University of Groningen

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Master Thesis

5-HMF production by enzymatic hydrolysis of biomass and heterogeneous catalysis with a Nb_2O_5 catalyst

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Abstract

The Cellic CTec2 enzyme is able to do enzymatic hydrolysis on canal grasses and rice straw in atmospheric conditions, under supercritical CO_2 conditions as well as after being treated in supercritical CO_2 conditions. The maximum yield for canal grasses was reached in atmospheric enzymatic conditions, while the maximum yield for rice straw was reached in supercritical CO_2 conditions. The maximum yields were 85% of both glucose and xylose from rice, 75% and 65% of the glucose and xylose in grasses. For regular enzymatic hydrolysis in atmospheric conditions, a buffer solution with pH = 5.0 seems to be a better solvent than water, while for supercritical enzymatic hydrolysis water seems to the better solvent.

Enzymatic hydrolysis in supercritical co2 conditions, was not effective for the canal grasses. It was however effective for rice, 50% to 85% for both glucose and xylose could be removed from the pellet. The reasoning for the difference in the effectivity could be the difference of structure between the two types of biomass or the negative effect that the supercritical CO_2 has on the lignocellulosic structure of the grass.

The Nb_2O_5 catalyst was synthesized in a novel supercritical CO_2 method with a surface are of $325m^2/g$. A benchmark reaction of glucose was able to reach a 40% 5-HMF with a turnover number of 0.19 at 120°C and a reaction time of 3h. The supernatant from the enzymatic hydrolysis as starting reagent could not reach the same values. Although some of the yields were in the range of the benchmark reaction, the turnover number was lower than the benchmark reaction. The amount of glucose in the supernatant was also lower, up to 100 times as low as the benchmark reaction.

Combining the enzymatic hydrolysis with the subsequent catalysis all under supercritical CO_2 conditions was not successful at this stage, no 5-HMF was found in the mixture after reaction. This is most likely caused by the biomass blocking the surface area of the catalyst by the solid biomass.

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Introduction

The need for renewable resources for our energy consumption and chemicals is growing by the day, as the current usage of fossil fuels is at an alarming rate of 70 billion barrels of oil annually [1], which can lead to irreversible environmental effects with great impact on human and animal life [2].

In 2015 the Paris climate change conference was held in which the United Nations and its members agreed upon keeping the global annual average temperature increase below 2°C [3]. This climate change conference has been held before with as prime example Kyoto in 1997, where the reduction of greenhouse gas emission was firstly agreed upon by a large majority of the developing countries. Unfortunately, the targets set by the Kyoto convention were not met, which brings doubts over the agreement signed in Paris and its credibility [3].

Decreasing the utilization of fossil fuels is the only option, since we get a great part of the carbon based products such as plastic and cosmetic from fossil fuels [4]. To establish a society which solely uses sustainable materials without decreasing our quality of life, vital alternatives have to be found. Renewable sources such as biomass from grasses, woody plants and agriculture crop residues are the key to solving these issues[5].

Chapter 1

Theoretical background

1.1 Biomass

Due to the need for utilization of renewable sources, biomass is therefore coined as solution for both the energy consumption as well as the production of chemicals. The production of syngas, a mixture of H_2 and CO, is currently done by the means of fossil resources but can also be produced by the means of biomass [6].

An ethical question to be raised is the competition of food versus energy, should we be using biomass for other purposes than for human consumption. It is therefore important to note that this thesis has its focus on so called second generation biomass, i.e. biomass that is not used for consumption. There are several sources for this type of biomass, agricultural waste is a prime example of this [7].

The structure of this type of biomass can be classified as lignocellulosic biomass which has polysachharides celullose, a polysaccharide of chains of glucose residues, and hemicellulose consisting of xylan chains, which is not a structure as simple as cellulose. The rest of composition can be accounted to lignin, a complex aromatic polymer. While there is a huge field which deals with extraction of compounds from lignin and other methods of utilization, this thesis will focus on obtaining the hexoses and pentoses from the cellulose and hemicellulose. The ratio of the cellulose, hemicellulose and lignin differs per biomass. Some might be richer in cellulose while others will have more hemicellulose. The type of biomass to be selected has to be widely available and its composition should be suitable for obtaining hexoses and pentoses, i.e. containing a large quantity of cellulose and hemicellulose. These hexoses and pentoses can be converted to building blocks or so called platform chemicals, specifically 5-hydroxymethylfurfural (5-HMF) and furfural. With these classifications, especially for its large availability, rice straw as well as canal grasses were selected as potential suitable biomass.

1.1.1 Utilization of Biomass

As the fossil resources are depleting, the need for sustainable routes to everyday chemicals is growing every day [1].The effective use of biomass comes into play as a solution for creating sustainable chemical routes. Biomass is commonly fermented to ethanol but there are many more options to harvest valuable chemicals. An overview of the options can be seen in figure 1.1.

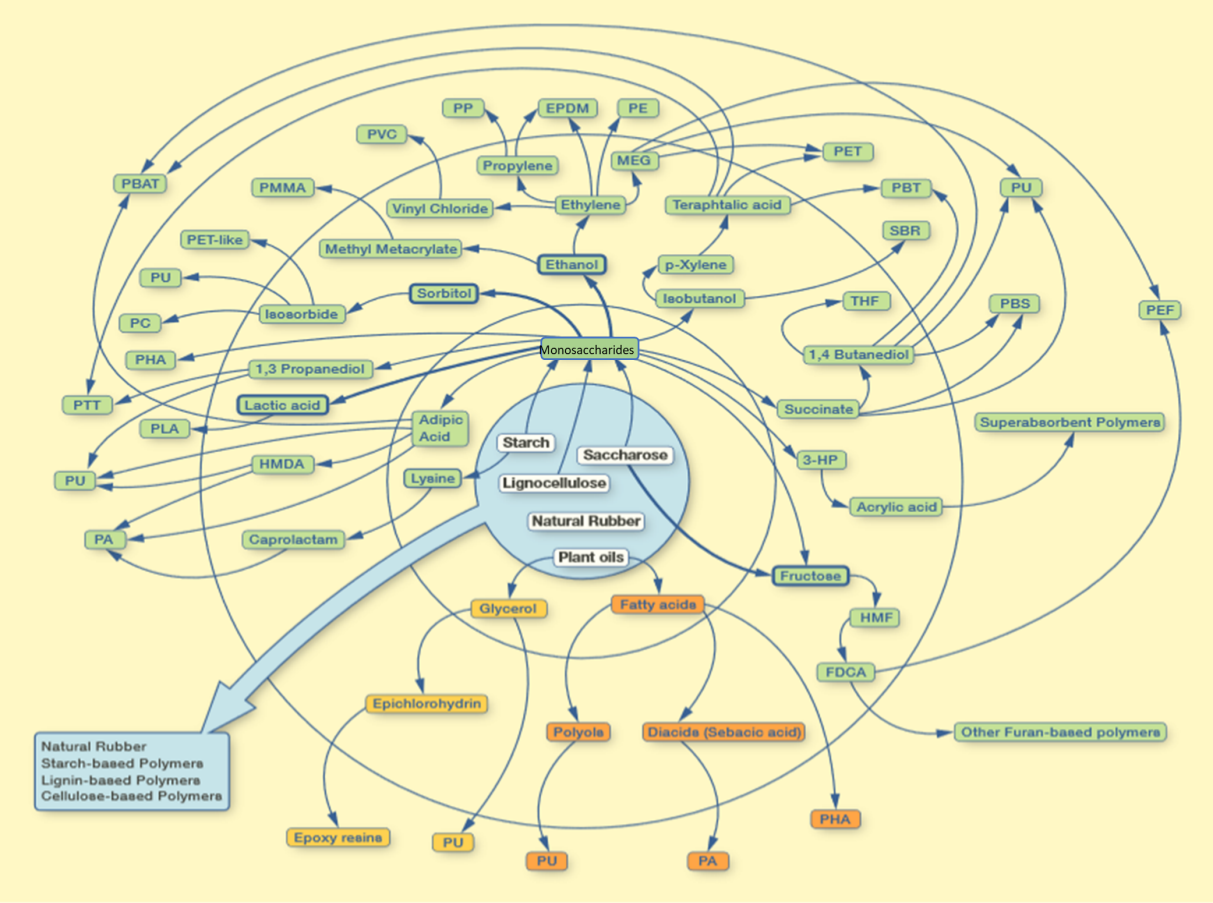

Figure 1.1: Scheme of valuable products that can be obtained from lignocellulosic biomass modified from [8].

1.2 Rice straw

Rice is produced on a large scale worldwide. Approximately 112 countries grow rice, making it an important food source for almost half of the total world population [9]. 90% of the rice is grown in Asia, especially in large countries such as China and India [9]. As the world population will continue to increase, the rice production is expected to reach 450 million annual tons in 2020 [10]. As the production of rice increases, so does the amount of agriculture waste that comes along with it. Nowadays most of this agriculture waste is burned, while it can be utilized in better ways than plainly burning it. Unfortunately, this is still common practice in rice producing countries [11]. In Thailand, another country where the major agriculture product is rice, 90% of the straw is burned with a total volume of approximately 9 million hectare annually. Burning of the straw is frequently done in the field, where due to the incomplete combustion a large amount of toxic fumes are emitted [11]. The biomass of rice which is interesting are the husks and the straws of the rice. The rice straw is the part which is left on the land after the rice has been harvested. The husk of the rice is the outer layer of the seed, which is obtained after processing the rice. The estimated value of both of these sources of biomass is 685 and 178 million tons annually for rice straws and husks respectively [9].

Rice straws are relatively easy to collect, as the biomass is left on the field to be collected. The rice husk on the other hand has to be processed in an industrial environment

to be effectively collected. Rice straw is regarded as an important source for many green alternatives such as organic chemical and bio-ethanol [12]. In the thesis, rice straw will be referred to as rice.

1.3 Canal grasses

Canal grasses was the other selected source of lignocellulosic biomass as it is an abundant material in the Netherlands. Having a local source as a potential biomass material can show that using a source that is very abundant can be used as well for enzymatic hydrolysis and further valorization. The canal grasses will be referred to in the results section as "grass".

1.4 Saccharification of biomass

Utilization of biomass can proceed in different ways, one of which is the saccharification of the plant material, specifically the enzymatic saccharification. This can then form the basis for the production of platform chemicals, which is very similar to a regular refinery but with biomass instead of crude oil. At first the biomass is, with use of enzymes, broken down into monosaccharides such as glucose, xylose and arabinose. Subsequent processing, such as heterogeneous catalysis, results into the production of platform chemicals. This approach is a green and renewable pathway to platform chemicals which has the potential to substitute the production of these chemicals from petrochemicals partially if not all together.

Multiple sources of biomass were selected in order to compare the effect of the pretreatment options on different sources.

1.5 Structure and composition of biomass

Lignocellulosic biomass primarily consists of three structures, cellulose, hemicelluloses and lignin along with smaller structures such as proteins and ash [13]. The distribution of these structures as well as the amount differs for different species of plants [13]. The sugars that can be obtained from these structures also differ, glucose is mainly obtained from cellulose, while xylose, arabinose and mannose are found in hemicellulosic structures. A schematic overview of the stucture of lignocellulosic can be seen in 1.2.

1.6 Complexity of inter linkages

1.6.1 Cellulose

Cellulose is a polysaccharide composed of D-glucose which are linked together by β-1,4-glucosidic bonds. The obtained structure is linear, thus allowing the chains to form inter- and intramolecular hydrogen bonds, which results in a crystalline structure. The structure makes it insoluble in most solvents, and resistant to microbial degradation [13]. The amorphous regions are more susceptible to enzymatic hydrolysis as the structure does not have the interactions that a crystalline structure has, which allows the enzymes to have better access to this backbone.

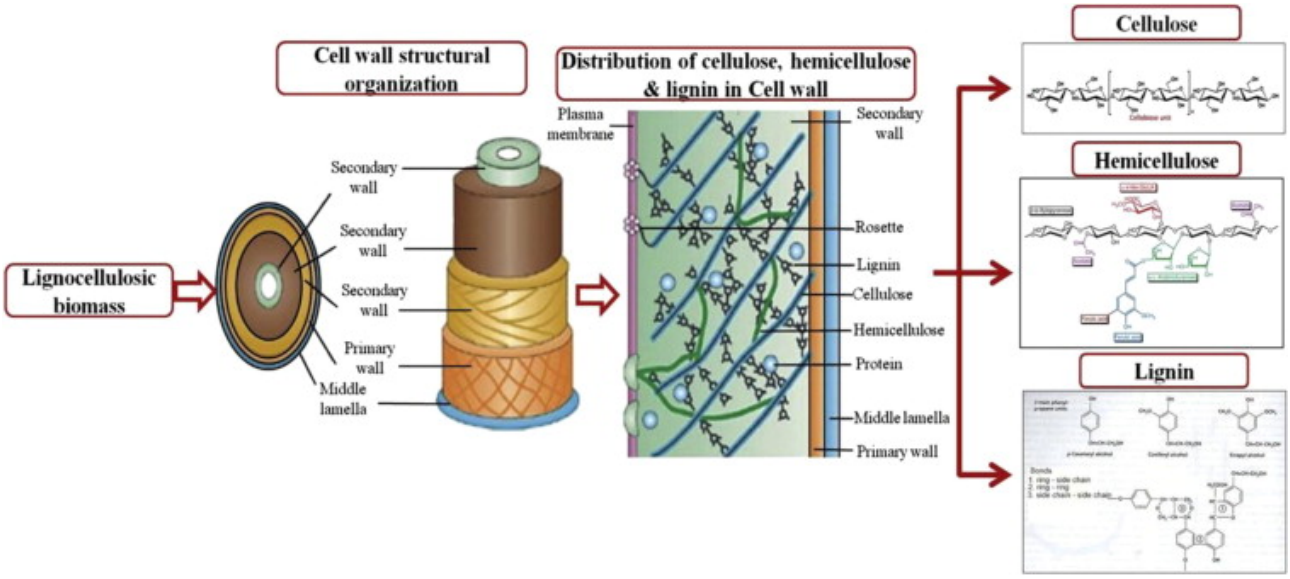

Figure 1.2: Schematical view of lignocellulose composition adapted from [14].

1.6.2 Hemicellulose

Hemicellulose consists of heteropolysaccharides composed of D-xylopyranosyl redidues. While cellulose is mostly a linear polysaccharide, hemicellulose is quite branched and is more amorphous than cellulose. Between different plant species, the structure and composition of hemicellulose varies [13]. In grasses, the structure mostly consists of arabinoxylan, which is a polyssacharide build up out of D-xylose monomers [15]. It has various side groups, esterified acetyl groups and arabinose groups can be found frequently. Xylan substituents can also create linkages with between itself as well as with other structures such as lignin. The linkages are made by the presence of ferulic acid in the xylan backbone, an ester linkage is formed between ferulic acid and an arabinofuranosyl residue [16]. The linkages formed with lignin are generated by ferulic acid as well, the phenolic compounds will link together by oxidative coupling. These interactions are referred to as Lignin Carbohydrates Complexes (LCC) [16].

1.6.3 Lignin

Lignin is a heteropolymer consisting of hydroxycinnamyl alcohols, resutling in a polymer that is very rigid [17]. As cells grow, lignin is deposited together with cellulose and hemicellulose primarily in the secondary cell wall Lignin, with its stable aromatic structure has interactions with cellulose and hemicellulose which make the structure even more recalcitrant, protecting the cellulose from (enzymatic) hydrolysis [17].

The structure of the biomass, with the specific elements explained in the previous section, lead to a recalcitrant nature. Protection of cellulose by lignin, intermolecular interactions in crystalline cellulose, specifically the hydrogen bonding between adjacent cellodextrin chains, cause the cellulose biopolymer to be resistant towards enzymatic hydrolysis. Cellulose is also embedded into lignin and hemicellulose networks, which proves to be another hindrance for the cellulose accessibility [5], [17]. As the composition of lignin, cellulose and hemicellose is different between different types of biomass, the hydrolysis of these different types will have other optimal conditions in terms of pH, pretreatment method etc.

1.6.4 Rice straw

The composition of rice straw consists mostly of cellulose and hemicellulose [12]. In terms of percentage dry matter it is roughly 35% and 29%, for cellulose and hemicellulose, respectively. Lignin composes of approximately 11% of the dry matter, the remaining dry matter consists of ash [12]. The xylan in rice-straw is an arabinoglucuronoxylan composed of the following groups: o-xylopyranose, L-arabinofuranose, Ferulic acid, o-glucopyranuronic acid, and 4-0-methyl-o-glucopyranuronic acid. The main chain of arabinoglucuronoxylan consists (1⟶4)-linked-1-o-xylosyl residues to which α-L-arabinofuranosyl stubs are attached at the O-3, and α-D-glucuronosyl or 4-0-methyl-α-D-glucuronosyl stubs at the 0-2 of the xylosyl residues of the main-chain [18].

1.6.5 Canal grasses

The canal grasses belong, just like the rice, to the poaecea family. Although they belong to the same family, the composition and the structure is not exactly the same. This can already be seen from the initial sugar composition, not only do the canal grasses contain more sugars overall as can be seen in figure 4.1, the ratio of the sugars is different. The lignocellulosic structure will therefore most likely differ from the rice, and the pretreatment methods and the enzymatic hydrolysis will as a result have different outcomes and effects.

1.7 Pretreatment of biomass

To combat the recalcitrant structure of the biomass, pretreatment it a necessity to obtain high yields of sugars from the polyssaccharides [5]. The effectivity for a pretreatment differs from the biomass it is used on, there is no single method that works on every type of biomass. The pretreatment is used to break lignin and polyssacharide crosslinks in order to reduce the crystallinity of the cellulose. This will result into a better accessibility of the enzymes [5]. There are different types of pretreatments, an overview can be found in table 1.1.

Table 1.1: An overview of frequently used pretreatment options, adapted from [5]

Category	**Pretreatment options**
Mechanical	Milling, steam explosion, hot water
Chemical	Acid treatment, alkaline treatment

Mechanical pretreatment options are usually more cost effective, while chemical pretreatment options result into higher yields but in the process chemical waste is produced. In the following section, the pretreatments used in this thesis will be introduced.

1.7.1 Supercritical CO_2

$CO_2$is a waste chemical in many processes in industry, most of which is released directly into the atmosphere [5]. It is an excellent chemical to be used as a supercritical solvent, as its critical point (T_c = 31.0, P_c = 73.9 bar) is relatively low compared to other solvents such as water [5]. Supercritical CO_2 (SC-CO_2) is primarily used as an extraction solvent but has recently gained some attention in other applications [19]. CO_2

is, compared to chemicals used in its stead, inexpensive and clean. The effect of SC-CO_2 is disputed, some claim that is has no effect on the structure of biomass, while others claim it changes the shape of lignocellulose to great extend by breaking the crystalline structure [19]. Using SC-CO_2 along with an aqueous buffer proved to increase the glucose release in cellulose type materials [19]. The mechanisms on how supercritical CO_2 is effective as a pretreatment can be divided into three parts. The first part is that the supercritical fluid penetrates the pores of the biomass as a results of the high pressure. As the pressure is released, the CO_2 ruptures the pores and surface area of cellulose is increased as a result [5], [20]. Secondly, the supercritical CO_2 loosens the crystallinity of the cellulose [21], but this claim has been disputed. The crystallinity of pure cellulose was indeed shown to have been lowered by the CO_2, but in a lignocellulosic biomass this was not the case. This was explained by the complexity of cellulose crystalline structure in lignocellulosic biomass, which is partially embedded in lignin, glycoproteins and hemicelluloses [22]. The third mechanism is that the CO_2 forms carbonic acid with the moisture residing in the biomass, increasing the acidity [20]. Pretreamtent using supercritical CO_2 seems to be most effective with a higher moisture content of the biomass, as more carbonic acid will be formed [22].

The SC-CO_2 will also have an effect when used with a aqueous solvent, as it will lower the pH of the liquid as it dissolves. Henry's law, which relates the partial pressure to a gas predicts the concentration in a liquid. This formula is shown in equation 1.1.

$$C = HP_g \tag{1.1}$$

With C being the concentration of the gas in the liquid, H being the Henry's solubility constant and P_g being the partial pressure of the gas.

By combining Henry's law with the pKa of carbonic acid, the pH of the water can be approximated. Henry's law however, required a dilution solution and ideal behaviour of the gas [23]. With supercritical CO_2 this is of course not the case. The conditions of the supercritical CO_2 cannot be called ideal to say the least, which is why Henry's law might not be applicable. Literature states that the pH of water under supercritical CO_2 is slightly lower than predicted. For a range of pressures of 70 - 200 atm CO_2 and a temperature range of 25-70°C, the pH was in the range of 2.80 - 2.95 [23]. This is between 0.2 and 0.3 lower than the predicted pH using the pKa of carbonic acid in combination with Henry's law.

By altering the pressure and the temperature, the density of the supercritical CO_2 changes but also its effectiveness as a pretreatment. Increase of the pressure results in a more powerful release, resulting into better penetration of the CO_2 and thus in a higher surface area. Increasing the pressure leads to higher yields in the subsequent enzymatic hydrolysis, but this effect increases differently for different biomass [5]. In some the yield increases by 50% when the pressure is doubled, while others increase by 5% with the same pressure increase [5]. Another case had its yield decreased when the pretreatment pressure was raised from 215 bar to 275 bar [5]. This phenomena, a decreasing yield as the pressure increases, is most likely caused by solubility of water in supercritical CO_2 [20]. As a result, the swelling of the biomass will be reduced.

Increasing the temperature will also increase the effectiveness of the pretreatment. This increase of the effectiveness depends just as the pressure on the type of biomass. For switchgrass for example the yield did not increase as the temperature was raised, but for corn stover it did [22]. The temperatures of the treatment are often higher than the of the critical point (31.0°C), in the range of 100-150°C. Higher temperatures will attribute

to thermal degradation, especially in the hemicellulose [20]. At low temperatures(50-80°C), in order to achieve similar effects to higher temperatures, a longer residence time (12-60h) is necessary [20].

1.7.2 Liquid hot water treatment

Pretreating biomass using hot water (T > 120°C) is effective in increasing the surface area of the biomass. A liquid hot water treatment is done by loading biomass with water into a closed chamber. This is subsequently heated to a desired temperature in the range of 140 - 180°C. Because the chamber is closed, pressure will build up in the system but is unable to release. This results in a volatile mixture inside the chamber, causing the structure of the biomass to crack which leads to more surface area [24].

1.7.3 Milling

Milling as a pretreatment option, is very attractive as it does not require any chemicals and it can be regarded as environmentally friendly [25]. Ball milling results into the pulverization of biomass, and reduces the crystallinity of cellulose. The enzymatic digestibility was subsequently improved by this reduction in crystallinity. The increase of the degestibility is measured by the yield of enzymatic hydrolysis, for which several different enzymes are used in literature. Increasing the milling time resulted into a higher yield of glucose and xylose. The increase of the yield stagnates as the milling time is increased, and this turning point is different for different types of biomass [25].

1.8 Cellic CTec2

Cellic CTec2(Novozymes A/S, Basgsvaerd, Denmark) is a commercial enzyme cocktail and is used to saccharify lignocellulosic biomass. Its activity and performance for hydrolysis of polysaccharides has been studied such as in [26]. The optimal conditions for CTec2, as described by novozymes and literature, is a temperature of 50°C and a pH of 5.0. [26]. Using a pH in the range of 4.8 - 5.0 is very common to be used on lignocellulosic substrates, but this might not always yield the best results [27]. For wheat straw, a lignocellulosic biomass, it was found that CTec2 yields the best results when the pH range is 5.8-6.0 [27]. Abdulsattar et al. attribute the better performance due to less inhibition of the enzyme by the lignin or by affecting the lignin cellulose binding. The pH optimum as provided by literature and the manufacturer are based on pure cellulose, which is why the pH optimum for lignocellulosic biomass differs. In this thesis, a sodium acetate buffer with a pH of 5.0 as well was water were used as solvents for the enzymatic hydrolysis.

1.8.1 Enzymatic hydrolysis

Enzymatic hydrolysis is, as the name states, hydrolysis by the means of enzymes. Different type of enzymes are used together to hydrolyze the different types of biomass structures [13]. Cellulose is treated with enzymes which cleave different parts of the cellulose. One class cleaves celliobiose units from the ends, another hydrolyses internal 1,4-glucosidic bonds in the chain and the last class which hydrolyse the obtained cellobiose

to glucose [13]. Hemicellulose is more complex due to various side groups, for which other classes of enzymes are used [13].

Although great improvements have been made in terms of efficiency and rate of enzymes, drawbacks still exist which has consequences for the overall yield. Product inhibition of the enzymes leads to a slower reaction [13]. Strategies have been developed to overcome these issues, by screening enzymes which are more resistant to higher sugar concentration for example [13]. Product removal by a subsequent reaction is also a possibility, fermentation of the formed glucose to ethanol is an example of this very strategy. Drawback of this method is that subsequent reaction might not have the same optimum reaction conditions. Enzymes such as cellulases work best at a temperature of 50°C while bacteria used for fermentation of glucose work best at a temperature of 37°C [13]. This problem might also arise for different subsequent reactions of the obtained sugars such as the production of 5-HMF. Enzymes that have a broader operation range are therefore preferred for this strategy, as this leads to a larger window of opportunity.

1.8.2 Enzymes in super-critical CO_2

The performance of enzymes in super-critical CO_2, which can differ in both temperature and pressure, has been tested for several enzymes, which were mostly lipases [28]. A couple of factors can be attributed to the improved or worsened performance of enzymes in super-critical CO_2. Pressure, pH and CO_2 inhibition are mentioned as reasons which influence the performance of the enzymes.

Increase of the enzyme activity occurs less than decrease of the enzyme activity by supercritical CO_2 [29]. Depressurisation is mentioned as the main cause of loss of activity, increasing the number of depressurization causes a gradual decrease of the activity [30]. The underlying theory of why certain enzymes will either benefit or not from treatment under supercritical CO_2 resides in the effect it has on the three dimensional structure of the protein [29]. While severe altercation of this structure will cause it to denature, small changes while keeping the structure largely the same can result into changes of stability and activity [31]. The change in structure may be a result from the CO_2 interacting with the free amino groups of the enzymes. CO_2 can form covalent complexes with the amino groups which affects the tertiary structure of the enzyme [32]. The supercritical CO_2 could also lower the activity by stripping water from the direct environment of the enzyme. As a minimum monolayer of water is necessary to activate the enzymes, removal of this monolayer results in lower activity. The supercritical CO_2 causes the water to partition between the mixture and the enzyme, thus lowering its activity [31].

Increase of activity is however, also a possibility. It has been shown that repetition of pressurization and depressurization with supercritical CO_2 increases the activity of certain lipases [29]. This increase was explained by possible purification, the impurities which limited the activity of the enzyme were simply extracted by the CO_2 as no conformational change was observed on fluorescence spectra [29]. A reduction of particle size, caused by the supercritical CO_2 was also indicated as explanation of the increased activity of the enzyme [33]. High diffusivity and low viscosity caused by the pressure and temperature of the supercritical CO_2 leads to enhanced mass transfer, which results into an increased activity of the enzyme [34], [35]. In general, increase of enzymatic activity in supercritical CO_2, although uncommon, occurs with a few enzymes. These are often lipases, but amylases and cellulases have been shown to work as well [29].

In practice, supercritical CO_2 is used in combination with enzymes for a variety of

reasons. Pretreatment to release and extract certain compounds from biomaterials is an example of that [35], [36]. α-amylase in combination with supercritical CO_2 was used to extract oleoresin from black pepper. Treating the α-amylase with supercritical CO_2 at 60°C and 300 bar increased the activity of the α-amylase by 25% [36]. Nucleus magnetic resonance (NMR) spectra of the treated enzymes showed clear differences with the non-treated enzymes. A singlet peak was modified to a doublet peak and a doublet peak was modified to a triplet peak and intensities of these peaks were different from the original enzyme as well. This suggests that conformational change has occurred in the protein structure and explains the increases activity. Increasing the amount of enzymes treated, from 2 to 10 mg, resulted in a lower activity than the untreated enzymes. The NMR spectra showed a single peak only, indicating that the supercritical CO_2 altered the protein structure in a negative way. Denaturation did not occur, since the enzymes retained activity, although it was approximately 25% of the activity of non-treated enzyme[36].

As the density of supercritical CO_2 changes when either the pressure or temperature is altered, the effect it has on the enzymes alters as well. A mixture of xylanase and cellulase in combination with supercritical CO_2 was used to scour and bleach flax roves. Enzyme dosage, temperature and pressure were varied to find the optimal conditions of the process. The effect of the pressure proved interesting, as the performance of the enzyme was increasing when the pressure was increasing from 10 MPa to 25 MPa [37]. Increasing the pressure above 25 MPa decreased the performance for the enzymes, as the tertiary structure will irreversible change under the conditions [37].

The effect of SC-CO_2 on lignocellulosic materials has been tested for different pressures and temperatures, as well as the effect on different moisture content within the lignocellulosic materials [19]. It was shown that SC-CO_2 only has effect on lignocellulosic material with a moisture content and that the effects scales linearly with the amount of moisture present [19].

1.9 5-hydroxymethylfurfural (5-HMF) as platform chemical

5-hydroxymethylfurfural or 5-HMF is listed as an important bio-based chemical by the U.S. Department of Energy for any prospective biorefinery [38]. 5-HMF is a chemical from which a wide range of product can be made that are important to industry. These are chemicals such as 2,5-icarboxylic acid (FDCA), used for production of bio-polyesters [39], biofuel precursors such as 2,5-dimethylfuran [40] and 5-ethoxymethylfurfural [41].

5-HMF can be obtained from different sugars, fructure and glucose are primarly used as precursors. Fructuce as a substrate is more reactive towards the production of 5-HMF than glucose [42]. The pathway from fructose to 5-HMF is a single step, dehydration of the fructose forms the 5-HMF. 5-HMF formation from glucose proceeds by a similar path way, first the isomerization of glucose to fructose occurs, followed by dehydration of fructose. This pathway can be seen in figure 1.3.

Although fructose is more selective, glucose is preferred as substrate as the production of fructose is costly and is oftenly made by the isomerization glucose [44]. Glucose on the other hand is easily obtainable, e.g. by the enzymatic hydrolysis of lignocellulosic biomass, specifically the cellulose [44]. As renewable raw material, this makes glucose a great precursor for the production of 5-HMF.

The catalysts used for the 5-HMF production are most often Brønsted acids (BA), but

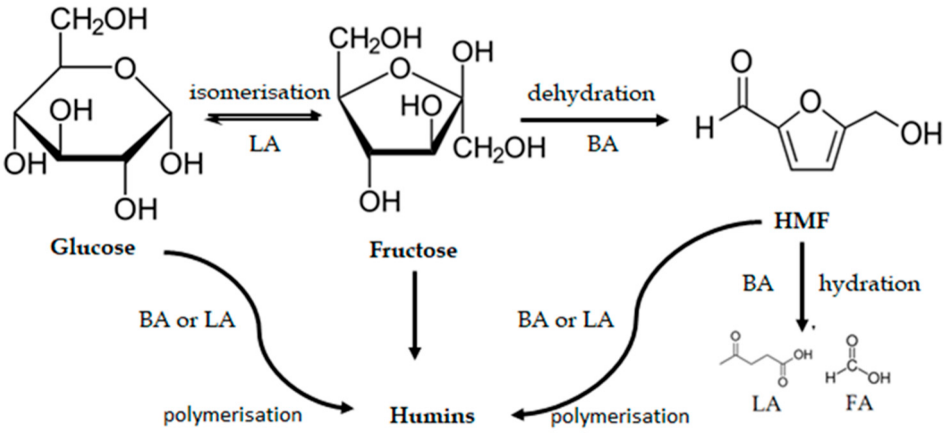

Figure 1.3: Pathway of glucose/fructose to the formation of 5-HMF. Side reaction are also depicted, with the involvement of Lewis acids (LA) and Brønsted acids (BA). Adapted from [43]

BA sites catalyse besides the dehydration to 5-HMF the subsquent rehydration to formic acid and levelunic acid [38]. Another undesired reaction is the formation of humins [45], which is the polymerization of 5-HMF which is also catalysed by BA sites. Humins can also form by the polymerization of 5-HMF and glucose which is catalysed by lewis acid (LA) sites. The use of water increases the frequency of these side reaction, which is why the production of 5-HMF is usually done in ionic liquids [46] or organic solvents such as DMSO [47]. Biphasic systems, where in situ extraction of the product is the goal is also used [48].

To catalyse the reaction of glucose to 5-HMF, a bifunctional catalyst that can catalyse both steps would be a good option. The first step, the isomerization from glucose to fructose, requires either lewis acid or lewis base functionality. The second step, the dehydration of fructose to 5-HMF requires BA catalytic sites. Niobium oxide (Nb_2O_5) is therefore proposed as catalyst for this pathway.

1.10 Niobium oxide (Nb_2O_5)

Niobium penta-oxide or Nb_2O_5, is an amorphous metal oxide consisting of NbO_4 tetrahedra and NbO_6 octahedra. It is synthesized by the hydrolysis of $NbCl_5$ and is a strong lewis acid and will be an brønsted acid in the aqueous phase [49] called niobic acid, the hydrated form Nb_2O_5 [43]. Nb_2O_5 in water, has both LA and BA catalytic sites. The NbO_4 tetrahedra lewis acid sites on the hydrated Nb_2O_5 will form NbO_4 - H_2O adducts with brønsted acid functionality [49]. It has been used to catalyse various reactions, including the dehydration of hexoses [43]. As the reaction of glucose to 5-HMF is in need of a catalyst that both has LA and BA functionality, Nb_2O_5 is therefore a great choice as catalyst for this reaction.

1.11 Supercritical CO_2 as synthesis method for catalyst

Using supercritical CO_2 as precipitation method is a rather novel technique to prepare nanoparticles [50]. Supercritical CO_2 as a medium is attractive for for the preparation for nanostructured oxide catalyst, such as Nb_2O_5, due to the properties of the solvent. The properties of supercritical CO_2 are between those of a gas and a liquid, and can be adjusted by altering the pressure and the temperature [50]. The dissolving ability, high diffusivity and low surface tension all make it an excellent solvent for the formation of nanoparticles. The relatively mild conditions at which CO_2 is in supercritical state (73.9 bar, 31.1°C) and low toxicity make it a very green solvent to use as well.

Chapter 2

Aim

The aim of this thesis can be summarized in a few points:

1. What is the influence of the solvent, water or buffer, on the yield of the enzymatic hydrolysis using CTec2?
2. Can the CTec2 enzyme blend survive in supercritical CO_2(T = 50.0°C, P = 80 bar) conditions?
3. Are the mild supercritical CO_2 conditions (T = 50.0°C, P = 80 bar) effective as a pretreatment for the canal grasses and the rice straw?
4. Does the yield of the sugars increase when enzymatic hydrolysis with CTec2 on canal grasses and rice straw in supercritical CO_2 conditions?
5. Can the enzymatic hydrolysis using CTec2 and the subsequent catalysis with the Nb_2O_5 catalyst be done simultaneously?

Chapter 3

Materials and methods

3.1 List of Materials

Cellic CTec2 was acquired from Sigma-Aldrich (Zwijndrecht, Netherlands) Rice straws were kindly provided by Facility management Breda (Breda, the Netherlands). Canal grasses were harvested in various locations around the city of the university (Groningen, the Netherlands). For the enzymatic reaction acetic acid and sodium acetate were used to prepare the buffer (pH = 5). CTec2 was used as enzymatic cocktail for the enzymatic hydrolysis. Glucose, xylose and arabinose were used to make standard curves for the HPLC. 4-Hydroxybenzoic acid hydrazide and sodium hydroxide were used for the reducing ends analysis. Sulfuric acid was used for acid hydrolysis. $NbCl_5$ was used as catalytic precursor for the synthesis of the N_2O_5 catalyst. Ethanol was used as solvent for the synthesis of the N_2O_5 catalyst. 5-hydroxylmethylfurfural(5-HMF) and furfural were used to make reference curves for GC and HPLC. CO_2 was used for reactions performed in the 10-block CO_2 reactor.

All chemicals used are purchased from Sigma-Aldrich if not specified otherwise.

3.2 Material preparation

3.2.1 Biomass preparation

The upper part of the rice straw were cut into small section and subsequently freeze dried. The dry straw was then ground using a coffee grinder. The straws were then milled for 1h and stored for further use.

Grass was collected in various locations around the city of Groningen.

Sampling was done as to get a representative of the vegetation of the location. At each location, a transect of 100m was set, after which at 4 intervals (0, 25, 50 and 100m) canal grasses were collected in an area of $0.5m^2$ at each interval. This was done at 4 locations in total, which can be seen in figure 3.1. The weight of the samples differed per location and were in the range of 315-918g in total. The samples were subsequently cut in small sections and mixed with the other samples of 1 location, to end up with 4 mixtures in total. 100g of this mixture was freeze-dried and milled for 1h. Then it was stored for further use.

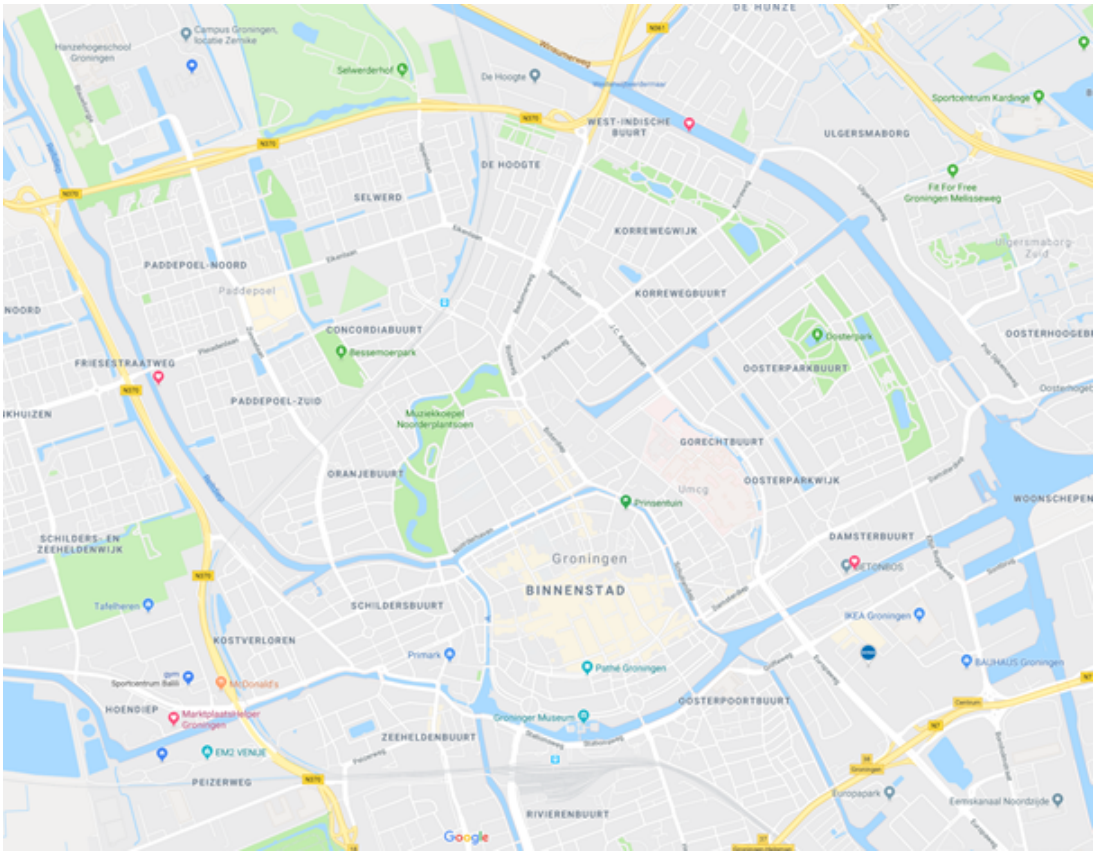

Figure 3.1: The selected locations where the canal grasses were collected. The four red markers indicate the area of collection.

3.2.2 Ball milling

100g of biomass was weighed, with a particle size between 425 en 250 microns. The ball mill, a Planetary Mono Mill Pulversitette 6 (Idar-Oberstein, Germany) was filled with 5 large ZrO_2 grinding balls with a diameter of 20 mm and 5 ZrO_2 grinding balls with a diameter of 5 mm. The biomass was milled for 1h or 6h with 10 minutes clockwise, 10 minutes rest and 10 minutes counterclockwise at 27x g which was repeated until the desired milling time was reached.

3.2.3 CTec 2 protein content

Quantification of he protein content of the CTec 2 enzymatic cocktail was done according to the Bio-Rad Bradford Dye Reagent microplate assay (Bio-Rad Laboratories Inc, Hercules, CA, USA), with bovine serium albumin as the standard. Ultrapure water was used as the blanc and the samples were done in triplicates.

3.3 Characterization and analysis

Numerous characterization and analysis methods were applied. As the methods are used for specific subjects within the thesis, either for the characterization or analysis regarding carbohydrates or regarding the catalyst this section is split into two sections, carbohydrates and catalyst respectively.

3.4 Analysis methods

For the analysis of the enzymatic reactions, two analysis methods were used as earlier described in the materials and methods section. The reducing ends analysis enables quick analysis, the complete protocol can be done within an hour. Reducing ends analysis measures the sugars by its reducing end position at the end of a carbohydrate chain, and it is used to detect endoacting enzymes by showing an increase of these reducing ends. It does not however differentiate between the length of the carbohydrates, one monosaccharide has the exact same outcome as one oligomer on this type of analysis.

Acid hydrolysis and subsequent HPLC is able to quantify the different sugars, as everything is broken down in to monosaccharides. It is noted that acid hydrolysis and subsequent HPLC on the pellet, compared with reducing ends analysis on the supernatant of that same pellet could hint the presence of bigger molecules such as oligomers. As an example, a low increase of reducing ends but a great amount of sugars removed from the pellet in the HPLC analysis point to the direction that the sugars are solubilized during the reaction, just not all as monosaccharides.

3.4.1 Carbohydrates

3.4.2 Acid hydrolysis

10mg of biomass was weighed and put into glass tubes. 0.45ml of 72 w/w% sulphuric acid was added to the tube and put into a 30°C water bath for 1 hour. 4.55ml of water was subsequently added to the mixture and put into a 100°C water bath for 3 hours. A sample was taken and diluted 20x with water. The sample was subsequently put into a HPLC for 20 minutes using solely the RID detector. With the use of an external standard with a range of 12.5μ g/ml - 50μ g/ml, the amount of glucose, xylose, and arabinose remaining in the pellet could be determined. Subsequently by using the initial amount of sugar in each biomass, the amount of glucose, xylose and arabinose that is released in the liquid phase could be determined.

Sugar composition

To determine the composition of the rice straws and canal grasses, in terms of their sugar composition, acid hydrolysis was performed on freeze dried 1 hour milled rice straws and canal grasses. In triplo, 10 mg of biomass was collected and acid hydrolysis as described above was performed. By determining the amount of glucose, xylose, and arabinose per the amount of dry weight the sugar content of the biomass could be determined.

PAHBAH reducing ends analysis

To evaluate the results of the enzymatic hydrolysis, reducing ends analysis was used. The specific reducing ends method or assay, PAHBAH [51], was carried out in duplicates. To prepare the solution for the assay, a 5% w/w stock solution of 4-hydroxybenzoic acid hydrazide (PAHBAH) was made in 0.5M HCl. To perform the assay, the stock solution was mixed with 0.5M NaOH in a 1:4 ratio. 50 μ L of samples were mixed with 200μL of the assay mixture in a 96 well plate. The plate was subsequently covered with a lid and put in a water bath of 70°C for 30 minutes. The lid was subsequently removed and the

plated was inserted in the SPectraMax Plus 384 spectrophotometer (Molecular Devices, LLC, San Jose, CA, USA) and the absorbance was measure at 405 nm. The results were quantified using D-glucose with concentrations in the range of $12.5 - 50 \mu g/mL$. Xylose standards were tested to check whether the absorbance was different from the glucose standards, but this was not the case.

HPLC

High performance liquid chromatography (HPLC) was done on an Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA) equipped with RID and VWD (210 nm) detectors with a Bio-rad Aminex HPX-87H ($300mm \times 7.8mm \times 9\mu m$) at an operating temperature of 60°C. Isocratic elution was done with 5 mM H_2SO_4 in miliq water as eluent at a flowrate of 0.55 ml/min. The injection volume of each sample was $5\mu L$.
To quantify the results, linear standard curves for glucose, xylose and arabinose were made. They had the following formula:

$$A = mx - b \tag{3.1}$$

By re-arranging the formula the amount of glucose, xylose and arabinose could be determined.

3.4.3 Catalyst

3.4.4 Synthesis of niobium oxide catalyst

To synthesize the Nb_2O_5 catalyst, the same procedure was followed as described in Tao et al. [50]. 1.0 gram of $NbCl_5$ was dissolved in 5 ml of ethanol. 10 mL of miliq water was added dropwise and the solution was subsequently transferred to a high-throughput supercritical CO_2 reactor (Integrated Lab Solutions, Berlin, Germany) as shown in A.3. The mixture was heated to 40°C and subsequently stirred for 3 hours at 800 rpm. The temperature was then raised to 80°C and the reactor was filled with CO_2 to a pressure of 140 bar. This was set to react for 3 hours. A schematic overview of this synthesis can be seen in figure 3.2.

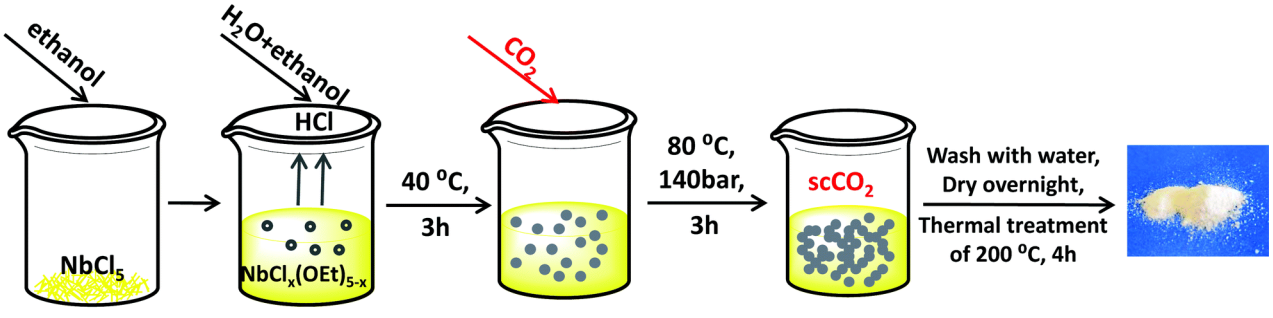

Figure 3.2: Synthesis of the nb2o5 catalyst using supercritical CO_2. Adapted from [50].

3.5 Material characterization

Various techniques were used to characterize the Nb_2O_5 catalyst. N_2-Physisorption isotherms were recorded on a Micromeritics ASAP 2420 apparatus at $-196°C$. The

samples were degassed at 200°C for 5 h under reduced pressure before N_2 adsorption. The BET method was used to evaluate the surface area. X-ray diffraction patterns (XRD) were recorded on a Bruker D8 phaser diffraction meter equipped with Cu $K\alpha$ radiation ($\lambda = 1.5406\mathring{A}$). The 2θ range of the reflection geometry was between 5 and 80°.

3.5.1 XRD

X-ray defraction or XRD, is a type of spectroscopy which determines the crystallinity of a material by shooting x-ray beams onto a surface and subsequently measuring the refraction. By measuring the angles of the refraction the faces of crystalline can be determined.

3.5.2 Brunuaer-Emmett-Teller (BET) adsorption analysis

BET analysis is a method to determine the surface area of a catalyst. c.a. 100 mg of catalyst was weighed and its weight was noted with 3 decimals accuracy. The catalyst was put in a glass tube which is put under vacuum and cooled down to -? using liquid nitrogen. Nitrogen is subsequently pumped into the system after which the absorption and desorption of the catalyst are measured. With BET analysis, the presence of micro-, meso-, and macropores can also be determined.

3.6 Experimental

3.6.1 Regular enzymatic hydrolysis

50 mg ± 2 mg of the different biomass, rice straw or canal grasses, with various or without pretreatments were weighed and put into 15ml falcon tubes. 1w/w% (protein/biomass) of CTec2 was added. The total liquid volume, either 50mM sodium acetate buffer or water, was 5ml. The enzymatic hydrolysis was carried out in an incubator at 50°C. The falcon tubes were stirred using a head over tails device. The reaction time varied between 4 and 24 hours. The reaction was subsequently quenched in a water bath of 100°C for 10 minutes. The mixture was centrifuged for 10 minutes at 10,000 G and a sample was taken from the liquid phase for PAHBAH reducing ends analysis. The supernatant was discarded and the solid residue was washed with water and subsequently centrifuged twice. The residue was subsequently frozen and freeze dried for further characterization of the sugar composition. When referred to regular enzymatic hydrolysis, these are the conditions under which the enzymatic hydrolysis was performed.

3.6.2 Supercritical CO_2 pre-treatment

3.6.3 Biomass

50 mg ± 2 mg of biomass was weighed into a glass vial equipped with a screwcap. The cap was equipped with a septum through which two small needles were installed. The needles provided an inlet for the CO_2 to enter the vial. Subsequently 5 mL of water or buffer was added to the vial, along with a stirring magnet. The vials were loaded into the CO_2 reactor and the lid was closed. The pressure was subsequently raised to 80 bars and the temperature was set at 50°C. When both the pressure and temperature was

reached, stirring was turned on for 4 hours. After 4 hours, the pressure was lowered by 3 bar/min and the reactor was simultaneously cooled down to room temperature. After the pressure returned to atmospheric the vials were put in a freezer at -20°C to be stored for later analysis.

Enzymes

The enzymes were treated within the same conditions as in section 3.6.3. The amount of enzyme used was 1w/w% (protein/biomass) calculated for 50mg of biomass. The total liquid volume was 5ml.

3.6.4 Supercritical CO_2 enzymatic hydrolysis

For the enzymatic hydrolysis performed in supercritical CO_2, the same procedure was followed as in the supercritical CO_2 pre-treatment with the following adjustments. 1w/w% (protein/biomass) of CTec2 solution was added to a final liquid volume of 5 mL. After 4 hours, the temperature was raised to 100°C to denature the enzyme and quench the reaction. The reactor was subsequently depressurized and cooled down after which the samples were put in a freezer at -20°C to be stored for later analysis.

3.6.5 Catalytic testing

Benchmark reaction

The catalytic tests for the production of 5-HMF and furfural were done in ACE sealed pressure resistant glass tubes from ACE incorperated (Vineland New Jersey, USA). For the production of 5-HMF and furfural, a reaction mixture consisting of 40 mg glucose, xylose or arabinose with 4 mL biphasic solvent ($V_(H_2O) : V_(MIBK) = 1 : 3$) and 40 mg of catalyst was weighed in the ACE glass tube. The organic solvent, MIBK, was introduced for the in-situ extraction of the products to prevent further hydrolysis and the formation of humins. The tubes were subsequently sealed and placed into an oil bath of 120°C. After continuous stirring for 3 hours, the reaction was quenched by placing the tubes into a water bath at room temperature. The mixture was subsequently transferred to a centrifuge tube and centrifuged at 10,000G for 10 minutes. A sample from the organic phase was taken and analysed by Thermo trace gas chromatography equipped with a Restek Stabilwax-DA column (30 m length, 320μ m internal diameter). The aqueous phase was diluted 10x and analysed using an Agilent 1200 high-performance liquid chromatography (HPLC) with a Bio-rad Aminex HPX-87H column. The HPLC was operated at a temperature of 60°C using a Waters 410 differential refractive index detector and a UV detector with 5mM aqueous sulphuric acid as the mobile phase with a flow rate of 0.55mL/min. The injection volume of the sample was set at 50μ L.

Supernatant reaction

The same procedure was used as described in the benchmark reaction, but instead of using 1 ml H_2O and a fixed weight of sugar, 1 ml of the supernatant of an enzymatic reaction was used. This supernatant was collected by centrifuging the enzymatic sample at 15,000g for 10 minutes to separate the liquid from the solid phase.

3.6.6 Gas chromatography

As the reaction mixture of the heterogeneous catalysis contains MIBK, an organic solvent, analysis with the use of gas chromatography was done to determine the amount of 5-HMF and/or furfural in the organic phase. Firstly a reference curve was made using 250μ L, 500μ L and 1;2.5; 5; 7.5; 10; and 20 mg/ml of 5-HMF and furfural in MIBK.

To calculate the yield, the productivity (Prod), turn over number (TON) and turn over frequency (TOF), the following formulas were used:

$$Yield = \frac{Moles_{5-HMF}(mol)}{Moles_{Glucose}(mol)} \times 100\% \tag{3.2}$$

$$Prod = \frac{mass_{5-HMF}(g)}{mass_{catalyst}(g) \times reactiontime(h)} \tag{3.3}$$

$$TON = \frac{Moles_{5-HMF}(mol)}{moles_{catalyst}(mol)} \tag{3.4}$$

$$TOF = \frac{TON}{reactiontime(h)} \tag{3.5}$$

Chapter 4

Results and discussion

4.1 Sugar composition

The sugar composition of the rice straws and canal grasses was determined using acid hydrolysis with subsequent HPLC analysis. In figure 4.1 below the composition is shown with respect to glucose, xylose and arabinose as a fraction of the total dry weight of its respective biomass.

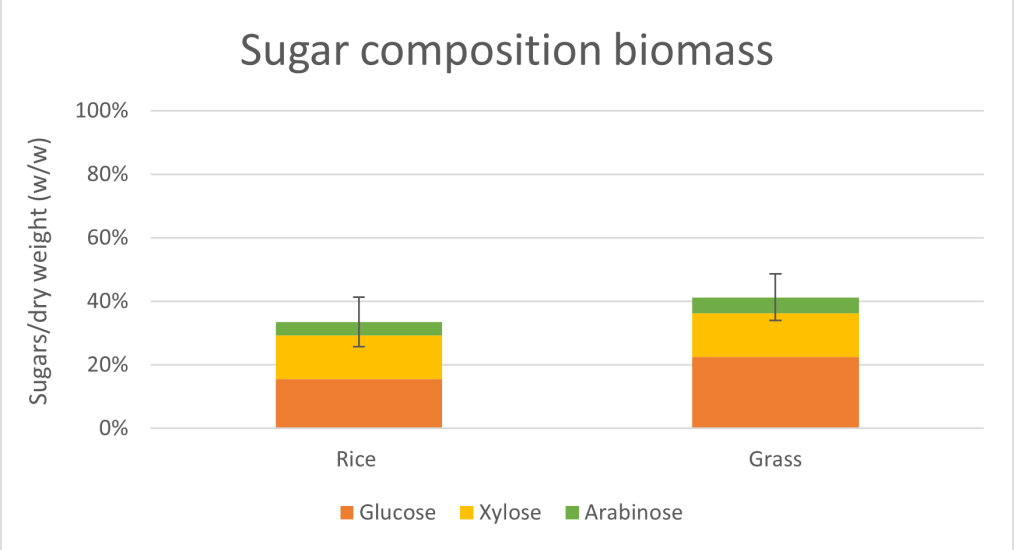

Figure 4.1: Composition with respect to glucose, xylose and arabinose as a fraction of the total dry weight of its respective biomass

In figure 4.1 It can be observed that the grass contains more sugars than rice does, 40% and 32% of the total dry weight, respectively. Grass has relatively more glucose than xylose and arabinose. For rice, the amount of glucose and xylose is very similar. Arabinose is the least present sugar in both types of biomass. Because grass contains relatively more glucose, it is likely a more suitable biomass to be used for the production of 5-HMF.

4.2 Regular enzymatic hydrolysis

At first, both the canal grass and the rice straw underwent regular enzymatic hydrolysis as described in the material and methods section 3.5.1. The goal was to see what the effect of the solvent is on the sugar yield. The difference between using buffer or water as solvent is the pH. The sodium acetate buffer has a pH of 5.0 while water has a pH of 7. As most cellulase reaction are done in the pH range of 4.8 - 5.0, the regular enzymatic hydrolysis in this thesis was also performed in that range. Water was used to see what the performance would be at such a pH, as well as the for any possible industrialization of the reaction as water would be a more preferable solvent than a buffer. As mentioned in theoretical background, CTec2 tends to perform better in a pH range of 5.8 - 6.0 for lignocellulosic biomass [27], which could result in water being a better solvent than the pH = 5.0 buffer. For supercritical co2 enzymatic hydrolysis it is expected that the buffer is a better option, since the pH would be more stable when carbonic acid starts to form.

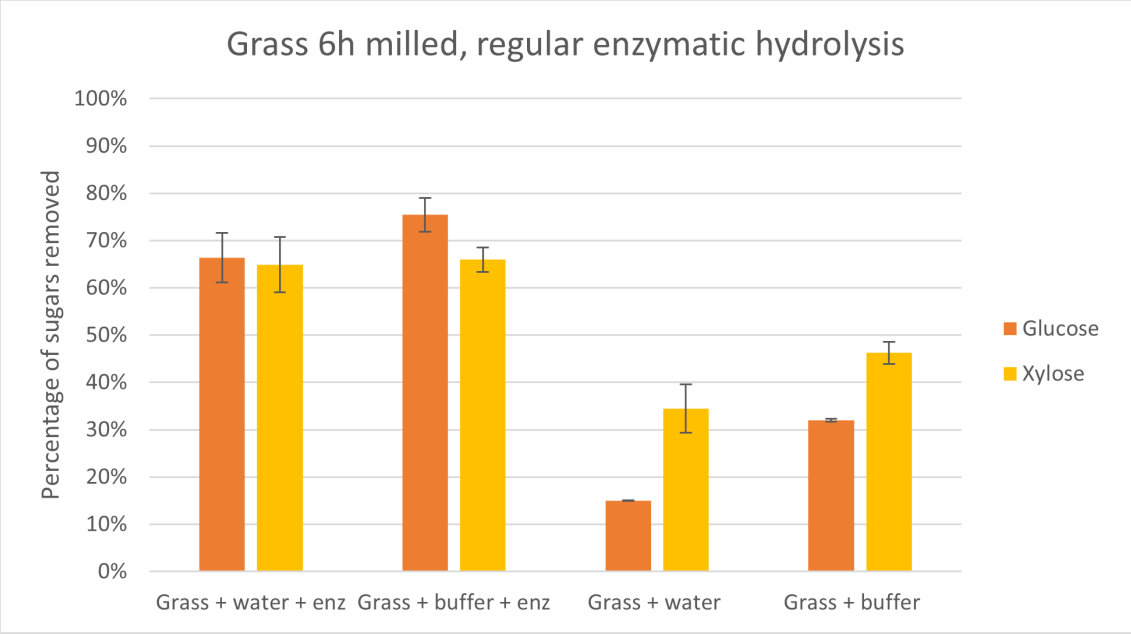

Figure 4.2: Percentage of sugars removed of 6h milled grass with regular enzymatic hydrolysis. 4h incubation, 50°C, 1 w/w% of CTec 2 enzyme.

From figure 4.2 it can be seen that with water as a solvent, 70% of the available glucose is released, and nearly 60% of the xylose. With buffer as a solvent the values are lower, 52 and 45% for glucose and xylose respectively.

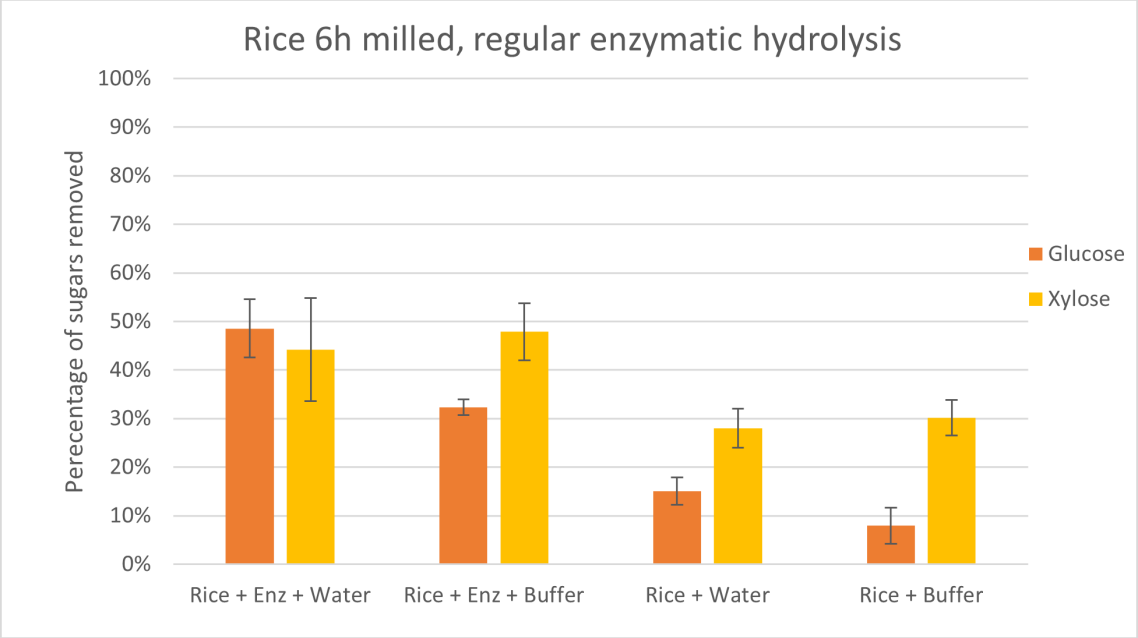

Figure 4.3: Percentage of sugars removed of 6h milled rice with regular enzymatic hydrolysis. 4h incubation, 50°C, 1 w/w% of CTec2 enzyme.

In figure 4.3 it can be seen that with buffer as a solvent, more than 70% of the total available reducing ends released in the liquid phase. This is in contrast with water as a solvent, where only 53% of the total reducing ends are released in the liquid phase.

From these two figures, it is concluded that under the regular enzymatic hydrolysis circumstances, canal grasses release more sugars with water as a solvent, whereas rice straws releases more with buffer as a solvent. In general, with only milling as pretreatment, it seems that CTec2 is more effective on the grasses than the rice, regardless of the pH of the solvent. The lignocellulosic structure of the grass could be more accessible for the enzymes than the structure of the rice, which would explain the difference in percentage of sugars removed. CTec2 tends to adsorb to lignin which reduces the activity, this could be a reasoning for the lower yields in different types of biomass as the lignin composition is different [52].

4.3 Enzymes resist supercritical CO_2

In order to check whether the CTec2 enzymes would still be active in supercritical CO_2 conditions, various experiments were done to verify this. The CO_2 has the ability to inhibit the enzyme and change its tertiary structure, which could alter the activity of the enzyme and in its worst case denature the enzyme [31]. The CTec2 was therefore tested in supercritical CO_2, by the means of treatment and subsequent regular enzymatic hydrolysis, as well as performing the enzymatic hydrolysis directly in supercritical CO_2 conditions.

It was found that treating the enzymes under supercritical CO_2 did not denature the enzymes. The CTec2 retained its activity after treatment which can be seen in figure 4.6. Roughly 65% of the activity is retained, this can seen from the difference in yields. This could point in the direction that the CO_2 affects the enzymes mildly, by either effecting the protein structure or by the acidity as it dissolves into the aqueous phase. Doing the

enzymatic hydrolysis in supercritical CO_2 conditions also yielded results. This can be seen in figures 4.4 and 4.3.

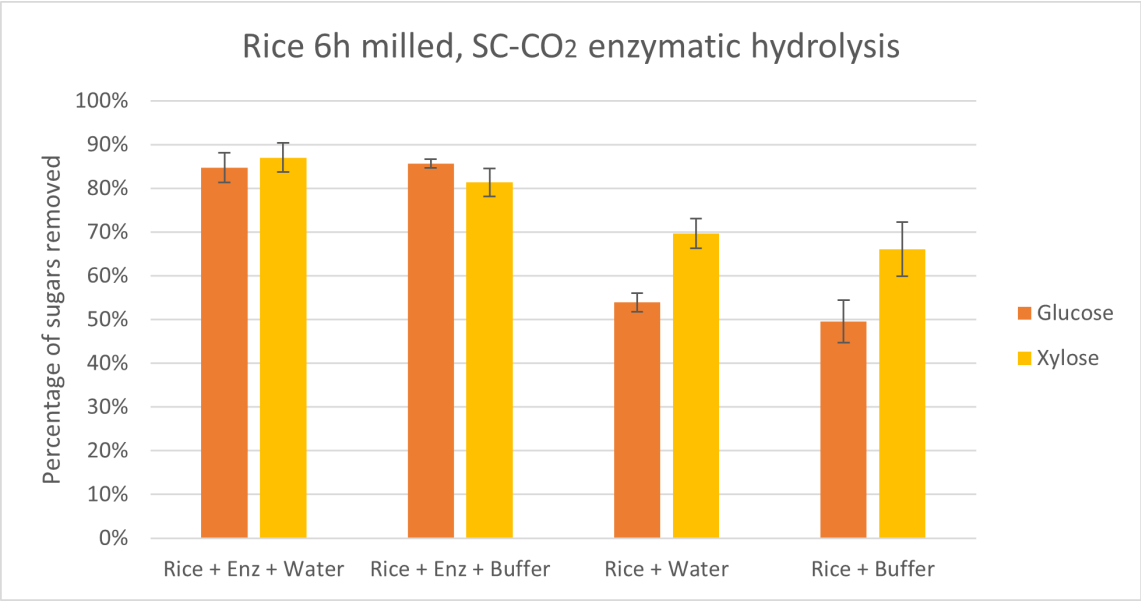

Figure 4.4: Percentage of sugars removed from 6h milled rice in supercritical enzymatic reaction. 4h incubation, 50°C, 1 w/w% of CTec2 enzyme. supercritical CO_2 conditions: T = 50°C, P = 80 bar

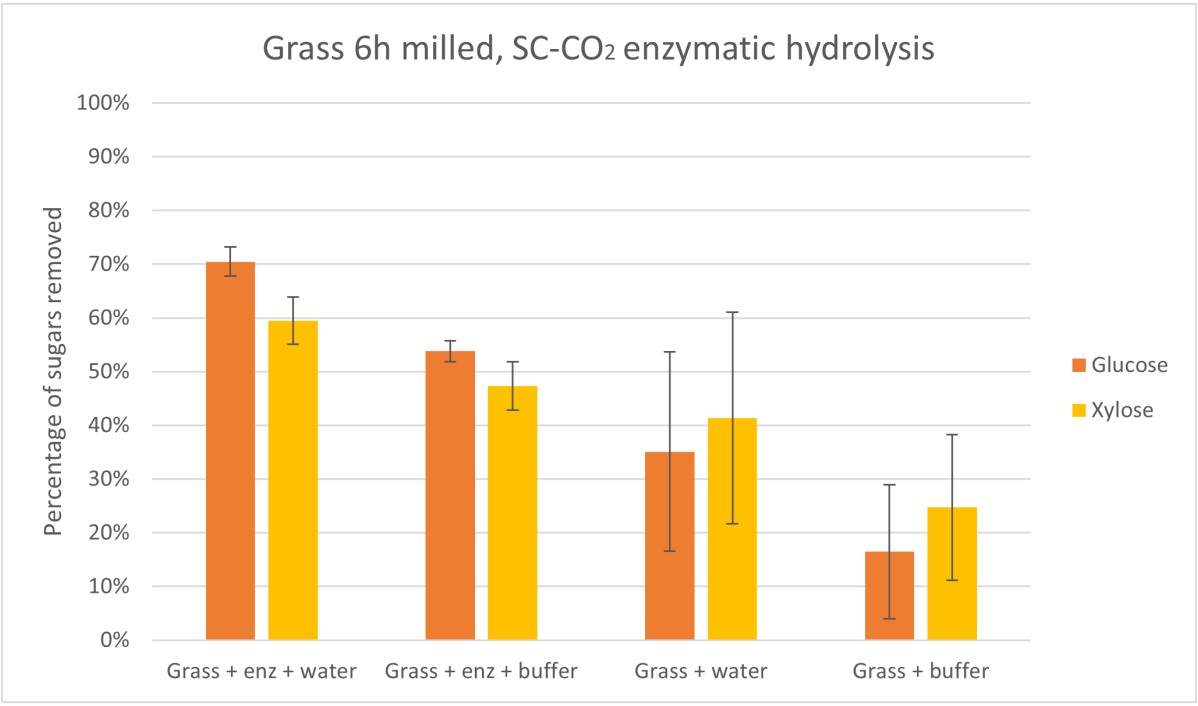

Figure 4.5: Percentage of sugars removed from 6h milled grass in supercritical enzymatic reaction. 4h incubation, 50°C, 1 w/w% of CTec2 enzyme. supercritical CO_2 conditions: T = 50°C, P = 80 bar

From figures 4.4 and 4.5 the removed glucose and xylose under supercritical CO_2 enzymatic reaction for rice and grass respectively. The graphs show that the enzymes still

perform hydrolysis while under supercritical CO_2, as the percentage of sugars removed in the enzymatic samples, 80-85% in rice; 45-70% in grass are higher than in the non-enzymatic samples, 50-70% in rice; 15-40% in grass regardless of which solvent is used. The effects are more noticeable in the rice than in the grasses, this could be a result of the difference of composition and structure of the respective types of biomass.

Thus it is concluded that the CTec2 enzymes work both under supercritical conditions and after being treated in supercritical conditions. This indicates that the stability of the enzyme under pressure is retained, as well as in an acidic environment due to the dissolving CO_2 (pH = 2.88).

4.3.1 Pretreatment under SC-CO_2

Pretreating the biomass in supercritical CO_2 was done in an attempt to break down the lignocellulosic structure to allow for better access of the CTec2 enzymes. The supercritical CO_2 has three ways of affecting the structure. Entering the pores during pressurization and breaking them after depressurization, loosening the overall crystalline structure of the cellulose, and breaking the structure down by forming carbonic acid and thus increasing the acidity.

An overview of the yields of various experiments on the grass is shown in figure 4.6. The results could not be accurately produced for the rice, which is why only the grass samples are shown. Comparing the SC-treated grass with SC-treated enzymes samples vs the SC-treated biomass with non treated enzymes samples indicate that the pretreatment is not having any positive effect on the yield. In fact it shows that the pretreatment has a negative effect on the sugar yield, as the percentage of sugar removed is lower than when the grass is not pretreated in supercritical CO_2.

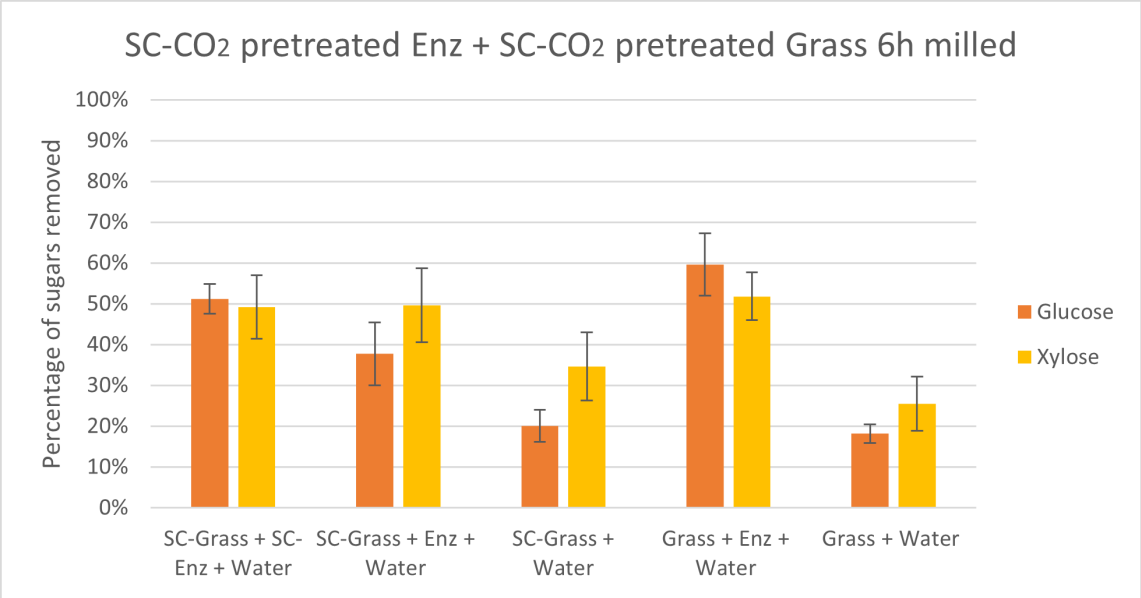

Figure 4.6: Percentage of sugars removed from supercritical CO_2 pretreated 6h milled grass with treated/non treated enzymes in regular enzymatic reaction. 4h incubation, 50°C, 1 w/w% of CTec2 enzyme. supercritical CO_2 conditions: T = 50°C, P = 80 bar. SC refers to supercritical CO_2, SC-Grass e.g. means that the biomass has been treated in supercritical CO_2

The conditions for supercritical CO_2 pretreatment, (T = 50.0°C, P = 80 bar), seem not sufficient enough to make the biomass more prone to enzymatic hydrolysis. This could be explained by the time that the CO_2 molecules need to penetrate the structure [20]. It was shown that at mild supercritical CO_2 conditions(T = 50-80°C; P < 100 bar) a residence time of at least 12h was needed, which is three times as much a the residence time here.

The method of depressurization has to be considered as well. In literature, supercritical CO_2 pretreatment is most often referred to as supercritical CO_2 explosion, which has close resemblance to steam explosion. After pressurization and a selected residence time, the pressure is suddenly released. This sudden release of pressure is what causes the rupture of the pores of the biomass, the first mechanism of the effectiveness of supercritical CO_2 pretreatment [5], [20]. In this case, the depressurization was slow, the pressure of 80 bar was released in a time span of 1.5h with the largest pressure drop being 10 bar maximum.

The other two mechanisms, loosening of the crytalline cellulose structure and the formation of carbonic acid, were not sufficient to weaken the lignocellulosic structure either in these conditions. A longer residence time or harsher conditions of the supercritical CO_2 might have sufficient effect to increase the yield of enzymatic hydrolysis.

It is unclear why the pretreatment has a negative effect on the yield of the subsequent enzymatic hydrolysis. As supercritical co2 impact the structure, it might be possibility that it changes the structure in a way that the enzymatic hydrolysis is less effective.

4.3.2 Enzymatic hydrolysis under SC-CO_2

As it was clear that the enzymes were working under supercritical CO_2, the next step was checking whether doing the enzymatic hydrolysis in the supercritical CO_2 would result into higher sugar yields. As mentioned earlier, the supercritical CO_2 has multiple potential effects on the enzymatic hydrolysis. Firstly the supercritical CO_2 could possible swell the biomass by CO_2 entering the pores, which leads to an increase in the surface area of the biomass. Decreasing in the crystallinity of the cellulose would be another potential effect, which would lead to better access ability of the enzymes. Another effect of the supercritical CO_2 is correlated with its solubility in water. Before supercritical conditions are met (73.9 bar, 31.1°C), the solubility increases as the pressure of the CO_2 increases. As Henry's law dictates, the solubility of the CO_2 increases as the pressure increases (insert formula from theory). Henry's law however, does not hold for supercritical fluids as it is designed for ideal gasses [23]. To get an estimate of the pH of the liquid under the reaction conditions of the supercritical CO_2. At a pressure of 80 bars and a temperature of 50°C, the pH of water was found to be 2.88 as described in [23].

From the pretreatment it could be concluded that the supercritical CO_2 did not have a sufficient effect on the biomass, so the potential benefit it would have on the enzymatic hydrolysis would have to be the activity of the enzyme, increased by the supercritical CO_2.

The positive effects versus the negative effects of supercritical CO_2 on the enzymes have to be considered. The pH of the solvent is lowered by the supercritical CO_2, specifically to 2.88 at the conditions used, as shown in [23]. The the CTec2 enzyme does not work optimally in this pH. The effects of the acidity of the supercritical CO_2 are more clear in the samples with water as a solvent, as the buffer solution will be less susceptible to a pH drop. The potential benefits of the supercritical CO_2 on the enzymes, such as the

enchanced mass transfer caused by the high diffusity and low viscosity of the supercritical CO_2, could lead to better performance of the enzymes.

Figures 4.7 and 4.8 show the effect of the supercritical CO_2 on the enzymatic hydrolysis compared to the regular enzymatic hydrolysis. For rice, the glucose and xylose yields are very similar in both with water and buffer as solvent.

Although the supercritical CO_2 shows, when figures 4.7 and 4.8 are compared, that it indeed does have a beneficial effect to the yield of the enzymatic reaction. This could be accounted to the enhanced mass transfer, for the canal grasses it does not seem to have the desired effect. This can be seen in figures 4.2 and 4.5. In the figures it can be seen that with the regular enzymatic hydrolysis the maximum yield of sugars removed from the pellet was 75% and 66% for glucose and xylose respectively. Under supercritical CO_2 conditions, this was only 70% and 59% for glucose and xylose respectively. For rice, this is 85% for both glucose and xylose compared to 50% and 40% in regular conditions. The maximum yields are also achieved with different solvents, in the regular conditions buffer seems the best solvent, while water seems the best solvent under supercritical conditions. This could be related to the effect of the acidity, which effects are more severe in the samples that had water as a solvent in contrast to a buffer which would keep the pH at a higher level initially.

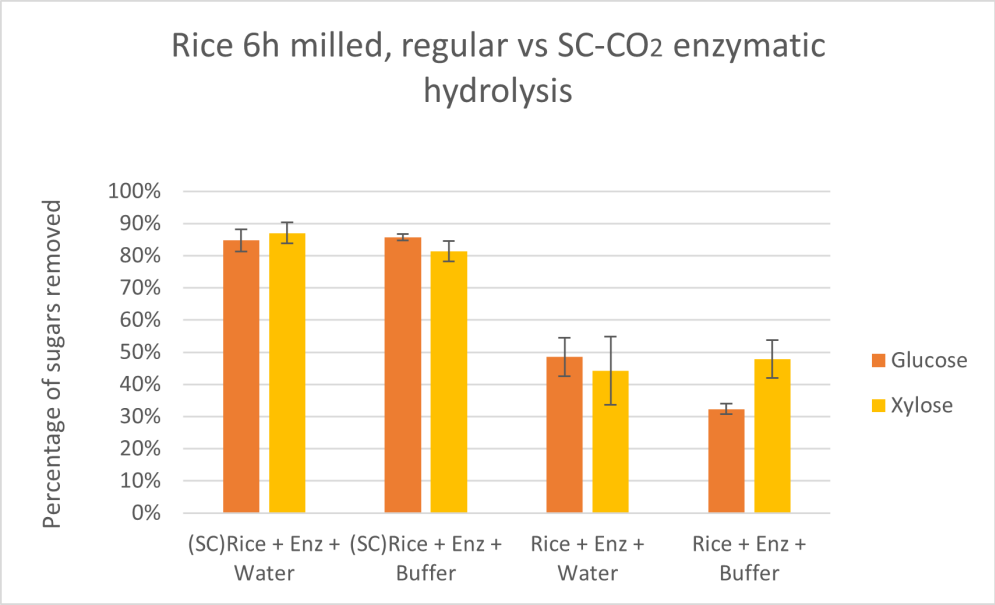

Figure 4.7: Percentage of sugars removed from 6h milled grass with in regular enzymatic reaction versus supercritical CO_2 enzymatic reaction. 4h incubation, 50°C, 1 w/w% of CTec2 enzyme; supercritical conditions: T = 50°C, P = 80 bar

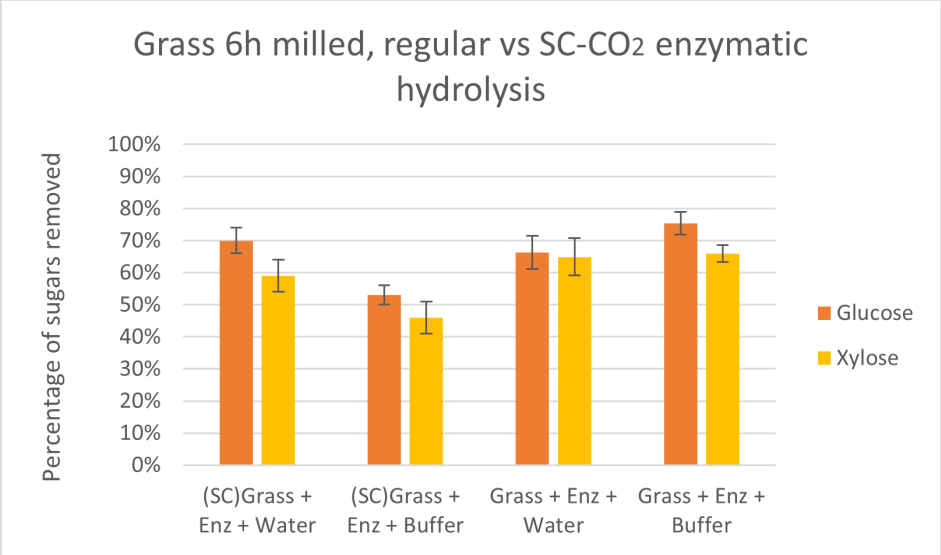

Figure 4.8: Percentage of sugars removed from 6h milled rice with in regular enzymatic reaction versus supercritical CO_2 enzymatic reaction. 4h incubation, 50°C, 1 w/w% of CTec2 enzyme; supercritical CO_2 conditions: T = 50°C, P = 80 bar

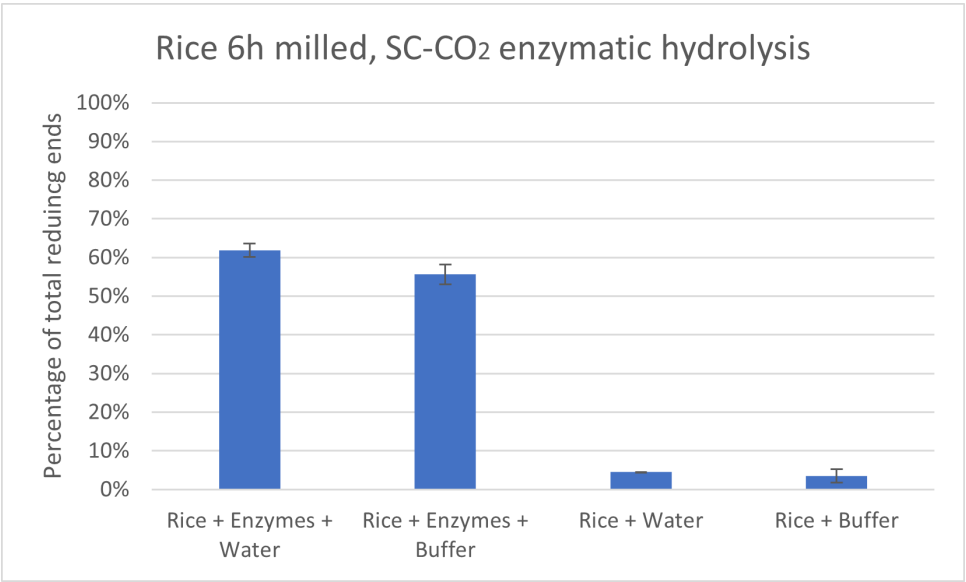

Figure 4.9: Percentage of total available reducing ends of 6h milled rice straw with supercritical enzymatic hydrolysis. 4h incubation, 50°C, 1 w/w% of CTec2 enzyme. supercritical CO_2 conditions: T = 50°C, P = 80 bar

Another interesting observation was made in regard to the effect of the supercritical CO_2, especially the control samples where no enzyme was added. When figures 4.4 and 4.9 are compared, the difference between the respective non-enzymatic samples is large, only 5% of the available reducing ends are observed in the liquid phase while the acid hydrolysis analysis shows that roughly 50% and 70% of glucose and xylose is removed from the pellet. Based on these differences it can be concluded that the supercritical CO_2 conditions break down the solubilized sugars into smaller fragments as it generates more reducing ends.

The control samples, where no enzyme is added to the reaction mixture, shows at least 50% of sugars removed from the pellet, as can be seen in figures 4.4. It is noticeable that this effect is mostly seen in the rice whereas for grass the effect is lower. This adds to the reasoning that the yield under the supercritical CO_2 conditions is caused mostly by the acidity.

4.4 Control experiments

Some control experiments were carried out to rule out any external factors that could have effect on the results of the experiments or to benchmark the selected biomass. Firstly the new selected biomass, the rice straw, was used to test the liquid hot water pretreatment as stated in [53]. The pretreatment and subsequent enzymatic hydrolysis was successful, the graph can be seen in Appendix A.1. Besides the liquid hot water treatment, the effect of diluting the enzymes and storing them in a refrigerator overnight on the enzymatic hydrolysis performance was tested. As the experimental states, after treating the enzymes in supercritical CO_2, the vials were stored in a refrigerator overnight. To make sure that the effect does not come from the refrigerator an experiment was performed where diluted enzymes were stored overnight and compared to regularly stored enzymes. The effect of the dilution and refrigeration was negligible, the graph can be seen in Appendix A.2.

4.5 Heterogeneous catalysis

4.5.1 Characterization

As described in the material and methods section, various methods of characterization were used to characterize the catalyst. The results will be discussed in this section.

N_2 physisorption

To determine the surface area of the catalyst as well as the isotherm type, N_2 physisorption was carried out.

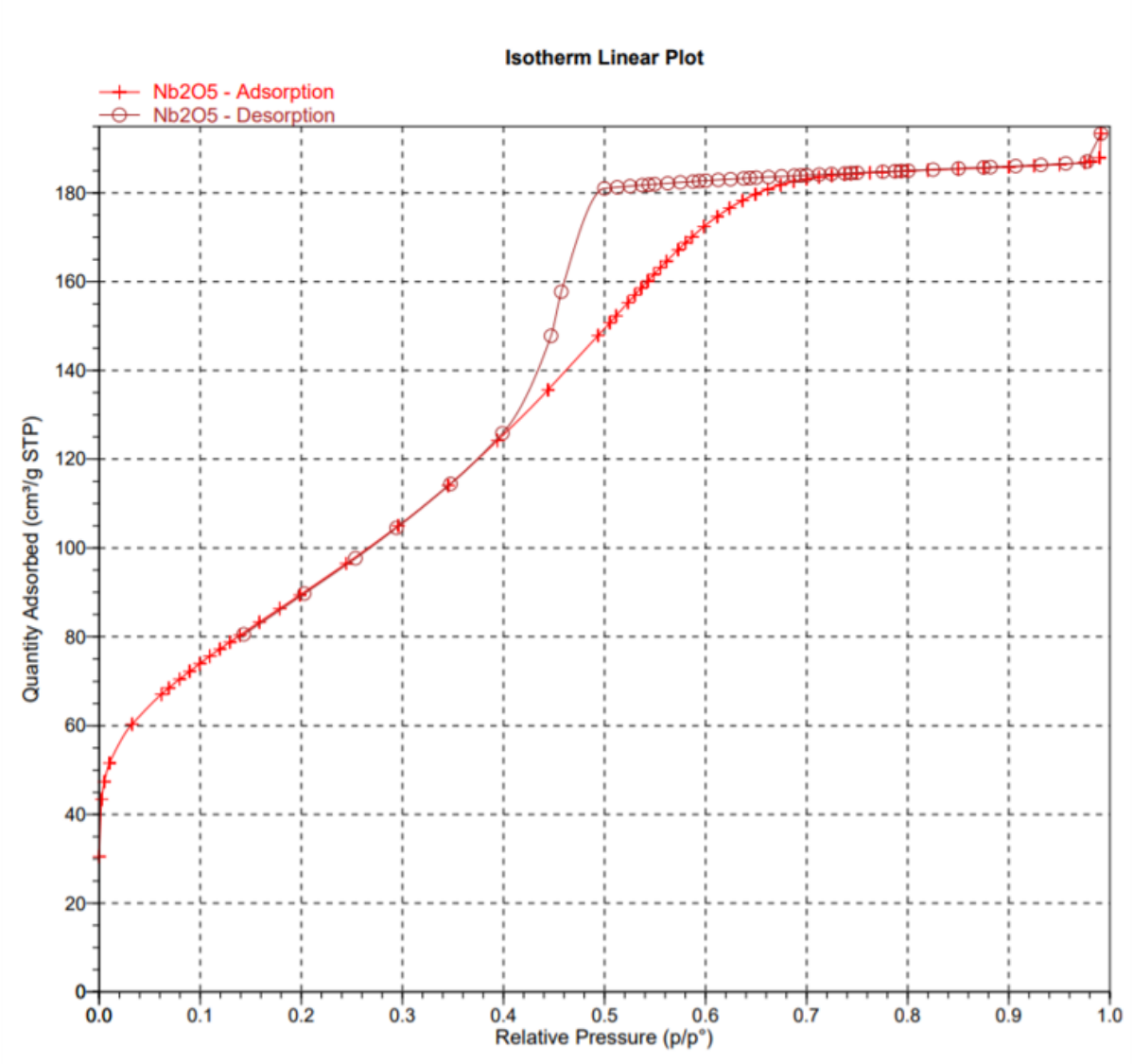

Figure 4.10: N_2 adsorption-desorption isotherms of the Nb_2O_5 catalyst

In figure 4.10 the adsorption-desorption isotherm of the Nb_2O_5 catalyst can be seen. The isotherm belong to type IV, and hysteresis loops are present at high p/p^0 values. These are a result of the interparticle void spaces, that are present in the mesopore scale. The surface area was found to be $325 m^2/g$ which is very comparable to the Nb_2O_5 catalyst synthesized by the same method as described in [50].

X-ray diffraction

To determine the crystallinity of the Nb_2O_5 catalyst, XRD spectroscopy was performed on the catalyst.

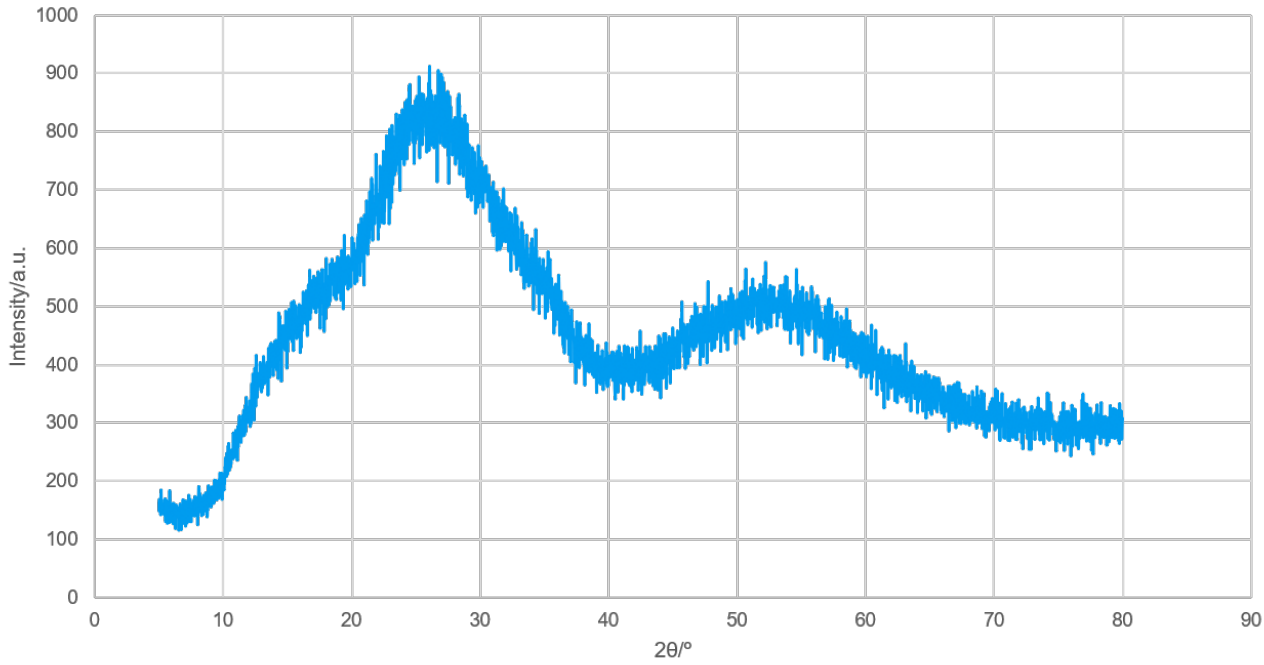

Figure 4.11: X-ray diffraction patterns of the Nb_2O_5 catalyst

In figure 4.11 the XRD spectrum can be seen. As the spectrum shows no specific crystalline structure, it can be concluded that the catalyst is quite amorphous. The broad peaks at 28°and 53°correspond to the NbO_4 tetrahedra and NbO_6 octahedra. The amorphous character of the catalyst is also an explanation for the high surface area. The XRD spectrum was, just as the N_2 physisorption spectrum, very similar to the one shown in [50].

4.6 Catalytic reaction

4.6.1 Benchmark reaction

Table 4.1: Sugar benchmark reaction with turnover numbers (TON) and turnover frequencies (TOF) of subsequent catalysis with Nb_2O_5. Reaction conditions: T = 120°C and t = 3h.

Benchmark	**Amount (mMol)**	**TON**	**TOF**	**Yield**
Glucose 40 mg	2.22E-01	5.67E-01	1.89E-01	34%
Xylose 40 mg	2.66E-01	1.46E-01	4.86E-02	8%
Arabinose 40 mg	2.22E-01	1.24E-01	4.12E-02	7%

The benchmark reaction show turnover numbers of below 1 with yields ranging from 7-34%. As glucose is reacted to 5-HMF, xylose and arabinose are reacted to furfural. As

the yields for the conversion to 5-HMF are higher this is most likely due to the nature of the catalyst, as the Nb_2O_5 catalyst has a mix of LA and BA catalytic sites. The formation of furfural from xylose or arabinose is done with lewis acid catalysts [54]. As a large portion of the lewis acids sites of Nb_2O_5 form brønsted acid sites, this could be a reason why the difference in yield is so substantial.

4.6.2 Supernatant reaction

The reaction selected to use for further catalysis were the supercritical CO_2 enzymatic samples of 6h milled rice and grass as well as the regular enzymatic samples of rice and grass. The supernatants were analysed using HPLC and the following concentrations of the sugars were determined, and can be found in table 4.2. Only a trace amount of arabinose was found, which could be neglected for the catalysis. The trace amount is not surprising, as it is the least present sugar in both the grasses and the rice as can be seen in figure 4.1.

Table 4.2: Sugar concentrations after enzymatic hydrolysis; turnover numbers and turnover frequencies of subsequent catalysis with Nb_2O_5 Reaction conditions: T = 120°C and t = 3h.

Supernatant	**Glucose (mMol)**	**Xylose (mMol)**	**TON**	**TOF**	**Yield**
SC-Rice water	7.04E-03	1.40E-03	2.10E-02	7.01E-03	7%
SC-Rice buffer	7.31E-03	1.29E-03	5.21E-02	1.74E-02	14%
SC-Grass water	1.06E-02	1.47E-03	8.51E-02	2.84E-02	15%
SC-Grass buffer	6.13E-03	7.30E-04	1.11E-02	3.70E-03	3%
Rice water	7.23E-03	8.35E-04	1.09E-01	3.63E-02	41%
Rice buffer	8.47E-03	1.14E-03	1.28E-01	4.26E-02	35%
Grass water	1.06E-02	1.88E-03	1.15E-01	3.83E-02	27%
Grass buffer	1.04E-02	1.82E-03	3.69E-01	1.23E-01	81%

The sugar concentrations are lower than the ones used in the benchmark reactions. A lower catalyst loading (5 mg $\pm 2mg$) was therefore used.

When comparing the turnover number and turnover frequency of the benchmark reactions and the supernatant reaction it is observed that the TON and TOF of the supernatant reaction are much lower than the numbers of the benchmark reactions. The catalyst loading in mg/mg of sugar was higher than the benchmark, but this did not lead to higher TON or TOF. The TON between the samples is also quite large, with the regular enzymatic samples in both solvents having higher TON, almost 10 times higher than samples from the supercritical enzymatic hydrolysis.

Although both the rice and the grass contain 10-12% of xylose and 15-20% of glucose, the concentrations in the supernatant were not in the same ratio. The xylose was 5-10 times less present in the liquid phase than glucose. This adds to the hypothesis that the released xylose from the pellet is not present as monosaccharides in the supernatant, but rather as larger sugar molecules.

The yields differ from each other, which was not expected as the molar amount of the glucose is very similar in each sample. At such low molar amounts it is unclear why the difference is so large. The turnover number is therefore a better point of comparison, as both the amount of starting reagent and the exact catalyst weight differ per sample.

4.6.3 All together

To check whether other factors might influence the total reaction, small steps were taken check whether that is the case. In a supercritical enzymatic reaction, MIBK was added to the mixture (V_{H_2O}:V_{MIBK}; 1:1) and the pahbah reducing ends results were compared with the normal reaction. The results were very similar and thus it was assumed that the addition of MIBK had no effect on the enzymatic hydrolysis. This was expected as MIBK does not dissolve in the aqueous phase.

Unfortunately no 5-HMF was detected in both the organic phase and the water phase. as sugar concentrations in the range of the supernatant samples, as depicted in table 4.2, the bottleneck has to be in the catalytic phase of the total reaction. As the biomass is still present in the reaction mixture, blockage of the catalytic site by leaching of the biomass could occur, preventing the dehydration to occur.

To tackle this issue, the reaction chamber should be split in a two phases where only liquid can pass through, such that the biomass and the catalyst will not interfere with each other.

Chapter 5

Conclusion

5.1 Enzymatic hydrolysis

The CTec2 enzyme is able to perform enzymatic hydrolysis on rice straw and canal grasses under supercritical CO_2 conditions as well as after being treated in supercritical CO_2 conditions. The maximum yield for canal grasses was reached in normal enzymatic conditions, while the maximum yield for rice straw was reached in supercritical CO_2 conditions. These maximum yields were 75% and 65% of the glucose and xylose in grasses and 85% of both glucose and xylose from rice. For regular enzymatic hydrolysis, a buffer solution with pH = 5.0 seems to be a better solvent than water, while for supercritical enzymatic hydrolysis water seems to the better solvent. In rice 20% more glucose was released from the pellet with water instead of buffer was used as a solvent. In grass 10% more glucose was released with buffer as solvent instead of water. This could be related to the difference in the respective lignocellulosic composition of the two types of biomass.

For enzymatic hydrolysis in supercritical co2 conditions, the yield was not improved for the canal grasses. It did however improve for rice, 50% to 85% for both glucose and xylose could be removed from the pellet. The difference in effectivity could be caused by the difference of structure between the two types of biomass or the effect of the supercritical CO_2 that negatively impacts the lignocellulosic structure of the grass.

5.2 Heterogeneous catalysis

The Nb_2O_5 catalyst was successfully synthesized with a novel method using supercritical CO_2. A surface area of $325 m^2/g$ was found. The catalyst was able to convert glucose in 5-HMF with a turnover number of 0.19 and a yield of 40% with a reaction time of 3h and a temperature of 120°C. Using the supernatant from the enzymatic hydrolysis was not as successful. Although some of the yields were in the range of the benchmark reaction, with one outlier even having a higher yield, the turnover number was found to be lower than the benchmark reaction. The amount of glucose in the supernatant was also lower, 100 times as low as the benchmark reaction.

The amount of monosaccharides released by the enzymatic hydrolysis is low, which hinders the production of 5-HMF in the subsequent reaction. For a design of an effective process, an increase of this concentration would be necessary, which can be accomplished by increasing the solid/liquid ratio of biomass to solvent.

Combining the enzymatic hydrolysis under supercritical CO_2 conditions with the sub-

sequent catalysis was not successful at this stage, no 5-HMF was found in the mixture after the reaction were combined. This is most likely caused by blockage of the catalyst by the solid biomass. A new reaction setup should be designed to tackle this problem which could lead to successful combination of these two reaction

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Appendix A

Appendix

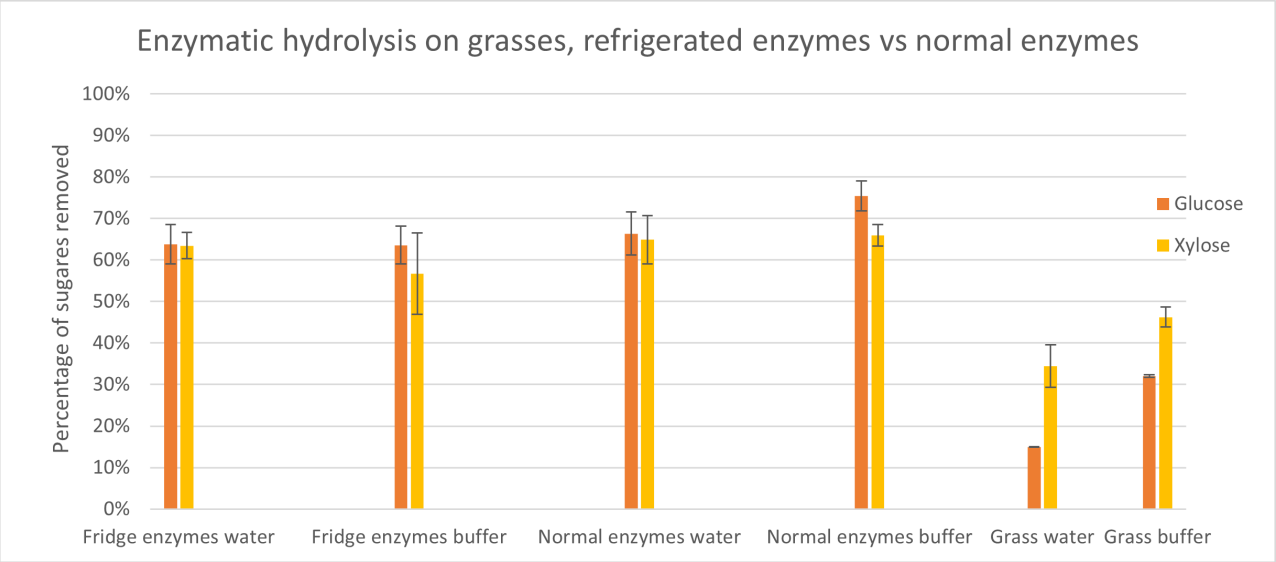

Figure A.1: The effect of dilution and refrigeration on enzymatic hydrolysis

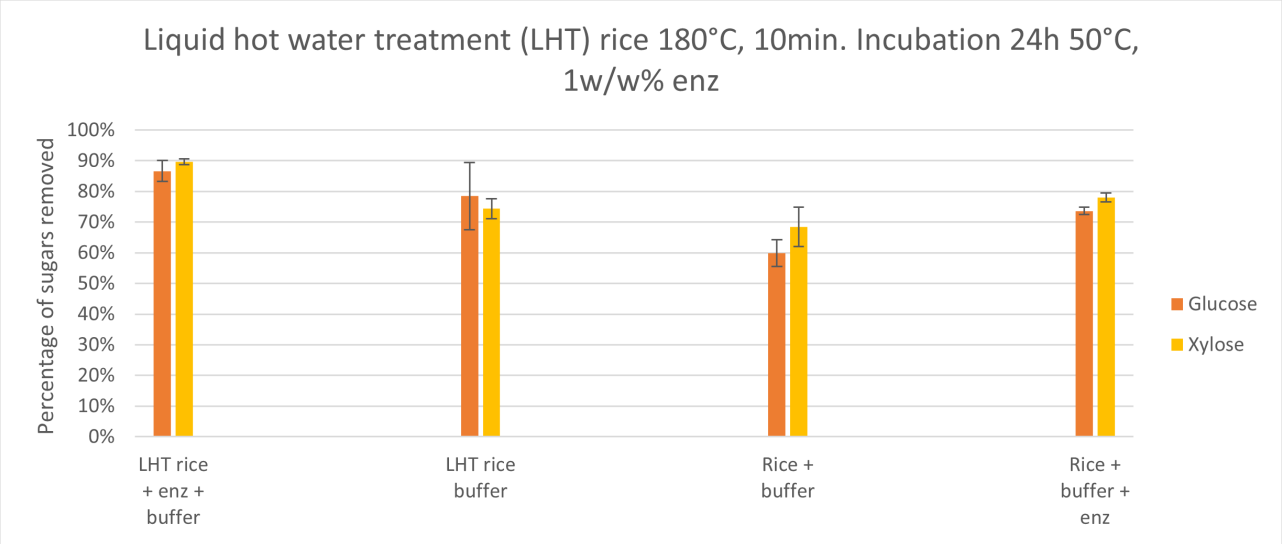

Figure A.2: Liquid hot water pretreatment on the new biomass, rice straw.

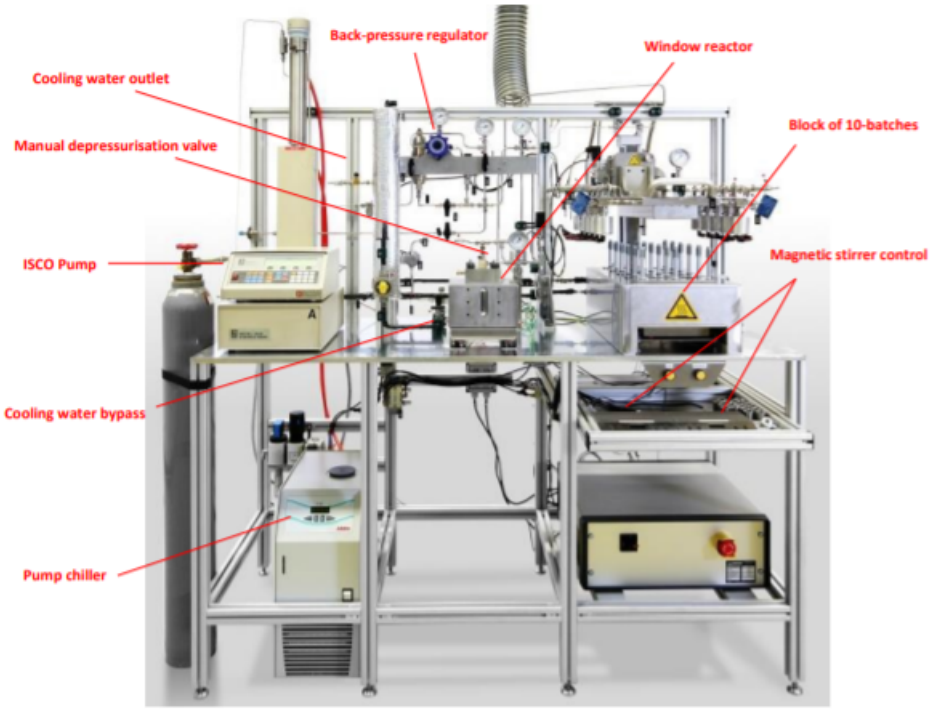

Figure A.3: High throughput CO_2 reactor. Adapted from [50]