Impact of *Ustilago maydis* Corn Infection on OrganoCat Pretreatment

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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.*

Introduction

Significant steps are being taken to move from today's fossil-based economy to a more sustainable economy based on biomass, namely by the development of biorefineries [1, 2]. 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 [3]. Lignocellulose is mainly composed of cellulose, hemicellulose, lignin and smaller amounts of pectin, protein, extractives and ash [1, 4].

Corn is the most demanded grain in the world and is among the fastest-growing in yearly volume [5, 6]. Nowadays, the annual corn production and demand is higher than 1 billion tons, surpassing rice and wheat in annual volume by more than 25% [6]. Corn stover is an agricultural residue categorized as a lignocellulosic biomass source and it is defined as the above-ground non-grain portion of the crop, which comprises the cob, leaves, husk and stalk of the corn plant [7, 8]. Studies in USA [9] estimate that between 105 to 117 million dry tons of corn stover can be sustainably collected per year.

In Mexico, corn plantations are wittingly infected with the plant pathogen fungus *Ustilago maydis*, which is the causal agent of the corn disease called smut [10]. The resulting corn smut galls are served as a typical Mexican food, called huitlacoche, that has been consumed by humans for centuries [10, 11]. 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 [10,11]. Even though the effects of smut disease in corn grain composition and nutritional value are well documented in literature [10], there is no information about the impact of smut infection in the composition of corn agricultural residues, neither the fate of such residues.

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 [12]. 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 [4,13–15]. Pretreatment methods can be divided into four categories: physical, chemical, physicochemical and biological [4, 16].

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 (Dominguez de Maria *et al.*, EP 2 489 780 A1) [17, 18]. OrganoCat pretreatment consists in subjecting lignocellulosic biomass to mild organic acid-

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catalyzed hydrolysis in a biphasic reaction mixture comprising an aqueous phase and an organic phase, composed of 2-methyltetrahydrofuran (2-MTHF). The mild conditions (125-140 °C, 2.5-3.5 hours) and the use of oxalic 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. According to reported assessments [19], the OrganoCat process may become economically feasible, as long as all fractions are pertinently valorized. Several plant materials have been successfully pretreated by OrganoCat process: beech wood [18, 20, 21], mate tea leaves and reed [20], rice straw [22], the energy plants Sida, Szarvasi, Silphium and Miscanthus [23], corn straw [23] and palm tree empty fruit bunch, EFB [24].

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. This work aims to evaluate the impact of corn leaves infected with *U. maydis* on OrganoCat pretreatment efficiency, compared to uninfected corn leaves.

Materials and Methods

Corn Leave Substrates

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). Corn seeds were planted in a greenhouse for one week and the plants were then injected with water, wild type or GMO *U. maydis*. After one or two weeks, the symptoms of the plant were analyzed. Then, the plants were left in the absence of light for 24 h in order to reduce starch. Finally, the leaves grown for three weeks were harvested. To obtain a powdered biomass, two different grinding methods were used: (1) the corn leaves were ground with a Retsch MM 400 ball mixer mill for 10 s at 30 Hz and then dried overnight at 60 °C; (2) the corn leaves were first dried at 60 °C and then ground with a Retsch RM 100 mortar grinder.

OrganoCat: Lignocellulose Fractionation

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. 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. The cellulose-enriched pulp was washed with MiliQ water until neutral pH and dried overnight at room temperature to constant weight.

If the intended analysis of the organic phase was to determine the total amount of degradation products (furfural and 5-hydroxymethylfurfural, 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.

Enzymatic Hydrolysis

An enzymatic hydrolysis with the commercial cellulase Accellerase© 1500 (Genencor) was performed to the raw substrates and to the cellulose-enriched pulps obtained from OrganoCat pretreatment. In 1.5 mL Eppendorf vials, 20 mg pulp were suspended in 1 mL citrate buffer (pH 4.5) 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 and 72 h. The hydrolysis was carried out in an Eppendorf Thermomixer Comfort at 50 °C and 750 rpm for 1 h or 72 h. Glucose concentration was determined by using a glucose (HK) assay kit obtained from Sigma–Aldrich and a BioTek Power Wave HT UV/Vis spectrometer. 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.

Lignin Quantification and Analysis

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 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. 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 (S), guaiacyl (G) and *p*-hydroxyphenyl (H).

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 OrganoCat.

Compounds Identification and Quantification: The compounds of interest were identified in NMR spectra by knowing the typical chemical shifts of their nuclei (^{1}H NMR - [25], HSQC NMR - [26]). The signal corresponding to the internal standard was integrated and normalised according to the number of protons giving rise to the signal, the integrals of the compounds of interest (furfural and 5-HMF) were then compared with those of the mesitylene and their mass was determined.

Lignocellulose Compositional Analysis

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. Cellulose content was determined via concentrated sulfuric acid hydrolysis, followed by anthrone colorimetric assay to quantify glucose formed during hydrolysis. Glucose mass was then converted to cellulose mass. Hemicellulose monosaccharides content was determined by high pressure anion-exchange chromatography (HPAEC). Before HPAEC, the raw substrates and the 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. These analysis were performed by Pauly *et al.* and further details about the methods can be consulted in [27].

Results and Discussion

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 [18], 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 [17,18], 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: (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.

To evaluate OrganoCat, five product fractions were quantified and converted to weight% (wt%) yields relative to the initial loading of biomass: cellulose-enriched pulp, hemicellulose sugar hydrolysate, 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.

Infected Corn: Wild Type *vs* GMO

Two different lines of corn infected with *Ustilago maydis* were studied: corn infected with wild type strain FB1xFB2 (WT infected corn) and corn infected with the genetically modified strain SG200 (GMO infected corn). Wild type strain FB1xFB2 is the pathogenic strain of the fungus obtained by mating haploid sporida FB1 with haploid sporida FB2 [28]. SG200 is a genetically modified strain of *U. maydis* that was designed for studies of dimorphism and mating genetic regulation [28]. 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 [28,29]. 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.

Raw Biomass

Raw WT infected corn is composed of 17.7% $\pm$ 0.2% cellulose, 10.4% $\pm$ 0.2% hemicellulose and 7.4% $\pm$ 0.4% while raw GMO infected corn is composed of 15.9% $\pm$ 1.8% cellulose, 9.7% $\pm$ 0.6% hemicellulose and 7.0% $\pm$ 0.6% lignin. GMO infected corn presents lower cellulose content than WT infected corn, 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. 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. This way, only one OrganoCat reaction condition was analyzed (140 °C + 1 h) for both substrates.

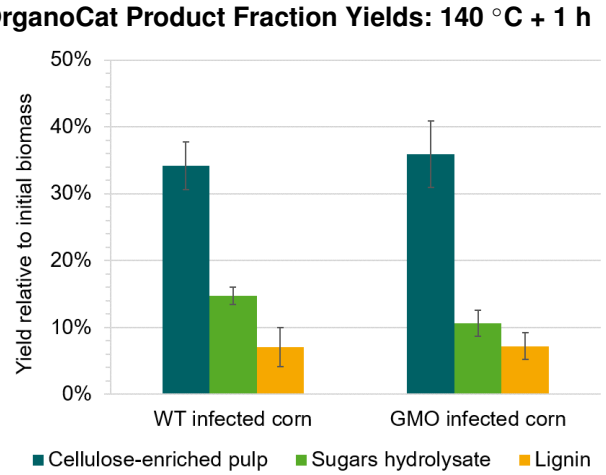

Figure 1: Yields (wt%), relative to the initial loading of biomass (100 g/L in the aqueous phase), of each product fraction (cellulose enriched-pulp, hemicellulose sugar hydrolysate and lignin) obtained applying OrganoCat to WT infected corn and GMO infected corn with the reaction condition 140 °C +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

Observing figure 1, GMO infected corn presents higher 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, 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 [30].

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. 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. Furfural and 5-HMF yields were 0.0% for both substrates.

Cellulose-Enriched Pulp Analysis

Compositional Analysis: Cellulose is observed to be the main mass component in the OrganoCat pulp of both substrates (figure 2). Compared to the raw substrate, 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

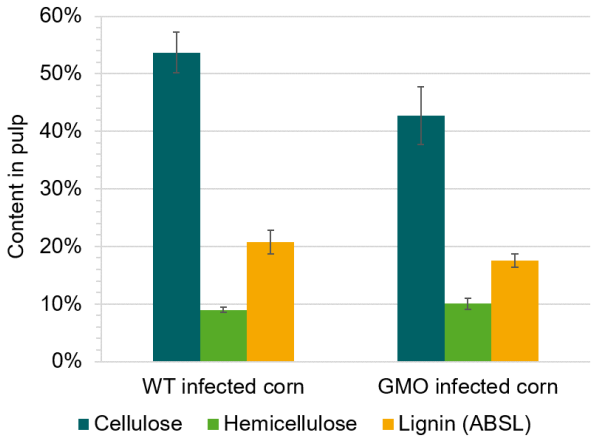

Figure 2: Cellulose, hemicellulose and lignin (ABSL) content (wt%) in cellulose-enriched pulps obtained applying OrganoCat pretreatment to WT infected corn and GMO infected corn with the reaction condition 140 °C + 1 h.

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, 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).

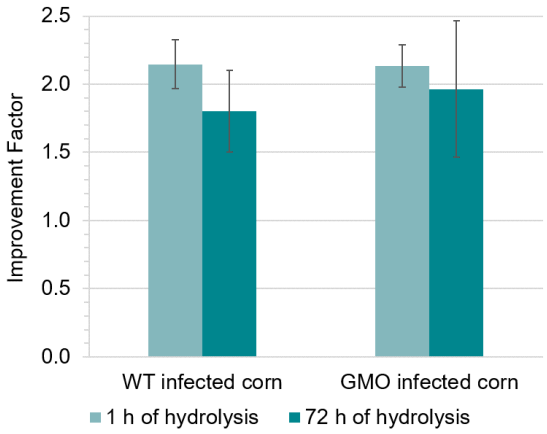

Figure 3: Improvement factor (comparing to raw biomass hydrolysis) of glucose concentration obtained after 1 h and 72 h of enzymatic hydrolysis (Accellerase© 1500, 50 °C) of the cellulose-enriched pulps obtained applying OrganoCat pretreatment to WT infected corn and GMO infected corn with the reaction condition 140 °C + 1 h.

Enzymatic Hydrolysis: Observing figure 3, the improvement factor (IF) of glucose concentration 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.

To conclude, no significant differences between the raw substrates were observed, however 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.

Uninfected Corn *vs* Infected Corn

Raw Biomass

Raw uninfected corn is composed of 20.0% ± 1.5% cellulose, 7.9% ± 0.6% hemicellulose and 5.9% ± 0.7% while raw infected corn is composed of 15.9% ± 1.8% cellulose, 9.7% ± 0.6% hemicellulose and 7.0% ± 0.6%. Raw infected corn presents lower cellulose content and higher hemicellulose and lignin content compared to raw uninfected corn. 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.

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), since 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 [31]. 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. Moreover, 52%-54% of the raw biomass corresponds to components removed during destarched alcohol-insoluble residue (dAIR) preparation which includes 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.

OrganoCat Product Fraction Yields

The figures 4a and 4b 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.

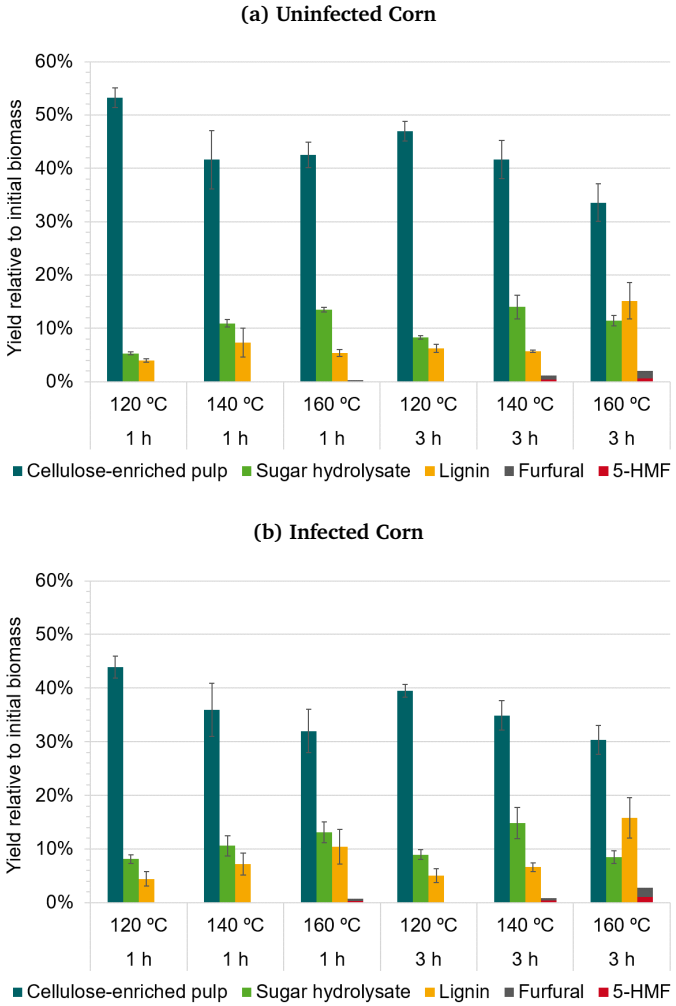

Figure 4: Yields (wt%), relative to the initial loading of biomass (100 g/L in the aqueous phase), of each product fraction (cellulose enriched-pulp, hemicellulose sugar hydrolysates and lignin) and degradation products (furfural and 5-HMF) obtained applying OrganoCat with 6 different reaction conditions (presented bellow the bars) to (a) uninfected corn and (b) infected corn.

Cellulose-enriched pulp: This product fraction 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. 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.

For infected corn, 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. 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.

Hemicellulose sugar hydrolysate: The depolymerization of hemicellulose was monitored via the formation of soluble sugars extracted to the aqueous phase, which were quantified by high pressure anion-exchange chromatography (HPAEC). 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: 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 destarched alcohol-insoluble residue (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.

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.

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: 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 [32]. In a recent paper [32], 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 [33] 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).

Lignin: In this work, 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. 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% ± 3.4%). 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% ± 3.8%) at the harshest condition.

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, 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.

Cellulose-Enriched Pulp Analysis

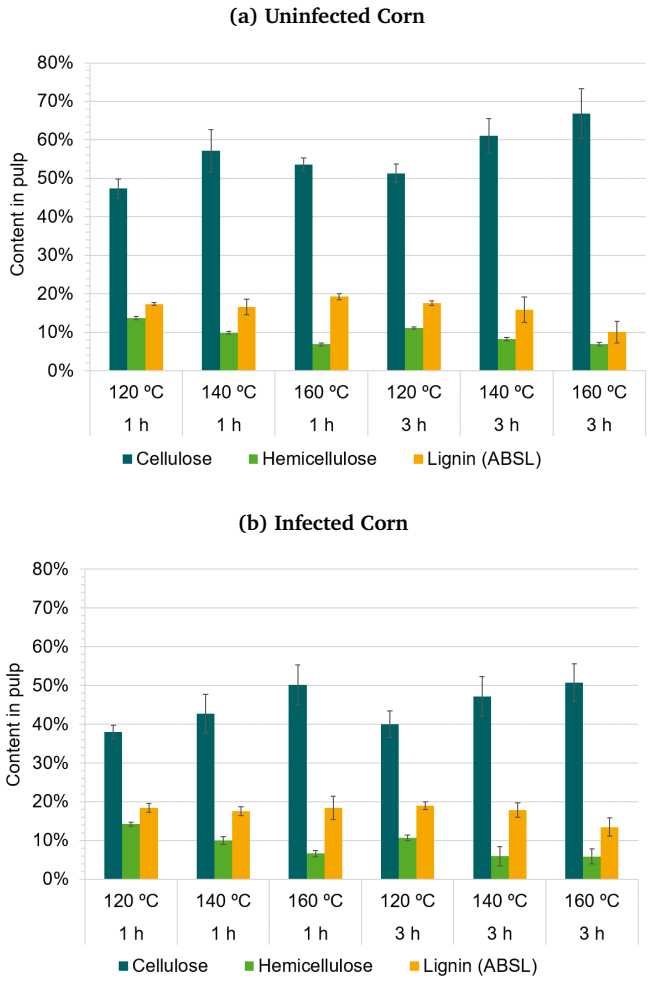

Figure 5: Cellulose, hemicellulose and lignin (ABSL) content (wt%) in cellulose-enriched pulps obtained applying OrganoCat with 6 different reaction conditions (presented bellow the bars) to (a) uninfected corn and (b) infected corn.

Compositional Analysis: The analysis of the pulp composition (figure 5) 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, 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.

Comparing both substrates, uninfected corn pulps present higher cellulose content than infected corn pulps, however the increase of cellulose content in pulp compared to the raw biomass was similar for both. 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.

Enzymatic Hydrolysis: Observing figure 6, the improvement factor (IF) of glucose concentration 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.

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.

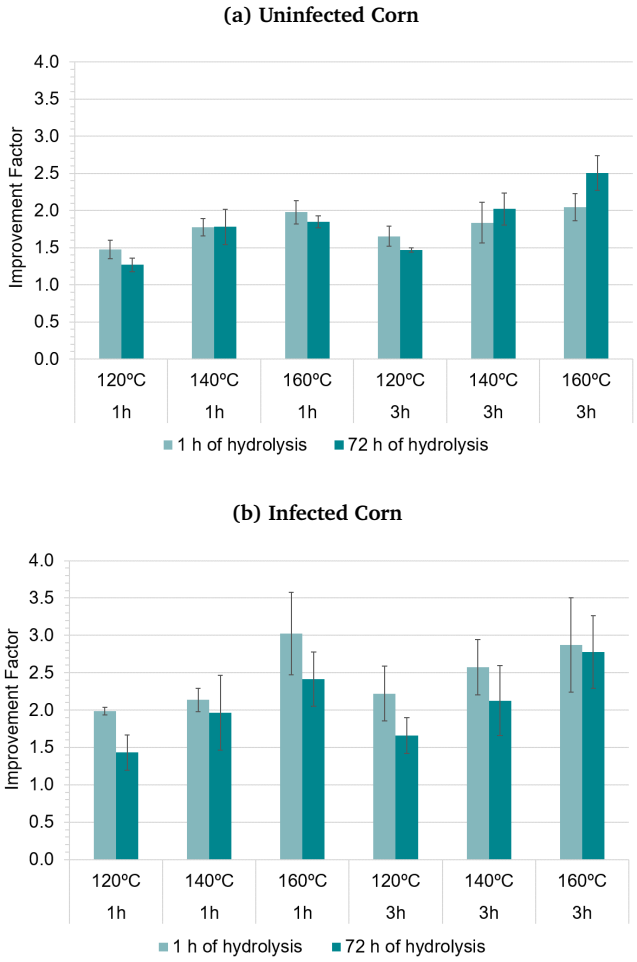

Figure 6: Improvement factor (comparing to raw biomass hydrolysis) of glucose concentration obtained after 1 h and 72 h of enzymatic hydrolysis (Accellerase© 1500, 50 °C) of the cellulose-enriched pulps obtained applying OrganoCat with 6 different reaction conditions (presented bellow the bars) to (a)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

Lignin Analysis

In uninfected corn lignin (figure 7a), for the mildest reaction condition (140 °C + 1 h), S and G units present similar ratios. 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.

In infected corn lignin (figure 7b), for the mildest reaction condition, S and G units present similar ratios. 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. 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

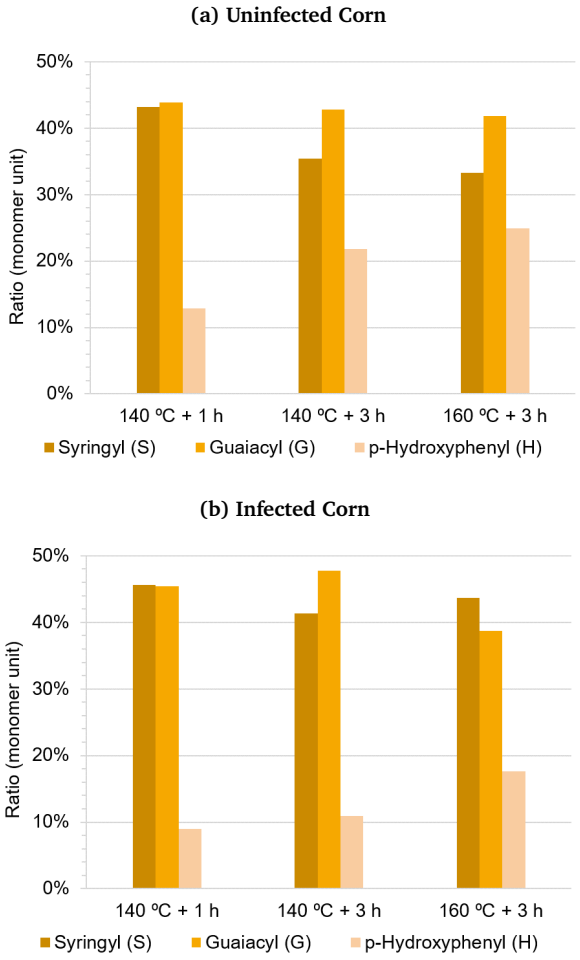

Figure 7: Normed ratio of each lignin monomer unit (syringyl, guaiacyl and *p*-hydroxyphenyl) in the lignin fraction obtained by applying OrganoCat pretreatment with the reaction conditions 140 °C + 1 h, 140 °C + 3 h and 160 °C + 3 h to (a) uninfected corn and (b) infected corn. Only one replicate per condition was analyzed.

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 analyzed.

For both substrates, an increase 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.

Conclusions and Future Work

The main differences in corn leaf composition caused by *U. maydis* infection were a decrease in cellulose content, while increasing hemicellulose and lignin content. 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.

After screening six OrganoCat condition sets, it was observed that increasing reaction temperature and time leads to a decrease in cellulose-enriched pulp yield, while sugar hydrolysate and lignin extraction yields increase 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. 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. For both substrates, the improvement factor (IF) of the enzymatic hydrolysis was always higher than 1.0, which suggests that OrganoCat pretreatment leads to an enhancement of the enzymatic hydrolysis. Moreover, for both substrates, increasing reaction temperature and time lead to an increase in IF values due to enhanced amorphization and delignification of the lignocellulose.

Analyzing OrganoCat lignin fraction, it was observed that increasing reaction temperature and time lead to a 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. Compared to uninfected corn, infected corn lignin fraction presents higher S unit ratios and lower H unit ratios for every reaction condition analyzed.

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).

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References

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

[2] Gírio, F., S. Marques, F. Pinto, A. C. Oliveira, P. Costa, A. Reis, and P. Moura: *Biorefineries in the world*. In *Lecture Notes in Energy*, volume 57, pages 227–281. 2017.

[3] Chen, H.: *Lignocellulose biorefinery engineering*. In *Lignocellulose Biorefinery Engineering*, pages 1–17. 2015.

[4] Kumar, P., D. M. Barrett, M. J. Delwiche, and P. Stroeve: *Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production*. Industrial and Engineering Chemistry Research, 48:3713–3729, 2009.

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

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

[7] Cherubini, F. and S. Ulgiati: *Crop residues as raw materials for biorefinery systems - A LCA case study*. Applied Energy, 87:47–57, 2010.

[8] Templeton, D. W., A. D. Sluiter, T. K. Hayward, B. R. Hames, and S. R. Thomas: *Assessing corn stover composition and sources of variability via NIRS*. Cellulose, 16:621–639, 2009.

[9] Graham, R. L., R. Nelson, J. Sheehan, R. D. Perlack, and L. L. Wright: *Current and potential U.S. corn stover supplies*. Agronomy Journal, 99:1–11, 2007.

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

[11] Feldbrügge, M., R. Kellner, and K. Schipper: *The biotechnological use and potential of plant pathogenic smut fungi*. Applied Microbiology and Biotechnology, 97:3253–3265, 2013.

[12] Jedrzejczyk, M., E. Soszka, M. Czapnik, A. M. Ruppert, and J. Grams: *Physical and chemical pretreatment of lignocellulosic biomass*. In *Second and Third Generation of Feedstocks*, pages 143–196. 2019.

[13] Capolupo, L. and V. Faraco: *Green methods of lignocellulose pretreatment for biorefinery development*. Applied Microbiology and Biotechnology, 100:9451–9467, 2016.

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

[15] Alvira, P., E. Tomás-Pejó, M. Ballesteros, and M. J. Negro: *Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review*. Bioresource Technology, 101:4851–4861, 2010.

[16] Putro, J. N., F. E. Soetaredjo, S. Y. Lin, Y. H. Ju, and S. Ismadji: *Pretreatment and conversion of lignocellulose biomass into valuable chemicals*. RSC Advances, 6:46834–46852, 2016.

[17] Dominguez De Maria, P., T. vom Stein, P. M. Grande, W. Leitner, and F. Sibilla: *Integrated process for the selective fractionation and separation of lignocellulose in its main components*, August 2012.

[18] Stein, T. vom, P. M. Grande, H. Kayser, F. Sibilla, W. Leitner, and P. Domínguez de María: *From biomass to feedstock: One-step fractionation of lignocellulose components by the selective organic acid-catalyzed depolymerization of hemicellulose in a biphasic system*. Green Chemistry, 13:1772–1777, 2011.

[19] Viell, J., A. Harwardt, J. Seiler, and W. Marquardt: *Is biomass fractionation by Organosolv-like processes economically viable? A conceptual design study*. Bioresource Technology, 150:89–97, 2013.

[20] Grande, P. M., J. Viell, N. Theyssen, W. Marquardt, P. Domínguez De María, and W. Leitner: *Fractionation of lignocellulosic biomass using the OrganoCat process*. Green Chemistry, 17:3533–3539, 2015.

[21] Weidener, D., H. Klose, W. Leitner, U. Schurr, B. Usadel, P. Domínguez de María, and P. M. Grande: *One-step lignocellulose fractionation by using 2,5-furandicarboxylic acid as a biogenic and recyclable catalyst*. ChemSusChem, 11:2051–2056, 2018.

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

[23] Damm, T., P. M. Grande, N. D. Jablonowski, B. Thiele, U. Disko, U. Mann, U. Schurr, W. Leitner, B. Usadel, P. Domínguez de María, and H. Klose: *OrganoCat pretreatment of perennial plants: Synergies between a biogenic fractionation and valuable feedstocks*. Bioresource Technology, 244:889–896, 2017.

[24] Grande, P. M., D. Weidener, S. Dietrich, M. Dama, M. Bellof, R. Maas, M. Pauly, W. Leitner, H. Klose, and P. Domínguez de María: *OrganoCat fractionation of empty fruit bunches from palm trees into lignin, sugars, and cellulose-enriched pulp*. ACS Omega, 4:14451–14457, 2019.

[25] AIST: *Spectral database for organic compounds SDBS*. `https://sdbs.db.aist.go.jp/sdbs/cgi-bin/cre_index.cgi`.

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

[27] Rosa, M., M. J. Abraham-Juárez, M. W. Lewis, J. P. Fonseca, W. Tian, V. Ramirez, S. Luan, M. Pauly, and S. Hake: *The maize mid-complementing activity homolog cell number regulator13/narrow odd dwarf coordinates organ growth and tissue patterning*. Plant Cell, 29:474–490, 2017.

[28] Bölker, M., S. Genin, C. Lehmler, and R. Kahmann: *Genetic regulation of mating and dimorphism in Ustilago maydis*. Canadian Journal of Botany, 73:320–325, 1995.

[29] Brefort, T., G. Doehlemann, A. Mendoza-Mendoza, S. Reissmann, A. Djamei, and R. Kahmann: *Ustilago maydis as a Pathogen*. Annual Review of Phytopathology, 47:423–445, 2009.

[30] Ciolacu, D., F. Ciolacu, and V. I. Popa: *Amorphous cellulose - Structure and characterization*. Cellulose Chemistry and Technology, 45:13–21, 2011.

[31] Pordesimo, L. O., B. R. Hames, S. Sokhansanj, and W. C. Edens: *Variation in corn stover composition and energy content with crop maturity*. Biomass and Bioenergy, 28:366–374, 2005.

[32] Shi, N., Q. Liu, H. Cen, R. Ju, X. He, and L. Ma: *Formation of humins during degradation of carbohydrates and furfural derivatives in various solvents*. Biomass Conversion and Biorefinery, 2019.

[33] Feather, M. S. and J. F. Harris: *Dehydration reactions of carbohydrates*. Advances in Carbohydrate Chemistry and Biochemistry, 28:161–224, 1973.