TÉCNICO LISBOA

Impact of *Ustilago maydis* Corn Infection on OrganoCat Pretreatment

Catarina Cabrito Soares

Thesis to obtain the Master of Science Degree in

Biological Engineering

Supervisors:

Prof. Walter Leitner
Prof. José António Leonardo dos Santos

Examination Committee

Chairperson: Prof. Tiago Paulo Gonçalves Fernandes
Supervisor: Prof. José António Leonardo dos Santos
Member of the Committee: Dr. Susana Santos Moita de Oliveira Marques

November 2019

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at Institut für Technische und Makromolekulare Chemie (ITMC) of Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen, Germany, during the period March-August 2019, under the supervision of Prof. Dr. Walter Leitner and Dr. Philipp Grande, and within the frame of the Erasmus programme. The thesis was co-supervised at Instituto Superior Técnico by Prof. José Santos.

Acknowledgments

I would like to thank Prof. Walter Leitner for accepting me at ITMC and Prof. José Santos, my supervisor in IST, for his guidance and feedback. I would like to thank everyone from iBiomass BioSc project, namely Dr. Vera Göhre and Dr. Vicente Ramirez, without whom my experimental work and some of the conclusions made would not be possible. A special thank to Dr. Vicente Ramirez for sharing with me his data and for letting me present some of it in my thesis.

Still in ITMC, I have three special thanks to give: to Dr. Philip Grande, my lab supervisor, to Dennis Weidener and to Dennis Wiecha, my lab coworkers. Because of the three of you, I felt not only my german skills improved but also my chemistry knowledge.

Philipp, thank you so much for accepting me in your group, for all your help, guidance and amazing feedback. You have taught me a lot about my thesis topic and about scientific research in general: to always see the bright side of the results, how to structure my thoughts and how cooperation projects work. You were tireless and always comprehensive, I could not ask for more.

Tall Dennis, thank you for teaching me all the lab techniques and tricks, for helping me everytime I messed up something in the lab and for sharing with me your theories about the results I had. Most importantly, thank you for being so easygoing, always making fun of everything (specially my corn) and for teaching me how germans drink beer.

Small Dennis, thank you for making my days in the lab way funnier. You were always in a good mood, playing (sometimes) good music, laughing everytime I was clumsy or fell from the chair and sharing your thoughts about GoT and Grey's Anatomy. You were very helpful and always trying to teach me, specially in the first few days when I did not know any method or where the material was.

To my friends from Técnico, I have a special thank to give. From each of you I have learned something that I apply in my day-to-day life and that I have applied in this internship. We all share the same passion for learning and for improving ourselves and the world we live in. Bea and Renato, I could not thank you enough. I am sure you agree that all the lab reports and group projects we did together taught us so much and were a key factor for writing quality thesis. To all of you, thank you for 5 years of real friendship!

To my family goes the biggest thank you, not only for my thesis but for my entire degree. You have always supported and trusted me, you were always proud of myself even when I was not. You have taught me all my values, my working principles, how to treat other people, see the world and deal with new adventures, which were incredibly important in these 5 years. I am so lucky to have all of you and I cannot thank you enough, specially to my mum, dad and brother Duarte!

Abstract

In the biorefinery context, an efficient pretreatment step is a key factor for the full valorization of lignocellulosic biomass. Corn stover is already used as a feedstock in biorefineries, however valorization of corn stover infected by *Ustilago maydis* (smut disease) has not yet been reported. This would be especially relevant in Mexico, since there corn plantations are wittingly infected, as the smut corn galls are considered a delicacy. In this thesis, uninfected and infected corn leaves were treated with OrganoCat process to evaluate the impact of corn smut infection on this pretreatment efficiency.

Compositional analysis of both substrates suggested a decrease in cellulose content and increase in hemicellulose and lignin content due to corn smut infection. Uninfected and infected corn leaves were screened for six process condition sets combining different reaction times and temperatures. Higher reaction times and temperatures lead to increased amorphization and delignification of the lignocellulose, but also to the degradation of extracted sugars into furfural and 5-hydroxymethylfurfural. The pretreatment of infected corn resulted in lower cellulose-enriched pulp yields and similar extraction of hydrolysed hemicellulose sugars and lignin, compared to uninfected corn. Compositional analysis of the pulps partially confirmed the results observed. Enzymatic hydrolysis of the pulps suggested an enhancement in cellulose accessibility due to OrganoCat pretreatment, and its improvement was higher for infected corn leaves. The monomers of the obtained lignin fractions were qualitatively analyzed.

Overall, the results suggested that OrganoCat pretreatment is an efficient fractionation method for both uninfected and infected corn leaves and that it can be be tuned to yield high delignification, high fermentable sugars or to a comprise between high extraction and low sugar degradation.

Keywords: pretreatment, fractionation, OrganoCat, corn leaves, corn smut, enzymatic hydrolysis.

Resumo

No contexto da biorefinaria, um pretratamento eficiente é de elevada importância para a valorização completa da biomassa linhocelulósica. Os resíduos agrícolas da planta do milho já são utilizados como matéria-prima nas biorefinarias, contudo a valorização dos resíduos infetados pelo fungo *Ustilago maydis* (doença carvão-do-milho) ainda não foi reportada na literatura. Isto seria especialmente relevante no México, onde as plantações de milho são infetadas intencionalmente, uma vez que os tumores do milho são considerados uma iguaria. Nesta tese, folhas de milho não-infetadas e infetadas foram tratadas com o processo OrganoCat para avaliar a influência da doença carvão-do-milho na eficiência deste pretratamento.

A análise composicional dos dois substratos sugeriu que esta infeção causa a diminuição do conteúdo em celulose e o aumento do conteúdo em hemicelulose e lignina. Folhas de milho não-infetadas e infetadas foram estudadas para seis condições de reação combinando diferentes durações e temperaturas. O aumento da duração e da temperatura do processo levou ao aumento da amorfização e deslignificação da linhocelulose, mas também à degradação dos açúcares extraídos em furfural e 5-hidroximetilfurfural. O pretratamento das folhas infetadas resultou em menores rendimentos de polpa enriquecida em celulose e numa extração semelhante dos açúcares hidrolisados e da lignina, em comparação com as folhas não-infetadas. A análise composicional das polpas confirmou parcialmente os resultados observados. A hidrólise enzimática das polpas sugeriu um aumento na acessibilidade da celulose devido ao pretratamento, sendo que as folhas infetas mostraram uma melhoria mais elevada. Os monómeros das frações de lignina obtidas foram analisados qualitativamente.

No geral, os resultados sugerem que o pretratamento com o OrganoCat é um método de fracionamento eficiente para folhas de milho não-infetadas e infetadas e que pode ser ajustado para uma alta deslignificação, um alto teor de açúcares fermentáveis ou um compromisso entre uma alta extração e uma baixa degradação dos açúcares extraídos.

Palavras-Chave: pretratamento, fracionamento, OrganoCat, folhas de milho, carvão-do-milho, hidrólise enzimática.

Contents

List of Figures

List of Tables

List of Abbreviations

1G	First generation
2-MTHF	2-Methyltetrahydrofuran
2G	Second generation
5-HMF	5-Hydroxymethylfurfural
ABSL	Acetyl bromide soluble lignin
AFEX	Ammonia fiber explosion
Ara	Arabinose
ARP	Ammonia recycle percolation
dAIR	Destarched alcohol-insoluble residue
DMSO-d_6	Hexadeuterodimethyl sulfoxide
FA	Ferulate
FAO	Food and Agriculture Organization of the United Nations
FDCA	Furandicarboxylic acid
G	Guaiacyl
Gal	Galactose
GalA	Galacturonic acid
GC-MS	Gas chromatography combined with mass spectrometry
GHG	Greenhouse gas
Glc	Glucose
GMO	Genetically modified organism
H	*p*-Hydroxyphenyl
HCl	Hydrochloric acid
HK	Hexokinase
HPAEC	High pressure anion-exchange chromatography
HSQC	Heteronuclear single-quantum correlation

ILs	Ionic liquids
LCC	Lignin-carbohydrate complex
LHW	Liquid hot water
NMR	Nuclear magnetic resonance
PAHBAH	*p*-Hydroxybenzoic acid hydrazide
***p*CA**	*p*-Coumarate
S	Syringyl
SE	Steam explosion
USA	United States of America
Xyl	Xylose

List of Nomenclature

Abs	Absorbance
c	Concentration
CF	Correction factor
I	Signal intensity (NMR)
IF	Improvement factor
M	Molar mass
m	Mass
N	Number of nuclei
r	Relative response of monosaccharides to PAHBAH reagent
x	Fraction

1 Introduction

1.1 Context: Biorefineries

Nowadays, the production of energy, fuels and high-value chemicals is still highly dependent on fossil resources [1, 2]. However, fossil resources depletion, its rising cost and environmental concerns, associated with increasing world population, have become major reasons to develop sustainable sources of renewable energy and chemicals [3]. Significant steps are being taken to move from today's fossil-based economy to a more sustainable economy based on biomass [4]. Biomass annual worldwide production is estimated around 150 billion tons and it accounts for approximately 12% of the global energy supply today, although it is mostly used for low-grade heat applications [5, 6].

A key factor in the realization of a successful biobased economy is the development of biorefinery systems which aim for a greater utilization of the biomass feedstock, for an enhanced mitigation of greenhouse gas (GHG) emissions, for producing fewer wastes and residues and for greater energy efficiency and product income [4, 7]. A biorefinery is defined as a facility (or network of facilities) where biomass conversion processes and equipment are integrated to sustainably separate biomass resources into their building blocks which are converted to energy and a spectrum of marketable products, such as high-value chemicals and biofuels [8]. To economically compete with conventional petroleum refineries, the valorization of all the biomass fractions and wastes in a biorefinery is of high importance [4].

The simplest (less complex in terms of design) biorefineries use first generation (1G) feedstocks as raw materials, *i.e.*, sugar-based crops (*e.g.* sugar beet, sugarcane), starch crops (*e.g.* cereals, grains, such as corn, cassava or wheat) or oleaginous crops (*e.g.* rape, soy) [7]. These biorefineries are already maturely established, and the majority of the commercial facilities belonging to this category produce 1G biofuels, *i.e.*, biodiesel or bioethanol [7]. However, 1G biorefinery feedstocks cause several concerns: competition for land and water used for food and fiber production, their potential availability is limited by soil fertility and yields per hectare, the effective savings of CO_2 emissions and fossil energy consumption are limited by the high energy input required for crop cultivation and conversion, and the need for fertilization leading to pollution of water [8].

These limitations are expected to be partially overcome by developing second generation (2G) biorefineries, which use more sustainable nonfood crop feedstocks, in particular lignocellulosic biomass [7, 8].

1.2 Lignocellulosic Biomass

Lignocellulosic biomass includes various species, is widely distributed and is the most abundant organic compound on Earth, representing the major portion of the world's annual production of renewable biomass [4, 9]. Lignocellulosic biomass sources include mainly forestry and agricultural resources [9]. Forestry resources are produced mainly in reforestation, forest protection, forest cutting and other processes (*e.g.* wood chips, sawdust, bark, pulp and paper industrial residue) [4, 9]. Agricultural resources include agricultural crops, agricultural residues and surpluses (*e.g.* corn stover, wheat and rice straw) and so-called energy crops (*e.g.* switchgrass, Miscanthus, willow) [4, 9].

1.2.1 Structure and Composition

The importance of lignocellulosic biomass is related to lignocellulose, which is the primary building block of plant cell walls [9, 10]. Lignocellulose is mainly composed of cellulose, hemicellulose, lignin and smaller amounts of pectin, protein, extractives (*e.g.* soluble nonstructural sugars, chlorophyll and waxes) and ash [4, 10]. The ratios between these constituents can vary from one plant species to another and within a single plant, varying with age, stage of growth and other conditions [10]. Usually the cellulose content may vary between 30% and 50%, hemicellulose from 20% to 40% and lignin from 10% to 30% [11].

Structural organization of these polymers in the plant cell wall consists of a microfibrilar cellulose skeleton surrounded by organized hemicelluloses, with lignin filling the empty spaces in between [12]. Extractives are found in cell lumen, cellular voids or channels [12]. Figure 1.1 shows the structural organization of lignocellulose components in the plant cell wall.

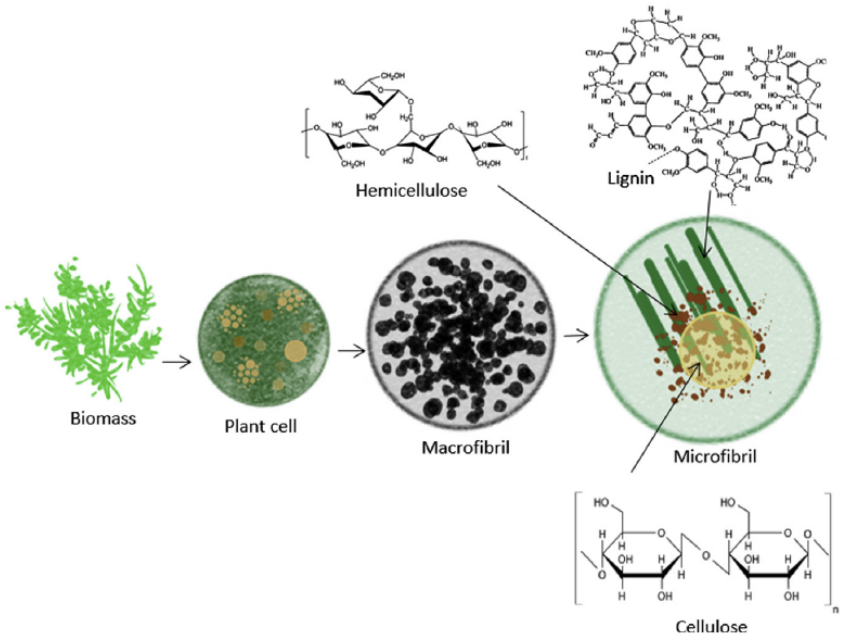

Figure 1.1: Lignocellulose structure and composition. Adapted from [13].

Cellulose

Cellulose is a linear homopolymer composed of D-glucose subunits linked by β-(1,4)-glycosidic bonds, with the general formula $(C_6H_{10}O_5)_n$ where n is the degree of polymerization [14]. Each glucose residue is rotated by 180 degrees relative to its neighbours, this way, the repeating stereochemical unit is the disaccharide, cellobiose [15]. Contrary to starch, which serves as a glucose storage polymer, cellulose plays an exclusively structural role: its high tensile strength allows plant cells to resist osmotic pressure and mechanical stress [15].

The individual cellulose chains are packed together due to intra and intermolecular hydrogen bonds, forming thin, flattened, rod-like structures named microfibrils [15]. Aggregation of microfibrils form fibrils and aggregation of fibrils leads to cellulose fibres [15]. Microfibrils form highly ordered, crystalline domains interspersed by more disordered, amorphous regions [15, 16]. Crystalline cellulose comprises the major proportion of cellulose, whereas only a small percentage of cellulose chains form amorphous regions, which are more easily hydrolyzed [15, 16].

Hemicellulose

Hemicellulose is a complex carbohydrate structure that consists of a homopolymer or heteropolymer backbone with short branches of sugars linked by β-(1,4)-glycosidic bonds and, occasionally, β-(1,3)-glycosidic bonds [10]. The building blocks in hemicellulose are pentoses (xylose and arabinose), hexoses (mannose, glucose and galactose) and uronic acids (glucuronic, methylgalacturonic and galacturonic acids) [4, 17]. Other sugars, such as rhamnose and fucose, may also be present in smaller amounts and the hydroxyl groups of sugars can be partially substituted with acetyl groups [4].

The type and amount of hemicellulose varies widely, depending on plant materials, type of tissues, growth stage, growth conditions, storage and method of extraction [14]. The most relevant hemicelluloses are the xylans and the glucomannans, with xylans being the most abundant [4]. For hardwood (*e.g.* oak and walnut) and agricultural plants, like grasses and straw, xylan is the main component of the hemicellulose (constituting about 20% to 30% of the biomass) while in softwood (*e.g.* pine and spruce) it is the glucomannan [4, 17].

Hemicellulose is usually linked to other cell wall components, such as cellulose, cell wall proteins, lignin, and phenolic compounds, by covalent and hydrogen bonds, and by ionic and hydrophobic interactions [4]. In fact, hemicellulose polysaccharides are interspersed with the microfibrils of cellulose, conferring both rigidity and flexibility to the structure of the cell wall [14, 18]. Compared to cellulose, hemicellulose has a lower molecular weight, its branches with short lateral chains of different sugars are easily hydrolyzed and is a relatively amorphous component, which makes it easier to break down with chemicals and/or heat [17, 19].

Lignin

Lignin is not a constitutionally defined compound, but is accepted to be a physically and chemically heterogeneous material consisting of representative phenylpropane monomeric units which conjugate by different bonds to form a three dimensional polymer without an ordered and regular macromolecular structure [14, 20]. These monomeric units (figure 1.2) are *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) and are derived from the monolignols *p*-coumaryl, coniferyl and sinapyl alcohols, respectively [21]. The major linkages between lignin monomer units are β-O-4, β-β and β-5 while the main types of lignin-carbohydrate complex

(LCC) linkages are believed to be phenyl glycoside bonds, esters and benzyl ethers [21]. Lignin in lignocellulosic biomass is essential for mechanical support, resistance to environmental stress, water transport and plant pathogen defense [4, 21].

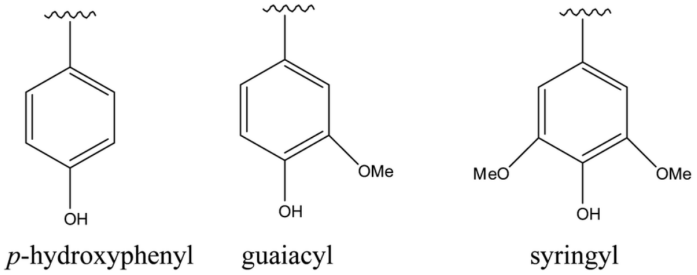

Figure 1.2: Structure of lignin monomer units: *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S). Adapted from [22].

Although the fundamental unit structure of lignin is now well-identified, its polymeric structure has not been fully elucidated yet [20]. The major obstacles are that lignin cannot be isolated in a chemically unaltered form, due to the tight physical binding and chemical linkages between lignin and cell wall polysaccharides, and the lack of ideal techniques that could provide adequate and quantitative information for the entire lignin structure and various LCC linkages [21]. Currently, two-dimensional nuclear magnetic resonance and pyrolysis gas chromatography mass spectrometry are the established analytical techniques for detailed structural lignin analysis [4]. Besides this, lignin composition and structure will be different not only between species, but also between different tissues of an individual plant [4]. In softwood lignin, guaiacyl is the predominant building unit, while in hardwoods the ratio guaiacyl/syringyl shows considerable variation [4].

Lignin is amorphous, not very soluble in acidic or neutral water and optically inactive which makes its degradation very tough [17]. Like hemicellulose, lignin usually starts to dissolve in water around 180 °C under neutral conditions, however its solubility in acid, neutral or alkaline environments depends on lignin main monomer unit [17]. While cellulose and hemicellulose are polysaccharides that can be hydrolyzed to sugars and then fermented to ethanol, for example, lignin is not as easily converted in fermentation processes, but it may be useful for chemical or energy production [19].

The vast majority of current industrial lignin applications have been developed for energy production and for lignosulfonates, which are isolated from acid sulfite pulping and have construction, mining, animal feed and agriculture uses [4]. This limited industrial use of lignin is mainly due to the easy use as energy source, the impurities in technical lignin sources, tendency to form condensed structures, inferior performance compared to synthetic compounds, unique reactivity, lack of high-purity lignins availability and a large variety of different types of lignins [23]. As lignin is the most abundant aromatic renewable resource on earth, it is one of the few resources that could fulfill the amounts needed to replace the main aromatic compounds used in current industry (phenol, terephthalic acid and BTX, *i.e*, a mixture of benzene, toluene and xylenes) [4].

1.2.2 Application in Biorefineries

Lignocellulosic biomass has some characteristics that make it an interesting alternative feedstock for the production of fuels, chemicals and other products, such as being renewable, richness, biodegradable and not directly competing with food resources [9]. Nowadays, there are more than 40 lignocellulosic biorefineries operating across Europe [24].

In general, some of the products that can be produced from lignocellulosic feedstocks include bio-based chemicals (*e.g.* furfural, levulinic acid, xylose, xylitol and oxalic acid), bio-based energy (*e.g.* fuel ethanol, bio-butanol alcohols, biogas, and biodiesel), and bio-based materials (*e.g.* plywood, lignin-modified phenolic resin adhesive, lignin-modified phenolic resin foam insulation board and polyurethane foam) [9]. Lignocellulosic feedstocks have been widely used in the paper industry, the textile industry, and herbal and organic chemical processing, however only single-product production and single-conversion technology are implemented, causing a great amount of resource waste and environmental pollution [9].

Two principal conversion technologies are generally used for valorisation of lignocellulose in the biorefining industry and may be classified as biochemical and thermochemical [24]. Biochemical conversion of lignocellulose involves the hydrolysis of carbohydrates to soluble sugars, followed by microbial fermentation or by direct anaerobic digestion, while the thermochemical route involves direct combustion, pyrolysis, gasification or torrefaction [24]. Due to the recalcitrant structure of lignocellulose, before biomass conversion, it is necessary to add a pretreatment step able to make cellulose, lignin, and hemicellulose more accessible for enzymes or chemicals, facilitating subsequent processing of biomass [13].

1.3 Corn: *Zea mays*

1.3.1 Overview

Corn (*Zea mays*) is a member of the Poaceae or grass family (grain crops) greatly responsible for the beginning of formal agriculture and establishment of primitive societies [25, 26]. It has originated in Mesoamerica and, through extensive selection by prehistoric plant breeders, was converted to its modern form from a low-yielding ancestor species, which is believed to be the teosintes (*Zea mexicana*), a wild grass [25, 27, 28]. Corn was disseminated by indigenous peoples throughout North and South America, where further selection adapted it to a wide range of soil and climate conditions, from high valleys with low temperatures to lowland tropics [27]. After the discovery of America, corn quickly spread around the world, becoming a staple crop in several world regions, mainly in Africa, and a model organism for scientific research [25, 27].

The typical corn plant is a tall (1 to 4 m) annual grass [29] and presents the morphology shown in figure 1.3. The male reproductive organ, named tassel, is found at the top of the stalk and grows pollen-bearing anthers. The female reproductive organ can be found in the middle of the stalk and is constituted by the ear or cob, protected by a number of leaves called husks (not shown in the figure), and the silks at the top of the ear [27, 29]. In nature, reproduction of *Z. mays* occurs by pollination: pollen grains produced by anthers fall from the tassel onto the silks, germinating and fertilising the ovule inside the ear which develops into a corn seed (also named kernel or grain) [27, 29]. After planting, 130 to 150 days are necessary to have corn cobs ready to harvest, which bear between 400 and 600 seeds per cob [27, 29]. Some of the factors that affect corn plant productivity and grain yields are water supply, sunlight availability, extreme temperatures, poor plant nutrition and loss of leaf area to insects, diseases or hail [27].

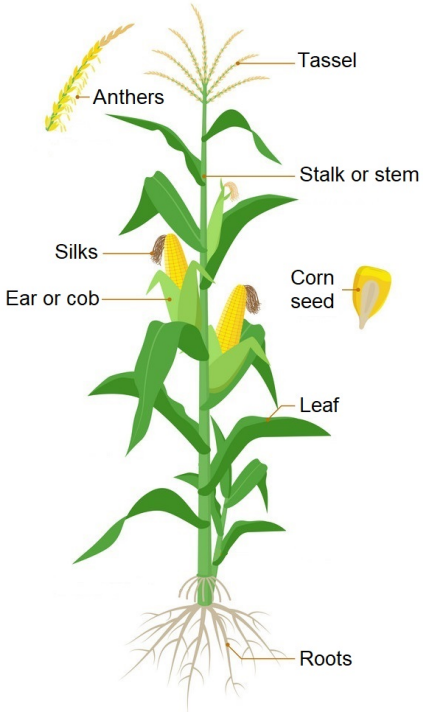

Figure 1.3: Morphology of *Zea mays* (corn) plant. Adapted from [30].

Corn is the most demanded grain in the world and is among the fastest-growing in yearly volume, being grown on every continent except Antarctica [25, 31]. Corn top producers are the United States of America (USA), China and Brazil [26]. Nowadays, the annual corn production and demand is higher than 1 billion tons, surpassing rice and wheat in annual volume by more than 25% [31]. According to Food and Agriculture Organization of the United Nations (FAO) [32], in 2017, 1134.7 million tons of corn were produced worldwide with the following distribution per continent: 51% in America, 32% in Asia, 10% in Europe and 7% in Africa. USA alone was responsible for 33% of the total corn production in 2017.

In terms of nutritive composition, the typical yellow dent corn seed contains, on a dry basis, 72% starch, 9.5% protein, 4.3% oil, 2.6% sugar and 1.4% ash [33]. Most of the corn seed sold for commercial purposes is hybrid seed, which is genetically uniform and very high-yielding [27]. In the mid-1990s, genetically modified (GM) corn hybrids were first produced and, in current commercial production, two types of traits derived from genetic engineering methods are available: insect resistance and herbicide tolerance [26, 27]. In 2012, 35% of the corn hectares grown globally were GM corn, however, legislation regulating such crops varies among countries [26].

Corn grain is used for food, animal feed, bioethanol production and industrial products, including biodegradable foams, plastics, and adhesives [26]. About 17% of the current worldwide corn production is converted into first generation bioethanol, mainly in the USA that uses 40% to 56% of its corn for this purpose [7, 31]. Additionally, the agricultural residues of corn crops are also used in several areas as described in section 1.3.2.

1.3.2 Agricultural Residues: Corn Stover

Corn stover is an agricultural residue categorized as a lignocellulosic biomass source, in contrast to the corn grain that is a starch source [19]. Corn stover is defined as the above-ground non-grain portion of the crop, *i.e.*, it comprises the cob, leaves, husk and stalk of the corn plant [34].

Pordesimo *et al.* [35] determined that the distribution of mass in corn stover is approximately 51% stalk, 21% leaf, 15% cob and 13% husk at the time of grain physiological maturity (118 days after planting). Shortly after maturity being reached, compositional analysis of corn stover fractions showed a rapid drop in soluble solids and an increase in lignin and xylan. Over time, the greatest fluctuation in composition was observed for the leaves, with prominent fluctuations in the analysis for structural glucan, xylan, and lignin. These were justified by the leaf tissue having greater metabolic activity due to photosynthesis. Moreover, the detected changes in the chemical composition over corn plant maturation did not translate into marked differences in energy content among the stover fractions.

In [34], corn stover composition was analysed with near-infrared spectroscopy by studying 508 commercial hybrid corn stover samples collected from 47 sites in USA from three harvest years. Table 1.1 shows the overall composition of the corn stover. In this study, the major components of the corn stover were glucan, xylan, solubles composite and lignin. Regarding the sugars, glucan is derived largely from cellulose, but is also present in starch and hemicellulose, while xylan, arabinan, galactan and mannan (summing a total of 23.5%) are derived largely from hemicellulose. This study also showed that the harvest year had the strongest effect on corn stover compositional variation, followed by location and then variety.

Table 1.1: Corn stover composition (%) presented on a dry weight basis. Data from [34].

Component	Content (% dry weight)
Sugars	
Glucan	31.9 ± 2.0
Xylan	18.9 ± 1.3
Arabinan	2.8 ± 0.3
Galactan	1.5 ± 0.2
Mannan	0.3 ± 0.1
Solubles	17.9 ± 4.1
Lignin	13.3 ± 1.1
Ash	3.9 ± 0.9
Protein	3.7 ± 0.8
Acetyl	2.2 ± 0.3

As previously mentioned, using agricultural residues as raw materials in a biorefinery is a promising alternative to fossil resources for energy and chemicals production, however, the removal of agricultural residues from fields gives rise to concerns in terms of soil quality, decrease in soil organic carbon, soil erosion, crop yields and other environmental implications [19, 36, 37]. This way, for a sustainable collection, a certain amount of residue must be left on the field, depending on tillage practice, topography, soil type, crop rotation

and others [36]. Studies in USA [38] estimate that between 105 to 117 million dry tons of corn stover can be sustainably collected per year.

Corn stover is used for forage, farm animal bedding, bioethanol and chemicals production [25, 26]. Corn cobs, in specific, have already been used to furfural production [39]. Some other possible applications are the production of composite products and use in pulp and paper industry, namely producing a ”pure” cellulose pulp (with low content of hemicellulose and lignin) that can be used to produce high-value cellulose derivatives, such as rayon, cellulose acetates and cellulose nitrates [36]. For use of corn stover in biorefineries, specifically for the production of biofuels, a 2018 study [40] estimated that the feedstock (corn stover) has associated costs of 30 $/ton and associated greenhouse gas emissions of 0.095 kg CO_2-eq/kg.

Bioethanol production is the main corn stover application found in literature, however there is scarce information of industrial practices [41]. Even so, three companies in USA are know to produce bioethanol from corn stover with a production capacity between 20 and 30 million gallons (76 to 114 million liters) per year [42]. A recent study [41] evaluated countless scientific publications on the production of bioethanol from corn stover, concluding that the main differences between the technological configurations are found in the pretreatment phase. The following processes of hydrolysis and fermentation into ethanol were largely identical in the different technological configurations, although a range of operating conditions was reported. Regarding hydrolysis step, enzymatic hydrolysis was found to be the dominating process, though acid and hydrothermal technologies are also available. Recently, several research has been carried out on the pretreatment of corn stover, including physical, chemical and biological processes [43]. The different pretreatment processes are described in detail in section 1.4.

1.3.3 Corn Smut by *Ustilago maydis*

Ustilago maydis, a plant pathogen fungus of the family *Ustilaginaceae*, is the causal agent of the corn disease called smut [44]. *U. maydis* has a very narrow host range, infecting only corn and its ancestor teosinte, and presents a biotrophic growth, *i.e.*, depends on living tissue for proliferation and development [45, 46]. Over time, *U. maydis* has become an eukaryotic model organism for diverse topics, such as DNA recombination, signalling, RNA biology, cell biology and biotrophic plant–pathogen interactions [45].

Figure 1.4 presents a schematic overview of *U. maydis* life cycle, which consists of two phases: an apathogenic and a pathogenic [47]. During apathogenic phase, the fungus displays an unicellular haploid yeast-like morphology (haploid sporida) that obtains nutrients from dead organic matter [44, 48]. This haploid sporida multiples by budding and can be cultured on laboratory media, however is unable to infect the host [48, 49]. To generate the infectious form, mating of two sexually compatible haploid sporidia has to occur, forming a pathogenic dikaryotic filament whose growth and development is dependent upon the plant [44, 48, 49]. This dikaryotic filament infects and colonizes the plant, giving rise to diploid, thick-walled resting spores (teliospores) by karyogamy, *i.e.*, fusion of the two haploid nuclei [48]. Teliospores germinate outside the host by meiosis, originating four haploid sporida that start the life cycle again [44].

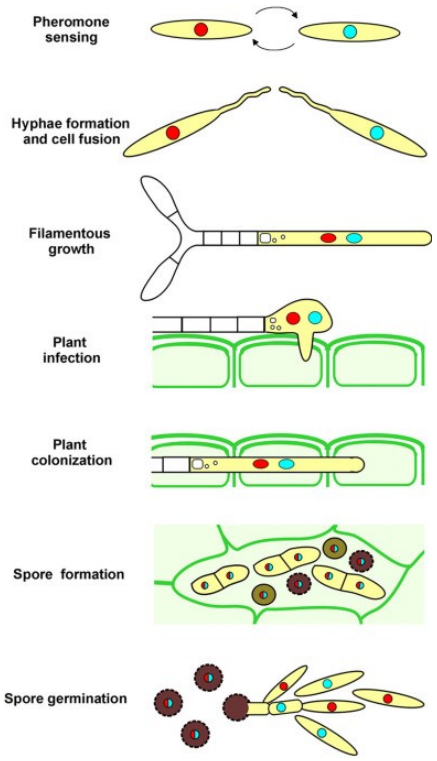

Figure 1.4: Life cycle of *Ustilago maydis*. Adapted from [50].

Haploid cell fusion, pathogenicity and sexual development in *U. maydis* are controlled by two mating factors/loci named a and b [48, 49]. Whereas there are only two a alleles (variant forms of a gene), named a1 and a2, the number of known b alleles is around 30 [44]. The a locus encodes a pheromone and receptor system that allows haploid cells of the opposite mating type to sense each other and to fuse [51, 52]. The fate of the resulting dikaryon depends on the b locus, which encodes a pair of homeodomain proteins, named bW and bE [49, 52]. When derived from different b alleles, bE and bW proteins dimerize and the bE/bW complex form triggers filamentation as well as sexual and pathogenic development [52]. In order to mating occur is necessary that both haploid sporida carry different a and b alleles [49].

The plant infection with *U. maydis* induces the formation of tumours filled with masses of diploid teliospores in the stem, leaves and flowers of the host [49]. One of the first symptoms of disease is a change in leaves colour due to chlorosis, a condition in which leaves produce insufficient chlorophyll, and/or due to the production of anthocyanins pigment (infected tissues appear to be red) [48]. Infection is local and associated with regions of undifferentiated cells (meristematic regions). The fungus can be distributed by elongation of these regions during plant growth [48]. As the disease develops, outgrowths of tissue, named galls or tumours, are formed on aerial plant parts which become filled with sooty black teliospores [48, 49].

In Mexico, corn plantations are wittingly infected with *U. maydis* since the resulting corn smut galls are served as a typical Mexican food, called huitlacoche, that has been consumed by humans for centuries [44, 45]. Huitlacoche contains proteins, carbohydrates, fats, minerals and vitamins that contribute to its nutritional value [44]. In comparison to corn, which has a deficiency in lysine, huitlacoche contains proteins with balanced levels of essential amino acids [44]. The fungus also contains compounds with antioxidant properties which can, therefore, be included in the food delicacy [44]. Nowadays, there is an increasing

industrial market for this delicacy with customers in Latin America and the USA, mainly due to its exclusive flavor different from any other known food [44, 45]. Even though the effects of smut disease in corn grain composition and nutritional value are well documented in literature, there is no information about the impact of smut infection in the composition of corn agricultural residues, neither the fate of such residues.

1.4 Pretreatments for Lignocellulosic Biomass

1.4.1 Importance and Challenges

In the last decade, more than 600 review papers concerning the pretreatment of lignocellulosic biomass have been published, showing that the interest in this field is continuously growing [13]. Pretreatment is crucial for the preparation of lignocellulosic biomass for its subsequent processing into value-added chemicals and biofuels [13]. The pretreatment step should weaken the cellulose recalcitrant structure, making cellulose, lignin, and hemicellulose more accessible for enzymes or chemicals, and facilitate further processing of biomass by efficient removal of lignin, depolymerization of hemicellulose, reduction of cellulose crystallinity and increase of the surface porosity [13].

Pretreatment is considered the most expensive process in biorefineries, but it has great potential for improvements in efficiency and lowering of costs through further research and development [10]. The key factors for biomass pretreatment that should be considered are: (1) preventing the degradation (or loss) of biomass, (2) preventing the generation of inhibiting compounds for subsequent steps, (3) efficient recovery of lignin, (4) possibility of large-scale feedstock processing, (5) being robust by allowing high yields regardless of the type and origin of biomass, (6) reducing the cost of equipment and (7) being sustainable by minimizing heat, power, chemical requirements and waste formation [10, 43, 53, 54].

Due to the recalcitrant nature of lignocellulose, current pretreatment processes are often conducted under harsh conditions (high temperatures and pressures), which induce the formation of degradation products that correspond to a loss of carbon source and may inhibit the enzymes and/or microorganisms required for the subsequent biochemical conversion of sugars [53, 55]. To eliminate inhibitory compounds, it might be necessary to add a detoxification step, such as neutralization, overliming, adsorption, ion exchange and enzymatic detoxification [2].

The degradation compounds formed during pretreatment include furans, phenolics, organic acids, as well as mono- and oligomeric pentoses and hexoses [55]. Thermal degradation of biomass and, in particular, decomposition of carbohydrates, is a complex process that includes several different reactions occurring simultaneously, such as dehydration, depolymerization, fragmentation, rearrangement, re-polymerization, condensation and carbonization [56]. Depending on the reaction conditions, glucose can be converted to 5- hydroxymethylfurfural (5-HMF) and/or levulinic acid, formic acid, and different phenolics at elevated temperatures [55]. Correspondingly, xylose can follow different reaction mechanisms resulting in formation of furan-2-carbaldehyde (furfural) and/or various C-1 and C-4 compounds [55]. At least four routes for the formation of 5-HMF from glucose and three routes for furfural formation from xylose are possible [55]. Moreover, the sugar degradation products can also form humins which are then difficult to valorize [57].

From a holistic perspective for biomass processing, the valorization of all lignocellulosic components would fully exploit the potential of biomass in economical, robust, large-scale and sustainable solutions for biorefinery concepts [53, 58]. This implies the fractionation and separation of hemicellulose, cellulose and lignin

for subsequent transformation in the production of biofuels, commodities and other high-value products [58]. Pretreatment using a fractionative, lignin-extracting method has a number of advantages, including lower enzyme loadings in the saccharification step and higher saccharification yields for both hardwoods and softwoods [6].

Due to the existing drawbacks in each of the pretreatment methods, combining two or three methods could significantly enhance the digestibility of biomass, increasing the yield of desired products [13]. Apart from developing new pretreatment approaches or to improve and combine the existent ones, another option is to genetically design plants with tailored properties for the provision of feedstock [59].

1.4.2 Current Technologies

Pretreatment methods can be divided into four categories (figure 1.5) - physical, chemical, physicochemical and biological - which are further described below.

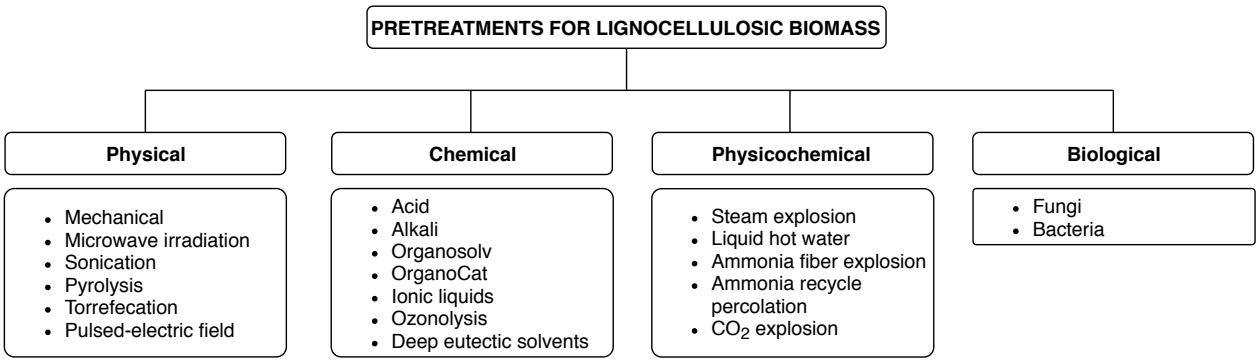

Figure 1.5: Pretreatments for lignocellulosic biomass. Based on [2, 10].

Physical Pretreatments

Physical pretreatment consists of an increase in the temperature or pressure causing changes in the structure of lignocellulosic materials (*e.g.* specific surface area, particle sizes, crystallinity index and polymerization degree) that reduce biomass recalcitrance [13]. It avoids the use of chemicals, thus reducing the generation of waste and inhibitors for subsequent reactions [60]. Mechanical, microwave irradiation and sonication pretreatments are the most common techniques used [13]. Other physical pretreatments include pyrolysis, torrefecation and pulsed-electric field [10, 60].

Mechanical pretreatment includes comminution and extrusion. Comminution is a combination of chipping (reduces the heat and mass transfer limitations), grinding and/or milling techniques (reduce the particle size and cellulose crystallinity due to shear forces) [10]. In extrusion, the biomass is transported along the extruder by a driving screw and it undergoes mixing, heating and shearing upon pressure release, leading to the disruption of the amorphous and crystalline cellulose matrix in the biomass [43, 60]. The main disadvantage of mechanical pretreatments is the high energy consumption, which contributes to high processing costs of lignocellulose materials [13].

Microwave irradiation is an alternative to conventional heating: microwave radiation absorbed by matter has appropriate energy to excite the vibration of molecules, generating thermal energy, but not enough to

break chemical bonds [13]. The main advantages of microwave heating compared to conventional heating are: lower energy consumption, shorter reaction times and avoided contact with the feedstock [61]. However, for prolonged microwave pretreatment the degradation of polysaccharides increases [13]. For a more effective breakdown, addition of mild alkaline reagents is possible [60].

In sonication pretreatment, ultrasound waves produce both physical and chemical effects that alter the lignocellulose morphology: it leads to the formation of small cavitation bubbles which rupture the cellulose and hemicellulose fractions thereby increasing the accessibility to cellulose degrading enzymes for effective breakdown into simpler reducing sugars [60]. The main advantages are shorter processing time, lower operation temperature and a lower amount of chemicals used during further valorization [13].

Chemical Pretreatments

Chemical pretreatment is characterized by the use of organic or inorganic compounds which, through interaction with the intrapolymer or interpolymer bonds of lignin, hemicellulose, and cellulose, lead to a disruption of the structure of lignocellulosic materials [13]. Depending on the chemical substances used during treatment, various mechanisms of biomass decomposition can occur [13]. The most commonly used methods are based on the application of acids, alkalis, ionic liquids (ILs), oxidizing agents and organosolv treatment [13]. However, in a green biorefinery, it is necessary to develop technologies that aim at eliminating the use and/or generation of chemicals - organic solvents, ozone and ILs are considered green solvents since they are non-toxic and do not produce hazardous wastes [43].

Acid pretreatment involves the use of sulfuric, nitric or hydrochloric acids to remove hemicellulose components and expose cellulose for enzymatic digestion [11]. Based on the type of end application, two types of acid pretreatments are developed: high temperature (above 180 °C) for short duration (1–5 min) and low temperature (<120 °C) for long duration (30–90 min) [60]. Acid pretreatment main disadvantages are the corrosive and toxic nature of most acids and the generation of high amount of inhibitory products (*e.g.* furfurals, 5-hydroxymethylfurfural, phenolic acids and aldehydes) [60]. Even though, it is the most widely employed pretreatment method on industrial scale [60].

Alkali pretreatment involves the use of bases (*e.g.* sodium, potassium, calcium and ammonium hydroxide) to remove lignin and various uronic acid substitutions on hemicellulose that lower the accessibility of enzymes to the hemicellulose and cellulose [11, 59]. This pretreatment is most effective with low lignin content biomass, like agricultural residues [62]. Alkali pretreatments are the most widely used in the pulp and paper industry: a relevant industrial example is the Kraft process, in which lignocellulose is treated with sodium hydroxide and sodium sulfide to yield almost pure cellulose fibers [59]. Due to the strongly basic conditions, loss of polysaccharides by peeling and hydrolytic reactions can occur as well as condensation of the lignin, which reduces the carbon efficiency of the pretreatment and can lead to less valuable products [59]. Comparing to acid pretreatment, alkali is less caustic, is carried out under milder conditions, some of them even at ambient temperature, and generates lower amounts of inhibitory products [63].

Organosolv pretreatments use an aqueous-organic mixture at temperatures of 100-250 °C and pressures of 10-45 bar to solubilize hemicellulose and extract lignin [59]. For an enhanced hydrolysis, acids, alkalines, and inorganic salts are often used as catalysts [2]. The process yields two streams: (1) a solid cellulose pulp and (2) a mixture of dissolved sugars and lignin, from which the latter is precipitated by diluting the liquid fraction with water [59]. The advantages of the organosolv pretreatment are high efficiency, mild conditions,

easy solvent recovery and the possibility of its recycling, relatively high purity of biomass fractions and the possibility of their separation. The main disadvantage is the high costs of solvents and their recovery, which is of great importance since solvents may be inhibitory to the growth of organisms, enzymatic hydrolysis and fermentation [13, 64]. A frequently observed challenge is the sugar degradation forming furfural and 5-HMF, as well as subsequent condensation reactions of dissolved lignin [59]. To overcome some of the organosolv drawbacks, OrganoCat pretreatment was designed (section 1.5).

Ionic liquids (ILs) are known to be able to dissolve pure cellulose as well as lignocellulose [59]. Subsequently cellulose is re-precipitated by addition of a cellulose antisolvent, which is miscible with the IL (*e.g.* water), becoming more accessible for further conversion steps [59]. The main disadvantages of ILs are its high costs and the difficulty of recovering and reusing them [59].

Physicochemical Pretreatments

Between physicochemical pretreatments, steam explosion, liquid hot water, ammonia-based and CO_2 explosion are the most effective and environmentally friendly available processes [43]. These were optimized for a wide variety of feedstock and have been tested on pilot scale for industrial applications [43]. The disadvantage of using harsh conditions, typical for these methods, is balanced with the advantage of obtaining high sugar yields, without addition of chemicals or with addition of non-toxic chemicals that can be recycled [43].

Steam explosion (SE) is one of the most commonly used methods for the pretreatment of lignocellulosic biomass [43]. SE pretreatment uses hot saturated steam at high pressure (0.7-4.8 MPa) and temperature (160-240 °C) for several seconds to a few minutes followed by pressure release [62]. Hemicellulose is the predominant fraction of the carbohydrates solubilized in the liquid phase during pretreatment, while the lignin is transformed as result of the high temperature [62]. The hydrolysis of hemicellulose is carried out by the acetic acid produced from the acetyl groups of hemicellulose [60]. The main advantages of SE are limited use of chemicals, low energy requirement, no recycling cost and environment friendly, while its main disadvantages are the possibility of formation of fermentation inhibitors at high temperature, incomplete digestion of lignin-carbohydrate matrix and the need to wash the hydrolysate which decreases the sugar yield [60].

Liquid hot water (LHW) pretreatment is similar to steam explosion, but uses liquid water at 160–240 °C and 3.5–4 bar instead of steam [59]. LHW pretreatment results in hemicellulose hydrolysis and removal of lignin, rendering more accessible cellulose in the biomass while avoiding the formation of fermentation inhibitors that occur at higher temperatures [62]. LHW main advantages are lack of chemicals, low-temperature requirement and minimum formation of inhibitory compounds [60]. However, the amount of solubilized product is high and it requires large amount of energy in downstream processing due to the large amount of water involved [62].

Ammonia fiber explosion (AFEX) and ammonia recycle percolation (ARP) are two ammonia-based pretreatments in which lignocellulosic material is exposed to ammonia at a given temperature and high pressure which causes swelling and phase change in cellulose crystallinity of biomass in addition to the alteration and removal of lignin [62]. In AFEX pretreatment, biomass and ammonia are enclosed in a high pressured reactor and the pressure is released rapidly to create an explosion effect, while in ARP pretreatment, ammonia flows through biomass in the reactor and it is recycled after the pretreatment [2]. Due to the difference in contact

of ammonia and biomass, usually ARP results in lower recovery of hemicellulose and high delignification, while AFEX results in lower lignin removal [2].

CO_2 explosion pretreatment has similar principles to steam explosion [2]. Supercritical CO_2 penetrates the biomass at high pressure and it is believed that once dissolved in water, CO_2 will form carbonic acid, which helps in the hydrolysis of hemicellulose [62]. As a consequence, this method is not effective on biomass with no moisture content [62]. The main advantages are the low cost of CO_2 as a pretreatment solvent, no generation of toxins, the use of low temperatures and high solids capacity, however, the high cost of equipment that can withstand high pressure conditions is a strong limitation to the application of this process on a large scale [62].

Biological Pretreatments

Biological pretreatment employs the use of microorganisms, mainly three groups of fungus (white-, brown- and soft-rot fungi) and four classes of bacteria (actinomycetes, α-proteobacteria, β-proteobacteria and γ-proteobacteria), that synthesize cellulolytic, hemicellulolytic, and ligninolytic systems to degrade lignin, cellulose, and hemicellulose [2, 60]. This pretreatment is cost-effective and easy to operate, requires low energy inputs, no chemical addition and milder conditions, resulting in few inhibitors generation, and it does not cause environmental pollution [43]. However, biological pretreatment is affected by many disadvantages, namely the low hydrolysis rate obtained, the necessity of a large sterile area, the slow growth rate of the fungi that limits scale up applications and the need for monitoring the microorganisms growth [43]. Extensive studies on the use of microorganisms for pretreatment of lignocellulosic material have been conducted by various research groups, but its use is still far from industrial application [2]. Current efforts in biological pretreatments are in combining this technology with other pretreatments and in developing novel microorganisms for rapid hydrolysis [65].

1.5 OrganoCat Pretreatment

1.5.1 Motivation

To circumvent some drawbacks of current chemical pretreatments, namelly organosolv, an integrated process for the selective fractionation and separation of lignocellulosic biomass into its main components was proposed, named OrganoCat. Sections 1.5.1 and 1.5.2 were written based on the scientific publication where OrganoCat concept was initially proposed [58] and on the patent describing this pretreatment and fractionation process (Dominguez de Maria *et al.*, EP 2 489 780 A1) [66].

Organosolv pretreatment is a promising approach, however it has some drawbacks that still need to be addressed. One of the challenges is that strong inorganic acids (*e.g.* sulfuric acid) are frequently required as a catalyst. This leads to corrosion problems and to the necessity of adding a neutralization step before further biomass processing, which, usually, causes a significant formation of wastes and increases pretreatment costs. Dicarboxylic acids have been suggested as a substitute for the use of sulfuric acid in chemical pretreatments [67]. Dicarboxylic acids have attractive chemical and practical features, such as controlled stepwise acidity, biodegradability, convenient handling and storage with limited corrosive behaviour, and many of them can be derived from bio-based resources.

Using dicarboxylic acids, an efficient depolymerization of cellulose is obtained applying high temperatures (above 160 °C), however such conditions lead to degradation problems. On the other hand, at mild temperatures (under 130 °C) selective and efficient hydrolysis of hemicellulose occurs, while crystalline cellulose regions are not accessible. This way, dicarboxylic acids, namely oxalic acid (WO 2002/075043 A1), have been assessed for pulp production in the paper industry via selective hemicellulose hydrolysis. However, as a significant amount of lignin remains in the cellulose pulp, further processes of bleaching and delignification are still needed, leading to waste formation. For an economic and environmental competitive pretreatment the reduction and/or valorization of formed wastes is fundamental. Therefore, an efficient and mild method that allows the lignocellulose fractionation into its three main components without degrading them, allowing their recovery and further conversion, is still needed.

Other challenges of organosolv are related with the organic solvents used. Most of the solvents applied are alcohols, which, at high temperatures and acidic conditions, have been reported to form ethers by dehydration (etherification). This leads to solvent losses and hampered recycling. Furthermore, most of the solvents applied are miscible with water, at least during pretreatment conditions, which creates the necessity of using strong conditions of pressure and temperature to efficiently remove lignin from pulp fibers. This can lead to more degradation problems. An interesting approach to overcome these challenges is the use of inert and bio-based, water-immiscible solvents.

1.5.2 Concept

Taking these challenges into account, OrganoCat pretreatment consists in subjecting lignocellulosic biomass to mild organic acid-catalyzed hydrolysis in a biphasic reaction mixture comprising an aqueous phase and an organic phase, which is immiscible with the aqueous phase, *i.e.*, two distinct liquid phases are visible. The overall process concept is shown schematically in figure 1.6.

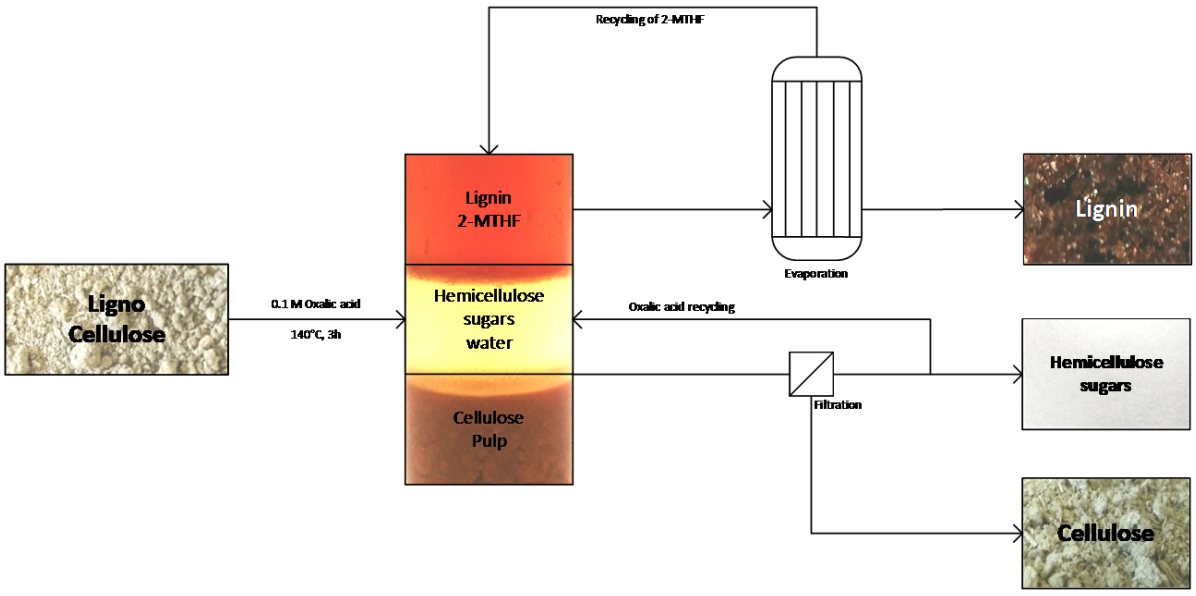

Figure 1.6: General scheme of OrganoCat pretreatment. Provided by Dennis Weidener, M.Sc. (ITMC, RWTH Aachen).

The mild conditions and the use of a dicarboxylic acid as catalyst lead to the selective hydrolysis of the amorphous hemicellulose, yielding soluble sugars in the aqueous stream. As the hemicellulose of the biomass is removed by depolymerization, the remaining biopolymers (cellulose and lignin) separate from each other due to their several differences in structure and solubility properties. The cellulose stays insoluble as a solid pulp, and can be recovered by filtration and directly converted to glucose by enzymatic hydrolysis. Lignin is extracted *in situ* into the organic phase of the biphasic system. All reagents and solvents in the process are bio-based materials and able to be recovered and recycled.

Typically, oxalic acid is used as catalyst in OrganoCat, which can be recovered from aqueous solutions by crystallization, with a concentration in the aqueous phase of 0.1 M. Regarding the organic solvent, cyclic ethers have been reported as efficient lignin solvents [68]. From these, 2-methyltetrahydrofuran (2-MTHF) is the usual solvent used for OrganoCat since it is inert, exhibits a miscibility gap with water, can be derived from biomass and has a boiling point (80 °C) that allows straightforward separation and recycling by distillation [69].

The reaction is typically performed at temperatures between 125-140 °C and with a duration between 2.5-3.5 hours. Bellow this range the reaction might be slower than desired, leading to few extraction of hemicellulose sugars, while, above this range, cellulose depolymerization and sugar degradation might occur. Another important reaction condition is pressure since it affords the biphasic liquid system even if the process temperature is above the boiling point of the constituents of the biphasic system. The typical pressure used is 10 bar.

1.5.3 State of the Art

OrganoCat was first validated by pretreating beech wood at different reaction temperatures (85, 125, 140, 145 and 150 °C), times (3 and 6 h) and pressures (10 and 20 bar) using oxalic acid (0.1 M in the aqueous phase) and 2-MTHF [58]. The set of conditions 140 °C, 3 h and 10 bar defined the optimal compromise for the selective fractionation of beech wood without significant pulp, sugar and lignin degradation [58].

Besides beech wood [58, 70, 71], other plant materials have been successfully pretreated by OrganoCat process: mate tea leaves and reed [70], rice straw [72], the energy plants Sida, Szarvasi, Silphium and Miscanthus [73], corn straw [73] and palm tree empty fruit bunch, EFB [74]. Spruce was also tested, however, it proved less suitable for the OrganoCat process due to its low content in hemicellulose and high lignin content [70].

According to economic assessments [75], the OrganoCat process was found to provide the potential for a competitive approach as compared to other Organosolv-like approaches even under the initial non-optimized conditions. This study showed that OrganoCat may become economically feasible, as long as all fractions are pertinently valorized. Furthermore, the raw material loading and the solvent recycling were identified as important factors affecting capital investment and operating costs.

In that respect, biomass loadings of 100 g/L have been demonstrated to be efficiently fractionated within 3 h whereby the mild conditions assure that no significant amounts of by-products (*e.g.* furans) are formed. Moreover, removing the cellulose pulp by filtration allowed the re-use of the aqueous and organic phase without product separation in repetitive batch mode. In this way, (at least) 400 g/L biomass can be processed in 4 cycles, leading to greatly improved biomass-to-catalyst and biomass-to-solvent ratios. Economic analysis

of the process revealed that the improved biomass loading significantly reduces capital and energy costs in the solvent recycle, indicating the importance of process integration for potential implementation. Finally, OrganoCat concept proved to be scalable since the results from bench-scale screening and optimization could be successfully transferred to a 3 L scale. [70]

One of the (economic) bottlenecks of OrganoCat process is the cost and challenging recovery of oxalic acid [70]. Likewise, the thermal decomposition of oxalic acid at 140 °C may represent another issue for its broader (re)use in biorefineries [71, 76]. In that respect, the use of 2,5-furandicarboxylic acid (FDCA) as a catalyst for OrganoCat process was recently studied [71], with particular focus on the lignin quality, on the enzymatic hydrolysis of the cellulose pulp, and on the noncellulosic sugar extraction. An efficient recovery and recycling of FDCA was found to be possible.

1.6 Aim of Studies

For a sustainable biobased economy, valorization of agricultural residues into different marketable products in the 2G biorefinery concept is a key factor. Corn agricultural residues are widely studied, literature presents its physicochemical characterization, worldwide production, its currents applications and also possible applications in the biorefinery concept. However, agricultural residues of corn crops infected with *Ustilago maydis* (smut disease) are rarely mentioned in literature.

Even though, in a worldwide perspective, amounts of infected corn agricultural residues might not be significant, in Mexico these amounts are certainly higher since corn plantations are wittingly infected with *U. maydis*, as the smut corn galls are considered a delicacy. Nowadays, these infected agricultural residues are likely incinerated, but, in the context of a biobased economy, it would be interesting to study the possibility of converting such residues into marketable products. Finding an appropriate pretreatment method for this feedstock should be one of the first studies since pretreatment step is a key factor in lignocellulosic biomass valorization. As OrganoCat is an innovative pretreatment approach that was already tested for grass plants, including corn straw, it might be an appropriate method to pretreat infected corn agricultural residues.

Furthermore, the infection of corn plants with *U. maydis* likely leads to changes in the chemical composition and structure of lignocellulose. Such changes can affect the pretreatment step, either by facilitating or hindering it. In the case of facilitating pretreatment, *U. maydis* could be an asset for the valorization of conventional corn agricultural residues.

Considering the hypothesis mentioned above, the main goal of this thesis is to evaluate the impact of corn leaves infected with *U. maydis* on OrganoCat pretreatment efficiency, compared to uninfected corn leaves. In that respect, other goals of the thesis include the screening of 6 OrganoCat conditions (temperature and time) and the evaluation of lignocellulose disentanglement and delignification by enzymatic hydrolysis of the cellulose-enriched pulp. Three corn leaves substrates were analyzed for the OrganoCat pretreatment: uninfected corn, corn infected with wild type *U. maydis* (WT infected corn) and corn infected with genetically modified *U. maydis* (GMO infected corn).

The experimental work for the present thesis was carried out under SEED FUND 2.0 project iBiomass of Bioeconomy Science Center (BioSC) with the title ”Improve maize biomass for processing applying OrganoCat

technology" [77]. The iBiomass project was a partnership of three working groups: (1) Göhre *et al.*[1], responsible for smut fungal infection and genetic modification of *U. maydis*, (2) Pauly *et al.*[2], responsible for corn genetics and the chemical analysis of lignocellulosic material, and (3) Leitner *et al.*[3], responsible for optimizing effectiveness and adaptation of OrganoCat to different plants.

[1]Dr. V. Göhre, Institute for Microbiology, HHU Düsseldorf
[2]Dr. M. Pauly and Dr. V. Ramirez, Institute for Plant Cell Biology and Bioechnology, HHU Düsseldorf
[3]Prof. W. Leitner, Institute for Technical and Macromolecular Chemistry, RWTH Aachen University

2 Materials and Methods

2.1 Chemicals

Table 2.1 presents all the necessary chemicals for the experiments performed, which were used without further purification.

Table 2.1: Chemicals used for the experiments performed, indicating their name, supplier, purity and usage.

Chemical	Supplier	Purity	Usage
2-methyltetrahydrofuran	Carl Roth	≥99%	OrganoCat solvent.
Oxalic acid anhydrous	Sigma-Aldrich	≥97.0%	OrganoCat catalyst.
Sodium citrate tribasic dihydrate	Sigma-Aldrich	≥99%	Citrate buffer for enzymatic hydrolysis. PAHBAH reagent for sugar quantification.
Citric acid	Sigma-Aldrich	99%	Citrate buffer for enzymatic hydrolysis.
D-(+)-glucose anhydrous	Carl Roth	≥99%	Calibration curve for glucose assay.
p-hydroxybenzoic acid hydrazide	Sigma-Aldrich	≥97%	PAHBAH reagent for sugar quantification.
Hydrochloric acid	Carl Roth	Technical, ≥30%	PAHBAH reagent for sugar quantification.
Calcium chloride anhydrous	Sigma-Aldrich	≥93.0%	PAHBAH reagent for sugar quantification.
Sodium hydroxide	Carl Roth	≥98%	PAHBAH reagent for sugar quantification.
D-(+)-xylose	SAFC	≥99%	Calibration curve for sugar quantification.
Hexadeuterodimethyl sulfoxide	Eurisotop	99.80%	Solvent for NMR analysis.
Mesitylene	Sigma-Aldrich	98%	Internal standard for NMR analysis.

2.2 Corn Leave Substrates

In this thesis, three types of corn (*Zea mays*) leave substrates were analyzed: corn leaves injected with water (uninfected corn), corn leaves injected with wild type strain FB1xFB2 of *U. maydis* (WT infected corn) and corn leaves injected with the genetically modified strain SG200 of *U. maydis* (GMO infected corn).

All three substrates were grown and supplied by Göhre *et al.* The substrates preparation started with the plantation of corn seeds in a greenhouse for one week, followed by injection of the plants with water, wild type *U. maydis* or GMO *U. maydis* depending on the type of sample being prepared. After one or two weeks, the symptoms of the plant were analyzed, namely the absence or presence of tumours, tumour size, amount

of healthy tissue, changes in leaves colour and absence or presence of anthocyanin pigment as a measure of stress. Then, the plants were left in the absence of light for 24 h in order to reduce starch content and, therefore, its interference with the polysaccharide content in cell wall. Finally, the leaves grown for three weeks were harvested.

To obtain a powdered biomass, two different grinding methods were used: some samples were ground by the thesis author and others by Göhre *et al.* The method used by the author started with cutting the corn leaves into 1 cm pieces and then grinding them with a Retsch MM 400 ball mixer mill for 10 s at a frequency of 30 Hz. During the grinding, both the equipment and the biomass were cooled down with liquid nitrogen to prevent biomass degradation due to the high temperatures caused by collisions of the ball with the leaves. After grinding, the powdered biomass was dried overnight at 60 °C until constant weight. On the other hand, Göhre *et al.* method, consisted on first drying the leaves at 60 °C and then grinding them with a Retsch RM 100 mortar grinder. For each type of substrate, the set of leaves ground with the same method was named batch.

The substrate uninfected corn consisted of a pool of two batches (one ground with the ball mixer mill and another with the mortar grinder) while the substrate WT infected corn consisted on only one batch ground with the mortar grinder. Finally, three types of substrate GMO infected corn were used for the experiments: one batch ground with the ball mixer mill, one batch ground with the mortar grinder and a pool of these two batches.

2.3 OrganoCat: Lignocellulose Fractionation

For the fractionation of the lignocellulosic substrates, the OrganoCat pretreatment was used. This consists of a biphasic system of water and biogenic 2-methyltetrahydrofuran (2-MTHF), using oxalic acid as a catalyst [58]. Applying OrganoCat to lignocellulosic biomass yields three separated product streams: an organic phase, an aqueous phase and a solid cellulose-enriched pulp. 2-MTHF was used without further purification and a solution 0.1 M of oxalic acid was prepared by weighing 9.00 g of oxalic acid in a Sartorius CP324S analytical balance and adding 1 L of MiliQ water.

For the pretreatment, in a glass inlay with a stirring bar, 400 mg of ground substrate were suspended in a biphasic mixture of 4 mL of 0.1 M oxalic acid aqueous solution and 4 mL of 2-MTHF. The glass inlay was inserted in a 20 mL high pressure reactor, which was closed and pressurized with argon (10 bar) to prevent 2-MTHF from evaporating. On a Heidolph MR Hei-Tec heating plate, with stirring of 650 rpm, the reactor was heated to 120, 140 or 160 °C for 1 or 3 h, depending on the reaction conditions being analyzed.

After cooling the reactor to room temperature and depressurizing, it was opened and the suspension in the glass inlay was centrifuged in a Hettich EBA 200 centrifuge for 5 min, since the solid residue was dispersed in the aqueous phase due to being powdered. Both organic phase (top phase) and aqueous phase (bottom phase) were, separately, removed with 5 mL disposable syringes. In order to remove the remaining particles of pulp in the aqueous phase, a disposable syringe filter (PTFE, pore size of 0.45 μm) was used, after which the aqueous phase was stored at 4 °C and later analyzed with PAHBAH colorimetric method. The cellulose-enriched pulp was washed with MiliQ water until neutral pH and dried overnight at room temperature to constant weight. After this, the pulp was weighted and an enzymatic hydrolysis with a commercial enzyme (Accellerase© 1500) was performed as described in section 2.4.

If the intended analysis of the organic phase was to determine the total amount of degradation products (furfural and 5-HMF), the organic phase was stored at 4 °C and later analyzed with Nuclear Magnetic Resonance (NMR) spectroscopy. If the intended analysis was to determine the mass of the organic extractives, the organic solvent (2-MTHF) was evaporated using a Heidolph Hei-VAP Precision rotary evaporator set at 1 mbar with a bath at 40 °C. The last traces of solvent were evaporated at 10^{-3}/10^{-2} mbar using a Schlenk line and a Edwards E2M12 vacuum pump. The dried organic extractives were weighted and analyzed with NMR spectroscopy.

For each set of conditions (temperature + time) at least 4 replicates were performed: 3 to quantify the organic extractives and 1 to quantify furfural and 5-HMF in the organic phase. From now on, these will be named organic extractives replicates and degradation products replicates, respectively.

2.4 Enzymatic Hydrolysis of Cellulose-Enriched Pulp

To test the accessibility of the cellulose in the pulp after delignification, an enzymatic hydrolysis with the commercial cellulase Accellerase© 1500 (Genencor) was performed to the cellulose-enriched pulps obtained from OrganoCat pretreatment. Accellerase© 1500 cellulase is an enzyme complex intended specifically for the lignocellulosic biomass processing industries and that contains multiple enzyme activities, mainly exoglucanase, endoglucanase, hemi-cellulase and β-glucosidase. Its best operational stability occurs at a temperature range of 50-65 °C and a pH range of 4.0-5.0.

A 0.1 M citrate buffer at pH 4.5 was prepared by weighing 13.40 g of sodium citrate and 10.45 g of citric acid and adding 1 L of MiliQ water. To confirm the pH a Hanna Instruments pH 211 pH meter was used. In 1.5 mL Eppendorf vials, 20 mg of cellulose-enriched pulp were suspended in 1 mL citrate buffer and 10 μL of commercial cellulase was added. For each pulp, 3 samples were prepared as described, one (”zero time”) was immediately quenched by heating the reaction mixture to 100 °C for 10 min and the other two were hydrolysed for 1 h (initial reaction rate) and 72 h (final hydrolysis yield). The hydrolysis was carried out in an Eppendorf Thermomixer Comfort at 50 °C and 750 rpm for 1 h or 72 h. The glucose concentration was then determined as described in section 2.5.1.

2.5 Analytical Methods

2.5.1 Glucose (HK) Assay

The glucose formed by hydrolysis of the cellulose-enriched pulp was quantified using a glucose (HK) assay kit obtained from Sigma–Aldrich. This is an enzymatic method based on glucose phosphorylation catalyzed by hexokinase (HK), followed by glucose-6-phosphate oxidation with NAD^+ as co-factor. The amount of NADH formed during the oxidation is directly proportional to glucose concentration and its absorbance can be measured at 340 nm.

To measure within the reliable range of absorbances (0.03 to 1.6), the resulting solutions from the enzymatic hydrolysis were diluted either by 20 or 50 fold with MiliQ water in 1.5 mL Eppendorf vials and mixed in a VWR VV3 vortex mixer for 15 min. In a microplate, 50 μL of diluted sample and 200 μL of glucose assay reagent were added and the absorbance was measured at 340 nm with a BioTek Power Wave HT UV/Vis spectrometer. The absorbance was measured every 5 min over a period of 1 h, in order to obtain a curve

of absorbance *vs* time. To determine the amount of glucose in the sample, the average of the values of absorbance of the constant part of the curve mentioned was converted into concentration of glucose with a calibration curve (appendix A.1) built with commercial D-(+)-glucose for the range of concentrations 0.0 to 0.5 g/L.

As mentioned in section 2.4, for each cellulose-enriched pulp, 3 hydrolysis were performed (0 h, 1 h and 72 h). The ”zero point” was measured to determine the concentration of glucose present in the reaction mixture before hydrolysis. To obtain the actual concentration of glucose hydrolysed from the pulp (c_{Gluc}) in the samples of 1 h and 72 h, the concentration of glucose present in the ”zero point” sample was subtracted from the concentrations at the measuring points 1 h and 72 h (equation 2.1).

$$c_{Glc} = c_{Glc}(\text{after 1/72 h of hydrolysis}) - c_{Glc}(\text{before hydrolysis, 0 h}) \tag{2.1}$$

The determined glucose concentrations due to hydrolysis were then converted to an improvement factor (IF) relative to the hydrolysis of the raw biomass, *i.e.*, glucose concentrations due to hydrolysis of the pretreated biomass were divided by the glucose concentrations due to hydrolysis of the raw biomass (equation 2.2).

$$IF = \frac{c_{Glc}(\text{pretreated biomass, 1/72h})}{c_{Glc}(\text{raw biomass, 1/72h})} \tag{2.2}$$

2.5.2 PAHBAH Colorimetric Method

The total amount of reducing-end sugars in the aqueous phase was determined with PAHBAH colorimetric method, which is based on the instability of reducing-end sugars in hot alkaline solution, producing yellow anionic species in the presence of *p*-hydroxybenzoic acid hydrazide (PAHBAH) [78].

To prepare PAHBAH reagent, two reagents, A and B, were mixed in a ratio 1:10. Reagent A was prepared by dissolving 1.00 g of PAHBAH in 6 mL of MiliQ water, after which 1 mL of concentrated hydrochloric acid (HCl) was added and the volume was made up to 20 mL with MiliQ water. Reagent B was prepared by dissolving 2.50 g of trisodium citrate, 0.22 g of calcium chloride and 4.00 g of sodium hydroxide in 200 mL of MiliQ water. Reagent A and B were stored at room temperature.

Since PAHBAH reagent is unstable at room temperature, it was always freshly prepared (by mixing reagent A with B) and stored in ice during the necessary timeframe. For the measurement, a sample of the aqueous phase was diluted 20 fold with MiliQ water in 1.5 mL Eppendorf vials and mixed in a vortex mixer for 15 min. In an Eppendorf vial, 50 μL of diluted sample were mixed with 100 μL of PAHBAH reagent and heated in an Eppendorf Thermomixer Comfort at 100 °C and 750 rpm for 10 min. In a microplate, 10 μL of this mixture and 250 μL of MiliQ water were added and the absorbance was measured at 410 nm with a BioTek Power Wave HT UV/Vis spectrometer.

To determine the amount of sugars in the sample, the values of absorbance were converted into concentration with a calibration curve (appendix A.1) built with commercial D-(+)-xylose for the range of concentrations 0 to 3 g/L. Since PAHBAH reagent was freshly prepared whenever needed, a control solution of 1 g/L of xylose was measured with every set of samples in order to adjust the measured sample absorbance to the calibration curve by calculating the difference between the absorbance of a 1 g/L xylose solution determined with the calibration curve (Abs=0.411) and the measured absorbance of the control solution.

Since PAHBAH method was calibrated for xylose the determined concentration of sugars was overestimated, mainly because glucose has a higher response to the method [78] and it is present in the aqueous phase in a significant amount. Therefore, a correction factor (CF) was determined to obtain a more accurate concentration of sugars in the aqueous phase (equation 2.3). This takes into account the relative response of the monosaccharides to PAHBAH reagent (r), presented in annex B.1, and their mass fractions (x) in the aqueous phase, determined with HPAEC results (section 2.5.4). Only the monosaccharides identified and quantified with HPAEC were considered in equation 2.3: xylose (Xyl), glucose (Glc), arabinose (Ara), galactose (Gal) and galacturonic acid (GalA).

$$CF = \frac{x_{Xyl}}{r_{Xyl}} + \frac{x_{Glc}}{r_{Glc}} + \frac{x_{Ara}}{r_{Ara}} + \frac{x_{Gal}}{r_{Gal}} + \frac{x_{GalA}}{r_{GalA}} \tag{2.3}$$

To obtain the total mass of sugars in the aqueous phase it was necessary to consider the dilution factor used for PAHBAH and the total volume of the phase, which was assumed to remain constant during the reaction (4 mL).

2.5.3 NMR: ^{1}H and ^{1}H-^{13}C-HSQC

Nuclear Magnetic Resonance (NMR) measurements were conducted on a Bruker AS400 (400 MHz) spectrometer and allowed for the analysis of the organic phase samples: for both organic extractives replicates (solvent was evaporated) and degradation products replicates (solvent was not evaporated). Hexadeuterodimethyl sulfoxide (DMSO-d_6) was used as solvent [79, 80] and mesitylene as the internal standard.

Analysis of Organic Extractives Replicates

A defined amount of dried organic extractives was dissolved in 450 μL of DMSO-d_6 and 10 μL of mesitylene was added. These samples were analyzed with ^{1}H NMR for the direct quantification of the remaining furfural and 5-HMF present in the dried organic extractives (some furfural and 5-HMF are evaporated together with the solvent). Defining lignin as the organic extractives except furfural and 5-HMF, the mass of lignin was determined by subtracting the mass of furfural and 5-HMF to the mass of dried organic extractives.

Moreover, for uninfected corn and GMO infected corn, qualitative ^{1}H-^{13}C-HSQC (heteronuclear single-quantum correlation) allowed for the evaluation of lignin composition by identifying its three monomers units: syringyl, guaiacyl and *p*-hydroxyphenyl. Only one technical replicate of the OrganoCat reaction conditions 140 °C + 1 h, 140 °C + 3 h and 160 °C + 3 h was analyzed with this 2D NMR method.

Analysis of Degradation Products Replicates

The samples for measurement were prepared by adding 10 μL of mesitylene to the organic phase of the replicates. From this mixture, 50 μL was added to 400 μL of DMSO-d_6. These samples were analyzed with ^{1}H NMR for the direct quantification of the total furfural and 5-HMF that were formed during the OrganoCat reaction and extracted to the organic phase.

Compounds Identification and Quantification

The compounds of interest were identified in NMR spectra by knowing the typical chemical shifts of their nuclei. For ^{1}H NMR, the chemical shifts corresponding to the protons of mesitylene, furfural and 5-HMF were obtained from the Spectral Database for Organic Compounds SDBS [81]. Appendix A.2 presents

detailed information about each signal analyzed: structure of the compound, proton(s) responsible for the signal and typical chemical shift. Only one signal per compound was analyzed. For HSQC NMR, the typical regions in the spectra (chemical shift in ^{1}H and ^{13}C NMR) for each lignin monomer unit were obtained from [79]. Appendix A.3 presents the aromatic region of the HSQC spectra obtained, with the identification of the molecules and respective carbon(s) responsible for the signals.

The principle of quantitative NMR is that a signal intensity (I) in the NMR spectrum is directly proportional to the number of nuclei (N) responsible for that particular resonance [82]. This way, the signal corresponding to the internal standard (mesitylene) was integrated and normalised according to the number of protons giving rise to the signal (3 in case of mesitylene), the integrals of the compounds of interest (furfural and 5-HMF) were then compared with those of the mesitylene and their mass (m) was determined using the equation 2.4, where M represents the molar mass.

$$m_{compound} = \frac{I_{compound}}{I_{mesitylene}} \times \frac{N_{mesitylene}}{N_{compound}} \times \frac{M_{compound}}{M_{mesitylene}} \times m_{mesitylene} \tag{2.4}$$

As the HSQC NMR method used was qualitative, it was not possible to obtain an absolute mass of syringyl, guaiacyl and *p*-hydroxyphenyl present in the organic extractives. This way, each monomer signal intensity was converted to moles (equation 2.4) and presented in normed ratios relative to the total moles of lignin monomers.

2.5.4 Compositional Analysis

The raw substrates and two of the OrganoCat product fractions (cellulose-enriched pulp and sugar hydrolysate) were further analyzed by Pauly *et al.* Cellulose, hemicellulose monosaccharides and lignin were quantified in the raw substrates and in the cellulose-enriched pulp fraction. For the sugars hydrolysate fraction, hemicellulose monosaccharides were quantified. In this section there is only a brief explanation of the methods used by Pauly *et al.*, further details can be consulted in [83].

For raw substrates, destarched alcohol-insoluble residue (dAIR) was prepared by removing starch and cytosolic components with several organic solvent washes. For cellulose-enriched pulps, this method was not applied since the cytosolic components have been removed with OrganoCat pretreatment.

Cellulose content, in dAIR from raw substrates and in cellulose-enriched pulp fraction, was determined via concentrated sulfuric acid hydrolysis, followed by anthrone colorimetric assay to quantify glucose formed during hydrolysis. Since one cellobiose molecule is converted to two glucose molecules, glucose mass (m_{Glc}) was converted to cellulose mass (m_{Cel}) by the equation 2.5, where M represents molar mass.

$$m_{Cel} = m_{Glc} \times \frac{M_{Cel}}{2 \times M_{Glc}} \tag{2.5}$$

Hemicellulose monosaccharides content was determined by high pressure anion-exchange chromatography (HPAEC). Before HPAEC, dAIR from raw substrates and cellulose-enriched pulps had to be hydrolyzed with trifluoroacetic acid. Lignin content was determined by solubilizing lignin with acetyl bromide and measuring the sample absorbance at 280 nm. With this method, only the acetyl bromide soluble lignin (ABSL) is quantified.

To convert cellulose, hemicellulose and lignin content in dAIR to content in the whole raw substrate, Pauly *et al.* determined the yield of substrate mass remaining after dAIR preparation for each substrate (dAIR/substrate): 48% for uninfected corn, 47% for WT infected corn and 46% for GMO infected corn. Each component content in dAIR was then multiplied by this mass fraction (dAIR/substrate).

2.6 Workflow Scheme

Figure 2.1 outlines the workflow followed for every experiment performed, consisting of OrganoCat pretreatment (reaction, decantation, evaporation and filtration), enzymatic hydrolysis of the cellulose-enriched pulp and analytical methods (NMR, PAHBAH colorimetric method, glucose assay and quantification of lignocellulose components).

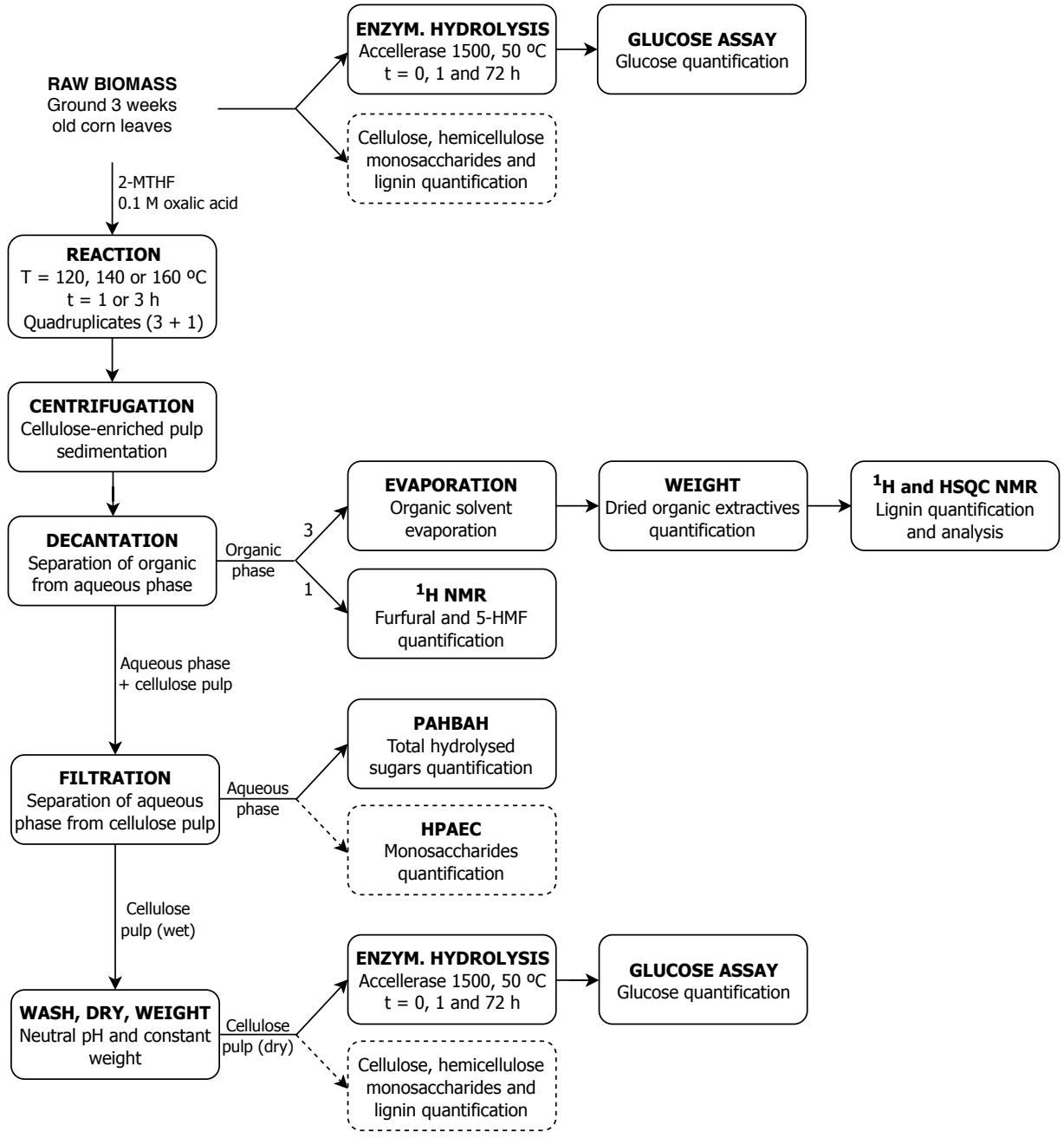

Figure 2.1: Workflow for every OrganoCat experiment performed. The number 3 represents the three technical replicates performed for dried organic extractives quantification, while the number 1 represents the replicate made for degradation products (furfural and 5-HMF) quantification. The dashed boxes present the analysis performed by Pauly *et al.*

3 Results and Discussion

3.1 General Considerations

In order to study and optimize OrganoCat pretreatment for the corn substrates, 6 reaction conditions, combining different reaction temperatures and times, were analyzed. Since 140 °C + 3 h is the optimal condition for beech wood, a prototypical lignocellulosic biomass [58], this condition and a set of milder and harsher conditions were studied for corn: the reaction temperatures analyzed were 120, 140 and 160 °C while the reaction times analyzed were 1 and 3 h. The remaining OrganoCat parameters were chosen according to the literature (mentioned in section 1.5), namely biomass loading (100 g/L in the aqueous phase), organic solvent (2-MTHF), catalyst (0.1 M oxalic acid) and pressure (10 bar).

After performing OrganoCat pretreatment it was possible to observe the expected three phases (figure 3.1): (A) an organic phase consisting of lignin and degradation products (furfural and 5-HMF) dissolved in 2-MTHF, (B) an aqueous phase consisting of hydrolysed hemicellulose sugars and oxalic acid dissolved in water and (C) a solid residue consisting mainly of cellulose.

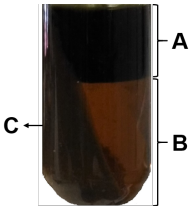

Figure 3.1: Photography of the resulting three phases from OrganoCat process with corn substrate. A - organic phase; B - aqueous phase; C - cellulose-enriched pulp.

To have representative results, at least four OrganoCat experiments for each condition were performed: three technical replicates in order to obtain the amount of the organic extractives after evaporating the organic solvent and one more experiment, without solvent evaporation, to obtain the amount of degradation products (furfural and 5-HMF) formed during the reaction.

To evaluate OrganoCat, five product fractions were quantified and converted to weight% (wt%) yields relative to the initial loading of biomass (400 mg of raw biomass, 100 g/L in aqueous phase): cellulose-enriched pulp, hemicellulose sugar hydrolysates, lignin, furfural and 5-HMF. For every set of substrate-condition, each product fraction yield presented in this thesis is an average of the product fraction yields determined for all the replicates. Since at least four replicates were performed for each substrate-condition set, the standard number of replicates used to obtain each average product fraction yield were: 4 replicates for cellulose-enriched

pulp and hydrolysed sugars, 3 replicates for lignin and 1 replicate for furfural and 5-HMF. However, for some sets of substrate-condition were performed more than 4 replicates and, therefore, the number of replicates used to determine the product fraction yields was higher. This way, appendix A.4 shows the number of replicates used to determine each product fraction yield for each set of substrate-condition. For every average yield determined with more than two replicates, the standard deviation was also calculated and presented as an error margin.

As previously mentioned, three corn substrates were studied: uninfected corn, WT infected corn and GMO infected corn. In section 3.2, the two infected corn substrates are briefly studied and compared in terms of raw biomass composition, OrganoCat product fraction yields obtained for the reaction condition 140 °C + 1 h and cellulose-enriched pulp composition. GMO infected corn was used to evaluate in detail the impact of corn smut infection in OrganoCat pretreatment. This evaluation is shown in section 3.3, where uninfected corn and GMO infected corn are studied in detail and compared in terms of raw biomass composition, OrganoCat product fraction yields obtained for 6 reaction condition sets, cellulose-enriched pulp composition and lignin composition.

3.2 Infected Corn: Wild Type *vs* GMO

Two different lines of corn infected with *Ustilago maydis* were supplied by Göhre *et al.* for the analysis with OrganoCat: corn infected with wild type strain FB1xFB2 (WT infected corn) and corn infected with the genetically modified strain SG200 (GMO infected corn).

As explained in section 1.3.3, *U. maydis* is only pathogenic if two haploid sporida with different a and b alleles mate to form a dikaryotic filament. Wild type strain FB1xFB2 is the pathogenic strain of the fungus obtained by mating haploid sporida FB1 (alleles a1 and b1) with haploid sporida FB2 (alleles a2 and b2) [49]. SG200 is a genetically modified strain of *U. maydis* that was designed for studies of dimorphism and mating genetic regulation [49]. SG200 was design to express the proteins bW2 and bE1 and a specific pheromone (mfa2) was added to the a1 locus. This way, SG200 is a solopathogenic haploid strain, *i.e.*, it stimulates itself to grow filamentously and infect the host, therefore no needing for a mating partner [49, 52].

As FB1xFB2 and SG200 only differ on mating, no difference in pathogenicity between both strains and, therefore, no difference on the results obtained by applying OrganoCat to WT infected corn and GMO infected corn, were expected. To confirm this hypothesis both substrates were briefly studied and compared in terms of raw biomass and obtained OrganoCat product fractions for one reaction condition.

3.2.1 Raw Biomass

Cellulose, lignin and hemicellulose monosaccharides content in each raw substrate (before pretreatment) were determined by Pauly *et al.* as described in section 2.5.4: the content of each component was determined for the destarched alcohol-insoluble residue (dAIR) of the raw substrate, which was then converted to the content in the entire raw substrate by knowing the yield of substrate mass remaining after dAIR preparation (47% for WT infected corn and 46% for GMO infected corn). The results obtained are presented in figures 3.2a and 3.2b. The total hemicellulose content presented in figure 3.2a was determined by summing each monosaccharide content.

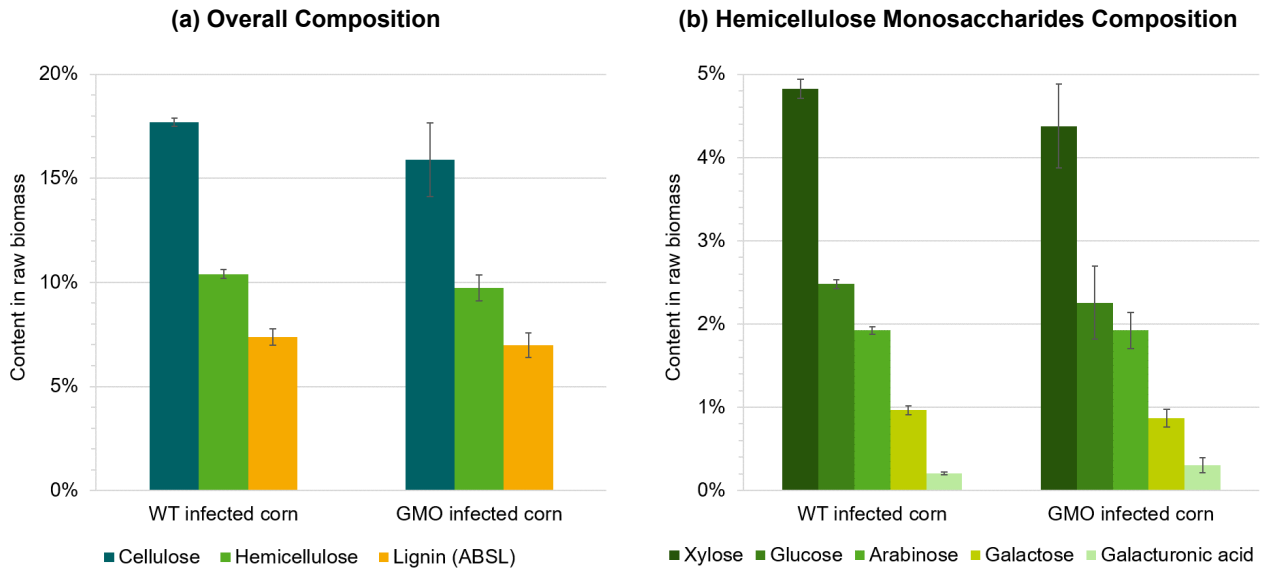

Figure 3.2: Composition of raw substrates (3 week old corn leaves): WT infected corn (4 replicates) and GMO infected corn (9 replicates). (a) Cellulose, hemicellulose and lignin (ABSL) content (wt%) in raw substrate. (b) Hemicellulose monosaccharides composition: xylose, glucose, arabinose, galactose and galacturonic acid content (wt%) in raw substrate.

Observing figure 3.2a, the main mass component in both substrates is cellulose, followed by hemicellulose and lignin, which is typical for lignocellulosic biomass [10]. GMO infected corn presents lower cellulose content (15.9% $\pm$ 1.8%) than WT infected corn (17.7% $\pm$ 0.2%), however, since the error margin associated to GMO infected corn is high, this difference was not considered significant. Both substrates present similar hemicellulose and lignin contents within the error margin, which are approximately 10% and 7%, respectively. Observing figure 3.2b, the main monosaccharide present in hemicellulose is xylose for both substrates. Glucose content, the second main component, is 1.9x lower than xylose content. GMO infected corn presents lower content in xylose, glucose and galactose, similar arabinose content and higher galacturonic acid content compared to WT infected corn. In terms of composition, both raw substrates were considered equal since the differences observed were not significant, especially within the error margin.

Generally, GMO infected corn presents higher error margins, even though a higher number of replicates to determine the average contents presented in the graphs was analysed for this substrate (9 to GMO infected corn and 4 to WT infected corn). This indicates that GMO infected corn is more heterogeneous.

Moreover, an enzymatic hydrolysis (Accellerase© 1500, 50 °C, 20 g/L), of 1 h and 72 h, was performed to the raw substrates and the glucose formed was quantified (table 3.1). After 1 h of hydrolysis, the glucose concentration is similar for both substrates, however, after 72 h, glucose concentration is 1.3x higher for WT infected corn. This is in accordance with the cellulose content in WT infected corn being higher comparing to GMO infected corn.

Table 3.1: Glucose concentration (g/L) obtained after 1 h and 72 h of enzymatic hydrolysis (Accellerase© 1500, 50 °C, 20 g/L) of the raw substrates WT infected corn and GMO infected corn.

Duration of	Glucose concentration (g/L)	
hydrolysis	WT infected corn	GMO infected corn
1 h	1.8 ± 0.0	1.6 ± 0.1
72 h	4.3 ± 0.2	3.4 ± 0.2

3.2.2 OrganoCat Product Fraction Yields: 140 °C, 1 h

Since both raw substrates were considered equal in terms of composition, not many differences were expected by applying OrganoCat pretreatment, therefore, only one OrganoCat reaction condition was analyzed (140 °C + 1 h) for both substrates. The OrganoCat product fraction yields obtained are presented in figure 3.3. Furfural and 5-HMF yields were 0.0% for both substrates, therefore, they are not shown in the graph.

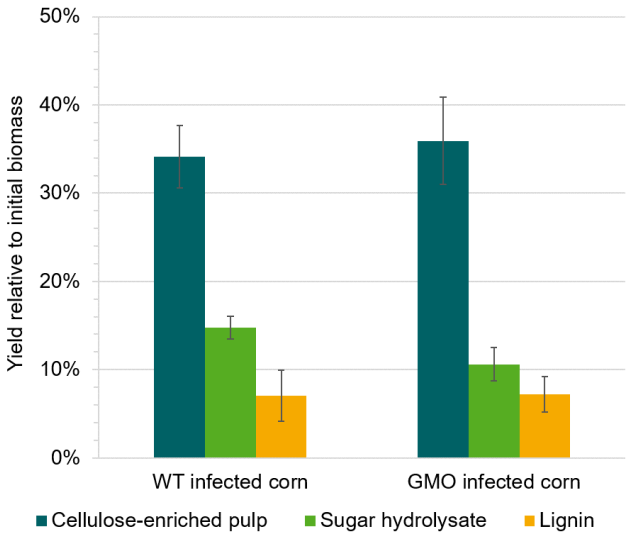

Figure 3.3: Yields (wt%), relative to the initial loading of biomass (100 g/L in the aqueous phase), of each product fraction (cellulose enriched-pulp, hemicellulose sugar hydrolysate and lignin) obtained applying OrganoCat to WT infected corn and GMO infected corn with the reaction condition 140 °C + 1 h.

Observing figure 3.3, GMO infected corn presents higher (1.1x) cellulose-enriched pulp yield than WT infected corn, however this difference is not significant considering the error margins. This is not consistent to the raw WT infected corn presenting higher cellulose content, which can indicate that during OrganoCat pretreatment of this substrate some of the cellulose was hydrolysed to glucose and extracted to the aqueous phase. This seems to be confirmed by the sugar hydrolysate yields: even though both raw substrates have similar hemicellulose content (figure 3.2a), approximately 10%, sugar hydrolysate yield is 1.4x higher and above 10% in WT infected corn. This can indicate that cellulose in WT infected corn is more amorphous, which is more easily hydrolysed [16].

Lignin yield is similar for both substrates (approximately 7%) as it would be expected, since lignin content in raw material is similar for both substrates (figure 3.2a). The lignin obtained via OrganoCat is the same

amount as was determined in the compositional analysis, which seems to indicate that OrganoCat pretreatment allowed for an almost complete delignification of the raw biomass. However, the lignin determined in the compositional analysis and the lignin extracted with OrganoCat can not be directly compared, as different extraction methods were used. It is likely that lignin yields from OrganoCat are higher than from the compositional analysis since, for this calculation, lignin was considered to be the organic extractives except furfural and 5-HMF.

3.2.3 Cellulose-Enriched Pulp Analysis

The cellulose-enriched pulps obtained from OrganoCat pretreatment were further analyzed: cellulose, hemicellulose and lignin content (figure 3.4a) were determined by Pauly *et al.*, as explained in section 2.5.4, and an enzymatic hydrolysis was performed to verify cellulose accessibility to enzymes by determining the glucose concentration after 1 h and 72 h of hydrolysis, as described in section 2.5.1. Figure 3.4b presents the improvement factor (IF) of glucose concentration comparing hydrolysis of the pulp to hydrolysis of the raw substrate (equation 2.2 in section 2.5.1).

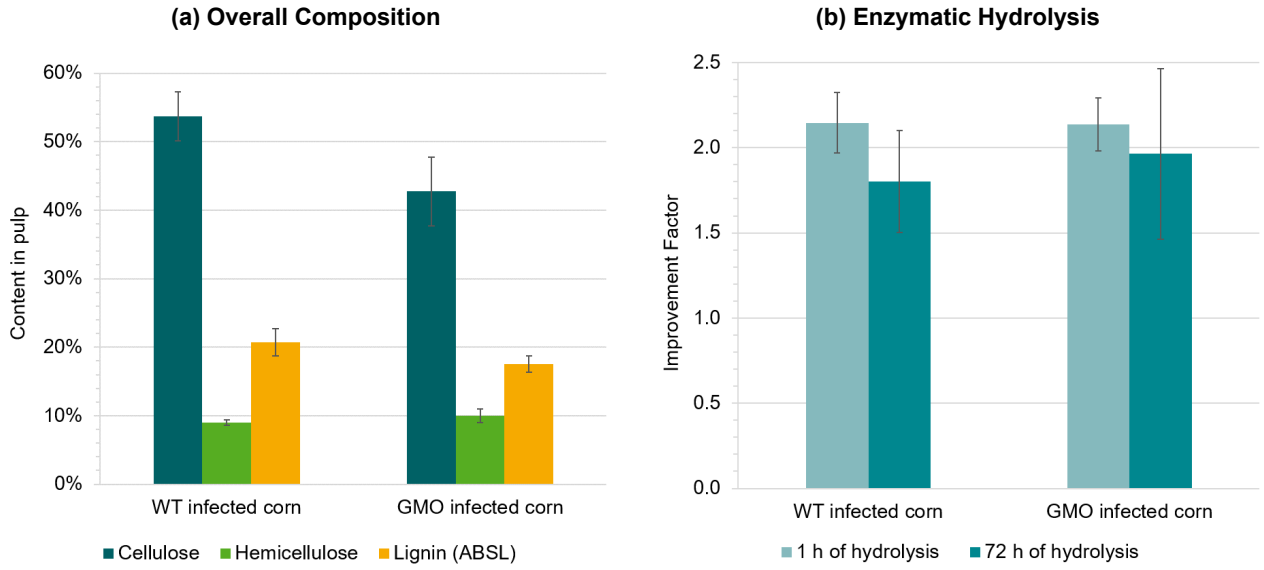

Figure 3.4: Analysis of cellulose-enriched pulps obtained applying OrganoCat pretreatment to WT infected corn and GMO infected corn with the reaction condition 140 °C + 1 h. (a) Cellulose, hemicellulose and lignin (ABSL) content (wt%) in cellulose-enriched pulp. (b) Improvement factor (comparing to raw biomass hydrolysis) of glucose concentration obtained after 1 h and 72 h of enzymatic hydrolysis (Accellerase© 1500, 50 °C) of the cellulose-enriched pulp.

Cellulose is observed to be the main mass component in the OrganoCat pulp of both substrates (figure 3.4a). Compared to the raw substrate (figure 3.2a), cellulose content is 3.0x and 2.7x higher in the pulp of WT infected corn and GMO infected corn, respectively. This indicates that the decrease of the other components content due to their extraction to the liquid phases lead to an increased cellulose content in pulp. In contrary to the raw substrate, lignin content is higher than hemicellulose content in the pulp, indicating that lignin extraction to the organic phase occurs after hemicellulose hydrolysis and monosaccharides extraction to the aqueous phase. This observation is also in line with OrganoCat product fraction yields (figure 3.3), since hemicellulose hydrolysed sugars present higher yields than lignin. Compared to GMO infected corn, WT

infected corn presents higher cellulose content (1.3x), lower hemicellulose content (0.9x) and higher lignin content (1.2x).

Observing figure 3.4b, the IF is always higher than 1.0, which means that the glucose concentration obtained by hydrolysis of the pulp is higher than the one obtained by hydrolysis of the raw substrate. This can be explained by the cellulose content in pulp being higher than in raw substrate, by improved accessibility of cellulose to enzymes in the pulp due to OrganoCat pretreatment and by the presence of less lignin in pulp since it can inhibit the enzymes. Moreover, in both substrates, IF is higher after 1 h of hydrolysis than after 72 h, which indicates that, the influence of pretreatment on the enzymatic hydrolysis of the cellulose is more notorious in the beginning of the hydrolysis. Comparing both substrates, no significant differences are observed due to high error margins.

Even though no significant differences between the raw substrates were observed, different results were verified after applying OrganoCat to WT infected corn and GMO infected corn, both in product fraction yields and in cellulose-enriched pulps. However, as the main goal of the thesis was to study the impact of corn smut infection in OrganoCat pretreatment and not to compare different infections, only GMO infected corn was analyzed in detailed and compared to uninfected corn since this substrate was available earlier in the laboratory. From now on, GMO infected corn will be named infected corn.

3.3 Uninfected Corn *vs* Infected Corn

In this section, the impact of corn smut infection in OrganoCat pretreatment is evaluated by comparing uninfected corn with infected corn in terms of raw biomass composition and obtained OrganoCat product fractions.

3.3.1 Raw Biomass

Cellulose, lignin and hemicellulose monosaccharides content in each raw substrate (before pretreatment) were determined by Pauly *et al.* as described in section 2.5.4: the content of each component was determined for the dAIR of the raw substrate, which was then converted to the content in the entire raw substrate by knowing the yield of substrate mass remaining after dAIR preparation (48% for uninfected corn and 46% for infected corn). The results obtained are presented in the figures 3.5a and 3.5b.

In figure 3.5a, it is observed that the main component in both substrates is cellulose, followed by hemicellulose and lignin, which is typical for lignocellulosic biomass [10]. Raw uninfected corn is composed of 20.0% $\pm$ 1.5% cellulose, 7.9% $\pm$ 0.6% hemicellulose and 5.9% $\pm$ 0.7% while raw infected corn is composed of 15.9% $\pm$ 1.8% cellulose, 9.7% $\pm$ 0.6% hemicellulose and 7.0% $\pm$ 0.6% lignin. Raw infected corn presents lower cellulose content (0.8x) and higher hemicellulose (1.3x) and lignin (1.2x) content compared to raw uninfected corn.

Observing figure 3.5b, in both substrates, the main monosaccharide present in hemicellulose is xylose, which is followed by glucose with a content of half of the xylose content. Raw infected corn presents higher content in all monosaccharides compared to raw uninfected corn: 1.1x more xylose, 1.2x more glucose, 1.9x more arabinose, 2.7x more galactose and 1.3x more galacturonic acid.

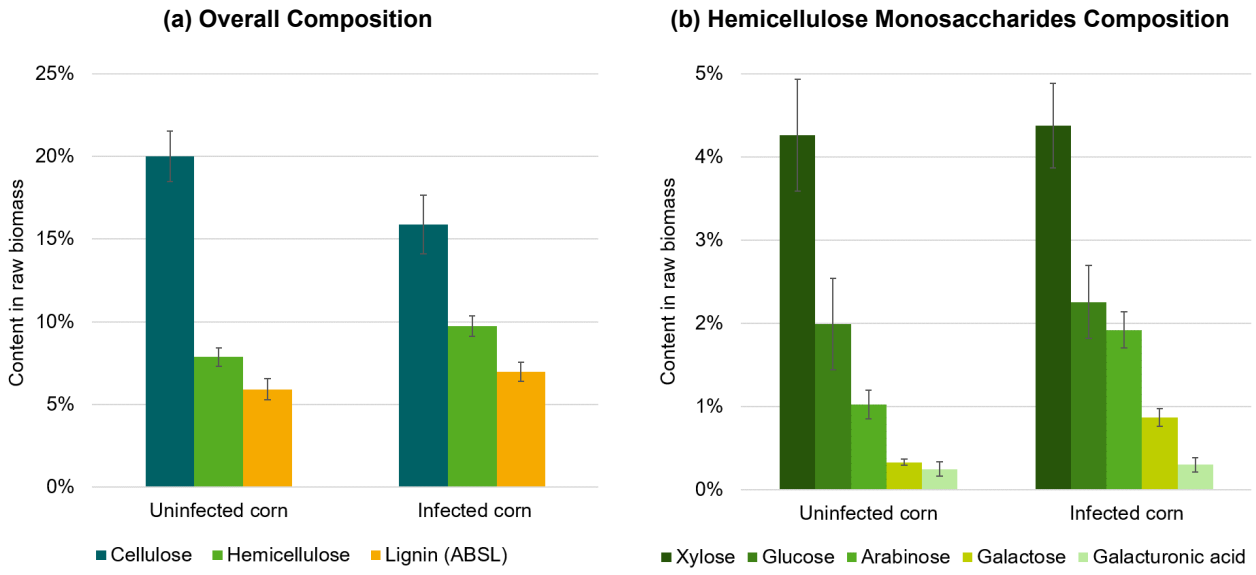

Figure 3.5: Composition of raw substrates (3 week old corn leaves): uninfected corn (11 replicates) and infected corn (9 replicates). (a) Cellulose, hemicellulose and lignin (ABSL) content (wt%) in raw substrate. (b) Hemicellulose monosaccharides composition: xylose, glucose, arabinose, galactose and galacturonic acid content (wt%) in raw substrate.

Some of the components content determined present high error margins (corresponding to over 5% of the average value), especially the monosaccharides content. This is likely explained by variations in substrate composition due to its type and growth stage, *i.e.*, leaves grown for approximately 3 weeks (young plant, not matured). As mentioned in section 1.3.2, the leaves are the corn stover fraction that present the higher fluctuation in composition over time, likely because the leaf tissue, being the site of photosynthesis, has greater metabolic activity. For infected corn, the presence of tumours and their heterogeneity is also responsible for variation of the biomass composition, leading to increased error margins.

According to the results observed, the infection does not lead to changes in main components total content (obtained by summing cellulose, hemicellulose and lignin content), which is approximately 33% for both uninfected and infected corn, but leads to changes in the ratios between the components. As previously mentioned, 52%-54% of the raw biomass corresponds to components removed during dAIR preparation which include starch and cytoplasmic material, such as proteins, lipids and nucleic acids. Further studies are needed to quantify each of these components and to identify and quantify the other components present in the raw biomass, which correspond to approximately 15% of the substrate.

Moreover, an enzymatic hydrolysis (Accellerase© 1500, 50 °C, 20 g/L), of 1 h and 72 h, was performed to the raw substrates and the glucose formed was quantified (table 3.2). Compared to uninfected corn, the glucose concentration after 1 h and 72 h of hydrolysis of the infected corn is 0.6x and 0.7x lower, respectively. This is in accordance with the cellulose content in infected corn being lower and, also, with the lignin content in infected corn being higher, since lignin can inhibit the enzymes.

Table 3.2: Glucose concentration (g/L) obtained after 1 h and 72 h of enzymatic hydrolysis (Accellerase© 1500, 50 °C, 20 g/L) of the raw substrates uninfected corn and infected corn.

Duration of	Glucose concentration (g/L)	
hydrolysis	Uninfected corn	Infected corn
1 h	2.6 ± 0.1	1.6 ± 0.1
72 h	4.7 ± 0.1	3.4 ± 0.2

To conclude, the main differences in corn composition caused by *Ustilago maydis* infection are a decrease by 21% in cellulose content, while increasing hemicellulose and lignin content by 23% and 19%, respectively. The increase of hemicellulose content in the infected corn is caused mostly by an increase in arabinose and galactose monosaccharides content.

3.3.2 OrganoCat Product Fraction Yields

After applying OrganoCat pretreatment to uninfected corn and infected corn, each product fraction was analyzed in order to obtain its yield relative to the initial loading of biomass (400 mg, 100 g/L in the aqueous phase). The figures 3.6a and 3.6b present the yields obtained for the fractions of cellulose-enriched pulp, hemicellulose sugar hydrolysates, lignin and degradation products (furfural and 5-HMF) for the reaction conditions tested for uninfected corn and infected corn, respectively.

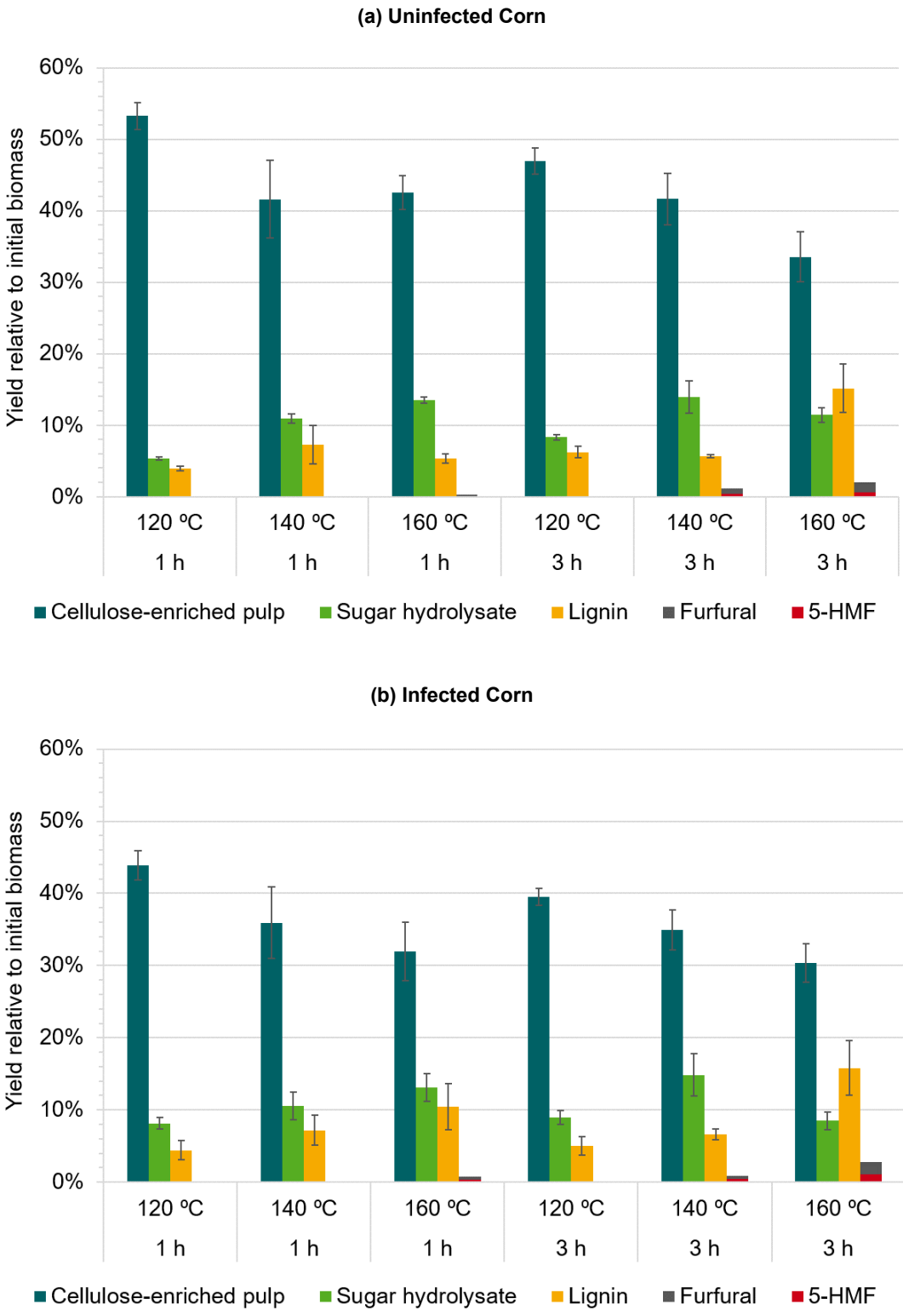

Figure 3.6: Yields (wt%), relative to the initial loading of biomass (100 g/L in the aqueous phase), of each product fraction (cellulose enriched-pulp, hemicellulose sugar hydrolysates and lignin) and degradation products (furfural and 5-HMF) obtained applying OrganoCat with 6 different reaction conditions (presented bellow the bars) to (a) uninfected corn and (b) infected corn.

Cellulose-enriched pulp

In figures 3.6a and 3.6b, cellulose-enriched pulp yield follows the expected trend: increasing reaction temperature and time leads to a decrease in cellulose-enriched pulp yield, since harsher conditions promote cleavage of lignocellulose linkages and extraction of hemicellulose hydrolysed sugars and lignin.

For uninfected corn, increasing reaction temperatures leads to lower cellulose-enriched pulp yields with the exception of the condition 160 °C + 1 h in which pulp yield is similar to the previous condition (140 °C + 1 h) considering the error margins. Increasing the temperature from 120 °C to 160 °C decreases the pulp yield by 20% for 1 h reactions and by 29% for 3 h reactions. With increased reaction time, a decrease in pulp yield is observed, with the exception of the condition 140 °C + 3 h in which the pulp yield is similar to the condition 140 °C + 1 h. It is further noted that the conditions 140 °C + 1 h, 160 °C + 1 h and 140 °C + 3 h present identical pulp yields within the error margins. As expected, the mildest condition (120 °C + 1 h) presents the highest amount of pulp while the harshest condition (160 °C + 3 h) presents the lowest amount of pulp.

For infected corn, increasing reaction temperatures from 120 °C to 160 °C leads to a decrease in pulp yield by 27% for 1 h conditions and by 23% for 3 h conditions. On the other hand, increasing reaction time from 1 h to 3 h does not seem to have a significant impact in pulp yield within the error margin, except for the condition 120 °C + 3 h where pulp yield decreases by 10% compared to the condition 120 °C + 1 h. As expected, the mildest condition (120 °C + 1 h) presents the highest amount of pulp while the harshest condition (160 °C + 3 h) presents the lowest amount of pulp, even though this is similar to the condition 160 °C + 1 h. Compared to uninfected corn, infected corn presents lower yields of cellulose-enriched pulp for all reaction conditions, which is in accordance with raw infected corn having less cellulose in its composition (figure 3.5a).

Hemicellulose sugar hydrolysate

The depolymerization of hemicellulose was monitored via the formation of soluble sugars extracted to the aqueous phase, which were quantified by two methods: high pressure anion-exchange chromatography (HPAEC), performed by Pauly *et al.*, and PAHBAH colorimetric method. With the first method it is possible to identify and quantify each monosaccharide present in the aqueous phase, while with PAHBAH method it is only possible to quantify the total amount of monosaccharides.

As explained in section 2.5.2, PAHBAH colorimetric method was calibrated for xylose. Since glucose has a higher response to PAHBAH reagent than xylose [78], the total sugar concentrations obtained were overestimated. This way, the ratios of each monosaccharide in the aqueous phase, obtained with the HPAEC method, and the relative response of each monosaccharide to the PAHBAH reagent were used to correct the total sugar concentration determined with this colorimetric method (equation 2.3 in section 2.5.2). In appendix A.5, the values for the corrected concentration for each set of substrate-condition and a comparison to the values of total sugar concentration obtained with HPAEC are presented. Observing these results, it is noticed that the corrected concentrations determined for PAHBAH method are, in the majority of the cases, significantly higher than the concentrations determined with HPAEC method. In some cases the corrected concentration determined for PAHBAH was twice as high or even three times as high. This seems to indicate that, even with the correction factor, PAHBAH colorimetric method is not an appropriate and accurate method to quantify the total amount of monosaccharides in a complex mixture of sugars. For simple mixtures of

sugars, PAHBAH colorimetric method appears to be accurate and appropriate as is the case of the sugar hydrolysates obtained applying OrganoCat to beech wood, which is mainly composed of xylose [58]. In consequence, only the sugar concentrations determined with HPAEC are presented in this thesis.

In figures 3.6a and 3.6b, sugar hydrolysate yields follow the contrary trends to the ones observed for cellulose-enriched pulp yield: higher reaction temperatures and times lead to higher sugar hydrolysate yields since more hemicellulose sugars are hydrolysed and extracted from the lignocellulosic biomass to the aqueous phase. However, the condition 160 °C + 3 h is an exception to this trend. Compared to its previous condition (140 °C + 3 h), the sugar hydrolysate yield decreases by 19% and 43% for uninfected and infected corn, respectively. This is explained by monosaccharide degradation occurring at reaction temperatures above 150 °C. Pentoses (xylose and arabinose) are converted to furfural while hexoses (glucose, galactose and galacturonic acid) are converted to 5-hydroxymethylfurfural (5-HMF). In the condition 160 °C + 1 h, even though the reaction temperature is higher than 150 °C, this decrease in yield compared to the previous condition (140 °C + 1 h) is not observed because the reaction time is not long enough for a relevant monosaccharide degradation to occur, since first the hemicellulose sugars need to be hydrolysed and extracted to the aqueous phase.

Comparing both substrates, the yields for each reaction condition are similar within the error margin, with the exceptions of the mildest and the harshest condition which present a higher and lower yield in infected corn, respectively. This is not in line with the composition of the raw substrates (figure 3.2a): infected corn presents higher hemicellulose content than uninfected corn, however OrganoCat with infected corn does not show higher extraction of hydrolysed monosaccharides from hemicellulose. To explain this fact, it is necessary to further analyze the structure and linkages between lignocellulosic components for both uninfected and infected corn, which is out of the scope of this thesis.

For both substrates, the highest extraction of sugars (approximately 14%) occurs at the conditions 160 °C + 1 h and 140 °C + 3 h, which present similar yields within the error margin. Compared to hemicellulose content in raw biomass, these sugar hydrolysate yields are significantly higher (even twice as high for uninfected corn). This is likely explained by the dAIR preparation used for the compositional analysis of the raw biomass. As the substrate used for OrganoCat was not pretreated before, some of the starch present in the biomass could have been hydrolysed to glucose and extracted to the aqueous phase, increasing sugar hydrolysate yields. Another hypothesis would be the hydrolysis of some of the cellulose to glucose during the OrganoCat process.

Degradation products: furfural and 5-HMF

As previously mentioned, at reaction temperatures above 150 °C, hemicellulose monosaccharide degradation into furfural and 5-HMF occurs, which are then extracted to the organic phase. These degradation products were quantified via ^{1}H NMR as described in section 2.5.3. In appendix A.2, detailed information (proton(s) responsible for the signal and chemical shift) about each molecule signal analyzed can be found.

For both substrates, there is no degradation of hemicellulose sugars in OrganoCat pretreatment at the mildest conditions (120 °C + 1 h, 140 °C + 1 h and 120 °C + 3 h) since the reaction time and temperature are not sufficient to hydrolyse the sugars. The maximum degradation products yield is observed at long reaction time and high temperature (160 °C + 3 h), followed by the condition 140 °C + 3 h. Furthermore, in both substrates, furfural yield is always higher than 5-HMF yield, which indicates that more pentoses were

degraded. This is in line with raw biomass monosaccharide composition (figure 3.5b): the sum of xylose and arabinose content (5.3% for uninfected corn and 6.3% for infected corn) is higher than the sum of glucose and galactose content (2.3% for uninfected corn and 3.1% for infected corn).

Comparing both substrates, higher furfural and 5-HMF yields are observed in infected corn for the reaction conditions 160 °C + 1 h and 160 °C + 3 h. This is in accordance with what was observed for the sugar hydrolysate yield: in infected corn there was a bigger decrease in sugar hydrolysates yield from the condition 140 °C + 3 h to 160 °C + 3 h, which indicates higher degradation of monosaccharides.

Assuming that the maximum extraction of hemicellulose sugars occurs at 140 °C + 3 h and that the only hemicellulose degradation products that are formed and extracted to the organic phase are furfural and 5-HMF, it would be expected that the decrease of sugar hydrolysate yield from the condition 140 °C + 3 h to 160 °C + 3 h could be explained by the increase in degradation product yield (sum of furfural and 5-HMF yields). However, comparing condition 160 °C + 3 h to 140 °C + 3 h, the difference between degradation product yields (0.9% for uninfected corn and 1.9% for infected corn) is significantly lower than the difference observed in sugar hydrolysate yields (2.6% for uninfected corn and 6.4% for infected corn).

The values for increase in degradation product yield and decrease in sugar hydrolysates yield are different enough to not be due to losses during the procedure, especially for uninfected corn, which is probably explained by furfural and 5-HMF not being the only products of hemicellulose sugars degradation. This way, formation of humins from furfural and 5-HMF (organic phase) or from monosaccharides (aqueous phase) and degradation of galacturonic acid are relevant hypothesis to future studies. The objective of the present thesis was not to identify the degradation products of OrganoCat with corn, therefore, only suggestions for further analysis are made.

Humins are formed by condensation/polymerization during degradation reactions of carbohydrates [57]. In a recent paper [57], formation of humins from glucose, xylose, furfural and 5-HMF in water and in tetrahydrofuran was studied at reaction conditions of 220 °C and 5 h. Using tetrahydrofuran as solvent, the formation of humins was not significant, while using water as solvent a high amount of humins were formed. This way, it is unlikely that a significant amount of furfural and 5-HMF present in the organic phase (2-MTHF as solvent) have been converted into humins. However, it is possible that some of the monosaccharides present in the aqueous phase undergo condensation/polymerization reactions to form humins, which are likely extracted to the organic phase but not detected as furfural or 5-HMF.

The degradation of galacturonic acid (GalA) is a relevant hypothesis to be further analyzed since studies [84] show that, within the degradation products of GalA in acidic solutions, 30% are furfural but the remaining 70% are not yet identified. To identify the degradation products of GalA in OrganoCat system it would be necessary to set up an OrganoCat reaction for the harshest condition (160 °C + 3 h) with the feedstock being only pure GalA. The ^{1}H NMR spectrum of the organic phase should be analysed by comparing it to the spectrum obtained for pure GalA (without acid catalysis) and to try to identify which peaks were formed by the degradation products and which ones were formed by some extraction of GalA to the organic phase. Gas chromatography combined with mass spectrometry (GC-MS) could be used to identify the molecule(s) responsible for the relevant peak(s).

Furthermore, in a high number of OrganoCat replicates, ^{1}H NMR spectra of the organic phase showed a clear peak with a chemical shift of 8.1 ppm and a relevant signal intensity that could not be identified. This

could correspond to other degradation product present in the organic phase. To test this hypothesis, an analysis of the OrganoCat organic phase samples with GC-MS to identify the possible molecule(s) responsible for the peak is suggested.

Lignin

Since lignin is a very complex molecule, that changes during extraction from lignocellulose, its structure and composition are not well defined, therefore, it is necessary to define lignin for each study in particular. For this thesis, lignin was defined as the dried organic extractives except the remaining degradation products (furfural and 5-HMF) after organic solvent evaporation. The dried organic extractives of the experiments with evaporation of the solvent (at least triplicates) were analyzed with ^{1}H NMR. In appendix A.2, detailed information (proton(s) responsible for the signal and chemical shift) about each molecule signal analyzed can be found.

Lignin yield is expected to follow the same trends as sugar hydrolysates yield. Higher temperatures should lead to higher lignin yields since harsher temperatures promote substrate delignification by cleaving linkages. Longer reaction times should lead to higher lignin yields because hydrolysis and extraction of hemicellulose sugars is the first reaction to occur (almost complete within the first hour of reaction) and only after lignin cleavage and extraction.

For uninfected corn, the expected trends are not as clearly observed, especially since in conditions 140 °C + 1 h, 160 °C + 1 h, 120 °C + 3 h and 140 °C + 3 h the lignin yields are similar within the error margin. The mildest and harshest conditions are the exception, at 120 °C + 1 h lignin yield is the lowest and at 160 °C + 3 h lignin yield is the highest (15.1% $\pm$ 3.4%), being 2.6x higher than the previous condition (140 °C + 3 h) and 2.8x higher than the condition 160 °C + 1 h.

For infected corn, with increasing temperatures it is clear that more lignin is extracted, however, with increasing reaction time, lignin yield does not increase in a significant amount, except for the condition 160 °C + 3 h. As in uninfected corn, the lowest lignin yield occurs at the mildest condition and the highest lignin yield (15.8% $\pm$ 3.8%) at the harshest condition, being 2.4x higher than the previous condition (140 °C + 3 h) and 1.5x higher than the condition 160 °C + 1 h.

Comparing both substrates, lignin yields are very similar, especially considering the error margin, with the exception of the condition 160 °C + 1 h, where lignin yield is 1.9x higher for infected corn than uninfected corn. Even though, raw infected corn has higher lignin content than raw uninfected corn, not more extraction of lignin in infected corn was observed. To understand this is necessary to further analyze lignin monomers content and its connectivity and linkage to the carbohydrates. For both substrates, some of the OrganoCat lignin yields are higher than the lignin content in the raw substrates. However, as mentioned in section 3.3.2, the lignin determined in the compositional analysis and the lignin extracted with OrganoCat can not be directly compared, as different extraction methods were used.

Finally, the mass balance of all the product fraction yields of each condition is between 60% and 62% for uninfected corn and between 54% and 57% for infected corn, indicating that there are several components present in the raw biomass that were not analyzed. To further close the mass balance, it would be necessary to quantify ash, moisture and acetylation degree and to further analyze the degradation products formed during the reaction.

3.3.3 Cellulose-Enriched Pulp Analysis

The cellulose-enriched pulps obtained from OrganoCat pretreatment were further analyzed: its cellulose, hemicellulose and lignin content (figures 3.7a and 3.7b) were determined by Pauly *et al.*, as explained in section 2.5.4, and an enzymatic hydrolysis was performed to verify cellulose accessibility to enzymes by determining the glucose concentration after 1 h and 72 h of hydrolysis as described in section 2.5.1. Figures 3.8a and 3.8b present the improvement factor (IF) of glucose concentration comparing hydrolysis of the pulp to hydrolysis of the raw substrate (equation 2.2 in section 2.5.1).

Overall Composition

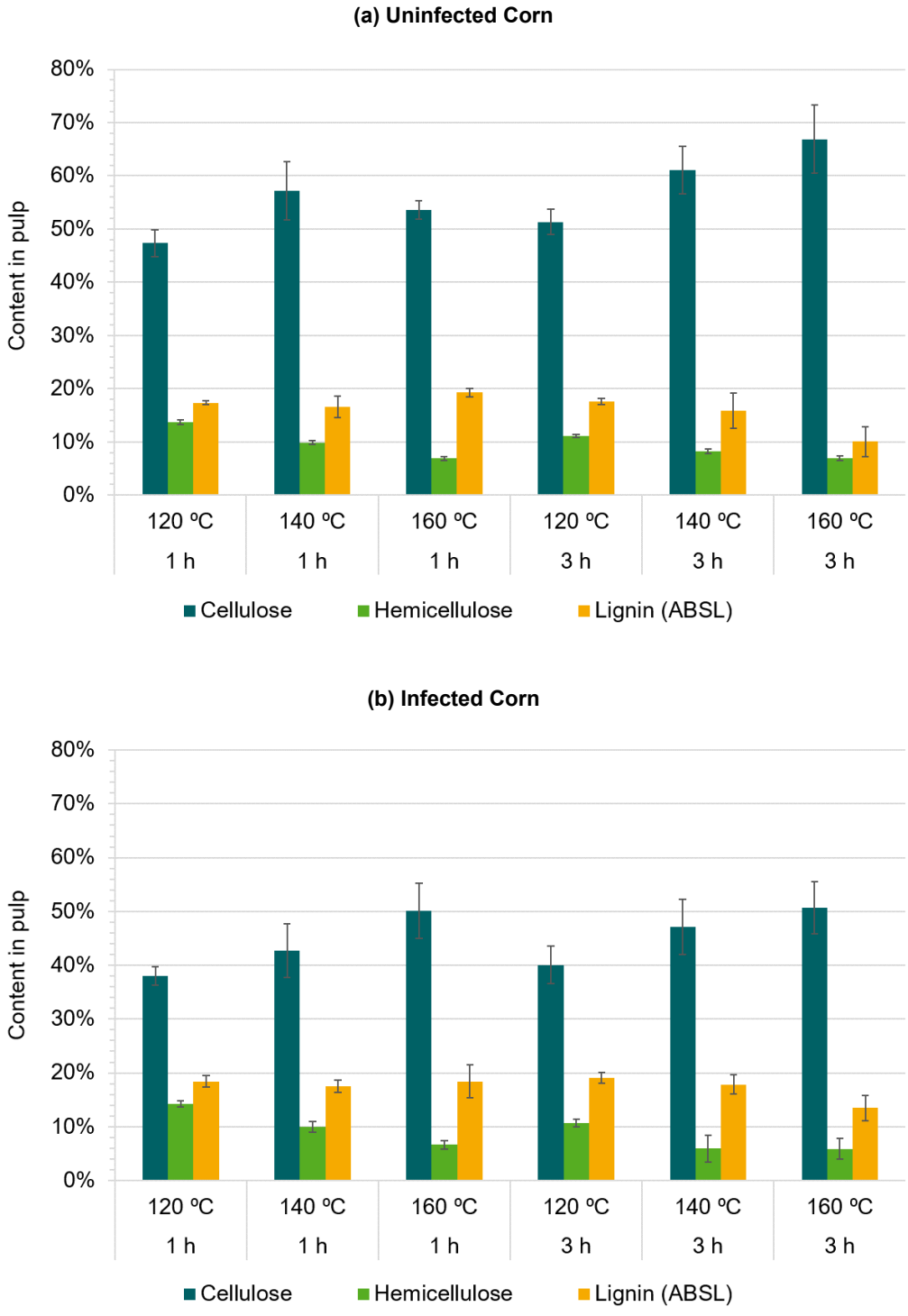

Figure 3.7: Cellulose, hemicellulose and lignin (ABSL) content (wt%) in cellulose-enriched pulps obtained applying OrganoCat with 6 different reaction conditions (presented bellow the bars) to (a) uninfected corn and (b) infected corn.

The analysis of the pulp composition verified the trends observed in OrganoCat product fraction yields. Increasing reaction temperature and time leads to an increase in cellulose content in the pulp and a decrease in hemicellulose and lignin content in the pulp since harsher conditions lead to more extraction of hemicellulose and lignin from the lignocellulosic biomass.

For both substrates, it is observed that cellulose content is higher in the pulp than in the raw substrate (figure 3.5a), which indicates that the pretreatment was successful since an increase in cellulose content means a decrease in other components content due to their extraction to the liquid phases. In contrary to the raw substrate, lignin content in pulp is higher than hemicellulose content in pulp, indicating that lignin extraction to the organic phase occurs after hemicellulose hydrolysis and monosaccharides extraction to the aqueous phase. This is in line with OrganoCat product fraction yields, since hemicellulose hydrolysed sugars present higher yields than lignin. Still regarding both substrates, a significant decrease in lignin content is only observed for the highest temperature and time condition (160 °C + 3 h) compared to the previous condition (140 °C + 3 h). Comparing the remaining conditions, lignin content is roughly similar within the error margins. This is in line with what was observed for OrganoCat lignin yield, which higher increment was from the condition 140 °C + 3 h to the condition 160 °C + 3 h. Once again this indicates that lignin extraction to the organic phase occurs after hemicellulose hydrolysis and monosaccharides extraction to the aqueous phase, therefore 1 h reactions are not enough to observe a high extraction of lignin.

In uninfected corn pulps, an exception to the general trend is observed: in 1 h reactions, increasing the temperature from 140 °C to 160 °C leads to a decrease in cellulose content and a slight increase in lignin content. Also in OrganoCat product yields (figure 3.6a) some unexpected trends between these conditions were observed: from 140 °C to 160 °C cellulose content was similar, instead of decreasing, and lignin content decreased, instead of increasing. These unexpected observations are likely explained by the significant error margins in condition 140 °C + 1 h, which could be decreased by the use of mature leaves or by performing more biological replicates. From the mildest condition to the highest temperature and time condition, an increase of 1.4x in cellulose content, a decrease of 2.0x in hemicellulose content and a decrease of 1.7x in lignin content is observed. Compared to raw uninfected corn, the cellulose content in the pulp increases between 2.4x (for the mildest condition) and 3.3x (for the highest temperature and time condition).

In infected corn cellulose-enriched pulps, an exception to the general trend is observed: in 160 °C reactions, increasing reaction time from 1 h to 3 h does not lead to an increase in cellulose content, even though the sum of hemicellulose and lignin content is lower for the condition 160 °C + 3 h, indicating that more extraction occurred. To better understand this fact it is necessary to identify and quantify the other components present in cellulose-enriched pulp. From the mildest condition to the highest temperature and time condition, an increase of 1.3x in cellulose content, a decrease of 2.4x in hemicellulose content and a decrease of 1.4x in lignin content is observed. Compared to raw infected corn, the cellulose content in the pulp increases between 2.4x (for the mildest condition) and 3.2x (for the highest temperature and time condition).

Comparing both substrates, uninfected corn pulps present higher cellulose content (47%-67%) than infected corn pulps (38%-51%), however the increase of cellulose content in pulp compared to the raw biomass was similar for both (2.4x-3.2x/3.3x). Hemicellulose and lignin content between both substrates are not significantly different, except for the conditions 140 °C + 3 h and 160 °C + 3 h, where hemicellulose and lignin content are lower in infected corn pulps. Infected corn pulps, having similar hemicellulose and lignin contents as uninfected corn pulps, contradicts the results observed for raw substrate composition and for OrganoCat

product yields. If raw infected corn has higher hemicellulose and lignin content than raw uninfected corn but there is not more extraction of hemicellulose and lignin with OrganoCat pretreatment for infected corn than uninfected corn, it would be expected that infected corn pulps had higher lignin and hemicellulose content. For a better understanding of these observations it is necessary to identify and quantify all the components of both substrates and to verify if more degradation in infected corn occurs. Moreover, using a less heterogeneous substrate (mature leaves or other parts of the plant) would decrease the variability observed between the replicates which would facilitate the analysis of the results.

Enzymatic Hydrolysis

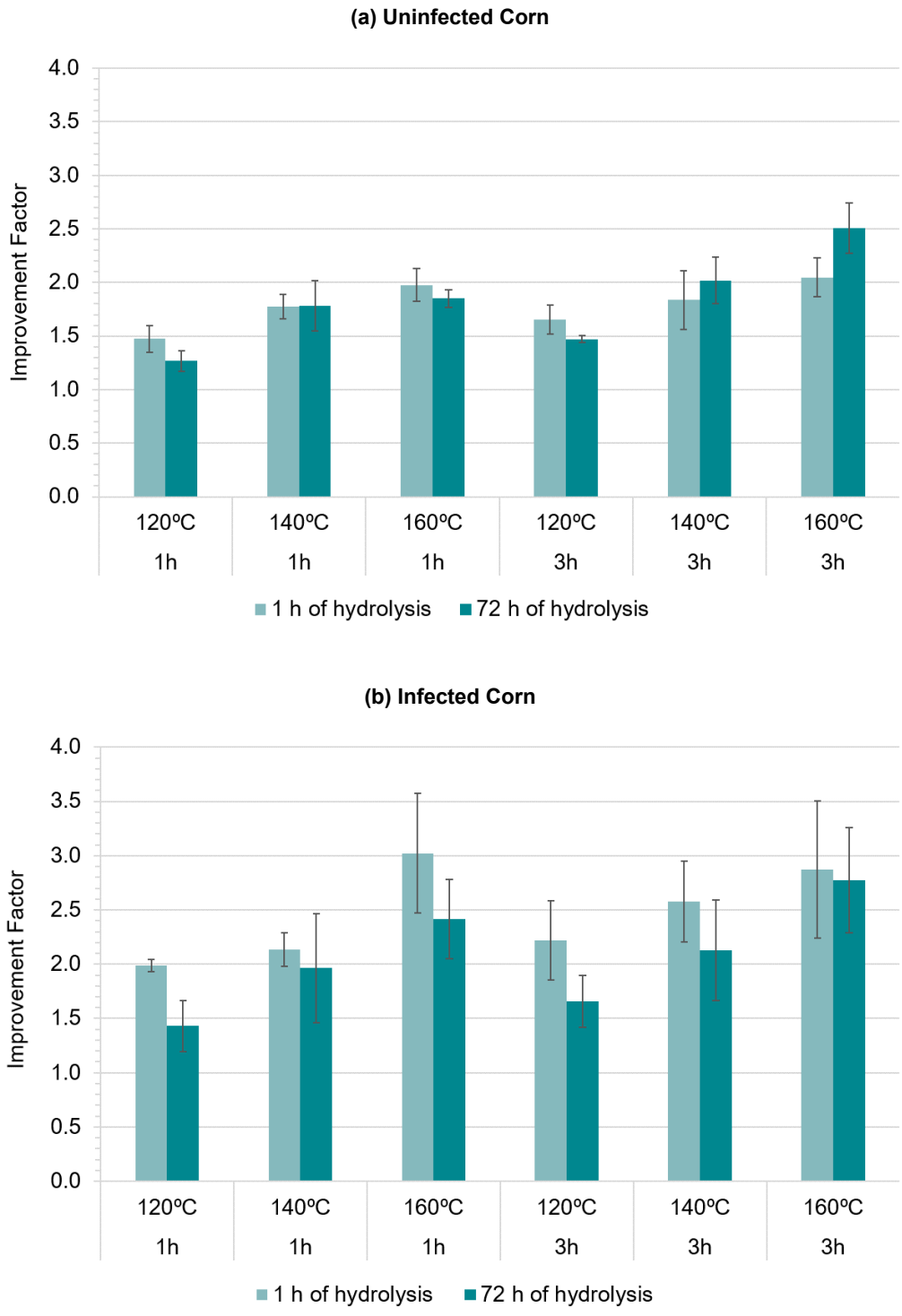

Figure 3.8: Improvement factor (comparing to raw biomass hydrolysis) of glucose concentration obtained after 1 h and 72 h of enzymatic hydrolysis (Accellerase© 1500, 50 °C) of the cellulose-enriched pulps obtained applying OrganoCat with 6 different reaction conditions (presented bellow the bars) to (a) uninfected corn and (b) infected corn.

Observing figures 3.8a and 3.8b, the IF is always higher than 1.0, *i.e.*, the glucose concentration obtained by hydrolysis of the pulp is higher than the one obtained by hydrolysis of the raw substrate, indicating that OrganoCat pretreatment leads to an enhancement of the enzymatic hydrolysis. This can be explained by the cellulose content in pulp being higher than in raw substrate, by improved accessibility of cellulose to enzymes in the pulp and by the presence of less lignin in pulp.

The enzymatic hydrolysis of lignocellulose is limited by several factors, including the crystallinity of cellulose and the lignin content [17]. During the first hour of hydrolysis only the very amorphous and easily accessible parts of the cellulose are hydrolysed, therefore, the amorphization of lignocellulose due to pretreatment is likely the most relevant factor affecting the improvement of the enzymatic hydrolysis. For longer hydrolysis, the effect of delignification is likely the most relevant factor since, a decrease in lignin content, increases the accessibility of the cellulose and decreases the inhibition of the enzymes by lignin. In most OrganoCat conditions, IF after 1 h of hydrolysis is higher than after 72 h, which suggests that the influence of pretreatment on the enzymatic hydrolysis of the cellulose is more notorious in the beginning of the hydrolysis. However, the difference between IF of 1 h and of 72 h of hydrolysis is not very significant due to the high error margins, especially for infected corn.

A similar trend is observed for both substrates: increasing reaction temperature and time leads to an increase in IF values due to enhanced amorphization and delignification of the lignocellulose. Compared to uninfected corn, infected corn pulps present higher IF for all conditions, which indicates that the effect of OrganoCat pretreatment in the amorphization and delignification of the lignocellulose is higher for infected corn. Moreover, infected corn presents higher error margins, which is explained by the heterogeneity of the tumour material on the leaves and by the different batches of substrate used for the OrganoCat, as mentioned in section 2.2.

3.3.4 Lignin Analysis

OrganoCat lignin fraction was analyzed by qualitative ^{1}H-^{13}C-HSQC NMR spectroscopy (section 2.5.3). This was used to determine the ratios between lignin monomers of the lignin fractions obtained applying OrganoCat to uninfected and infected corn with the reaction conditions 140 °C + 1 h, 140 °C + 3 h and 160 °C + 3h (only one replicate per condition was analyzed). Lignin monomer units are syringyl (S), guaiacyl (G) and *p*-hydroxyphenyl (H), and their structure is presented in figure 1.2. Appendix A.3 presents the aromatic region of the HSQC spectra obtained, with the identification of the molecules and respective carbon(s) responsible for the signals. Figures 3.9a and 3.9b (next page) present the normed ratio percentages of lignin monomer units for uninfected corn and infected corn, respectively.

In uninfected corn lignin (figure 3.9a), for the mildest reaction condition (140 °C + 1 h), S and G units present similar ratios (43.2%, 43.9%). However, with increasing reaction temperature and time, both S and G units ratio decrease while H unit ratio increases. This is likely to indicate that, with harsher reaction conditions, S and G units are converted to G and H units by loss of MeO groups. However G ratio decrease is not as sharp as S ratio decrease, which implies that not all S units were fully converted to H units, some remained as G units. That is also a possible explanation for G unit being the main monomer present in lignin fraction obtained with the reactions conditions 140 °C + 3 h and 160 °C + 3 h.

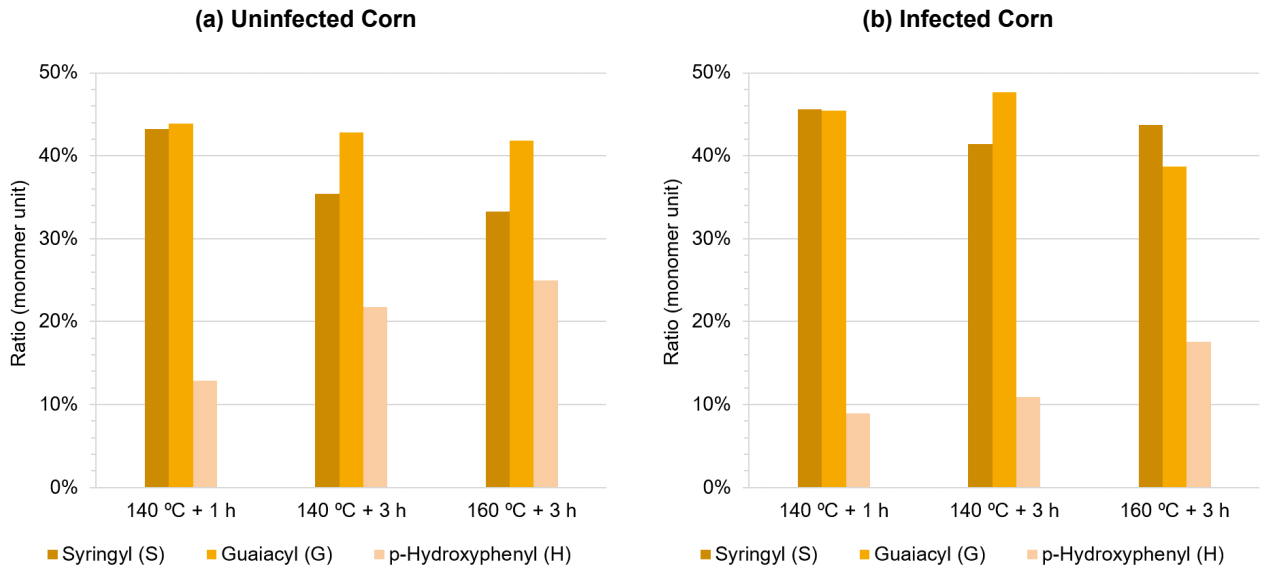

Figure 3.9: Normed ratio of each lignin monomer unit (syringyl, guaiacyl and *p*-hydroxyphenyl) in the lignin fraction obtained by applying OrganoCat pretreatment with the reaction conditions 140 °C + 1 h, 140 °C + 3 h and 160 °C + 3 h to (a) uninfected corn and (b) infected corn. Only one replicate per condition was analyzed.

In infected corn lignin (figure 3.9b), for the mildest reaction condition (140 °C + 1 h), S and G units present similar ratios (45.6%, 45.4%). However, contrary to uninfected corn lignin, no trend is observed for S and G units by increasing reaction temperature and time. At 140 °C, increasing the reaction time from 1 h to 3 h, leads to a decrease in S unit ratio, an increase in G unit ratio and a slight increase in H unit ratio. This implies that the conversion rate of S to G units is higher than the conversion rate of G to H units. On the other hand, at 3 h reactions, increasing the temperature from 140 °C to 160 °C leads to an increase in S unit ratio and a decrease in G unit ratio while increasing H unit ratio. This suggests that, at harsher conditions, the conversion rate of G to H units is higher than at milder conditions. While the decrease in G unit ratio is explained by its conversion to H unit, the increase in S unit would mean that more S units were formed, which is not a plausible hypothesis. This discrepancy is likely to be due to the method used being qualitative and because only one replicate for each set of substrate-condition was analyzed. Compared to uninfected corn, infected corn lignin fraction presents higher S unit ratios and lower H unit ratios for every reaction condition.

For both substrates, an increase (2x) in H unit ratio is observed from the mildest to the harshest condition. This is in line with the hypothesis of S and G units being converted to H units by cleavage of MeO groups during OrganoCat pretreatment. This hypothesis should be confirmed by quantifying each monomer unit and MeO groups using quantitative ^{1}H-^{13}C-HSQC NMR and by analyzing the lignin fraction of all the OrganoCat reaction conditions and the respective replicates.

Moreover, observing HSQC spectra, peaks of other relevant molecules in lignin structure were also identified: methoxyl group; β-O-4 and β-5 bonds, which link the monomer units; ferulate (FA) and *p*-coumarate (*p*CA), which link lignin to carbohydrates [79]. To better understand the composition, linkages and structure of the lignin in the raw substrates and of the lignin extracted with OrganoCat, these molecules would have to be analyzed and quantified via quantitative ^{1}H-^{13}C-HSQC NMR.

4 Conclusions and Future Perspectives

The main goals of the present thesis were achieved: (1) the influence of corn smut infection in OrganoCat pretreatment was evaluated, (2) six OrganoCat condition sets were screened for uninfected and infected corn to determine maximum extraction and minimum sugar degradation, and (3) the enzymatic hydrolysis of the cellulose-enriched pulp was found to be enhanced by OrganoCat.

Two types of infected corn leaves were studied: leaves infected with wild type *Ustilago maydis* (WT infected corn) and leaves infected with genetically modified *U. maydis* (GMO infected corn). The raw substrates presented similar contents of cellulose, hemicellulose and lignin. However, different results were verified after pretreating the substrates with the OrganoCat condition 140 °C + 1 h: GMO infected corn presented a lower sugar hydrolysate yield and the compositional analysis of its pulp showed a lower cellulose and lignin content, compared to WT infected corn. The improvement factor (IF) of the glucose concentration obtained after enzymatic hydrolysis of the pulp was similar for both substrates. GMO infected corn was the substrate used for the evaluation of the impact of corn smut infection on OrganoCat pretreatment, and, from this point on, it will be named infected corn.

Before pretreating uninfected corn and infected corn with OrganoCat, a compositional analysis was performed. The main differences in corn leaf composition caused by *U. maydis* infection were a decrease by 21% in cellulose content, while increasing hemicellulose and lignin content by 23% and 19%, respectively. The hemicellulose of corn leaves showed to be composed mainly by xylose, followed by glucose, arabinose, galactose and galacturonic acid. Hemicellulose content increase in the infected corn was caused mostly by an increase in arabinose and galactose monosaccharides content. Moreover, the corn smut infection lead to a decrease of the glucose concentration that can be obtained by enzymatic hydrolysis of the substrate which is in line with its lower cellulose content, compared to uninfected corn.

After screening six OrganoCat condition sets it was possible to observe some general trends for both substrates. Increasing reaction temperature and time lead to a decrease in cellulose-enriched pulp yield, while sugar hydrolysate and lignin extraction yields increased due to a better disentanglement and delignification of lignocellulose. OrganoCat with infected corn presented lower cellulose-enriched pulp yields which is consistent with the substrate having less cellulose in its composition. Sugar hydrolysate and lignin yields were similar between both substrates even though raw infected corn has higher hemicellulose and lignin content.

Degradation of the sugars present in the aqueous phase into furfural and 5-hydroxymethylfurfural (5-HMF) was observed for the reaction conditions 160 °C + 1 h, 140 °C + 3 h and 160 °C + 3 h. For both substrates, furfural yield was always higher than 5-HMF yield, which indicates that more pentoses were degraded. This

is in line with raw biomass monosaccharide composition: the sum of xylose and arabinose content was higher than the sum of glucose, galactose and galacturonic acid content.

The sum of all the product fraction yields of each condition was 60%-62% for uninfected corn and 54%-57% for infected corn, indicating that there are several components present in the raw biomass that were not analyzed. To further close the mass balance, it would be necessary to quantify ash, moisture and acetylation degree of the substrate and to further analyze the degradation products formed during the reaction, namely the degradation of galacturonic acid and the formation of humins.

The compositional analysis of the pulp confirmed that the cellulose content increased compared to cellulose content in the raw substrates, which indicates that the pretreatment was successful. Hemicellulose and lignin content between both substrates was not significantly different, which is surprising as raw infected corn presented higher contents of these components but their extraction in OrganoCat pretreatment was similar for both substrates.

An enzymatic hydrolysis was performed to the cellulose-enriched pulps and the improvement factor (IF) of glucose concentration comparing hydrolysis of the pulp to hydrolysis of the raw substrate was determined. For both substrates, the IF was always higher than 1.0, which indicates that OrganoCat pretreatment leads to an enhancement of the enzymatic hydrolysis. This can be explained by the cellulose content in pulp being higher than in raw substrate, by improved accessibility of cellulose to enzymes in the pulp due to amorphization and by the presence of less lignin in pulp. Moreover, for both substrates, a similar trend was observed: increasing reaction temperature and time lead to an increase in IF values due to enhanced amorphization and delignification of the lignocellulose.

OrganoCat lignin fraction was analyzed with ^{1}H-^{13}C-HSQC NMR and the normed ratio between its monomer units was determined. One general trend for both substrates was observed: increasing reaction temperature and time lead to an increase in the *p*-hydroxyphenyl (H) unit ratio. This is likely to indicate that, with harsher reaction conditions, cleavage of the methoxyl groups present in syringyl (S) and guaiacyl (G) units occurs, which are, therefore, converted into G and H units. For uninfected corn, this is confirmed by the decrease of S and G units ratio with increasing time and temperature, however such trend was not observed for infected corn. Compared to uninfected corn, infected corn lignin fraction presents higher S unit ratios and lower H unit ratios for every reaction condition analyzed: 140 °C + 1 h, 140 °C + 3 h and 160 °C + 3 h.

Most of the obtained results for OrganoCat yields and for the compositional analysis presented high standard deviations (corresponding to over 5% of the average value), especially infected corn. This is likely explained by variations in substrate composition due to its type and growth stage, *i.e.*, leaves grown for approximately 3 weeks (young plant, not matured). For infected corn, the presence of tumours and their heterogeneity is also responsible for variation of the biomass composition, leading to increased error margins. To decrease the variability observed between the replicates it is suggested to (1) use less heterogeneous substrate such as mature leaves or other parts of the plant (*e.g.* straw), (2) scale-up the biomass initial loading and (3) perform more technical replicates.

Overall, the results indicate that OrganoCat pretreatment is an efficient fractionation method for both uninfected and infected corn leaves. Moreover, OrganoCat showed to be an adaptable system since different reaction conditions seem promising for different applications. The pretreatment can be tuned to yield high

delignification (160 °C + 3 h), high fermentable sugars (140 °C + 3 h) or to a comprise between high extraction and no or low sugar degradation (140 °C + 1 h and 160 °C + 1 h).

Regarding follow-up works, it would be interesting to investigate field-grown infected corn samples, including a scale-up of the OrganoCat pretreatment for more representative results, to test other corn species and to test other corn infections.

References

[1] P. Dhepe, K. Tomishige, and K. Wu. “Catalytic conversion of biomass”. In: *ChemCatChem* 9 (2017), pp. 2613–2614.

[2] J. N. Putro et al. “Pretreatment and conversion of lignocellulose biomass into valuable chemicals”. In: *RSC Advances* 6 (2016), pp. 46834–46852.

[3] G. de Bhowmick, A. K. Sarmah, and R. Sen. “Lignocellulosic biorefinery as a model for sustainable development of biofuels and value added products”. In: *Bioresource Technology* 247 (2018), pp. 1144–1154.

[4] E. de Jong and R. J. A. Gosselink. “Lignocellulose-based chemical products”. In: *Bioenergy Research: Advances and Applications*. 2014, pp. 277–313.

[5] M. Balat and G. Ayar. “Biomass energy in the world, use of biomass and potential trends”. In: *Energy Sources* 27 (2005), pp. 931–940.

[6] A. Brandt et al. “An economically viable ionic liquid for the pretreatment of lignocellulosic biomass”. In: *Green Chemistry* 19 (2017), pp. 3078–3102.

[7] F. Gírio et al. “Biorefineries in the world”. In: *Lecture Notes in Energy*. Vol. 57. 2017, pp. 227–281.

[8] F. Cherubini. “The biorefinery concept: Using biomass instead of oil for producing energy and chemicals”. In: *Energy Conversion and Management* 51 (2010), pp. 1412–1421.

[9] H. Chen. “Lignocellulose biorefinery engineering”. In: *Lignocellulose Biorefinery Engineering*. 2015, pp. 1–17.

[10] P. Kumar et al. “Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production”. In: *Industrial and Engineering Chemistry Research* 48 (2009), pp. 3713–3729.

[11] P. Binod and A. Pandey. “Introduction”. In: *Pretreatment of Biomass: Processes and Technologies*. 2015, pp. 3–6.

[12] H. Pereira, J. Graça, and J. C. Rodrigues. “Wood chemistry in relation to quality”. In: *ChemInform* 35 (2004).

[13] M. Jedrzejczyk et al. “Physical and chemical pretreatment of lignocellulosic biomass”. In: *Second and Third Generation of Feedstocks*. 2019, pp. 143–196.

[14] R. C. Kuhad, A. Singh, and K. E. Eriksson. “Microorganisms and enzymes involved in the degradation of plant fiber cell walls.” In: *Advances in biochemical engineering/biotechnology* 57 (1997), pp. 45–125.

[15] P. Béguin and J. P. Aubert. “The biological degradation of cellulose”. In: *FEMS microbiology reviews* 13 (1994), pp. 25–58.

[16] D. Ciolacu, F. Ciolacu, and V. I. Popa. “Amorphous cellulose - Structure and characterization”. In: *Cellulose Chemistry and Technology* 45 (2011), pp. 13–21.

[17] A. T.W.M. Hendriks and G. Zeeman. “Pretreatments to enhance the digestibility of lignocellulosic biomass”. In: *Bioresource Technology* 100 (2009), pp. 10–18.

[18] I. Spiridon and V. I. Popa. “Hemicelluloses: Major sources, properties and applications”. In: *Monomers, Polymers and Composites from Renewable Resources*. 2008, pp. 289–304.

[19] F. Cherubini and S. Ulgiati. “Crop residues as raw materials for biorefinery systems - A LCA case study”. In: *Applied Energy* 87 (2010), pp. 47–57.

[20] H. Hatakeyama and T. Hatakeyama. “Lignin Structure, Properties, and Applications”. In: *Advances in Polymer Science* 232 (2010), pp. 1–63.

[21] T. Q. Yuan et al. “Characterization of lignin structures and lignin-carbohydrate complex (LCC) linkages by quantitative 13C and 2D HSQC NMR spectroscopy”. In: *Journal of Agricultural and Food Chemistry* 59 (2011), pp. 10604–10614.

[22] S. Chatterjee et al. “Synthesis and characterization of lignin-based carbon materials with tunable microstructure”. In: *RSC Advances* 4 (2014), pp. 4743–4753.

[23] A. Vishtal and A. Kraslawski. “Challenges in industrial applications of technical lignins”. In: *Bioresources* 6 (2011), pp. 3547–3568.

[24] S. S. Hassan, G. A. Williams, and A. K. Jaiswal. “Lignocellulosic Biorefineries in Europe: Current State and Prospects”. In: *Trends in Biotechnology* 37 (2019), pp. 231–234.

[25] S. García-Lara and S. O. Serna-Saldivar. “Corn history and culture”. In: *Corn: chemistry and technology*. 2019, pp. 1–18.

[26] M.P. Scott and M. Emery. “Maize: Overview”. In: *Reference Module in Food Science*. 2016.

[27] E. D. Nafziger. “Maize agronomy”. In: *Reference Module in Food Science*. 2016, 7 pages.

[28] P. C. Mangelsdorf. “The origin of corn”. In: *Scientific American* (1986), pp. 72–78.

[29] Australian Government Office of the Gene Technology Regulator. *The Biology of Zea mays L. ssp mays (maize or corn)*. Tech. rep. 2008.

[30] VectorStock. *Maize plant diagram infographic elements with the vector image*. URL: `https://www.vectorstock.com/royalty-free-vector/maize-plant-diagram-infographic-elements-with-the-vector-23593206`. (Accessed: 01.10.2019).

[31] E. L. Martinez and F. J. B. Fernandez. “Economics of production, marketing and utilization”. In: *Corn: chemistry and technology*. 2019, pp. 87–107.

[32] FAO. *FAOSTAT: Crops*. 2019. URL: `http://www.fao.org/faostat/en/#data/QC`. (Accessed: 29.09.2019).

[33] S. A. Flint-Garcia, A. L. Bodnar, and M. P. Scott. “Wide variability in kernel composition, seed characteristics, and zein profiles among diverse maize inbreds, landraces, and teosinte”. In: *Theoretical and Applied Genetics* 119 (2009), pp. 1129–1142.

[34] D. W. Templeton et al. “Assessing corn stover composition and sources of variability via NIRS”. In: *Cellulose* 16 (2009), pp. 621–639.

[35] L. O. Pordesimo et al. “Variation in corn stover composition and energy content with crop maturity”. In: *Biomass and Bioenergy* 28 (2005), pp. 366–374.

[36] K. L. Kadam and J. D. McMillan. “Availability of corn stover as a sustainable feedstock for bioethanol production”. In: *Bioresource Technology* 88 (2003), pp. 17–25.

[37] S. A. Barber. “Corn residue management and soil organic matter”. In: *Agronomy Journal* 71 (1979), pp. 625–627.

[38] R. L. Graham et al. “Current and potential U.S. corn stover supplies”. In: *Agronomy Journal* 99 (2007), pp. 1–11.

[39] F. A. Riera, R. Alvarez, and J. Coca. “Production of furfural by acid hydrolysis of corncobs”. In: *Journal of Chemical Technology & Biotechnology* 50 (1991), pp. 149–155.

[40] E. M. Hernandez and K. S. Ng. “Design of biorefinery systems for conversion of corn stover into biofuels using a biorefinery engineering framework”. In: *Clean Technologies and Environmental Policy* 20 (2018), pp. 1501–1514.

[41] Y. Zhao, A. Damgaard, and T. H. Christensen. “Bioethanol from corn stover – A review and technical assessment of alternative biotechnologies”. In: *Progress in Energy and Combustion Science* 67 (2018), pp. 275–291.

[42] L. Kemp. *Cellulosic ethanol from corn stover: can we get it right?* Tech. rep. Natural Resources Defense Council (NRDC), 2015.

[43] L. Capolupo and V. Faraco. “Green methods of lignocellulose pretreatment for biorefinery development”. In: *Applied Microbiology and Biotechnology* 100 (2016), pp. 9451–9467.

[44] C. G. León-Ramírez, J. A. Sánchez-Arreguín, and J. Ruiz-Herrera. “Ustilago maydis, a delicacy of the aztec cuisine and a model for research”. In: *Natural Resources* 5 (2014), pp. 256–267.

[45] M. Feldbrügge, R. Kellner, and K. Schipper. “The biotechnological use and potential of plant pathogenic smut fungi”. In: *Applied Microbiology and Biotechnology* 97 (2013), pp. 3253–3265.

[46] J. Kämper, R. Kahmann, and M. Bölker. “Insights from the genome of the biotrophic fungal plant pathogen Ustilago maydis”. In: *Nature* 444 (2006), pp. 97–101.

[47] M. Feldbrügge et al. “Regulation of mating and pathogenic development in Ustilago maydis”. In: *Current Opinion in Microbiology* 7 (2004), pp. 666–672.

[48] B. J. Saville and S. A. Leong. “The molecular biology of pathogenesis in Ustilago maydis”. In: *Genetic Engineering: Principles and Methods*. 1992, pp. 139–162.

[49] M. Bölker et al. “Genetic regulation of mating and dimorphism in Ustilago maydis”. In: *Canadian Journal of Botany* 73 (1995), pp. 320–325.

[50] U. Fuchs et al. “Endocytosis is essential for pathogenic development in the corn smut fungus Ustilago maydis”. In: *Plant Cell 18*. 2006, pp. 2066–2081.

[51] M. Bölker, M. Urban, and R. Kahmann. “The a mating type locus of U. maydis specifies cell signaling components”. In: *Cell* 68 (1992), pp. 441–450.

[52] T. Brefort et al. “Ustilago maydis as a Pathogen”. In: *Annual Review of Phytopathology* 47 (2009), pp. 423–445.

[53] S. I. Mussatto and G. M. Dragone. “Biomass pretreatment, biorefineries, and potential products for a bioeconomy development”. In: *Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery*. 2016, pp. 1–22.

[54] P. Alvira et al. “Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review”. In: *Bioresource Technology* 101 (2010), pp. 4851–4861.

[55] H. Rasmussen, H. R. Sørensen, and A. S. Meyer. “Formation of degradation compounds from lignocellulosic biomass in the biorefinery: Sugar reaction mechanisms”. In: *Carbohydrate Research* 385 (2014), pp. 45–57.

[56] K. Werner, L. Pommer, and M. Broström. “Thermal decomposition of hemicelluloses”. In: *Journal of Analytical and Applied Pyrolysis* 110 (2014), pp. 130–137.

[57] N. Shi et al. “Formation of humins during degradation of carbohydrates and furfural derivatives in various solvents”. In: *Biomass Conversion and Biorefinery* (2019).

[58] T. vom Stein et al. “From biomass to feedstock: One-step fractionation of lignocellulose components by the selective organic acid-catalyzed depolymerization of hemicellulose in a biphasic system”. In: *Green Chemistry* 13 (2011), pp. 1772–1777.

[59] P. Domínguez De María, P. M. Grande, and W. Leitner. “Current trends in pretreatment and fractionation of lignocellulose as reflected in industrial patent activities”. In: *Chemie-Ingenieur-Technik* 87 (2015), pp. 1686–1695.

[60] A. K. Kumar and S. Sharma. “Recent updates on different methods of pretreatment of lignocellulosic feedstocks: A review”. In: *Bioresources and Bioprocessing* 4 (2017), pp. 1–19.

[61] E. T. Kostas, D. Beneroso, and J. P. Robinson. “The application of microwave heating in bioenergy: A review on the microwave pre-treatment and upgrading technologies for biomass”. In: *Renewable and Sustainable Energy Reviews* 77 (2017), pp. 12–27.

[62] V. B. Agbor et al. “Biomass pretreatment: Fundamentals toward application”. In: *Biotechnology Advances* 29 (2011), pp. 675–685.

[63] J. S. Kim, Y. Y. Lee, and T. H. Kim. “A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass”. In: *Bioresource Technology* 199 (2016), pp. 42–48.

[64] X. Zhao, K. Cheng, and D. Liu. “Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis”. In: *Applied Microbiology and Biotechnology* 82 (2009), pp. 815–827.

[65] G. Brodeur et al. “Chemical and physicochemical pretreatment of lignocellulosic biomass: A review”. In: *Enzyme Research* 2011 (2011), pp. 1–17.

[66] P. Dominguez De Maria et al. “Integrated process for the selective fractionation and separation of lignocellulose in its main components”. Patent EP2489780A1 (RWTH Aachen). Aug. 2012.

[67] J. W. Lee and T. W. Jeffries. “Efficiencies of acid catalysts in the hydrolysis of lignocellulosic biomass over a range of combined severity factors”. In: *Bioresource Technology* 102 (2011), pp. 5884–5890.

[68] W. Brown. “Solution properties of lignin. Thermodynamic properties and molecular weight determinations”. In: *Journal of Applied Polymer Science* 11 (1967), pp. 2381–2396.

[69] V. Pace et al. “2-Methyltetrahydrofuran (2-MeTHF): A biomass-derived solvent with broad application in organic chemistry”. In: *ChemSusChem* 5 (2012), pp. 1369–1379.

[70] P. M. Grande et al. “Fractionation of lignocellulosic biomass using the OrganoCat process”. In: *Green Chemistry* 17 (2015), pp. 3533–3539.

[71] D. Weidener et al. “One-step lignocellulose fractionation by using 2,5-furandicarboxylic acid as a biogenic and recyclable catalyst”. In: *ChemSusChem* 11 (2018), pp. 2051–2056.

[72] A. Morone, R. A. Pandey, and T. Chakrabarti. “Evaluation of OrganoCat process as a pretreatment during bioconversion of rice straw”. In: *Industrial Crops and Products* 99 (2017), pp. 7–18.

[73] T. Damm et al. “OrganoCat pretreatment of perennial plants: Synergies between a biogenic fractionation and valuable feedstocks”. In: *Bioresource Technology* 244 (2017), pp. 889–896.

[74] P. M. Grande et al. “OrganoCat fractionation of empty fruit bunches from palm trees into lignin, sugars, and cellulose-enriched pulp”. In: *ACS Omega* 4 (2019), pp. 14451–14457.

[75] J. Viell et al. “Is biomass fractionation by Organosolv-like processes economically viable? A conceptual design study”. In: *Bioresource Technology* 150 (2013), pp. 89–97.

[76] J. Higgins et al. “Theoretical study of thermal decomposition mechanism of oxalic acid”. In: *Journal of Physical Chemistry A* 101 (1997), pp. 2702–2708.

[77] BioSC. *Improve maize biomass for processing applying OrganoCat technology*. 2018. URL: `https://www.biosc.de/iBiomass_en`. (Accessed: 15.10.2019).

[78] M. Lever. “A new reaction for colorimetric determination of carbohydrates”. In: *Analytical Biochemistry* 47 (1972), pp. 273–279.

[79] H. Kim and J. Ralph. “Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d 6/pyridine-d5”. In: *Organic and Biomolecular Chemistry* 8 (2010), pp. 576–591.

[80] K. Cheng et al. “Solution-state 2D NMR spectroscopy of plant cell walls enabled by a dimethylsulfoxide-d6/1-ethyl-3-methylimidazolium acetate solvent.” In: *Analytical chemistry* 85 (2013), pp. 3213–21.

[81] AIST. *Spectral database for organic compounds SDBS*. URL: `https://sdbs.db.aist.go.jp/sdbs/cgi-bin/cre_index.cgi`. (Accessed: 22.10.2019).

[82] S. K. Bharti and R. Roy. “Quantitative 1H NMR spectroscopy”. In: *Trends in Analytical Chemistry* 35 (2012), pp. 5–26.

[83] M. Rosa et al. “The maize mid-complementing activity homolog cell number regulator13/narrow odd dwarf coordinates organ growth and tissue patterning”. In: *Plant Cell* 29 (2017), pp. 474–490.

[84] M. S. Feather and J. F. Harris. “Dehydration reactions of carbohydrates”. In: *Advances in Carbohydrate Chemistry and Biochemistry* 28 (1973), pp. 161–224.

A Appendices

A.1 Calibration Curves

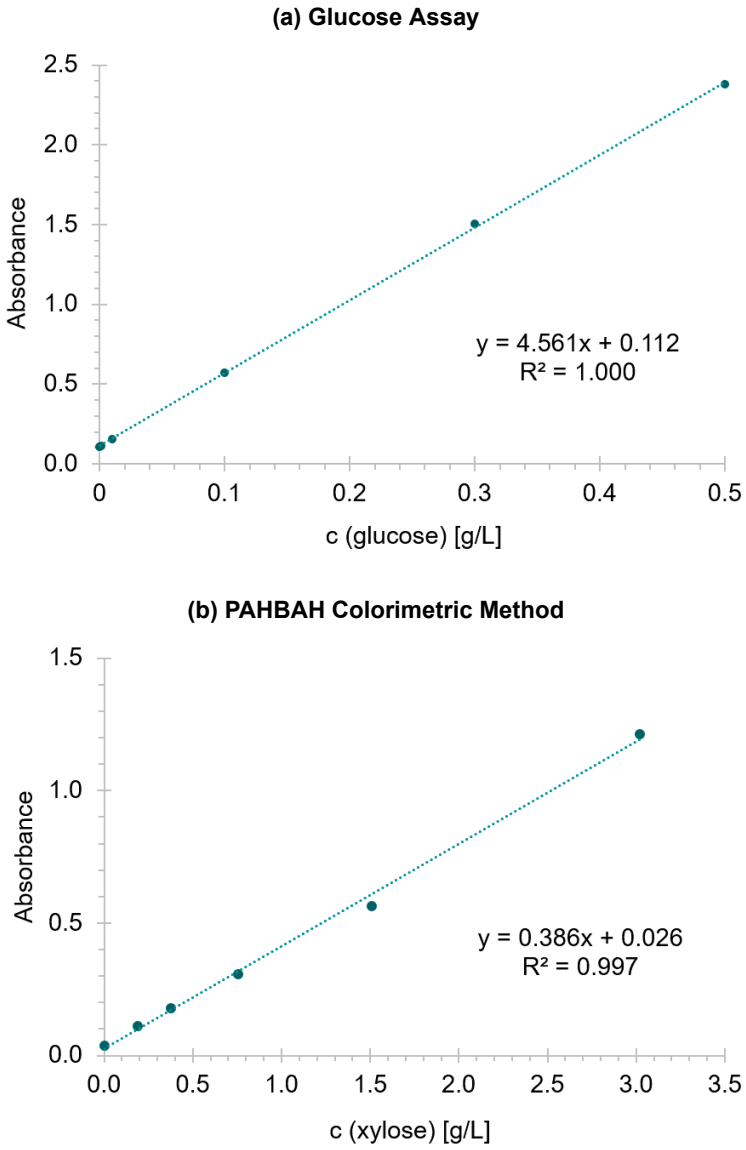

Figure A.1: Calibration curve for: (a) glucose (HK) assay from Sigma-Aldrich, built with commercial D-(+)-glucose; (b) PAHBAH colorimetric method, built with commercial D-(+)-xylose.

A.2 ^{1}H NMR Measurements

(a) Mesitylene **(b) 5-HMF** **(c) Furfural**

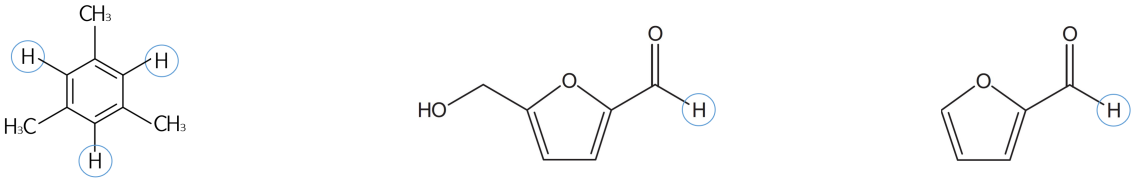

Figure A.2: Structure of (a) mesitylene, (b) 5-hydroxymethylfurfural and (c) furfural. The proton(s) responsible for the ^{1}H NMR (400 MHz, DMSO-d_6) signal analyzed for each compound is marked with a circle.

Table A.1: Information about the ^{1}H NMR (400 MHz, DMSO-d_6) signal analyzed for mesitylene, 5-HMF and furfural: the expected chemical shift (ppm) provided by the Spectral Database (mesitylene: 90 MHz, $CDCl_3$; 5-HMF and furfural: 300 MHz, $CDCl_3$), the range of chemical shifts observed and the number of protons responsible for the signal.

Compound	Chemical shift (ppm)		Number of protons
	Spectral Database [81]	Observed	responsible for signal
Mesitylene	6.78	6.75 - 6.78	3
5-HMF	9.53	9.54 - 9.56	1
Furfural	9.66	9.61 - 9.63	1

A.3 ^{1}H-^{13}C-HSQC NMR Measurements

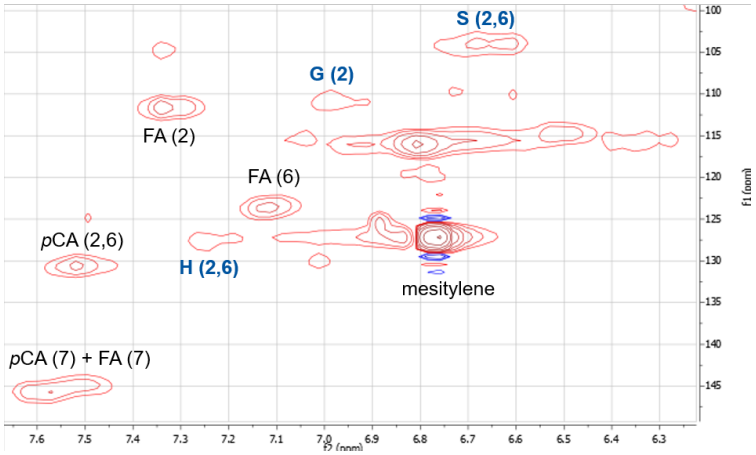

Figure A.3: Aromatic region of ^{1}H-^{13}C-HSQC NMR spectra with identification of the peaks corresponding to: syringyl (S), guaiacyl (G), *p*-hydroxyphenyl (H), ferulate (FA) and *p*-coumarate (*p*CA). The numeric identification of the carbon(s) responsible for the signal is in brackets. The only signals analyzed in this thesis were the ones correspondent to lignin monomer units (S, G and H), which are highlighted in blue. The x axis corresponds to the chemical shift (ppm) of the ^{1}H spectra and the y axis to the ^{13}C spectra.

A.4 Number of Replicates for OrganoCat Experiments

Table A.2: Number of replicates used to determine the average OrganoCat yields of the five product fractions analysed (cellulose-enriched pulp, sugar hydrolysate, lignin, furfural and 5-HMF) for each set of substrate-condition. As WT infected corn was only analyzed for the condition 140 °C + 1 h the number of replicates are not presented in this table.

Reaction conditions		**Uninfected corn**			**GMO infected corn**		
Time	Temperature	Pulp, Hydrolysate	Lignin	Furfural, 5-HMF	Pulp, Hydrolysate	Lignin	Furfural, 5-HMF
1 h	120 °C	4	3	1	4	3	1
	140 °C	4	3	1	4	3	1
	160 °C	6	4	2	6	4	2
3 h	120 °C	4	3	1	4	3	1
	140 °C	4	3	1	5	3	2
	160 °C	6	4	2	5	3	2

A.5 HPAEC *vs* PAHBAH Colorimetric Method

Table A.3: Comparison of sugar hydrolysate yield (wt%) determined by high pressure anion-exchange chromatography (HPAEC) with the one determined by PAHBAH colorimetric method for each set of substrate-condition.

Reaction conditions		**Uninfected corn**		**Infected corn**	
Time	Temperature	HPAEC	PAHBAH	HPAEC	PAHBAH
1 h	120 °C	5.3 ± 0.2	16.2 ± 0.6	8.2 ± 0.8	14.8 ± 4.2
	140 °C	10.9 ± 0.7	17.9 ± 2.3	10.6 ± 1.9	20.1 ± 3.4
	160 °C	13.5 ± 0.5	17.3 ± 2.3	13.1 ± 1.9	18.2 ± 2.7
3 h	120 °C	8.3 ± 0.3	22.2 ± 0.6	9.0 ± 1.0	16.7 ± 1.9
	140 °C	14.0 ± 2.3	23.9 ± 4.2	14.9 ± 2.9	21.2 ± 2.1
	160 °C	11.4 ± 1.0	13.8 ± 2.8	8.5 ± 1.2	9.4 ± 4.8

B Annexes

B.1 Sugar Relative Response to PAHBAH

Table B.1: Response of galacturonic acid, glucose, arabinose and galactose to PAHBAH colorimetric method relative to xylose. Data provided by Dr. Philipp Grande, Forschungszentrum Jülich GmbH.
*Galacturonic acid relative response was not determined, therefore it was assumed to be the same as xylose, according to [78].

Monosaccharide	**Relative response to PAHBAH (r)**
Xylose	1.00
Galacturonic acid	1.00*
Glucose	1.50
Arabinose	0.86
Galactose	0.70