the genera (Berlin et al., 2010; Perdereau et al., 2013). More recently, annotated genome assemblies have been published for several *Salix* species as well, including *S. purpurea* (Zhou et al., 2018) and *S. viminalis* (Almeida et al., 2020).

In Europe, one of the most important *Salix* species for bioenergy use is *S. viminalis* (Karp et al., 2011; Larsson, 1998). *S. viminalis*, also known as *basket willow* or *common osier*, is a shrubby willow, which, as its name implies, has traditionally been used for basketry. The species is included in most commercial European bioenergy varieties, with species such as *S. schwerinii* crossed in for rust and insect tolerance (Larsson, 1998).

1.3 Aims

Although *Salix* is in many ways an attractive biorefinery feedstock, it is not currently used for this purpose, and research on many aspects related to its use and improvement is still lacking. Genetic parameters for biomass recalcitrance and AD conversion performance are especially understudied areas. Thus, the overarching aim of the works included in this thesis was to expand the knowledge of *S. viminalis* as a feedstock for biofuel production. Specifically, the research presented herein seeks to provide insights regarding the breeding of tailored *Salix* variants for biofuel (paper **I**), and to understand the influence of biomass traits and chemical components (papers **I** and **II**), agricultural practices (papers **III** and **IV**), and conversion methodology and technology (papers **II** and **III**) on conversion performance.

2 Biomass composition and recalcitrance

Biomass recalcitrance refers to the ability of plant tissues to resist deconstruction. Plants have evolved properties on multiple scales to resist degradation (McCann and Carpita, 2015). For example, the tree is covered in a layer of bark, containing high amounts of lignin and antimicrobial compounds, acting as a first line of defense. Cells in the secondary cell wall, which harbor most of the easily utilizable energy, are protected by a lignin barrier through which access is restricted. Within the cell walls, cellulose polymers are tightly packed and enveloped in a complex polymer matrix, limiting accessibility to depolymerizing enzymes. A necessary product of evolution, biomass recalcitrance has enabled plants to withstand harsh environments and to counter microbial attacks. While helpful for plant survival, this general resistance presents a significant hurdle for second-generation biofuel conversion processes, which, by their very nature, rely on deconstructing lignocellulosic biomass. Designing feedstocks of lower recalcitrance is thus considered a key aspect of attaining financially viable biorefineries (Himmel et al., 2007; Wyman, 2007).

Willow wood is classified as *hardwood*, as is wood from all other angiosperm trees, whereas wood from gymnosperms such as conifers is classified as *softwood*. Biomass from hardwoods, softwoods, and herbaceous species are similar in many regards relating to biomass structure, although differences exist regarding certain components. This chapter provides a basic overview of the constituents of lignocellulosic biomass, which is required for understanding biomass recalcitrance and for comprehending research on biorefinery feedstocks, with a focus on hardwoods.

2.1 Structure of lignocellulosic biomass

The bulk of the energy contained within lignocellulosic plants is located in the secondary cell walls, which form the structural parts of the plant such as the straw or wood. While the primary cell walls are plastic and develop as cells grow, the secondary cell walls are rigid and formed along the interior of the cell when it is fully formed. Due to their firm structure, secondary cell walls function as essential load-bearing structural supports, which remain after cell death. Ding and Himmel (2008) provide an in-depth description of cell wall structure and its analysis.

Lignocellulose consists of three major classes of chemical constituents, which can be crudely classified as cellulose, non-cellulosic carbohydrate polymers (chiefly the *hemicelluloses*), and lignin, as well as minor amounts of extractives, proteins, and ash. The carbohydrate polymers and lignin make up the largest part of lignocellulosic biomass, and are intricately intertwined within the secondary cell walls of the plant. The arrangement and interconnectedness of these components confers many important properties to the plant, such as rigidity and strength, and also enable crucial features such as water transport (Himmel, 2008).

The levels of cellulose, hemicellulose, and lignin vary with species, harvest age, and to a certain degree the method of quantification. Typical values for *Salix* are 40–45 % cellulose, 15–30 % hemicelluloses, and 20–25 % lignin (Weih et al., 2020). Below, I briefly describe these individual components.

2.1.1 Carbohydrate polymers

The carbohydrate polymers in lignocellulosic biomass can be grouped into cellulose, hemicelluloses, and other non-cellulosic polysaccharides. Cellulose is generally considered the most important polymer for lignocellulosic biofuel production, as it is the most abundant and its degradation products are readily convertible. Cellulose exists within the cell walls in a largely crystalline state, with several molecules tightly packed in microfibrils. It is a linear molecule consisting of $\beta(1\rightarrow4)$-D-glucopyranosyl units, with average degrees of polymerization of 6000 and 14 000 in primary and secondary cell walls, respectively (Harris and Stone, 2008). Thus, the only depolymerization product of cellulose is glucose, a sugar molecule that is exceptionally well suited to microbial conversion into biofuel.

Hemicelluloses are heterogeneous, non-crystalline polymers which make up a considerable part of the carbohydrate contents of cell walls. These molecules are thought to surround and interact with the cellulose microfibrils, both covalently and via other interactions, as well as with the surrounding lignin (Section 2.1.2). In hardwoods, the main hemicellulosic polymer is 4-O-methylglucuronoxylan (Ebringerová and Heinze, 2000). This polymer has a backbone of $\beta(1\rightarrow4)$-D-xylopyranosyl residues with 4-O-methyl-α-D-glucopyranosyluronic acid and acetyl groups regularly substituted along the chain (Harris and Stone, 2008; Peng et al., 2012). Thus, when hardwood hemicelluloses are hydrolyzed, the main product is xylose. This pentose sugar

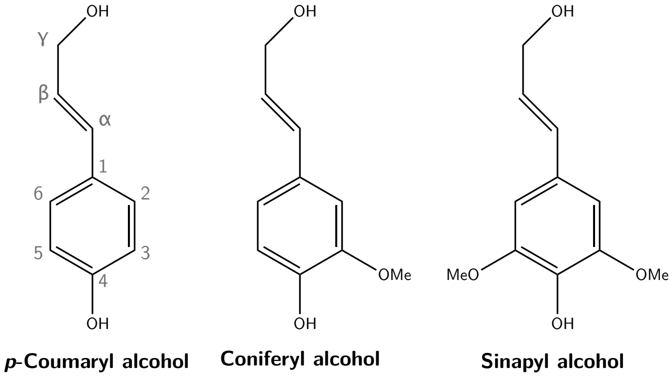

Figure 2: The three main monolignols *p*-coumaryl alcohol (making hydroxyphenyl (H) lignin when incorporated into the lignin polymer), coniferyl alcohol (guaiacyl (G) lignin), and sinapyl alcohol (syringyl (S) lignin). Carbon numbering is indicated on the *p*-coumaryl alcohol structure. Note the presence of a methoxy group on the 5-position of sinapyl alcohol, hindering formation of a recalcitrant C–C bond at this position.

cannot, in contrast to glucose, be utilized in all microbial conversion systems. Hardwoods also contain several other types of minor carbohydrate polymers, including pectins, although these are primarily located in the primary cell walls.

2.1.2 Lignin

Lignin is a complex aromatic heteropolymer which provides several important functionalities to the harboring plant. The complex, highly condensed, irregular nature of lignin makes it an especially challenging target for depolymerization. Notably, its hydrophobic nature enables water transport, and aids in partitioning cellulose, thereby increasing hydrogen bonding between individual cellulose molecules. The basic building blocks of lignin, the three *monolignols*, are 4-hydroxyphenylpropanoid units: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, distinguished by their degree of methoxylation at the 3 and 5 positions (see Figure 2). The residues of these compounds, when incorporated into the lignin molecule, are referred to as hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. In hardwoods, lignin contains both S, G, and to some degree H units, whereas softwood lignins do not contain S units (Abu-Omar et al., 2021). It is worth noting that although these are the “classical” monolignols, other molecules may also be incorporated into lignin (del Río et al., 2020).

Lignin is synthesized through the oxidative coupling of monolignols to the growing lignin polymer, a process which can form several bonding patterns

(Abu-Omar et al., 2021; Ralph et al., 2004). The proportions of these units, especially the S and G units, within the polymer influences its properties. The presence of methoxy groups in both the 3 and 5 positions on sinapyl alcohol (Figure 2) prevents the formation of a C–C (β–5) bond, which means that a syringyl unit on the polymer can only form a bond to another unit via the comparably more labile ether (β–O–4) bond (Ralph et al., 2004). Thus, it is generally believed that the S:G ratio is central to making lignin more readily degradable, especially in the context of biomass pretreatment (Li et al., 2016; Yoo et al., 2017; Section 3.1).

2.1.3 Minor components

Other than the major polymeric components, woody biomass contains a number of other constituents of importance for biofuel conversion systems. Depending on the species and part of the plant (e.g., wood or bark), biomass can contain a significant amount of nonstructural components which can be extracted using different solvents, termed *extractives* (Hillis, 1971). Extractive compounds are to a large degree phenolic and polyphenolic in structure, and many exist in glycosidic forms. Many extractive compounds are so-called *secondary metabolites*, and have been found to play important roles in plant defense against herbivores and pathogens (Bennett and Wallsgrove, 1994; Boeckler et al., 2011; Ullah et al., 2017). Members of the genus *Salix* are well-known for their ability to produce a wide range of phenolic secondary metabolites, including phenolic glycosides and flavonoids (Boeckler et al., 2011; Dou et al., 2018; Noleto-Dias et al., 2020, 2019; Palo, 1984; Pohjamo et al., 2003), and extractives content in *Salix* biomass is often 10 % or higher (Ray et al., 2012).

The composition of bark is different from that of wood, with higher amounts of extractives and lignin, and lower amounts of polysaccharides (Rowell et al., 2005; Serapiglia et al., 2009). The ash content of *Salix* biomass is typically low (Weih et al., 2020).

2.2 Compositional analysis methods

Compositional analysis of biomass is crucial to bioenergy research. Quantification of the components of biomass allows calculating process yields, evaluating effects of genetic perturbations, and measuring phenotypes in breeding and screening programs. Wet chemical analysis of gross biomass chemical composition dates back over a hundred years (reviewed in Sluiter et al. (2010)), and methods are generally based around a two-stage acid hydrolysis method coupled with gravimetric and chromatographic measurements.

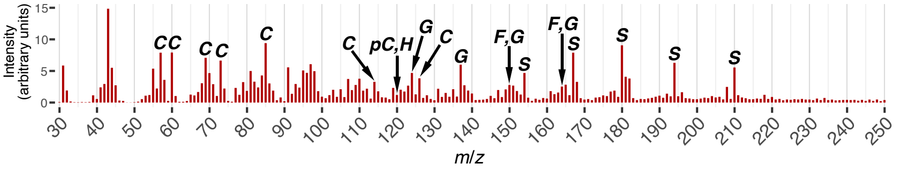

Figure 3: Example py-MBMS spectrogram showing peak intensities (arbitrary units) over the *m*/*z* range 30–250, with peaks diagnostic for certain biomass components highlighted. Legend: *C*, carbohydrate; *F*, ferulate; *G*, guaiacyl lignin; *H*, hydroxyphenyl lignin; *pC*, *p*-coumarate; *S*, syringyl lignin.

Although traditional wet chemistry methods of biomass analysis are routinely used, their relatively low-throughput, laborious nature and associated high costs limit their utility for certain research endeavors. Notably, large-scale phenotypic characterization, such as for quantitative genetics experiments, require methods of higher throughput and lower cost in order to be feasible. To meet these demands, several high-throughput biomass characterization techniques have been developed, including miniaturized two-stage acid hydrolysis coupled with high-performance liquid chromatography (HPLC), pyrolysis–molecular beam mass spectrometry (py-MBMS), and spectroscopic methods (Decker et al., 2018). High resolution thermogravimetric analysis (HR-TGA) is another method which has seen some use, especially in *Salix* composition research (Serapiglia et al., 2009). This method employs high-resolution weighing of a sample which is being gradually combusted. By measuring mass change at certain temperature intervals, individual components of the biomass (cellulose, hemicellulose, lignin) can be determined. This method is reasonably precise, and can be classified as intermediate-throughput.

A relatively recent development is the use of nuclear magnetic resonance (NMR) spectroscopy to replace the time-consuming HPLC quantification after miniaturized two-stage acid hydrolysis (Gjersing et al., 2013). By obviating the need for HPLC quantification, large sample sets can be analyzed with reasonably good precision.

In two of the papers included in this thesis (**I**; **II**) py-MBMS was utilized to gain key insights into aspects of the biomass composition of samples from a large population of natural *Salix* clones. In py-MBMS, a sample is pyrolyzed, i.e., thermally decomposed in an inert atmosphere, and its resultant vapors analyzed using mass spectrometry. This allows rapid inferral of lignin contents and S:G ratios (Sykes et al., 2009) directly from the spectra. To a certain extent, cell wall carbohydrates (Sykes et al., 2015) and extractives (Harman-Ware et al., 2021) may also be quantified, although multivariate modeling is needed for accurate measurements of these compounds.

A py-MBMS spectrum (Figure 3) represents the totality of the ions produced during pyrolysis; there is no separation. While this lack of separation enables very rapid sample analysis, it also makes spectra less interpretable. However, knowledge of decomposition patterns of common biomass components can allow inferring from a certain ion species its likely precursor compound(s) (Evans and Milne, 1987). Importantly, ions in a py-MBMS spectrum may arise from the decomposition of several compounds, such as is the case with several phenolic or aromatic species, including lignin, as well as for carbohydrates. In the case of such ions, a py-MBMS analysis may be considered hypothesis-generating, unless multivariate methods are constructed to elucidate relationships between ions and their parent compounds.

It should be noted that biomass compositional analysis is a complex and often expensive undertaking. There is no agreed-upon “gold standard” of analysis which gives a full quantification of all aspects of biomass composition, and all methods have their drawbacks and biases (see e.g., Happs et al. (2021); Wolfrum et al. (2009)). These inaccuracies can have multiple sources, both owing to the principle of the analysis method, direct *vs.* indirect measurements, throughput, operator skill, etc. The results of quantification are generally more comparable within than between studies. Although a thorough discussion on this topic lies outside the scope of this thesis, the reader should be aware of this fact when interpreting the data presented herein and in other work relating to biomass composition.

3 Biomass conversion

Several conversion processes may be used to transform lignocellulosic biomass into biofuels. These processes span a continuum from experimental, laboratory scale methods to time-tested processes with a large installed base. Biofuel production plants are sometimes referred to as biorefineries, a term that emphasizes their single-input, multiple-outputs characteristic. Proposed biorefinery modalities utilize different combinations of biochemical, thermochemical, chemical, and mechanical/physical conversion processes. As an example, a lignocellulosic ethanol plant may use coarse grinding (mechanical), steam pretreatment (physical, chemical), biomass hydrolysis (biochemical), and fermentation (biochemical) in order to produce ethanol, animal feed, and biogas, as well as residual lignin which can be used for heat or electricity generation (Cherubini et al., 2009).

3.1 Pretreatment

Biomass pretreatment, an operation which aims to partially fractionate biomass and reduce its recalcitrance (see Section 2) is common to most second-generation biorefineries utilizing biochemical conversion processes (Mosier et al., 2005). Although comminution (i.e., size reduction) can be considered a pretreatment step, I will use the term *pretreatment* to refer only to processes utilizing hydrothermal, thermochemical, or chemical reactions to facilitate biomass hydrolysis, and will thus consider biomass that has only been milled as non-pretreated.

Pretreatments which are considered economical for large-scale biorefineries generally employ high temperatures and pressures, combined with an acid or alkaline catalyst (Mosier et al., 2005). Through these processes, cellular structures are opened up and bonds between lignin, hemicelluloses, and cellulose are broken, and depending on the conditions either lignin or hemicelluloses are solubilized and/or degraded. Steam pretreatment is one relatively common pretreatment method, which is also used in the production of fiberboard. In this process, biomass is subjected to high pressure steam, sometimes followed by a rapid reduction in pressure which expels the pretreated biomass into a flash tank (steam explosion). Steam pretreatment units work well both in industrial settings and in smaller pilot-scale and lab-scale units, and there are even miniaturized steam pretreatment units in microtiter format (Decker et al., 2018; Section 3.1; **I**). Steam pretreatment can be catalyzed by addition of acids such as SO_2, or autocatalyzed by the release of acetic acid from hemicellulose degradation (Jönsson and Martín, 2016).

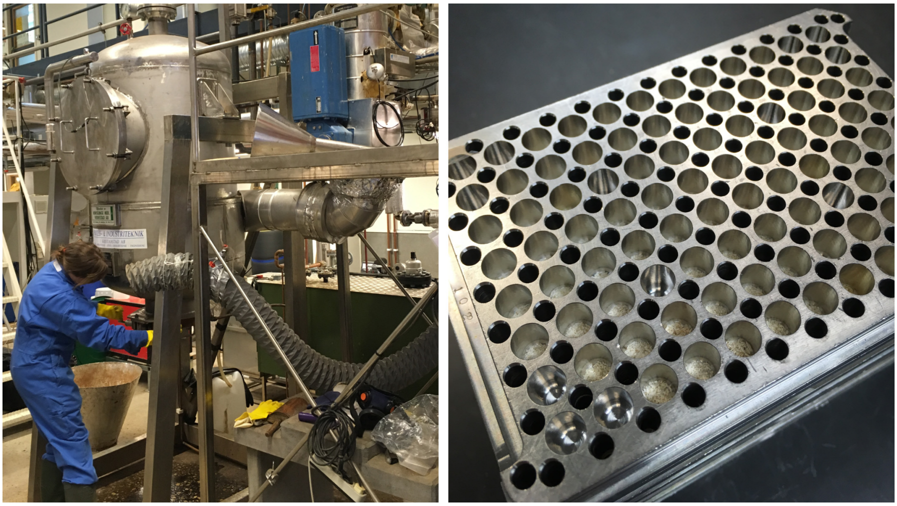

Figure 4: Pretreatment units of different sizes: kilogram-scale reactor at Lund University, Sweden (left), and milligram-scale reactor used for high-throughput recalcitrance screening at the National Renewable Energy Laboratory, Golden, CO, USA (right).

A major drawback of most types of pretreatments is the formation of compounds which inhibit the organisms used in the conversion of sugars into biofuels. Under acidic conditions, such as steam pretreatment, the main inhibitors formed from degradation of sugars are furfural and 5-hydroxymethyl-2-furaldehyde (5-HMF) (Jönsson and Martín, 2016). Acetic acid, released from the hydrolysis of acetyl groups adorning certain hemicelluloses (Section 2.1.1) may also act as potent inhibitors. The formation of inhibitors is partly dependent upon the type of pretreatment used and the severity (i.e., temperature and time) (Horn et al., 2011; Jönsson and Martín, 2016). Thus, the development of less recalcitrant feedstocks may benefit conversion processes both by reducing operating and capital costs on the pretreatment side, as well as by generating less toxic raw material for biofuel production.

3.2 Microbial conversion systems

For lignocellulosic biorefineries, arguably the two most commonly discussed categories of microbial conversion strategies are enzymatic saccharification followed by fermentation, and AD. In the former system (referred to as enzymatic hydrolysis (EH) in this thesis), pretreated biomass is hydrolyzed using a mixture of cellulolytic enzymes, with the resulting *hydrolysate* used as a substrate for the fermentation. In the latter system, the microbial consortium is itself able to deconstruct the biomass.

AD plants are associated with relatively low capital costs in comparison with EH plants, due to the requirements for additional equipment in the latter. EH plants typically require separate reactors for pretreatment, hydrolysis, and fermentation, waste handling facilities (which can itself be an AD plant), chemical storage, steam generators, etc., that an AD plant may often do without. The optimal size, in a financial sense, for an EH plant can thus be assumed to be larger than the corresponding AD plant (Nguyen and Prince, 1996; Walla and Schneeberger, 2008), allowing AD plants to be operated at both small (such as many plants operated on farms) and large scales.

The EH system may provide a greater control over output products: when hydrolysis and fermentation generally occur separately, any organism capable of fermenting the sugar stream may theoretically be used. Xylose fermentation and inhibitor tolerance are seen as major problems with this system, as commonly used microbial workhorses may not have these capabilities natively (Nieves et al., 2015). EH-like systems where hydrolysis and fermentation occur in the same reactor, known as simultaneous saccharification and fermentation (SSF) or consolidated bioprocessing (CBP), may reduce capital costs, but place further demands on the fermentative organisms (Hasunuma et al., 2013). During hydrolysis, cellulose and hemicelluloses are converted into simple sugars, which are used as substrates for the fermentation step. The main advantages of EH systems are arguably the range of possible products, product specificity provided by the potential use of engineered microorganisms, and throughput. Sensitivity analysis has shown that the financial viability of EH biorefining is particularly influenced by costs relating to the use of enzymes, and to sugar yields from biomass (Tao et al., 2014).

Conversely, the microbial agents responsible for conversion in an AD system are by definition less well-defined. In AD, an undefined microbial consortium is responsible for degrading complex material through a series of reactions to CO_2 and CH_4 (Angelidaki et al., 2011). Broadly speaking, this process starts with *hydrolysis*, which produces mono- and oligomeric sugars, peptides, amino acids, glycerol, etc. from more complex compounds. These molecules are further converted into short-chain organic acids, alcohols,

H_2, and CO_2 during *acidogenesis*. During *acetogenesis*, acetate is mainly formed from substrates such as sugars, acids, CO_2, and H_2. Finally, CH_4 and CO_2 are generated through *methanogenesis* by the splitting of acetic acid into these compounds or by the reduction of CO_2. Among the multitude of possible configurations of AD systems are the most common single-stage process, where all reaction steps occur in the same vessel; a sequential single-stage process where material from the first reactor is further degraded in a second, similar reactor (**III**; Boe and Angelidaki, 2009); and a two-stage process, where methanogenesis and acid formation occur in separate reactors (Schievano et al., 2014); but several other possibilities exist.

The outputs of traditional AD plants are biogas, a versatile energy carrier which can be burned for energy or upgraded into a transportable fuel consisting of pure methane (biomethane), and digestate, which can be directly used as fertilizer or further refined (Monlau et al., 2015). However, AD can also serve as a basis for more advanced biorefineries. One proposed method is based around the so-called *carboxylate platform* (Agler et al., 2011; Kleerebezem et al., 2015), which utilizes the two-stage AD system and converts the short-chain carboxylic acids via chemical and microbial means into high-value pure chemicals. Another suggested route makes use of the biogas itself as a substrate for the production of a diverse range of products such as protein-rich animal feed (Matassa et al., 2020), lactate (Henard et al., 2018), and other products such as ectoine, vitamins, biopolymers, and fuels (Strong et al., 2016). Biogas can also be converted into syngas via a reverse water–gas shift reaction, which can be converted into methanol (Ghosh et al., 2019) or to other products via the Fischer–Tropsch process (Hernandez and Martin, 2018).

The benefits of traditional AD systems include general substrate agnosticism, low pretreatment requirements, ease of product separation, and low operational expenditure. Drawbacks include long retention times, limited product spectrum, and low commercial value of the products. Proposed AD-based biorefineries aimed at an expanded product portfolio share most of the benefits of traditional systems, whereas drawbacks generally relate to their unproven, largely theoretical nature. Another important benefit of traditional AD systems is that their low capital and operational costs make very small-scale AD plants, such as smaller farm-scale operations, feasible. For lignocellulosic biomass conversion, this allows reducing the financial and energy costs associated with feedstock transport, which can be considerable.

3.3 Measuring biomass recalcitrance

Biomass recalcitrance is commonly evaluated using three classes of methods: EH assays, biomethanation potential (BMP) assays, and *in vitro* digestibility assays. The latter type of assay is used for estimating ruminal digestibility of feeds, and will not be discussed further in this thesis.

In EH assays, biomass is first subjected to one or several pretreatments (unpretreated biomass can be included as a control), after which it is hydrolyzed using cellulolytic enzyme preparations, commonly including several enzymes. Minimally, the enzyme mixture contains one or several cellulases, with cellobiase and xylanases being common additions. The enzyme mixtures used are often commercial mixtures such as Cellic CTec from Novozymes or Accellerase from Du Pont. EH assays, including the pretreatment step, can be scaled from the microscale up to pilot scale, depending on the question being investigated (Section 3.1).

For EH assays, it is important that the pretreatment step is of sufficient *severity* to open up the lignocellulosic matrix and allow access to hydrolytic enzymes. For hydrothermal pretreatments, the severity factor R_0 is often used, calculated according to the following equation:

$$R_0 = t \times \exp\left[\frac{T - 100}{14.75}\right]$$

In the above equation, T is the pretreatment temperature expressed in °C, and t is the time in minutes. The severity factor is most commonly expressed as its base-10 logarithm, $\log R_0$. The use of R_0 allows some degree of comparison between studies, and also allows readers (and authors) to estimate whether the pretreatment parameters are likely to be sufficient for the question at hand. Several published studies have unfortunately utilized suboptimal pretreatment parameters, compromising their results. For an overview of the history and evolution of this equation, see Garrote et al. (1999).

After pretreatment and hydrolysis, sugar concentrations in the hydrolysis slurry are measured, often using HPLC, although NMR and enzymatic assays are more suitable for high-throughput assays. Values can be expressed relative to the amount of dry biomass (preferably extracted when comparing between biomass species, to reduce influence of the variable amounts of extractives which may be present), or relative to the amount of carbohydrate polymers present in the native biomass. Although the latter presentation provides a better measure of recalcitrance, as it reduces the variability due to differing amounts of carbohydrate in the native biomass, the associated costs and scarcity of high-throughput biomass characterization platforms often precludes the use of this measure in high-throughput settings. An important drawback of EH assays is that the inhibitory effects of biomass hydrolysates on the converting organism is generally not accounted for, mainly due to the laborious nature of such tests.

Figure 5: Biomethanation potential (BMP) is commonly measured either automatically using the automated AMPTS system (top), or manually using rubber-sealed bottles (bottom) .

BMP assays can be used to evaluate biomass recalcitrance as it specifically relates to AD. In this assay, inoculum from an operating AD reactor is mixed with the substrate of interest, with gas production monitored throughout the process. Gas production is most commonly monitored manually using manometric methods combined with gas chromatography (GC), or by using automated systems such as the AMPTS (Badshah et al., 2012; Figure 5). Although automatic systems reduce the workload associated with BMP assays, a truly high-throughput system has not yet been developed.

When measuring gas production manometrically, pressure is measured using a manometer connected to a syringe inserted through the bottle's rubber septum. A headspace gas sample is collected for GC analysis, and the pressure in the bottle is subsequently equalized to atmospheric pressure. By knowing the overpressure and methane content of the gas, the amount of methane produced can be calculated. In the automated system, the produced gas is diverted through a strongly alkaline solution, causing dissolution of CO_2. The remaining gas, considered to consist of 100 % CH_4, is then measured volumetrically using a piston displacement system. Results from different methods are generally in agreement (Amodeo et al., 2020). For obtaining reproducible and accurate results, care must be taken when designing and reporting BMP assays. Holliger et al. (2016, 2021) provides a good summary of important considerations.

It is worth noting that BMP assays can be performed using either pretreated or non-pretreated material, as AD cultures are more effective biomass degraders than enzymatic extracts. Another important point to consider is that the BMP assay measures gas production from a complex microbial culture, making the assay a measure of both the degradability of the material and the function of the microbiota. Thus, a material which is highly degradable but at the same time contains compounds inhibiting the AD culture will yield a low BMP value. Moreover, BMP assays are inherently more variable than EH assays, being influenced both by assay parameters (e.g., substrate:inoculum ratios, organic loads (Holliger et al., 2016)) and inoculum source (Liu et al., 2017).

3.4 Comparative biomass recalcitrance

Most research evaluating biomass recalcitrance can be classified into one or more of the following categories: studies investigating the effects of genetic perturbations; surveys of natural or breeding populations; studies concerned with the effects of different pretreatment modalities and/or parameters; evaluations of potential product yields (e.g., ethanol per ha or methane per ton) using different species, cultivars, or agricultural practices; or investigations into the effects of biomass features such as chemical composition on recalcitrance.

Although considerable resources have been dedicated towards the study of the influence of biomass features on recalcitrance (such as lignin content, composition, and linkage patterns, cellulose degree of polymerization and crystallinity, carbohydrate polymer composition, surface porosity, etc.), the literature is still fraught with inconsistencies and conflicting results (Foston and Ragauskas, 2012). At the core of this problem is likely the highly complex nature of secondary cell walls, and the interactions of its components with regard to recalcitrance. Moreover, detailed study of molecular and ultrastructural features is a resource-intensive task, and no published study has exhaustively studied all possible aspects of the cell wall. However, it is possible that given enough adequately-powered studies with comparable methodology, concensus regarding certain well-studied characteristics (i.e., monolignol composition, lignin content) can emerge.

An understudied aspect of biomass recalcitrance is how it relates to different biological conversion systems, such as EH, AD, or pure cultures of cellulolytic microbial agents such as *Clostridium thermocellum*. Several differences between these systems, including the involvement of live microbes in the deconstruction of biomass, the necessity for pretreatment, and the reaction temperature, suggest a differential response to recalcitrance features. A better understanding of how biomass recalcitrance relates to conversion systems is vital to developing universal reduced-recalcitrance feedstocks.

Lignin content is generally considered to influence yields negatively in both EH and AD systems. However, less is known about the influence of the lignin S:G ratio on AD systems, especially those utilizing non-pretreated biomass. Whereas the S:G ratio is typically found to be positively correlated with sugar yields in EH systems using pretreated biomass, the inverse finding has been reported when using non-pretreated biomass (Min et al., 2013; Skyba et al., 2013). The fact that AD cultures can metabolize lignin monomers and oligomers into methane has long been established, however, native lignin is often considered a relatively inert substrate under AD (Mulat and Horn, 2018; Young and Frazer, 1987).

Whereas some studies have indicated that the S:G ratio decreases during AD, suggesting a preferential degradation of S lignin, data on the influence of monolignol composition on AD performance is scarce. Waliszewska et al. (2019) reported considerable reductions in S:G ratios after AD of sorghum and miscanthus, although no data was reported on the effect of lignin structure on methane yields. In a similar vein, Huang et al. (1998) reported on degradation of plant matter in peat bogs, and found a preferential degradation of syringyl monomers under anaerobic conditions, with the surprising finding that ether-linked S and G units were unaffected after 23 years of decomposition. On the other hand, Pawar et al. (2018) reported a possible positive correlation of G lignin with methane yields, albeit the finding was possibly confounded by cellulose content. Malayil and Chanakya (2019) demonstrated that S and H lignin can be degraded during AD, and that their removal correlated with a loosening of the cell wall structure, allowing deconstruction to commence.

In order to better understand the influence of the S:G ratio (as well as other biomass properties) on AD performance using *Salix* wood as a feedstock, a selection of *Salix* material from a natural population, previously characterized for biomass properties and EH yields, were subjected to BMP tests (**II**). Further details on the population and the EH characterization are provided in Section 4.4. The results indicated that sugar release yields were correlated with BMP values, suggesting that improvements in recalcitrance as measured using sugar release assays would also benefit AD systems, even using non-pretreated biomass. The variation in BMP values was considerably smaller than that of sugar release values, most likely due to the greater deconstruction capability and substrate agnosticity of the AD culture.

Interestingly, sugar release yields correlated most strongly with methane production rates and yields at the early stage of the process, with late methane production being essentially uncorrelated (**II**). The same was true for lignin S:G ratio and other biomass properties such as the weight of the main shoot. These findings suggest that *Salix* wood with a reduced recalcitrance in EH systems would provide benefits in AD systems by allowing shorter hydraulic retention times (HRTs), thereby increasing throughput and allowing for higher volumetric methane production per unit reactor volume and time.

3.5 *Salix* as an anaerobic digestion feedstock

Whereas the use of woody feedstocks for ethanol production via EH is at this point a relatively well-studied subject, less is known about the performance of such feedstocks, including *Salix* and closely related hardwood species, in AD. This lack of research interest likely stems to some degree from the historically low interest in AD systems in the United States, where much of the biofuel research on *Populus* is performed. Moreover, woody biomass is often believed to require pretreatment in order to perform well under AD (see e.g., Matsakas et al. (2016)), which would preclude its use in many biogas plants. However, Turick et al. demonstrated already in 1991 that BMP values for non-pretreated hardwood biomass, including several *Salix* species, could reach over 300 Nml CH_4 g^{-1} VS. Although this number is in the lower end of feedstocks currently in use for AD (Scarlat et al., 2018), the comparatively low moisture content of *Salix* biomass means that the BMP per unit wet biomass is relatively high.

Although pretreatment has been reported to improve the BMP of *Salix* (Estevez et al., 2012; Horn et al., 2011), it is possible that this finding may simply be an artifact of assay duration. In paper **II**, biomass recalcitrance was found to mainly influence rates rather than ultimate methane yields, i.e., lower-recalcitrance material was converted into methane faster than higher-recalcitrance material. Thus, if not allowing a sufficient duration for the BMP test, a difference in rate will be interpreted as a difference in ultimate methane yield, because the assay is terminated prematurely for the lower-rate material. As a consequence, in comparisons of pretreated and non-pretreated material, or of material pretreated at different severities, the apparent BMP differences may be exaggerated. Allowing longer HRTs should allow reaching similar ultimate conversion yields for pretreated and non-pretreated material. And indeed, when HRT was increased, the methane potential of non-pretreated

Salix biomass (**III**) was essentially identical to that reported for steam exploded material (Estevez et al., 2014).

Salix may be particularly beneficial as a feedstock for small-scale operations where the motivation is minimizing environmental footprint. In comparison with combustion, *Salix*-based AD provides greater climate benefits (Ericsson et al., 2014), while requiring substantially lower energy and agrochemical inputs compared to both perennial and annual bioenergy crops (Section 1.2). Many smaller farm-scale biogas plants primarily digest manure, in a mono-digestion setup (Ahlberg-Eliasson et al., 2017). Due to the high moisture content of this feedstock, the volumetric production of such an operation is low, as a consequence of suboptimal organic load in the digester. By complementing manure with a low-moisture material (a practice known as *co-digestion*), both volumetric and specific outputs can be improved. In paper **III**, we evaluated the effects of increased HRT in a manure–*Salix* co-digestion system. By increasing the HRT from 36 to 72 days, volumetric production was increased by 57 %, and compared to the manure mono-digestion system, volumetric production was increased by 119 %. Of note, these values were acquired using non-pretreated material. In small-scale scenarios, pretreatment equipment may be prohibitively expensive, and increased HRTs may be a more viable option.

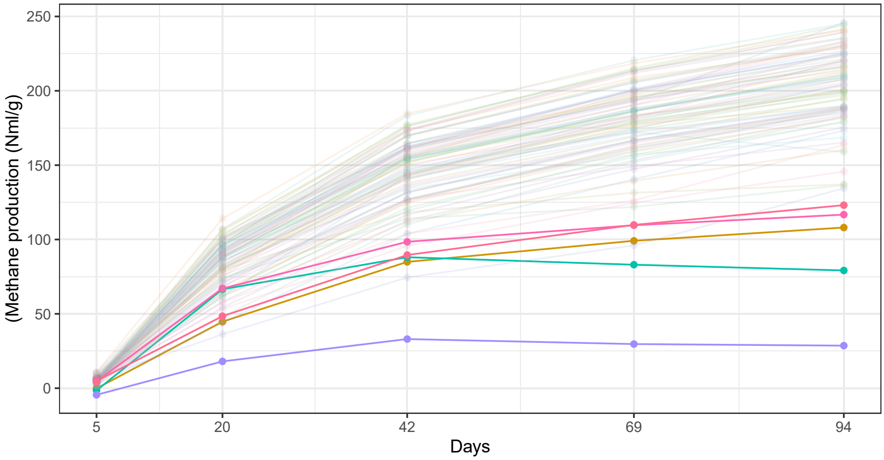

Figure 6: Methane production (Nml CH_4/g biomass) versus time from *S. viminalis* material from a natural population. Low-producing samples are highlighted.

3.6 Inhibitory compounds present in *Salix* biomass

A notable finding of paper **II** was that certain material (five out of 95 samples) appeared to inhibit the conversion process, as indicated by methane yields which were lower than $1.5 \times IQR$ (interquartile range; Figure 6). By comparing the averaged py-MBMS spectra of these samples against the averaged spectra of all other samples, notable features could be observed. The low-producing samples had lower intensities of peaks associated with C6 sugars and S lignin, features which would suggest lower yields and greater recalcitrance. However, these samples were also enriched in peaks indicative of phenolic or aromatic compounds. Due to the nature of py-MBMS, the exact origin of these peaks cannot be ascertained. However, as previously noted, many extractives found in biomass are of a phenolic nature, and a reasonable assumption is that the parent compounds of these peaks are secondary metabolites (Section 2.1.3).

It is well known that aromatic compounds can be potent inhibitors of the AD process. Sierra-Alvarez and Lettinga (1991) investigated the inhibitory effects of 34 aromatic compounds on aceticlastic methanogenesis and found that the inhibition strength was strongly related to substitution patterns, with more complex patterns imparting stronger inhibitory effects. *Salix* bark contains several substituted phenolic glycosides (Palo, 1984), which can constitute up to 10 % of the bark, by mass, with considerable inter- and intraspecific variation (Förster et al., 2010). Non-structural biomass constituents may thus be an important, but often overlooked, factor to consider when developing or investigating feedstocks for biorefinery purposes (discussed further in Section 4.5).

4 Breeding towards enhanced biofuel performance

Plant breeding is arguably one of the most important discoveries in human history. Enabling man to domesticate and improve wild species, breeding has become a defining feature of human societies since the neolithic. It was not until relatively recently, however, with the rediscovery of Gregor Mendel's work in the early 20th century, that plant breeding became an area of broad scientific interest (Reeves and Cassaday, 2002). Whereas breeders have refined yield and other agricultural traits of food crops for millennia, the same cannot be said for bioenergy crops. The lack of breeding progress coupled with the advent of new molecular tools and genomic resources should pave the way for rapid progress towards enhanced bioenergy crop varieties (Allwright and Taylor, 2016; Vermerris et al., 2007).

4.1 Estimating genetic variation

Plant breeders strive to refine the *phenotype*, i.e., the observable properties of a plant, by changing its genetic makeup, its *genotype*. The phenotype is not a direct translation of the genotype, however, complicating the work of the breeder. Phenotypic variance (V_p) can be divided into variation due to genetic (V_g), environmental (V_e), and genotype-by-environment ($V_{g\times e}$) factors:

$$V_p = V_g + V_e + V_{g\times e}$$

The genetic variance can itself be similarly divided into additive (V_a), dominance (V_d), and epistatic variance (V_i, for *interactive*). Additive variance refers to variance caused by additive effects of multiple alleles at different loci, and this is generally the type of genetic variance that breeders are interested in, as it is the one that is easiest to utilize in recurrent selection programs. Dominance variance is due to dominant alleles, causing the phenotype of the heterozygote to deviate from the mean of the homozygotes. Epistatic variance refers to variance stemming from interactions of multiple alleles at different loci.

Breeders are interested in the *heritability* of a trait, which refers to how much of the total phenotypic variance exhibited in a plant is due to genetic variance. Heritability can be described in the *broad sense*, H^2:

$$H^2 = \frac{V_g}{V_p}$$

This measure captures all genetic variation, even that which cannot easily be utilized by breeders. Thus, a more useful parameter for breeding purposes is the *narrow-sense heritability*, h^2, which only includes the contribution of additive genetic variation:

$$h^2 = \frac{V_a}{V_p}$$

Through high-resolution genetic mapping, so-called single-nucleotide polymorphisms (SNPs), which indicate positions in the genome where individuals differ, can be identified. SNPs can be used for genome-wide association studies (GWASs), where phenotypic differences are mapped to causative SNPs (Section 4.3), as well as for allowing more precise heritability estimates (Kruijer et al., 2015; Speed et al., 2012). Heritability estimates based on SNP data are referred to as SNP or chip heritabilities, and utilize SNP data to calculate relatedness between individuals in order to separate phenotypic variance into additive and environmental variance components (with environmental variance in this case encompassing all non-additive variance). Chip heritabilities are useful in cases where pedigrees are not otherwise known, such as when investigating natural populations.

Although heritability is important for breeding purposes, the coefficient of variation (CV) for a trait also provides valuable information. The CV is a standardized measure of standard deviation (SD), i.e., SD divided by the overall mean for the trait in question, and is commonly expressed as a percentage. Thus, CV quantifies the variance for a certain trait in a more easily interpreted manner. Similarly to variances, CVs can be separated into additive and environmental components (CV_a and CV_e). If both h^2 and CV_a are known for a trait, the estimated improvement that can be realized through breeding (genetic gain, ΔG) can be calculated (Cornelius, 1994; Falconer, 1996).

4.2 Phenotypic and genetic correlations

In addition to variances and heritabilities, correlations between traits are frequently investigated. Such correlations are typically reported as Pearson's correlation coefficients, and can be presented as phenotypic correlations (r_p or sometimes simply r), and, when kinship is known or can be estimated from genetic mapping data, genetic correlations (r_g, or r_a for additive genetic correlations). Genetic correlations reflect how much of the correlation between two traits is due to shared underlying genetic background.

Genetic correlations are especially useful for breeding purposes. As they provide breeders with information on the shared genetic basis for two traits, the breeder can both estimate whether one trait is likely to arise as a consequence of selecting for another, as well as for choosing indirect measurement traits for simplifying selection. As an example of the latter use case, a breeder may be interested in selecting for a certain hard-to-measure trait A that is highly genetically correlated with the readily measured trait B. If the breeder then selects for trait B, improvements in trait A may also be realized, while resources spent on measuring phenotypes are minimized.

4.3 Genome-wide association studies

With high-resolution SNP (and possibly including other markers such as insertions and deletions, *indels*) mapping comes the possibility to infer causality of a certain polymorphism to a trait. This methodology, known as GWAS or association mapping, has been widely employed in humans, animals, and plants. GWAS methodology allows identifying marker–trait associations in unrelated individuals, enabling marker discovery in natural populations. Markers can be identified either randomly using genotyping-by-sequencing approaches, by using a candidate gene approach targeting polymorphisms in genes believed to influence the trait of interest are identified, or by a combination of both strategies. For certain species, SNP chips have been developed which permit cheap and rapid identification of known SNPs.

Although GWAS methodology was initially heralded as a promising means of elucidating the genetic underpinnings of a wide variety of traits, the value of the method has come into question. GWAS critics point out that few verifiable results have been delivered by the use of the methodology outside of inbred populations, that many GWAS hits are likely false positives, and that the utility of the method is greatly reduced by the highly polygenic nature of most traits (Grattapaglia et al., 2018; Korte and Farlow, 2013).

4.4 Prospects for breeding improved *Salix* feedstock variants

Most efforts aimed at reducing the recalcitrance of lignocellulosic species have focused on lignin content reduction, monolignol composition, or both, usually via genetic modification methods. However, genetic perturbations of the lignin pathway are commonly associated with growth penalties in field trials (Chanoca et al., 2019). Moreover, establishing plantations of genetically modified trees may be associated with regulatory or public perception issues. Traditional plant breeding may thus be a more tenable strategy for generating low-recalcitrance bioenergy crop variants, especially in regions where regulations prohibit or severely restrict establishment of plantations of genetically modified organisms. Such recurrent selection breeding programs may be further accelerated through the use of molecular methodologies like marker-assisted breeding (MAB) (Neale and Kremer, 2011) or genomic selection (GS) (Grattapaglia et al., 2018). GS, where numerous markers scattered across the genome are used for predicting breeding outcomes, may be the more viable alternative for accelerated breeding, owing in part to the problem of identifying reproducible marker–trait associations as well as finding individual markers explaining a significant part of the phenotypic variation.

Large-scale surveys have found considerable variation in cell wall compositional and other biofuel-related properties in the wild, both in *Salix* and in the closely related genus *Populus*. Porth et al. (2012) investigated variation and heritability in a number of compositional and ultrastructural traits in 334 *P. trichocarpa* clones, using SNP markers to estimate chip heritability and genetic correlations. Compositional traits were measured using wet chemistry methods. In this study, many traits relevant to breeding for bioenergy purposes were found to have moderate to high heritabilities, including glucose, xylose, alpha cellulose, hemicellulose, and S monolignol content. Interestingly, hemicellulose and lignin contents displayed negative genetic correlations with alpha cellulose content and wood density, suggesting this highly favorable phenotype as a target for bioenergy-focused breeding in this species. In a similar vein, Studer et al. (2011) also reported considerable variation in lignin contents and S:G ratios in a population of natural *Populus* variants, and found both traits to correlate with biomass recalcitrance.

Muchero et al. (2015) investigated variation in a number of compositional traits (measured using py-MBMS), as well as sugar release after pretreatment using the miniaturized NREL assay, using both natural and backcrossed populations in multiple environments and material of different ages. Although considerable variation in biomass traits was reported, only the S:G ratio was reasonably correlated with the corresponding measure using wet chemistry. S:G ratio was also the compositional trait with the highest repeatability between environments. Although heritabilites were not estimated in this study, it was noted that environmental influences were overall large. Moreover, the authors report several SNP–trait associations that were stable across environments.

Figure 7: The *S. viminalis* association mapping population in Pustnäs, Uppsala. The shoots in the image are 1 year old. Photo credit: Nils-Erik Nordh.

In paper **I**, a number of traits of importance to *Salix* breeding for biofuel purposes where investigated, such as biomass yield, wood density, lignin S:G ratio, and sugar release using the miniaturized NREL assay, in a population of natural *S. viminalis* clones collected throughout Europe and Russia and grown in a randomized complete block experiment in Uppsala (Figure 7). Four replicates per clone where included in the study. Mirroring other studies, most traits exhibited wide variation (Figure 8). In this study, sugar release traits showed relatively low heritability ($h^2 = 0.23–0.29$). Lignin S:G ratio and wood density where more heritable ($h^2 = 0.42$ and 0.59, respectively).

Genetic and phenotypic correlations identified in paper **I** indicate that plant fresh weight, and especially the weight of the main shoot (which was used for the assays), was positively correlated with all measures of sugar release. Lignin S:G ratio was also correlated with sugar release, slightly more so in the phenotypic sense. This suggests that breeding for higher biomass yield, particularly through partitioning of biomass into fewer, larger shoots, may be a viable strategy for improving *Salix* biofuel performance. The prospect of using this natural variation as a source of beneficial traits in *Salix* breeding is high, as the genetic pool used for breeding is quite narrow compared to the variation found in the wild (Perdereau et al., 2013). Although py-MBMS data was available for this population, it could not be used for estimation of C5 and C6 sugar composition as correlations with wet chemistry data where very poor. Thus, it cannot be known how much of the variation in sugar release seen was

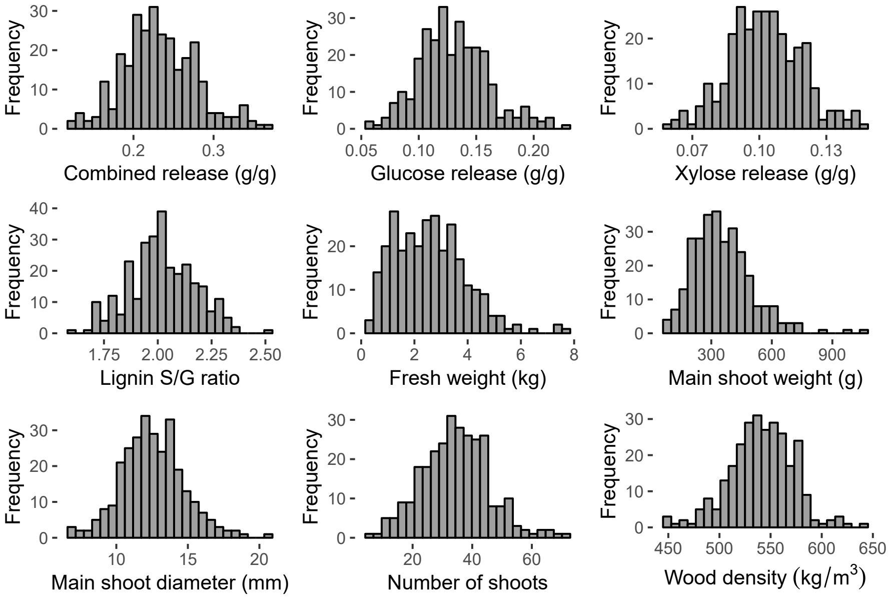

Figure 8: Histograms showing the trait variation in the association mapping population used in paper **I**. Sugar release values are relative to dry biomass. Values used for generating the histograms are clonal means.

due to variations in cell wall carbohydrates (discussed further in Section 5.1).

A GWAS was additionally performed as part of this study. Using a set of 19 411 SNP and indel markers generated through genotyping by sequencing (GBS) (including 1290 markers from an earlier candidate gene study), marker–trait associations for the investigated traits were evaluated. Few associations were found, however, with only one marker appearing below the multiple-testing-corrected α level of 0.05. This finding is likely representative of the polygenic nature of the traits in question. For example, biomass recalcitrance is influenced by a multitude of factors including several ultrastructural and chemical aspects, each of which is likely influenced by several genes. The effect of each polymorphism on such traits will, in most cases, be very small.

Overall, there are still open questions regarding the feasibility of developing *Salix* varieties with improved composition or reduced recalcitrance through traditional breeding. On the biomass composition side, many studies suffer from the use of imprecise compositional measurement methods (primarily py-MBMS) for quantifying cell wall carbohydrate composition. Moreover, genotype × environment interactions are poorly studied, and most studies evaluate genotypes only in a single locale. Thus, heritability estimates for valuable traits can differ widely. It does seem like lignin S:G ratio is under reasonably strong genetic control and that this trait is also to some degree consistent between environments (Muchero et al., 2015), making it a suitable target for breeders at this stage.

Wood density is another trait which has been shown to be strongly heritable in most tree species (Cornelius, 1994). On its own, higher density is a desirable trait in a biomass species. It reduces the bulkiness of the harvest, thus allowing more effective harvesting. Wood density in shrubs is linked to anatomical changes, reflected by changes in fiber sizes and cell wall areas (Martínez-Cabrera et al., 2009), and possibly formation of cellulose-rich tension wood (Berthod et al., 2015), which could influence recalcitrance. However, the effect of wood density on biomass recalcitrance is understudied. In papers **I** and **II**, weak positive correlations were found for sugar release and BMP on the one hand, and wood density on the other. A similar result, albeit with a slightly stronger positive correlation, was reported by Serapiglia et al. (2013), using hot water-pretreated *Salix* clones. Thus, wood density may be a viable target for improvement in *Salix* breeding programs geared towards biorefinery use.

4.5 Improving the secondary metabolite profile

A relatively unexplored area of research regarding the use of *Salix* as a biorefinery feedstock is the effect of secondary metabolites on the process. Other than being likely inhibitors of AD (Section 3.6) and EH processes (de Oliveira et al., 2017), these molecules have potential uses in the pharmaceutical, chemical, and other industries and could contribute to overall financial viability if extracted prior to biofuel conversion (Devappa et al., 2015; Tyśkiewicz et al., 2019). Thus, characterization of the secondary metabolites of willow biomass is an active research area, and several new compounds have recently been identified (see e.g., Noleto-Dias et al., 2020; Ward et al., 2020).

Of interest to breeders looking to create improved *Salix* variants for biorefinery use, the secondary metabolite profile of willow biomass seems to be under strong genetic control. Orians et al. (1996) reported on the heritability of two phenolic glycosides, salicortin and 2'-cinnamoylsalicortin, in *S. sericea* leaves. 2'-cinnamoylsalicortin concentration was considerably heritable ($h^2 = 0.59$), although salicortin concentrations were not ($h^2 = 0.20$), and there was no genetic correlation between the two. Muklada et al. (2020) investigated the influence on genotype on secondary metabolites in leaves of natural *Salix* accessions, grown under contrasting water treatment conditions. In their study, chemotype was strongly influenced by genotype, with smaller effects of water treatment reported.

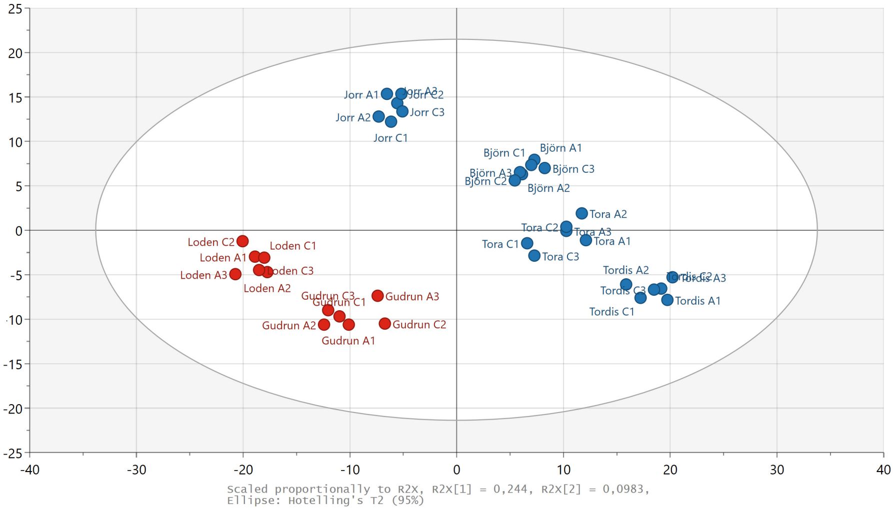

Figure 9: OPLS-DA plot of LC-MS profiles of methanolic extracts of six commercial varieties of *Salix* grown under two contrasting conditions. Samples cluster according to genotype, and segregate based on heritage. Color coding according to genetic background: *S. viminalis* in blue, *S. dasyclados* in red. Variation captured by the first and second components was 24.4 % and 9.8 %, respectively.

Harman-Ware et al. (2021) performed a large-scale survey of biomass constituents in *P. trichocarpa*, using py-MBMS to enable high-throughput screening of a large population. As discussed above (Section 2.2), the ability of py-MBMS to resolve the origins of ions identified in spectra varies with the parent compound. For phenolic secondary metabolites, the parent compound may at best be identified on a class-level, e.g., unknown phenolic metabolite. The heritability for certain ions diagnostic for phenolic secondary metabolites was found to be very high ($H^2 \leq 0.79$). The results of this study are further strengthened by the use of advanced multivariate modeling and simultaneous quantification of lignin contents, as lignin itself can have a confounding effect on the analysis of phenolic compounds in py-MBMS spectra.

In a similar albeit considerably smaller-scale approach, the secondary metabolite profiles of biomass from six commercial *Salix* varieties, grown under fertilized and unfertilized conditions (details on this field trial in **IV**) were analyzed in our lab (unpublished data). Briefly, ground air-dried biomass was extracted using 80% methanol and analyzed using liquid chromatography–mass spectroscopy (LC-MS). LC-MS has a much greater resolving power compared to py-MBMS, allowing a considerably higher resolution map of the secondary metabolites. Using orthogonal projections to latent structures–discriminant analysis (OPLS-DA), differences between clones could readily be identified (Figure 9), whereas the effect of fertilization regime could not easily be discerned. The clustering of samples on the OPLS-DA plot roughly conformed to relatedness between the clones, lending further evidence for the strong heritability of secondary metabolites in *Salix* spp.

Given that the secondary metabolite profile of willow biomass seems to be under considerable genetic control, breeding is likely an effective strategy for creating *Salix* variants with enhanced chemotypes for biorefinery use. However, further research is warranted to analyze which metabolites are the most influential on conversion performance and which compounds carry the highest value as purified chemicals.

5 Influence of agronomic practices

The agronomic practices employed in cultivating a SRC biofuel feedstock, such as the fertilization regime and cutting cycle length, affect biomass productivity, and, as a consequence, overall economics of the production and conversion system: energy economics, financial results, and climate impacts. Interestingly, these practices may also influence biomass properties, such as chemical composition and possibly recalcitrance, which will be discussed further in this chapter.

5.1 The importance of biomass yield for feedstock quality

Biomass yield has, alongside biotic and abiotic stress resistance, been the primary target for willow breeders aiming to improve willow for bioenergy use (Larsson, 1998), and for any species cultivated primarily for its biomass, it will likely continue to be the most important trait regardless of end use. It is likely that this trait will thus also be the primary breeding target even for willows targeted towards biofuel feedstock use, and that the focus on yield may have synergistic effects.

In a study investigating biomass composition and yield, Fabio et al. (2017) examined a number of *Salix* genotypes, mainly originating from breeding programs (although natural variants were also present) in several environments. HR-TGA was used for compositional analysis. The authors reported significant environmental influences on most traits, with hemicellulose being the most heritable of the cell wall compositional traits. Interestingly, the authors report a correlation between yield and cell wall composition, where low yielding replicates exhibited a correspondingly low carbohydrate:lignin ratio. This correlation held true for yield levels up to approximately 6 Mg ha^{-1} year^{-1}, suggesting a preferential allocation of carbon into lignin when conditions are suboptimal for growth. Most authors investigating the matter have arrived at similar conclusions, correlating cellulose and/or lignin content with biomass yield in willow (see e.g., **IV**; Gouker et al. 2021; Serapiglia et al.

2014; Stolarski et al. 2011; Szczukowski et al. 2002).

With (holo)cellulose yield per hectare being a function of biomass yield and composition, the importance of biomass yield for biorefineries cannot be overlooked. Happs et al. (2020) investigated the influence of biomass yield and cell wall carbohydrate composition on biorefinery financial viability in a large population of poplar natural variants, assuming perfect EH yields (i.e., no remaining recalcitrance at the chosen pretreatment severity). The conclusion of the authors was that the carbohydrate contents matter only when biomass yields are already at high levels. This could be attributed to the great variation seen in biomass yields in the population, which was considerably larger than the variation in cell wall carbohydrates. However, among the top 25 % of biomass yield, carbohydrate content was essentially as important as biomass production for biorefinery profitability. Ray et al. (2012) came to similar conclusions in willow, where the effect of biomass yield was found to trump sugar release when calculating per-hectare yields in a number of commercial and breeding program clones.

One likely contributor to increasing carbohydrate content in higher biomass-yielding willow is the resulting higher wood-to-bark ratio. The bark content of whole willow biomass is negatively correlated with stem diameter (Adler et al., 2005; Tharakan et al., 2005), and contains considerably less cellulose compared to wood (Guidi et al., 2009; Serapiglia et al., 2009). SRC willows can be said to accumulate biomass using one of two separate strategies: either through allocation of carbon into larger or more numerous shoots (Tharakan et al., 2005). For biorefinery purposes, breeding willows towards biomass allocation into fewer and larger shoots, preferrably of high density, may thus be beneficial due to the higher wood-to-bark ratio in larger shoots. In support of this recommendation, it was observed in papers **I** and **II** that both sugar release values and methane yields (per unit biomass) were more strongly associated with main shoot weights than with whole plant weights, and that the number of shoots was essentially uncorrelated with either measure.

On the whole, increasing biomass yields seems to improve conversion yields. This was shown in papers **I** and **II** for EH and AD processes in natural *Salix* variants, and also held true for four commercial clones in a relatively large field experiment (Figure 10; unpublished data). Moreover, increasing biomass yields has a favorable effect on the climate impact of *Salix* biomass production systems, due to a greater capacity for replacing fossil fuels (Kalita et al., *in review*). Thus, investigations into the effects of yield-increasing agricultural practices on biomass composition and conversion performance are warranted.

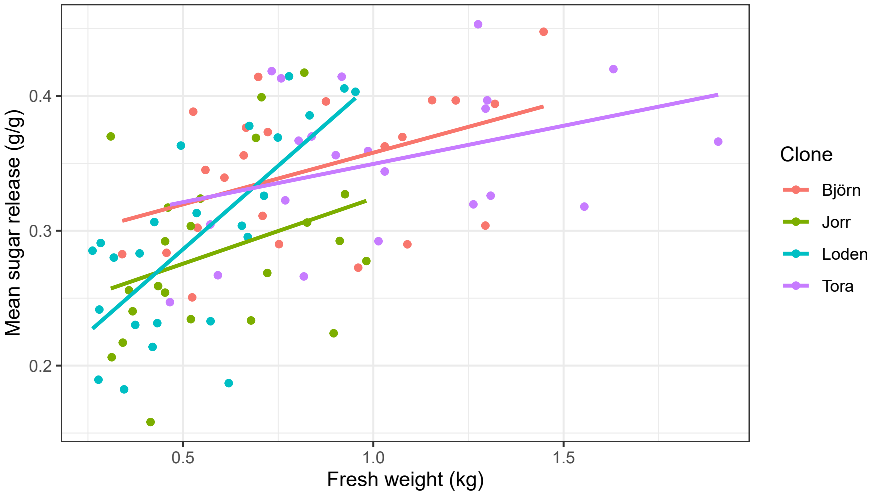

Figure 10: Sugar yield (g sugar per g biomass) vs biomass fresh weight in four commercial *Salix* clones. Independently of genotype, greater biomass yields are positively correlated with sugar yields. Lines indicate linear regression fits.

5.2 Effects of rotation length

Although biomass composition is to a certain degree influenced by genotype, agronomic practices which increase biomass yield, such as longer rotations, seem to generally produce biomass with a composition more favorable for biorefinery purposes. Szczukowski et al. (2002) investigated six commercial *Salix* clones in cutting cycles of 1, 2, and 3 years. With increasing rotation lengths, cellulose contents increased while water content decreased, whereas lignin and hemicellulose contents were unaffected. Estimating the yearly profit per hectare, the authors found that the three-year rotation length was about 70 % more profitable than the one-year rotation, mainly due to higher biomass yields and lower relative production costs. Similar effects were reported by other authors, with longer rotations leading to enhanced biomass yields and improved biomass composition (Guidi et al., 2009; Stolarski et al., 2011).

In paper **III**, the BMPs of six commercial *Salix* clones harvested one, two, and three years after coppicing were investigated. The results indicated that first-year clones had low BMP values, reaching their maximum potentials after 150 days. Assays for the two and three years old shoots did not finish even after 300 days, with two years old samples progressing slightly faster and thus reaching higher BMP values. As recalcitrance in *Salix* primarily affects rates rather than yields, this indicates that the two years old shoots were slightly less recalcitrant than the three years old shoots. That the one year old shoots finished earlier than the others indicates that these shoots contained a lower amount of biodegradable material. In very young shoots, the wood-to-bark ratio is likely to be very low, meaning that relatively little metabolizable material would be present for the AD process. The findings suggest that two years old shoots may be optimal for conversion via AD, and that in older shoots, physiological changes lead to a more recalcitrant phenotype. However, the differences in the resulting BMP values were relatively small, and would likely be offset by the higher costs associated with more frequent harvesting.

It is worth noting that the extended period of time needed for the BMP assays does not reflect the time needed for conversion in an actual AD reactor, where the conditions (co-substrate, stirring, etc.) are more suitable for the necessary microbial metabolism (see Section 3.5). When four years old biomass was used in a co-digestion experiment (**III**), a HRT of on average 76 days was adequate for reaching a methane production equal to 70 % of the BMP of the material.

5.3 Effects of nitrogen fertilization

Biomass yields can generally be increased through nitrogen fertilization. Interestingly, nitrogen fertilization has also been shown to impart physiological changes in *Populus* wood which imply that it might affect biomass recalcitrance. Consistent with the generally negative correlation between lignin concentration and biomass yield, nitrogen fertilization has been consistently shown to reduce the lignin concentration in secondary cell walls of *Populus* and *Eucalyptus* in greenhouse studies (Camargo et al., 2014; Euring et al., 2014; Luo et al., 2005; Pitre et al., 2007; Plavcová et al., 2013). Most of these studies have found that secondary cell wall thickness is reduced, and that fiber and vessel elements have larger diameters under high N conditions, and where S:G ratio is evaluated it is generally slightly reduced in the high N treatments. Similar results have been found in a field trial, suggesting that these changes carry over to real-world conditions (Luo et al., 2005). It is worth noting, however, that some authors have found the effects of N fertilization to be genotype-dependent (Euring et al., 2014; Luo et al., 2005).

In paper **IV**, effects of nitrogen fertilization on biomass composition and BMP were evaluated in six commercial *Salix* clones in a field trial. In this study, fertilized plants did not differ significantly in biomass composition from their unfertilized counterparts, whereas composition was found to be significantly influenced by genotype. Similar results were found for BMP, which was again influenced by genotype but not by fertilization. A somewhat surprising finding was that the lignin content was not correlated with BMP. Lignin content is often found to be negatively correlated with BMP, although it is far from a perfect predictor of ultimate methane yields. Several authors have reported weak negative correlations between lignin content and BMP in lignocellulosic material (see e.g., Komilis and Ham, 2003; Schievano et al., 2008; Tong et al., 1990), and similar results have been reported for mildly pretreated *Salix* in EH assays (Ray et al., 2012; Serapiglia et al., 2013). These findings again underscore the complexity of the biomass recalcitrance phenotype, and how it currently cannot be predicted only from proxy measures such as biomass composition.

6 Conclusions and future perspectives

As a biofuel feedstock, *Salix* lies between the lower-recalcitrance but often more resource-intensive grasses and the longer-rotation, more frugal, woody plants, providing fast turnover biomass with minimal agrichemical and energy inputs. Being a recently domesticated species, much of the genetic potential for improvement is likely yet unexploited. Biomass recalcitrance is a heritable trait which can be reduced through breeding (**I**), thereby creating future refined *Salix* varieties tailored for biorefinery purposes. Importantly for overall economics, the negative genetic correlations between biomass yield and recalcitrance indicate that breeding towards lower recalcitrance would not incur yield penalties. Moreover, *Salix* can be a suitable feedstock for both EH and AD based biorefineries, and recalcitrance improvements are not specific to one system (**II**), allowing for development of a universal *Salix* biorefinery feedstock.

Biomass recalcitrance is a very complex phenotype, and simple marker-assisted selection may not be a viable solution for developing low-recalcitrance crop varieties. For accelerated breeding, genomic selection strategies should be employed, including multi-locale studies of the recalcitrance phenotype. By comparing improved variants against a reference, causative phenotypic changes could likely be identified, improving our knowledge of the processes underlying biomass recalcitrance. Ideally, the characterization should be in-depth, including detailed characterization of both chemical (e.g., lignin and carbohydrate polymer structure, biomass composition) and physical (e.g., ultrastructural) differences.

A relatively understudied area of research concerns the process of decomposition of *Salix* or other woody material under AD. Using a method similar to that used by Mulat et al. (2018) to study degradation of hydrolysis lignin during AD, where the sample is placed in nylon bags during batch AD assays, the decontruction process can be characterized over time. By analyzing material at set timepoints or key points in the process (e.g., rate changes) and employing techniques such as advanced microscopy, compositional analysis including NMR, and metagenomic sequencing, insight could likely be gleaned on how the AD culture interacts with the biomass during hydrolysis, especially if *Salix* material of both high and low recalcitrance, as well as inocula from different sources, are evaluated.

The secondary metabolite profile of *Salix* can adversely affect conversion performance under AD, and possibly EH, systems (**II**; **IV**). Moreover, several secondary metabolites present in *Salix* biomass are of commercial value. With evidence suggesting that the production of secondary metabolites is under strong genetic control, an improved metabolite profile may be a viable target for breeding programs. Future research should be directed towards investigating which metabolites act as inhibitors in biofuel conversion processes, how they interact with AD communities, which easily-extractable compounds may provide added value as byproducts of *Salix* biorefining, and how abundant such compounds are in currently used commercial clones and breeding populations. Findings indicating that fertilization regime influences the production of certain secondary metabolites (**IV**; Hakulinen et al., 1995) also warrants deeper inspection.

Agronomic practices can influence the overall financial proposition of a *Salix* production system, as well as its energy economics, climate impact, and possibly even specific biofuel yields. To better understand these dynamics, studies using life cycle assessment (LCA) methodology should be performed, taking into account the effect of genotype, fertilization, and soil organic carbon allocation together with multiple conversion systems. Moreover, the effects of nitrogen fertilization on recalcitrance under several conversion systems should be studied in larger field trials, possibly using multiple genotypes and nitrogen addition levels.

Further study is also warranted regarding optimal conversion routes for *Salix*-based biorefineries. This not only entails techno-economic assessments of conversion systems (e.g., AD vs. EH), but also interconnected production systems. When different conversion routes and methodologies are included, such studies may inform the choice of optimal biorefinery modalities for *Salix* conversion. For example, several authors have reported better overall biofuel yields from lignocellulosic materials when serially treating material using EH and AD (Bahmani et al., 2016; Dererie et al., 2011; Monlau et al., 2015). Using different conversion routes after fractionation of the biomass, such as producing biogas from the lignin fraction (Khan and Ahring, 2020; Mulat and Horn, 2018), may also be an efficient method for optimizing yields, especially given that AD is already widely implemented in biorefinery settings for heat and power generation and waste handling.

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Popular science summary

Climate change is one of our time's defining questions. Humanity's use of crude oil and coal, natural resources formed over millions of years, has caused increased carbon dioxide (CO_2) emissions to the atmosphere. Carbon dioxide is a *greenhouse gas*, which means that it absorbs part of the sun's radiation, trapping its heat energy and increasing the Earth's temperature. The carbon in this molecule was at one point fixated and ended up in the oil and coal, however, the large time span between fixation and emission leads to an inbalance when we burn the fuels for energy, causing an elevation in atmospheric levels.

In order to not further spur climate change, the use of fossil energy must essentially cease, so that carbon dioxide emission and fixation can return to an equilibrium. Several energy sources can replace oil, coal, and gas—some better than others—and depending on the intended use, replacement can be relatively straightforward or quite complicated. The rapid increase in solar and wind power in the EU during the last two decades has led to a concomitant decrease in coal power, and essentially rendered coal power plants financially nonviable. Other sectors have faced larger obstacles. Perhaps chiefly among these hard-to-convert sectors are long-haul transport and flight, which require storing and transporting considerable amounts of energy.

The challenge with replacing these transportation fuels with renewables, such as electricity from renewable sources, is that the replacing energy carrier must be able to store a large amount of energy per unit weight. As an example, in order to store the amount of energy contained in one kg of gasoline, a battery weighing 5 to 10 kg is required. An alternative to storing energy as electricity is to convert plant matter, *biomass*, into liquid or gaseous fuels of high energy content. This conversion can be performed in *biorefineries*, which, analogous to petroleum refineries, convert plant matter into several end products, including fuels.

Plant matter, foremost corn and sugarcane, has been used for the production of fuel ethanol for a relatively long period of time. However, the use of edible raw material for fuel production has been criticized for contributing to world hunger and for increasing the divide between affluent and developing nations. Thus, the interest in using inedible plant matter, such as residues from agriculture and forestry, as feedstock for biorefineries has surged. Fuels produced from such materials are known as *second-generation* biofuels.

Rapid-growing willows (*Salix* species) make for excellent bioenergy crops. In contrast to most other tree species grown in temperate climates, *Salix* is cultivated in short rotation cycles, with harvesting of the biomass normally occurring every three years. This leads to a shortening of the time between fixation and emission of carbon dioxide compared to conventional forestry, where cycles often are 50 years or longer. For biofuels to have an immediate impact on carbon dioxide levels, short cycles are required.

Inedible plant matter is inedible for a reason: it is hard to digest. Over millions of years, plants have refined their defences against diverse challenges posed by their environments. Although both edible and inedible plant matter to a large degree consists of sugars, a grain contains sugars in the readily digestible form of starch, whereas the wheat straw is contructed from the considerably less palatable cellulose. Moreover, the cellulose is encapsulated in lignin, acting as a type of glue or insulation. Thus, the structural parts of plants cannot easily be deconstructed, and this property is known as *biomass recalcitrance*. Although biomass recalcitrance has allowed protection from pathogens and animals, it has also prohibited the widespread use of inedible biomass as biorefinery raw material.

In order to use *Salix* as a biorefinery feedstock this inherent resistance must be overcome. One way of accomplishing this goal would be to breed new variants with reduced recalcitrance. A breeding program can only be effective if the trait it aims to improve is under genetic control. Most biological traits are influenced both by genetics and environment—nature and nurture—and only part of the genetic component can easily be utilized by breeders. To assess how much of biomass recalcitrance is amenable to breeding, a collection of hundreds of Salix variants sourced in the wild in Europe was evaluated. In a mini-biorefinery, recalcitrance was evaluated and by using genetic markers we were able to reveal that there is potential for breeding Salix varieties better suited for the biorefinery.

In further experiments, we could deduce that a reduced recalcitrance in *Salix* would benefit not only production of ethanol, but also biogas. Biogas is an energy-rich gas mixture which can be used directly for heat and power generation, or which can be upgraded into a vehicle fuel. The biogas production process differs considerably from that used for ethanol, and it was not previously known how transferrable these recalcitrance improvements would be between the two systems. Other properties linked to recalcitrance, such as the composition of lignin and size of the shoots, were also consistently associated with recalcitrance under both systems.

To facilitate conversion of recalcitrant biomass, different types of *pretreatments* are commonly used. Steam explosion, where the biomass is subjected to high pressure steam and subsequently "exploded" upon pressure release is one example of such a pretreatment. Although this procedure significantly reduces recalcitrance, it requires costly equipment which can preclude its use in smaller-scale facilities, such as many biogas plants operated on farms. These farm-operated plants could benefit from using *Salix* in order to balance the biogas production process, which uses manure as a feedstock to a large degree. By using two biogas digesters in serial, we could reach conversion efficiency of a *Salix*/manure mixture comparable to that reported for steam exploded *Salix* in a similar process, and could thus allow for using this feedstock in farm-scale plants. Moreover, we could show that the choice of willow cultivar and harvest age affected conversion performance in biogas processes, whereas fertilization did not have an effect.

In conclusion, bioenergy willows are promising crops for use in future biorefineries, mostly due to its high biomass productivity and its frugal requirements for fertilizer and land. Through breeding, improved low-recalcitrance variants can be developed, which should further improve the proposition of using these crops as biorefinery feedstocks.

Populärvetenskaplig sammanfattning

Klimatet är en av vår tids ödesfrågor. Vår användning av olja och kol, miljontals år gamla naturresurser, har inneburit ökade utsläpp av koldioxid (CO_2) i atmosfären. Koldioxid är en s.k. *växthusgas*, vilken absorberar solstrålning och därför gör att värmen från solen hålls kvar kring jorden. Kolatomerna i denna molekyl har en gång lagrats in i oljan och kolet, men eftersom detta var för så länge sedan så uppstår en obalans när vi förbränner dem, och halterna i atmosfären ökar.

För att inte ytterligare spä på växthuseffekten måste vi i princip sluta använda dessa fossila resurser, så att utsläpp och inlagring av koldioxid kan komma i balans. Många energislag kan ersätta olja, kol och gas – vissa bättre än andra – men beroende på användningsområde är detta lättare eller svårare att åstadkomma. Den snabba ökningen av sol- och vindenergi under de senaste två decennierna har gjort att dessa energislag till stor del ersatt kolkraften i EU och gjort den olönsam. Inom andra områden har det gått långsammare. Framförallt gäller detta tillämpningar där mycket energi behöver lagras och förflyttas, som flyg och andra långväga transporter.

Svårigheterna att ersätta dessa bränslen med förnybara alternativ, exempelvis el från förnybara källor, har att göra med hur mycket energi som kan lagras per viktenhet. För att lagra lika mycket energi som finns i ett kg bensin krävs ett batteri som väger 5–10 kg. Ett alternativ till att lagra energin som el är att omvandla växtmaterial, *biomassa*, till flytande eller gasformiga bränslen med högt energiinnehåll. Denna omvandling kan göras i *bioraffinaderier*, vilka i likhet med oljeraffinaderier omvandlar växtmaterial till flera olika produkter inklusive bränslen.

Växtmaterial, främst majs och sockerrör, har använts för produktion av bränsleetanol under relativt lång tid. Användningen av ätliga råvaror för bränsleproduktion har dock kritiserats för att bidra till hunger och öka motsättningar mellan fattiga och rika länder. Därför har intresset ökat för att använda oätligt växtmaterial, som restprodukter från jord- och skogsbruk, som råvara för bioraffinaderier. De bränslen som framställs på detta vis kallas för *andra* *generationens* biodrivmedel.

Salix är en vedartad energigröda som är mycket snabbväxande. Till skillnad från de flesta andra träd som odlas i tempererade klimat odlas salix med korta skördecykler, där biomassan normalt skördas vart tredje år. Detta gör att tiden mellan inlagring och utsläpp av koldioxid blir betydligt kortare jämfört med drivmedel producerade från restprodukter från skogsindustrin, där skördecyklerna är minst 50 år och ofta längre. För att användningen av biodrivmedel ska kunna få en omedelbar effekt på koldioxidnivåerna i atmosfären krävs korta skördecykler.

Ett problem med att använda oätliga växtdelar som råvara i bioraffinaderier är att det som gör dessa växtdelar oätliga är att de är svårsmälta. Växter har under miljontals år utvecklat sina bärande delar för att stå emot olika typer av yttre påverkan, och detta innebär också att det är betydligt svårare att i ett bioraffinaderi bryta ner dem i sina beståndsdelar. Oätliga växtdelar är, i likhet med deras ätliga motsvarigheter, till stor del uppbyggda av sockerarter. Skillnaden ligger i *hur* dessa sockerarter använts för att bygga upp biomassan. I vetekärnan lagras sockret i form av relativt lättnedbrytbar stärkelse, medan vetestråt är uppbyggt av mer härdig cellulosa. Vidare så är cellulosan inbäddad i lignin, vilket fungerar som ett sorts klister eller isolering.

För att kunna använda salix som råvara för bioraffinaderier måste dess inneboende motståndskraft övervinnas. Ett sätt att uppnå detta kunde vara att förädla nya varianter med minskad strukturell härdighet. Ett förädlingsprogram kan bara vara effektivt om den egenskap som man önskar förändra står under genetisk kontroll. I biologiska varelser påverkas de flesta egenskaper av både arv och miljö, och av den ärftliga delen kan endast en del användas för förädling. För att undersöka hur mycket av härdigheten hos salix som kan användas i växtförädlingens tjänst utgick vi från en samling av hundratals salixindivider som hittats i det vilda i stora delar av Europa. I ett sorts mini-bioraffinaderi kunde härdigheten utvärderas, och med hjälp av genetiska markörer kunde vi se att det finns en potential för att förädla mindre motståndskraftig salix.

I vidare försök såg vi även att en minskad härdighet skulle vara till nytta inte enbart för produktion av etanol, utan även för framställning av biogas. Biogas är en energirik gasblandning som kan användas för att generera elektricitet och värme, men som också kan uppgraderas till drivmedel för bl.a. bussar. Biogasprocessen skiljer sig i stora delar från den som används för etanol, och det har inte tidigare varit känt i vilken grad biomassans motståndskraft varit specifik för antingen den ena eller andra omvandlingsmetoden. Vi såg också att egenskaper hos biomassan som samvarierade med härdigheten, som ligninets struktur och storleken på skotten, påverkade båda processerna på liknande vis.

Vid omvandling av motståndskraftig biomassa används ofta någon typ av *förbehandling*. Ångexplosion, där biomassan utsätts för ånga under högt tryck för att sedan “explodera” när trycket snabbt minskas är ett exempel på sådan förbehandling. När biomassan behandlas på detta sätt minskas dess härdighet betydligt, men utrustningen som krävs för att kunna förbehandla biomassa är dyr och kan därför inte användas på mindre anläggningar, exempelvis sådana som kan finnas i anslutning till jordbruk. Just gårdsanläggningar för biogas skulle kunna ha stor nytta av att använda salix för att balansera sina biogasprocesser, som till stor del använder gödsel som råvara. Genom att använda oss av seriekopplade rötkammare kunde vi uppnå en biogasproduktion från salix och gödsel som motsvarade den från förbehandlad salix. En sådan seriekopplad process kunde vara ett alternativ till förbehandling för denna typ av mindre anläggningar. Vidare kunde vi visa att valet av sort och skördecykelns längd påverkar utbytet från salix i biogasprocesser, medan gödsling inte hade någon effekt.

Sammanfattningsvis så är salix en lovande gröda för framtidens bioraffinaderier, framförallt för att den ger stora skördar trots relativt blygsamma krav på gödsling och odlingsmarkens kvalitet. Genom förädling kan mindre motståndskraftiga varianter tas fram, vilket ytterligare kan förbättra möjligheterna för förnybara drivmedel att konkurrera mot fossila motsvarigheter.

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ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

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To reduce greenhouse gas emissions, fossil fuels must be replaced by renewable resources. Fast-growing trees such as *Salix* provide ample biomass with a high turnover rate, making for an excellent feedstock for biofuel production. However, its use as a biorefinery feedstock is relatively unexplored. In this thesis, several factors relating to the genetic variation of biomass recalcitrance as well as those concerning the use of *Salix* as a substrate for microbial conversion are explored.

Jonas Ohlsson received his graduate education at the Department of Molecular Sciences at SLU, Uppsala, the university where he also received both his B.Sc. in Biology and M.Sc. in Food science.

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