Norwegian University
of Life Sciences

Master's Thesis 2019 60 ECTS
Faculty of Chemistry, Biotechnology and Food Science

Enhancing enzymatic saccharification of spruce by 2-naphthol impregnation during pre-treatment

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Acknowledgements

This study was completed at the faculty of Chemistry, biotechnology and Food Science, with Prof. Svein Jarle Horn and Line Degn Hansen as advisors.

I want to show my gratitude to my main supervisor Svein Jarle Horn for allowing me to experience how it is to work in the field of science. I was so lucky to get a project that was a bit challenging, working with some problems that have not yet been solved. Overcoming these challenges would not be possible without the encouragement and support I got from him.

I also want to thank my second supervisor Line Degn Hansen for her huge contribution to my work. She has been a great mentor, teaching me everything I needed to know when carrying out different tasks in the lab as well as introducing me to a great group of friends that have made this experience unforgettable. The completion of my thesis would not be possible without her.

In addition to my advisors I want to thank everyone in the BioRef group for being helpful whenever needed. It has been great working alongside all of them in the lab.

Special thanks to my fellow master students in our lab, Lars Jordhøy Lindstad and Pernille Margrethe Olsen. We have motivated each other through this time with the challenges it brought with it. We have had a lot of fun times, making the days in the lab overwhelmingly enjoyable.

I am forever grateful for the love and support I have gotten from my family. My father, mother, sister and niece have given me the priceless effort of believing in me when I have had troubles believing in myself. My parents have always been great role models though out my life, always pushing me towards pursuing knowledge and ensuring that it is possible to achieve whatever I put my mind to. I am also very grateful for the emotional support I have gotten from my sister. Infinite love and thanks to my family for being there for me through this amazing time.

Finally, I want to thank all my friends, both in and out of the lab. They have made sure that I get out once in a while to remind me to also be social during the time of my thesis. I have gotten a great number of new friends during my time as a master student and it has really given me the feeling that I belong, and for that I am forever grateful.

Abstract

Today, the production of fuels, chemicals and materials is highly dependent on unsustainable fossil reserves. Usage of fossil energy is the key contributor to emissions of greenhouse gasses (GHG). Buildup of GHG such as CO_2 in the atmosphere causes the temperature on earth to increase at an alarming rate, causing environmental problems around the globe. To prevent or slow down anthropogenic climate change it is time to look for other, more sustainable sources of energy. Woody biomass is a renewable and abundant resource that is rich in lignocellulose that can be used for production of fuels and chemicals. Lignocellulose is a recalcitrant matrix rich in cellulose, hemicellulose and lignin. To access the large amounts of sugars the recalcitrant nature of the biomass needs to be overcome using pre-treatment techniques.

There are many possible methods used to pre-treat lignocellulosic biomass. One efficient method is steam explosion which disrupts the lignocellulosic matrix by applying steam under high pressure followed by a rapid drop in pressure. To enhance the efficacy of the pre-treatment it is possible to impregnate the substrate or add a catalyst. Dilute sulfuric acid is frequently used. Dilute acid pre-treatment may cause both economic and environmental problems in downstream processing steps, such as the extra cost of neutralizing the pre-treated material.

Carbocation scavengers, such as 2-naphthol has been used as an additive during steam explosion. Steam explosion causes lignin to de-polymerise and form carbocations which are highly reactive and will repolymerise to form a lignin that is more inhibitory for enzymes. The carbocation 2-naphthol prevents this reaction and thus make enzymatic hydrolysis of the pre-treated material more efficient.

In this study, 2-naphthol impregnation of Norwegian spruce was tested together with steam explosions at different severities to find out how it affected glucose release. The different pre-treatments were assessed by enzymatic hydrolysis using the commercial cellulase cocktail Cellic® CTec 2 under several oxidative conditions to investigate the importance of the lytic polysaccharide monooxygenases (LPMOs) present in the cocktail. This study shows that positive effects of 2-naphthol is related to increased LPMO activity which again is positive for saccharification yield.

Sammendrag

Produksjon og forbruk av energi på jorda i dag er hovedsakelig fossilbasert. Forbruket av fossil energi er hovedfaktoren i utslipp av drivhusgasser som bygger seg opp i atmosfæren. Akkumulering av drivhusgasser som CO_2 i atmosfæren forårsaker en temperaturøkning på jorda som fører til en rekke naturkatastrofer. For å motvirke eller sakne hastigheten på de menneskeskapte klimaendringene er det på tide å se etter andre, mer bærekraftig energikilder. Treverk er en stor og fornybar kilde som inneholder store mengder lignocellulose som kan utnyttes for produksjon av drivstoff og kjemikalier. Lignocellulose er en rigid matriks bestående av sukker som kan fermenteres og lignin. For å komme til de fermenterbare sukkrene må man først bryte ned den rigide strukturen til lignocellulosen ved hjelp av forbehandling.

Det finnes flere ulike forbehandlinger som brukes til å bryte ned lignocellulose. Dampeksplosjon er en av forbehandlingene som brukes mest i dag. Dampeksplosjon bryter og lignocellulosen ved å tilføre dampl under høyt trykk etterfulgt av et raskt trykkfall. Ved å impregnere biomassen eller tilsette en katalysator kan man øke effektiviteten av forbehandlingen betraktelig. En vanlig katalysator under forbehandling er fortynnet svovelsyre. Denne katalysatoren skaper problemer nedstrøms i form av nøytralisering av behandlet biomasse, i tillegg til produksjon av store mengder salter. En måte å unngå dette på er å bruke en mildere katalysator, 2-naphthol.

2-naphthol er klassifisert som en ‘’carbocation scavenger’’. Når lignocellulosen depolymeriserer under dampeksplosjon dannes det lignin-karbokationer som lett kan reagere med hverandre og danne et nettverk som gjør sukkrene mer utilgjengelig. 2-naphthol har vist seg å motvirke denne repolymeriseringen noe som fører til at cellulosen blir mer tilgjengelig og man kan få mer glukose ut av lignocellulosen.

I dette studiet ble norsk gran impregnert med 2-naphthol for å se hvordan dette påvirket glukoseutbyttet etter rekke dampeksplosjoner med ulike temperaturer og trykk. Forbehandlingene ble vurdert med enzymatisk hydrolyse med en kommersiell cellulase cocktail ved ulike forhold for å aktivere lytisk polysakkarid monooxygenaser som finnes in cocktailen.

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1. Introduction

Today, our civilization is heavily dependent on fossil fuels for transport, heat and production of chemicals and materials. Burning coal, natural gas and petrol from refined crude oil is the greatest cause of CO_2 emissions (Höök M., Tang X., 2013). Ever since the industrial revolution, humans have exploited fossil reserves at an alarmingly increasing rate. From the year 1800 to the present day, tapping of fossil reserves has gone from insignificant to around 10 billion tons of oil equivalents every year. That accounts for about 85 million barrels of crude oil every day. This high exploitation of fossil fuels provides raw materials to produce an array of products, such as liquid fuels, plastics, paints and energy. Utilizing this high output of fossil fuels provides society with about 80% of the energy consumed today (oil 32.8%, gas 20.9%, coal 27.2%) (Höök M., Tang X., 2013). The remaining 20% is from nuclear, biomass, solar, wind and hydro power.

The high consumption of fossil fuels results in high rates of emissions. In 2008, anthropogenic emissions of CO_2 were calculated to be around 30 billion tons, with 60% originating from the fossil industry (Höök M., Tang X., 2013). CO_2 along with methane and nitrous gases are considered green-house gases (GHGs) which build up in the atmosphere. High levels of GHGs cause the temperature to rise on earth, leading to several environmental issues. According to The Intergovernmental Panel on climate change (IPCC), if the temperature increase surpasses 1.5°C above pre-industrial levels, sea levels will rise, causing extensive flooding. In addition, it is stated in a special report by IPCC that an increase of 2°C will result in destruction of all coral reefs around the globe. With these environmental threats, a transition from a fossil-economy to a bio-economy is highly needed (IPCC, 2019).

One way to slow down the increase of global temperatures is to turn towards greater utilization of biomass and waste in addition to hydro, wind and solar power. All these resources have a huge potential in energy production as they are renewable and abundant. In particular, liquid fuels originating from biomass can be directly implemented in existing infrastructure. By focusing more on research to improve access to the energy stored in biomass, it is possible to reduce the need for unsustainable sources in the future. There are different types of biomass, which will be described in the sections below. This thesis will focus on the use of second-generation biomass.

1.1 Biomass

Biomass can be categorized into two groups: first and second generation. First generation biomass is obtained from agricultural crops and is often referred to as the biomass fit for consumption by either humans or livestock (Müller G., 2017). Popular sources include corn, different grains and sugar-cane. This type of biomass is an easy feedstock to process due to its high content of sugars or starch, which makes it an excellent source of fermentable sugars. These types of biomasses are used for food and also often fermented with yeast to produce ethanol for human consumption. Some examples are bourbon whiskey made from corn, rum from sugar cane and Scotch whisky or beer made from grain (Kosar K. R., 2010). Similar processes are used for production of bioethanol which is a biofuel. This so-called first-generation bioethanol is mainly produced from sugar cane in Brazil and from corn in the US (Dias et al., 2010).

On the other hand, second generation biomass does not directly compete with food production. Wood and residuals from agriculture and aquaculture are all labelled as second generation. Examples from agriculture include corn stover, sugar cane bagasse and wheat straw. Wood is a great source of energy due to its abundance on Earth.

The plant cell wall in trees consists of cellulose, hemicellulose and lignin and is collectively referred to as lignocellulose. The cellulose and hemicellulose fraction can both potentially be utilized to produce bioethanol in the same way as first-generation biomass. However, these polymers are less accessible and to utilize the sugars from lignocellulose, more elaborate processing methods are needed. Utilizing the large amount of sugars in second generation biomass is more difficult to achieve because of the challenges posed by its recalcitrant structure. Exploring ways to use second generation biomass is therefore a hot topic because of the potential it has regarding production of fuels and chemicals from a source which does not compete with food supply.

In Norway, there is hardly any cultivation of corn and sugar cane. However, Norway is covered with approximately 12.2 million hectares of forest accounting for about 38% of the total land area, with 8.66 million hectares being productive forest (27% of total area) (Stokland et al., 2014). Converted to volume, 8.66 million hectares accounts for 780.9 million m^3 wood without bark. This massive amount of biomass consists of two main types of wood, softwood (gymnosperms) (604,2 m^3) and hardwood (angiosperms) (174,6 m^3) (Stokland et al., 2014). As the names suggest, there are differences between the two regarding composition and density.

As seen in Figure 1.1, hardwood and softwood have quite similar composition when it comes to the three main polymers in the cell wall, cellulose, hemicellulose and lignin. The difference is in the composition of hemicellulose; hardwoods contain high amounts of xylose while softwoods contain high levels of mannose (Figure 1.1) (hemicellulose will be explained more in detail in section 1.1.3.). Examples of hardwoods are birch, oak and ash. For softwood, the most common species are spruce and pine.

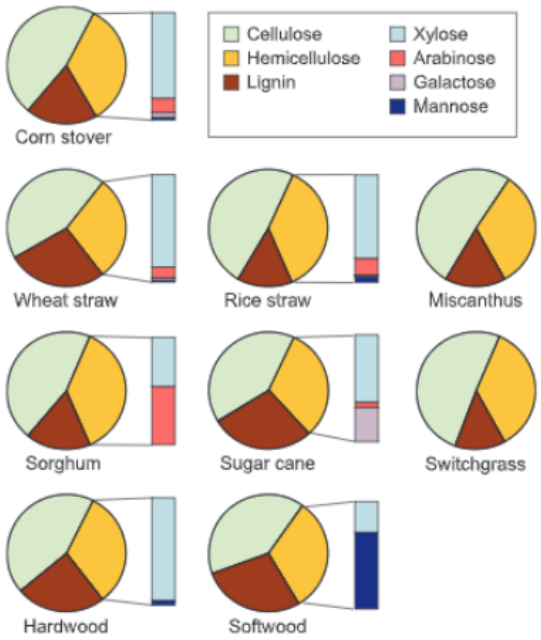

Figure 1.1: Pie-charts ilustrating the main building blocks; Cellulose, hemicellulose and lignin in different plant species. (Stokland et al., 2014)

1.2 Lignocellulose

As previously mentioned, plant cells are encapsulated by a cell wall containing large amounts of lignocellulose. Lignocellulose is a rigid structure consisting of interconnected lignin, hemicellulose and cellulose. The polymers bind together and form microfibrils, with cellulose tightly packed in the middle, surrounded by hemicellulose and lignin (Figure 1.1) (Meng et al., 2014). These fibres are bound together and provide the mechanical support that makes plants stand up.

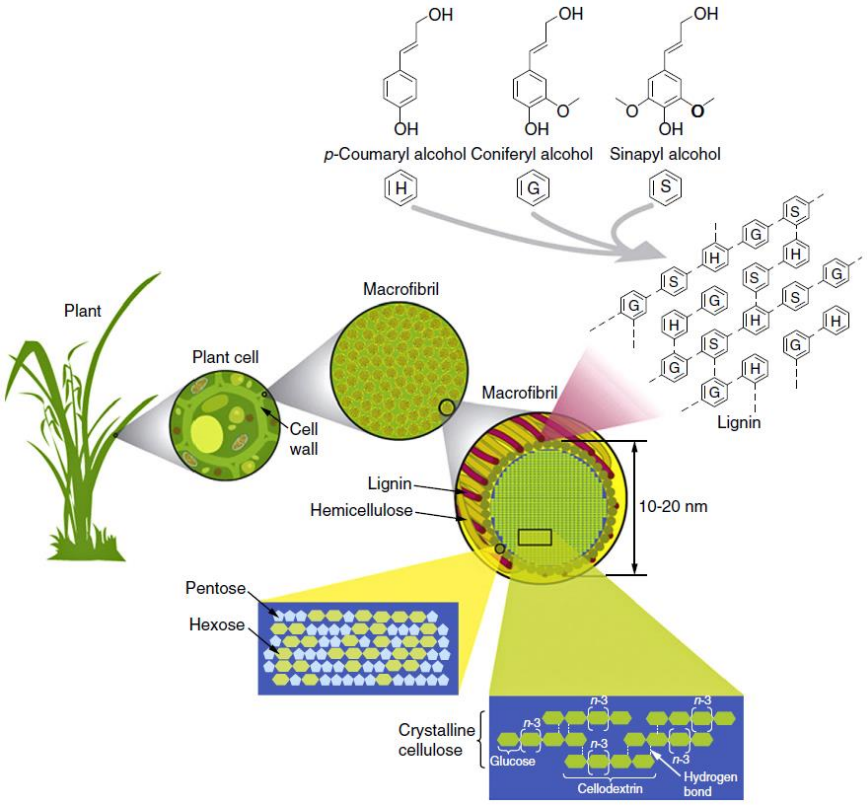

Figure 1.2: Magnifying the lignocellulose fibre showing the three components; lignin, hemicellulose and cellulose. (Meng et al., 2014)

1.3 Cellulose

Cellulose is a crystalline microfibril containing linear glucose polysaccharides held together by hydrogen bonds and van der Waals forces (Horn et al., 2012). The individual cellulose chain exclusively contains D-glucose bound together by β-1,4 linkages (Müller G., 2017). Every other glucose molecule is rotated 180 degrees, resulting in a repeating disaccharide unit called cellobiose. In nature cellulose is synthesized by large cellulose synthase complexes. Embedded in the plasma membrane, cellulose-synthases produce homogenous cellulose chains that aggregate to form crystalline cellulose microfibrils. These crystalline structures are usually of high order but can contain less ordered, amorphous regions (Müller G., 2017) (Figure 1.2).

1.4 Hemicellulose:

Cellulose microfibrils are wrapped in a layer of hemicellulose and lignin. In contrast to cellulose, hemicellulose is a heterogenous polysaccharide with branching, and is composed of different combinations of three hexoses (D-Glucose, D-galactose and D-Mannose) and two

pentoses (L-arabinose and D-xylose) in addition to D-glucuronic acid. The monosaccharides can form a backbone with β-1,4 bonds with branching formed via the hydroxyl groups at C2, C3 or C6 position. (Müller, 2017). The many different combinations of backbone and branching result in many different variants of hemicellulose. The composition of hemicellulose differs between plant species with the most common being xylan, xyloglucans and mannans (Figure 1.3) (Pauly M. & Keegstra K., 2008).

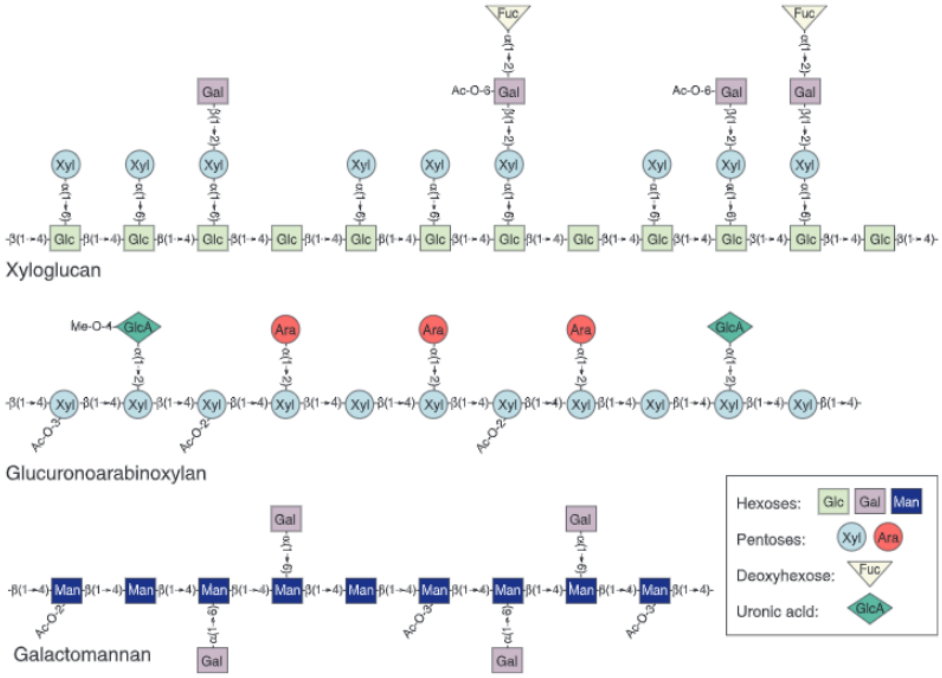

Figure 1.3: Three examples of hemicelluloses present in the cell wall. (Pauly M. & Keegstra K., 2008)

1.5 Lignin

In contrast to hemicellulose and cellulose, lignin is a heterogenous polymer consisting of three aromatic monolignols (phenylpropanoid precursors). P-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol differing in the amount of methoxylations (1, 2 & 3, respectively). The monolignols polymerizes via radical coupling with the help of enzymatic catalysis. Polymerization of lignin results in the formation of guaiacyl (G), syringyl (S) and p-hydroxyphenyl (H). These three constituents are linked together with high variability and different amounts of branching depending on plant species and positioning in the cell wall (Figure 1.4) (Muller, 2017). In softwood, lignin accounts for approximately one third of the total dry-weight.

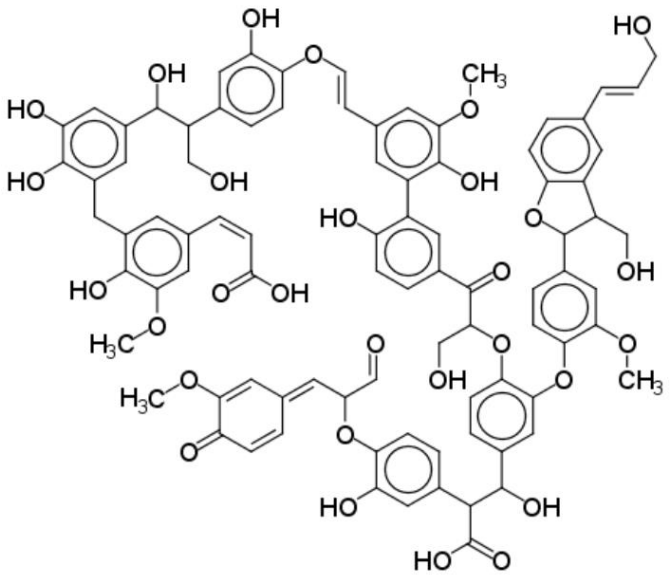

Figure 1. 4: Illustration of a lignin molecule (Kun et al., 2017)

1.6 - Pre-treatment

Due to the recalcitrant nature of lignocellulose, pre-treatment is a necessary step to open up the structure of woody biomass and enhance downstream processing by enzymes. The pre-treatment used in this thesis was steam explosion (SE) with and without addition of 2-naphthol or dilute sulphuric acid. A steam explosion unit is used to open up the structure of lignocellulosic biomass to make it more accessible for enzymes.

SE reduces the recalcitrance of lignocellulose by penetrating the biomass matrix with high pressure steam. When the desired residence time is reached, the pressure is released and this rapid pressure drop causes the steam to expand inside the biomass, ripping the fibre structure apart.

The SE process causes alterations in the lignocellulosic structure. Lignin will depolymerize and eventually repolymerize while hemicellulose and cellulose get partially degraded depending on the severity of the process. Repolymerization of lignin may have an inhibitory effect on enzymatic saccharification, which will be further explained later in this introduction. In addition, organic acids are released from the hemicellulose fraction, lowering the pH of the biomass.

SE treatment makes the biomass accessible for enzymes and make it possible to release monomeric sugars from the cellulose and hemicellulose fractions. However, it is important to use the right temperature and residence time to minimize formation of degradation products of sugars that may be inhibitory for down-stream fermentation processes. The combined effects of residence time and temperature during SE can be described by a single severity factor (R_0). The severity factor is calculated by the following equation (eq1): (Pielhop et al., 2017).

$$R_0 = t \cdot e^{\frac{T-100}{14.75}} \quad \text{(eq.1)}$$

High severity results in higher amount of mechanical disruptions in the lignocellulosic fibres.

1.7 Inhibitory compounds formed by pre-treatment

When exposing lignocellulosic biomass to harsh treatments like SE, several inhibitory compounds can be formed. Degradation of sugars and lignin have the possibility to form inhibitors such as furans, aliphatic acids and phenols (Müller G., 2017). Phenolic compounds such as vanillin and ferulic acid are derived from the aromatic building blocks of lignin, while aliphatic acids and furans are formed from degradation of monosaccharides during the treatment (Horn et al., 2011). All mentioned inhibitors can inhibit both enzymatic hydrolysis as well as downstream microbial fermentation. Together with feedback inhibition by accumulated sugars, phenols have the possibility to irreversibly inhibit cellulose degrading enzymes. In addition, lignin in itself can work as a physical barrier, blocking the enzymes working along the cellulose fibre (Pielhop et al., 2015). Recent studies have shown that the use of a carbocation scavenger during pretreatment can help overcome the inhibitory effect of lignin repolymerization (Pielhop et al., 2017).

1.8 Impregnation with 2-Naphthol

As mentioned above, lignin poses problems in bioconversion of lignocellulosic biomass. It is known that lignin is one of the main challenges when degrading lignocellulose, but the exact mechanism for lignin-inhibition of enzymatic hydrolysis still needs to be elucidated. One known problem is repolymerisation of lignin during thermal pre-treatment, especially in softwoods (Pielhop et al., 2015), which makes lignin more inhibitory for

cellulases. It has been observed that the lignin fraction in softwood is more branched resulting in lower enzymatic degradation of cellulose. In addition, irreversible adsorption of cellulases to repolymerised lignin from softwoods like spruce inhibits hydrolysis of polysaccharides (Pielhop et al., 2015).

When lignocellulosic biomass undergoes SE, organic acids like acetate are released when hemicelluloses are degraded (Müller, 2017). Depending on the biomass and severity of the treatment, pH will be reduced to 2-4. The moderate acidic environment created by the degraded hemicellulose results in formation of carbocations in the degraded lignin (Pielhop et al., 2017). As seen in Figure 1.5, the carbocation can either repolymerise with other lignin fragments, or further degrade. The positively charged fragment is an intermediate in the cleavage of the β-aryl-ether linkages within lignin resulting in further depolymerisation (Figure 1.5 [a-c]) (Pielhop et al., 2015). Fragments formed by this reaction can bind to lignin carbocations, forming large repolymerised molecules.

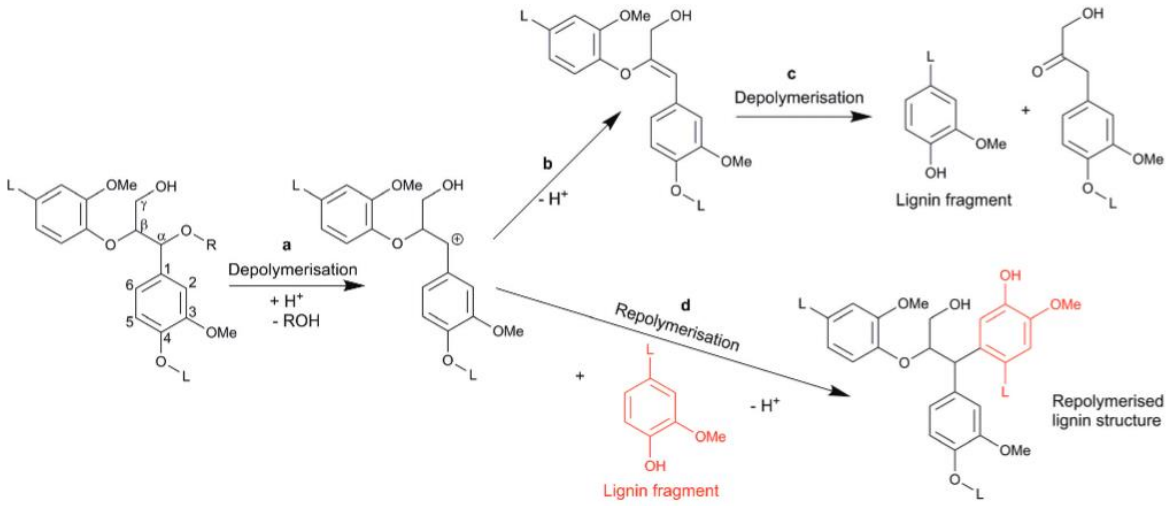

*Figure 1.5: Illustrates depolymerisation and repolymerisation of lignin during pre-treatment. **a** shows depolymerisation of lignin and formation of the carbocation. Path **b** and **c** illustrates further depolymerisation of the carbocation. Path d shows repolymerisation of lignin fragments. Note that the depolymerised fragment formed in path **c** can repolymerise with the carbocations forming larger lignin structures (Pielhop et al., 2015)*

To utilize these carbocations and reduce the amount of lignin re-polymerisation, recent studies show that addition of a carbocation scavenger before SE can be useful (Pielhop et al., 2017). A carbocation scavenger is a molecule that will compete with the aromatic lignin fragments for a reaction with the carbocation. As shown in Figure 1.5, the carbocation scavenger will react with the depolymerized lignin (Figure 1.5 a), preventing it from reacting with lignin fragments (Figure 1.5 b, d) (Pielhop et al., 2015).

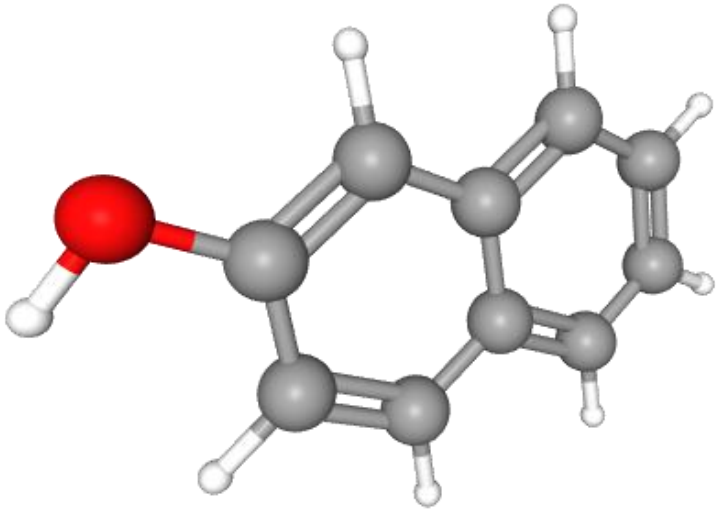

Figure 1.6: Illustration of 2-naphthol (PubChem Database, 2019)

Pielhop et al. have shown that impregnating woody biomass with the carbocation scavenger 2-naphthol has a positive effect on downstream enzymatic hydrolysis. This carbocation scavenger consists of two aromatic rings with an alcohol group on the C_2 carbon (Figure 1.6). There are two reasons why 2-naphthol is an effective carbocation scavenger. First, 2-naphthol is a strong nucleophile, resulting in a strong attack on the lignin carbocation. Second, the reaction between 2-naphthol and the carbocation results in only one electrophilic

substitution, hindering lignin crossing reactions (Figure 1.7) (Pielhop et al, 2015).

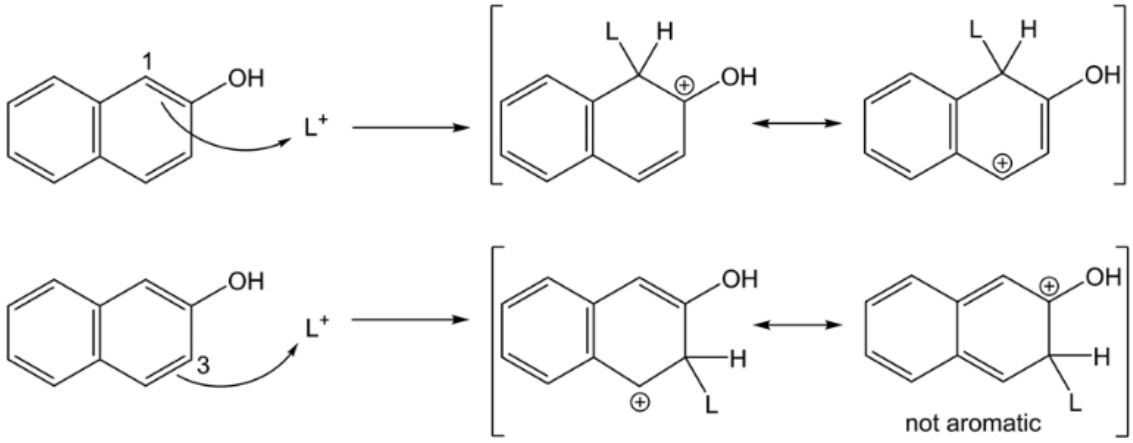

Figure 1.7: Showing the two possible nucleophilic attacks between 2-nathol and lignin carbocations. The substitution happens at C1 in the top reaction and at C3 in the bottom reaction (Pielhop et al, 2015).

At high severities, 2-naphthol has shown to increase saccharification of Norwegian spruce by over 50% when using a concentration of 205 mol 2-napthol/ mol lignin (Pielhop et al., 2017). During steam explosion, nearly all added 2-naphthol will react with lignin, leaving just a small residual concentration, shown to not hinder downstream fermentation by *Saccharomyces cerevisiae* (Seidel et al., 2019). In this thesis, the same concentration of 2-naphthol as in Pielhop et al., 2017 was used when steam exploding Norwegian spruce.

1.9 - Enzymes

When a tree falls, microbes and insects start to consume the nutrients in the wood. Decomposition of wood is a long process with many enzymes involved, each having their own catalytic activity. Brown- and white-rot fungi are good examples of microbes that slowly break down the recalcitrant lignocellulose in nature. By secreting lignin degrading enzymes together with Carbohydrate Active enZymes (CAZymes), the brown rot basidiomycetes fungi can utilize the sugars embedded in the recalcitrant lignin meshwork. White rot fungi contain Lignin-, Manganese- and versatile Peroxidases in addition to laccases have the catalytic power to break down lignin. When hemicellulose and cellulose is exposed, CAZymes start to degrade the polysaccharides (Müller G., 2017)

1.9.1 CAZymes

All discovered Carbohydrate active enzymes are recorded in the CAZy database (www.cazy.org). The CAZy database classify CAZymes based on several characteristics such as 3D structure, sequence similarities and catalytic mechanisms. Several enzyme classes in the CAZy database are related to degradation of lignocellulosic biomass. Four classes are particularly important: Glycoside hydrolases (GH), Carbohydrate binding modules (CBM), Carbohydrate Esterases (CEs) and Auxiliary Activities (AA). The biggest class is GH with a total of 166 families according the CAZy database (Müller G., 2017).

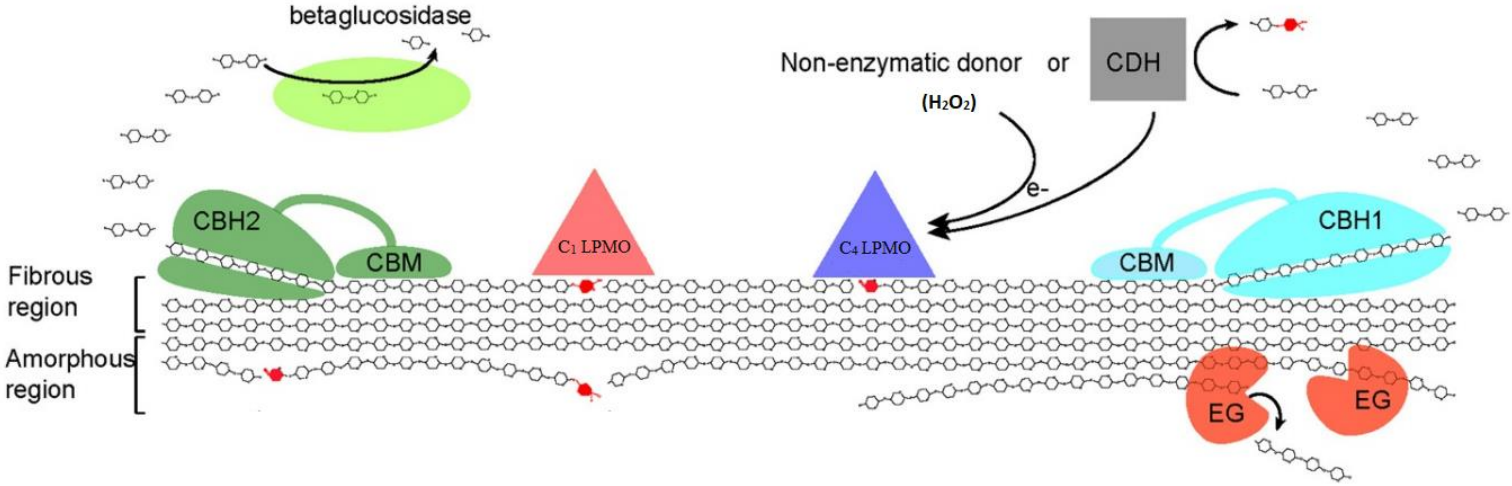

Figure 1.8: Illustration of how the different CAZymes work in synergy to degrade the cellulose fibre. (Horn et al., 2012)

GHs are known for cleaving β-1,4-bonds hydrolytically. Three important subclasses are cellobiohydrolase (CBH), β-glucosidase (BG) and endoglucanase (EG) (Horn et al., 2015). These three classes carry out the same hydrolysis (β -1,4) but on different locations in the cellulose fibre. Endo- 1,4-β-glucanases (EG) makes regions of the cellulose fibre more amorphous by hydrolysing internal bonds in cellulose chains (Figure 1.8) (Müller, 2017). This opens up the structure, making it possible for more GHs to bind. From the reducing or non-reducing end of the cellulose chains cellobiohydrolases (CBHs) work their way processively along the glucose-chain, cleaving every second β-1,4-bond, releasing cellobiose (Horn et al., 2015). Often CBHs are accompanied by a non-catalytic CBM domain. CBMs increase the enzymes affinity to the substrate. Concurrently with the production of cellobiose by CBHs, beta-glucosidases cleave the β-1,4-bond between the two glucose molecules, releasing glucose as its product (Müller, 2017). All mentioned GHs work in synergy as shown in Figure 1.8, degrading cellulose steadily, but there is a bottleneck. Both EGs and CBHs can only attack

single cellulose chains which are difficult to get hold of in a cellulose crystal (Horn et al., 2015).

1.9.2 LPMO – Lytic polysaccharide monooxygenase

In 2010, a new CAZyme was discovered, called lytic polysaccharide monooxygenase (LPMO) (Vaaje-Kolstad G., el al., 2010). By adding this enzyme to cellulase cocktails, the efficiency of hydrolysing lignocellulose increased. LPMOs make nicks in the crystalline regions of cellulose fibrils, creating more chain ends which can be accessed by other enzymes. LPMOs are a powerful mono-copper redox enzyme that bind to the flat, outer surface of cellulose, breaking glycosidic bonds (Bissaro et al., 2018). The enzyme consists of 8 to 10 beta-strands, connected by loops to form a β-sandwich. The loops holding the sandwich in place contain short α-helices that together form the flat binding site of the enzyme. In the middle of the planar binding cite lies a motif consisting of a single copper ion held in place by a highly conserved histidine brace (Figure 1.9) (Bissaro et al., 2018). Though LPMOs have a rigid structure, there is one loop between β-strand nr. 1 and 3 that is highly variable. The variations of this loop are thought to determine the regioselectivity of substrate binding (Chaplin A.K. et al., 2016)

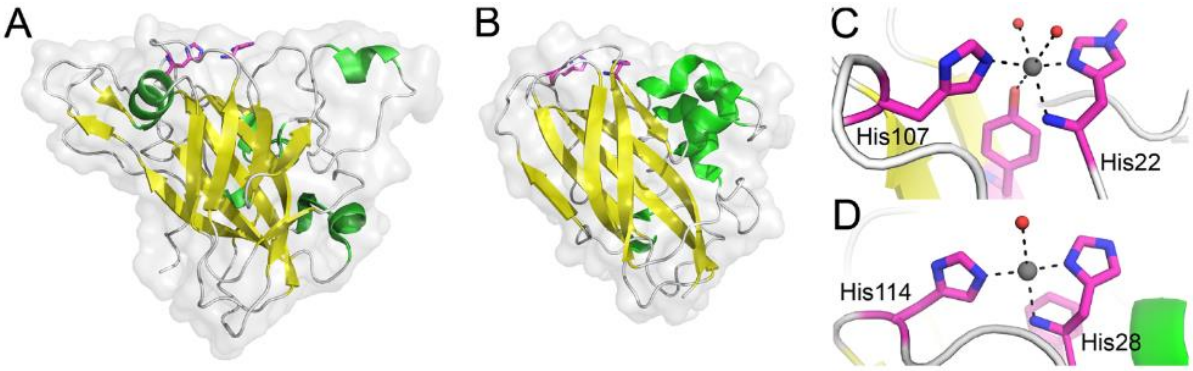

Figure 1.9: Illustration of the 3D structure of an LPMO from Termoascus aurantiacus (TaGH61A) (A) and an illustration of the highly conserved histidine brace in the avtive site(C). B and C show the struvture of chitin active CBP21 from Serratia marcescens and its active site respectively (Horn et al., 2011).

To carry out a glycosidic cleavage, LPMOs need two electrons and two protons. There are two suggested catalytic pathways, one consuming oxygen (O_2) and one consuming hydrogen peroxide (H_2O_2) (figure 1.10) (Bissaro et al., 2018). In both pathways the copper ion must be reduced by one electron to become its active state (from Cu(II) to Cu(I)). This can be done by a variety of reducing agents, such as ascorbic acid, oxidoreductases and even aromatic fragments derived from lignin (Müller, 2017). In the oxygen pathway, O_2 binds with the activated copper forming a LPMO-Cu(II) – radical oxygen complex. With the transfer of a 2nd electron and two protons, the complex can hydroxylate either C1 or C4, resulting in a spontaneous elimination reaction (Figure 1.10). Unlike the oxygen pathway, when LPMOs are in the presence of H_2O_2, no second transfer of a 2nd electron or protons are necessary. H_2O_2 is sufficient to carry out the complete reaction after the first electron have activated the LPMO (Bissaro et al., 2018). As seen in Figure 1.10, H_2O_2 binds the LPMO, releasing H_2O and leaving a single oxygen radical bound to the enzyme. This oxygen pulls of a proton from the substrate and hydroxylates the C1 or C4 carbon in the chain. Like the oxygen pathway, hydroxylation of the substrate results in spontaneous elimination, seen on the right-hand side of Figure 1.10 (Bisarro et al., 2018). It is suggested that the only real co-substrates for LPMOs is H_2O_2. In

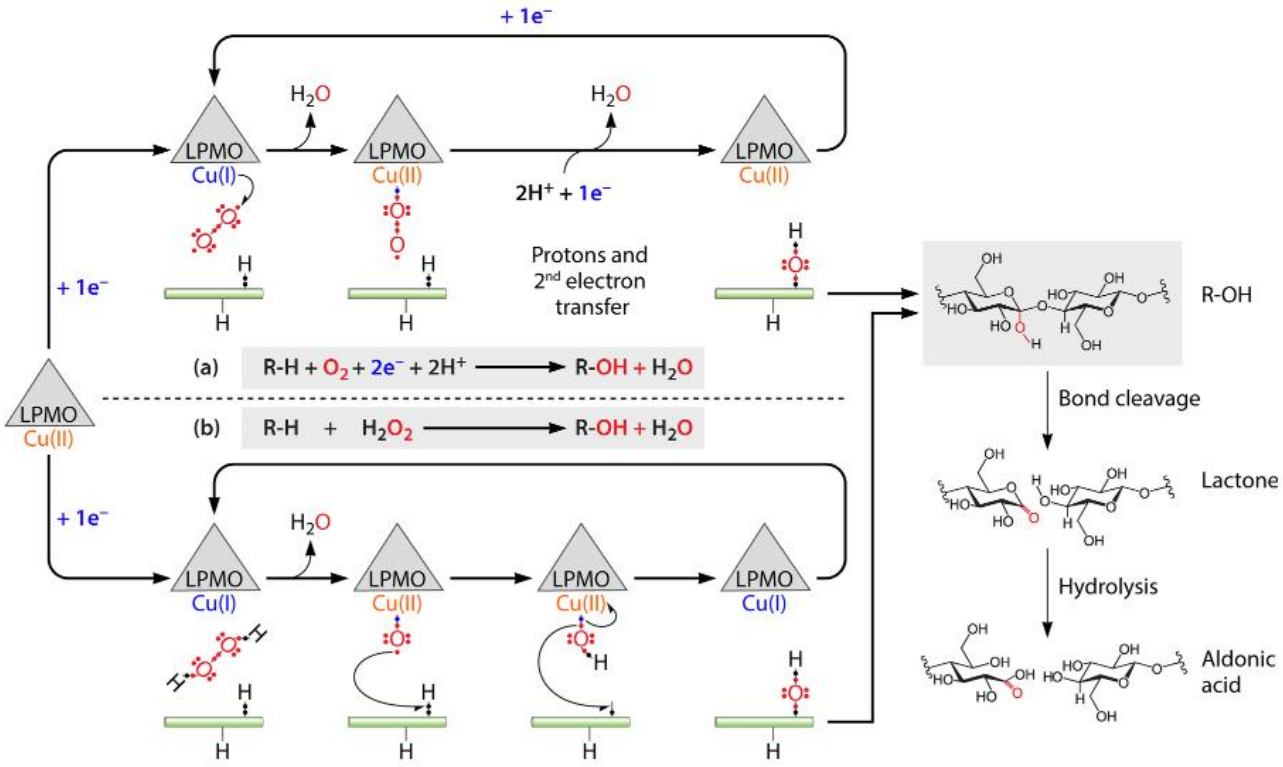

Figure 1.10: An overview of oxygen (a) and hydrogen peroxude (b) based glycosidic cleavage. Both pathways end up with a spontaneous elimination reaction. On the right hand side, a C1 oxidation is illustrated, forming a lactone that forms an aldonic acid after hydrolysis (Bissaro et al., 2018).

reactions with oxygen and electrons present H_2O_2 will be produced in-situ and used by the LPMO (Bissaro et al., 2018). This can be easily overlooked.

In a study done by Müller et al., H_2O_2 was shown to enhance LPMO catalysis of cellulose using the commercial enzyme cocktail Cellic© CTec2 (Müller et al., 2018). Results from the study suggest that H_2O_2 is the favoured co-substrate over O_2, and that oxygen is a precursor to form H_2O_2, rather than a co-substrate (Müller et al., 2018). It was also shown that too high concentrations of hydrogen peroxide resulted in inactivation of LPMOs. Oxidative self-inactivation can happen if LPMOs are exposed to excessive amounts of H_2O_2 or O_2. This can be a problem with LPMOs with poor substrate binding, exposing the copper-histidine brace for rapid oxidation (Bissaro et al., 2018). To prevent this suicide reaction, a steady, low concentration of H_2O_2 can be added over time (Müller et al., 2018). This discovery is relevant to industry because liquid H_2O_2 can be added at a steady pace using automated pumps, eliminating the need for aeration of reactors. An interesting observation has been that H_2O_2 addition to activate LPMOs seem to work best for pure cellulosic substrates. Substrates containing lignin seem to be more challenging, possible because of side reactions between lignin and H2O2, and the use of oxygen and in-situ generation of H_2O_2 may be a better solution (Bissaro et al., 2018).

In this thesis, the goal was to optimize SE treatment by the use of 2-naphtol impregnation to maximize enzymatic sugar release from spruce. A sub-goal was to understand the role of LPMOs in the saccharification step and if direct addition or in-situ production of $H_2O_2$was most beneficial.

2 Materials and methods

2.1 Materials

2.1.1 Equipment:

Table 2.1: Listing equipment used during the work of this thesis

Category	Equipment	Supplier
Appliances	Freezer -20°C	Bosch
	Refrigerator 4°C	Bosch
	Milli Q ® Direct water purification system Direct 16	Merck Millipore
	Water bath – Isotemp®GPD 20	Fisher Scientific
	Incubator – Multitron standard	Infors HT
	Heratherm Oven	Thermo scientific
	Autoclave	Certoclave
	IKA® Dry block heater 1	IKA
	Magnet stirrer	VELP® scientifica
Centrifuge	Heraeus Pico 21	Thermo Scientific
Filtration equipment	Membrane dry vacuum pump/compressor VCP 80	VWR
	Filter crucible – ROBU® H13 Borosilicat 3.3 15mL – Por.4	ROBU
	Multiscreen ® ^{HTS} 96-well plates 0.45µm Durapore ® membrane	Merck Millipore
Miscellaneous equipment	Glassware	Duran Group
	Magnet Teflon stirring bar	SP Science ware
	Pipette refill tips	Thermo scientific & VWR
Bottles, Tubes, vials and plates	Glass shake flasks 50mL	Wheaton, Milliville, USA
	Cellstar® Centrifuge tubes 15ml & 50ml	Greiner Bio-One
	Eppendorf-tubes	Axygen
	Microtiter® 96-well microplates	Thermo scientific
	Snap Ring Micro-Vials, 0.3mL and Snap Ring-caps	VWR
	Pressure tubes 50mL	KIMAX

2.1.2 Recipes of solutions, buffer and eluents:

Solutions:

- 5M NaOH
    - 20g of NaOH pellets in 80ml of dH2O in a beaker. The contents were then transferred to a 100mL volumetric flask where the remaining dH2O was added to make the total volume 100mL
- 1M NaOH
    - 40g of NaOH pellets were mixed in 800mL of dH20 in a volumetric flask. The volumetric flask was then filled to the 1L mark. This was also done on ice.
- 15mM NaOH
    - This solution was Made by mixing 0.675mL of 1M NaOH in 44.325mL dH2O.
- Enzyme dilutions
    - 2-, 4- and 8-times diluted suspensions of Cellic© CTec2 were made by mixing the CTec2 stock (81.9mg protein/ml) with dH_2O

Buffer:

- 500mM Sodium acetate buffer

  Made by mixing 2.860ml of glacial acetic acid in dH2O in a volumetric flask. The volumetric flask is then filled to the 1L mark. After mixing the pH was adjusted to 5 using a 5M NaOH solution followed by filtration through a 0.2µm PES membrane connected to a vacuum pump system.

Eluents:

- ICS-3000 ED1
    - Eluent A:
        - 10.4 mL 50% (w/v) NaOH
    - Eluent B:
        - 84.02 g CH3COONa
        - 5.2 mL 50% (w/v) NaOH
    - Eluent C:

- dH_2O

Eluent A was prepared by adding 2L of dH_2O into a 2L volumetric flask followed by transferring the dH_2O to an ICS eluent bottle. To prepare Eluent B, 83.02g of Sodium acetate (CH_3COONa) was added to a beaker on a magnet stirrer containing about 500mL dH_2O. When the sodium acetate had dissolved, the solution was transferred to a 1L volumetric flask. dH_2O was added to the volumetric flask to a total volume of 1L. The solution was then filtered through a 0.2µm PES membrane vacuum filtration system into an ICS eluent bottle. Eluent C was prepared by adding about 2L of dH_2O to an eluent bottle. Before the eluents could be used in the HPLC, they had to be degassed first, using a Sonics Vibra-Cell™ Ultrasinuc Precessor for 20 minutes. After degassing, 10.4ml NaOH 50% (w/v) was added to Eluent A and 5.2mL 10.4ml NaOH 50% (w/v) was added to Eluent B. After thorough mixing, the bottles were connected to the ICS-3000 system.

- ICS-3000 ED2
    - dH_2O (prepared same way as Eluent C for ICS-3000 ED1
- Rezex long column, mobile phase
    - 5mM H_2SO_4

2.2 Methods

The substrate chosen for this thesis was the stem wood of Norwegian spruce without bark, harvested locally, shredded (3-5mm chips) and dried in a drum dryer.

2.2.1 Milling

Figure 2.1

Milling of the substrate was necessary to reduce the particle size and make the substrate more homogenous. This was done by a cutting mill (SM2000, Retsch, Haan, Germany) (Figure 2.1) in the Biorefinery Laboratory at NMBU. Substrate was added through a funnel at the top of the milling unit, from there it automatically entered for the milling chamber. Three knives were rotating in the chamber, cutting the substrate into smaller particles. A metal sieve was placed underneath the knives and over the outlet to ensure desired particle size of 1mm. The sieve is shaped to fit inside the cutting chamber. Milled substrate was collected in a plastic bag attached below the milling chamber. To prevent overheating, milling was done in intervals of 30 minutes each followed by a cool down period of 30 minutes. With a lot of saw dust particles floating in the air, eye-protection, ear mufflers and a face mask were crucial to keep the sawdust away from eyes, ears and lungs.

2.2.2 Impregnation

After milling, half of the biomass was impregnated with 2-naphthol (Sigma-Aldrich St. Louis, USA). Acetone was used as the solvent to get 2-naphthol into the biomass. Flakes of 2-naphthol was mixed with acetone in a beaker using a magnet stirrer to make a final 2-naphthol concentration of 0.205M. When the flakes of 2-naphthol were fully dissolved, the solution was poured into a bucket containing 1750g of milled spruce. The bucket was placed in a fume hood and the 2-naphthol solution was mixed with the biomass using a spatula (Figure 2.2).

Figure 2.2: Snapshot from a video of mixing substrate and impregnation.

The wet substrate was then spread out on a sheet of plastic inside a fume hood and left for 12 hours to evaporate the acetone (Figure 2.3). During the drying process, the substrate was turned twice to ensure complete evaporation. When dry, the substrate was split in to 350g portions for the steam explosion treatment.

Figure 2.3: Picture of substrate after being spread out on a plastic sheet in a fume hood and left to dry.

2.2.3 Steam explosion

The equipment used for the pre-treatment is called a steam explosion unit. The unit consists of a steam boiler, a pressure vessel, a flash tank and a collection bucket as shown in Figure 2.4. On top of the pressure vessel a ball valve can be opened and closed, allowing addition of feedstock. After addition of feedstock the top valve is fully closed, and steam is added to the vessel which increases the pressure and temperature. Temperature and pressure increase to the desired set-point and the biomass stays for a set time (residence time). When that time is reached, a flash valve is manually opened at the bottom of the reactor and the pressure rapidly drops (explosion), moving the biomass into a collection bucket.

Before the pre-treatment of the samples started, the SE-unit was heated up to the desired temperature by adding steam to the empty reactors. Between treatment of samples, three blank explosions with only steam were carried out to flush the system of any residuals from previous samples. 350g dry matter (DM) of milled spruce was loaded into the pressure vessel followed by closing of the feed ball-valve. When the ball-valve was closed, another valve was opened to let steam enter the vessel, increasing the temperature and pressure. The addition of steam was automatically controlled by a valve that was regulated by measuring the pressure in the reactor and comparing it to the set point pressure. A timer was set to desired residence time when temperature and pressure reached the correct value. In this project, twelve explosions in total were carried out. Details about residence time and temperatures can be seen in Table 2.2.

Figure 2.4: Picture of the steam explosion unit with markers to show the main parts.

Table 2.2: Treatment conditions for all samples. The sample ID will be used to describe the samples further in this thesis.

Sample ID.	Substrate/Biomass	Temperature (°C)	Residence time (min)
190.1U	Norwegian spruce	190	10
190.2U	Norwegian spruce	190	10
190.3U	Norwegian spruce	190	10
190N	Norwegian spruce + 2-naphthol	190	10
200U	Norwegian spruce	200	10
200N	Norwegian spruce + 2-naphthol	200	10
200N5	Norwegian spruce + 2-naphthol	200	5
210U	Norwegian spruce	210	10
210N	Norwegian spruce + 2-naphthol	210	10
210A	Norwegian spruce + 1% H_2SO_4	210	10
220U	Norwegian spruce	220	10
220N	Norwegian Spruce + 2-naphthol	220	10

2.2.4 Dry-matter (DM)

Dry matter content of spruce feedstock and steam exploded spruce samples were determined by means of drying. Aluminium cups were weighed before and after addition of a small amount of sample. Weights were recorded before and after drying in an oven at 105°C over-night and the weight loss was assumed to be solely caused by water evaporation. cup-sample

2.2.5 pH regulation of hydrolysis reaction

Cellic © CTec2 has a pH optimum of 5. To make sure the pH was correct during EH, titration of all samples had to be done prior to hydrolysis. Suspensions with 10% DM were mixed in 50mL falcon tubes. With a pH-meter in the suspension, 1M NaOH was added with a pipette, 100µL at a time until the pH was 5. Amount of NaOH needed to reach a pH of 5 was recorded.

2.2.6 Enzymatic hydrolysis (EH)

The pre-treated biomass was hydrolysed with the commercial enzyme cocktail Cellic© CTec2 in 50ml rubber seal glass bottles as shown in Figure 2.5. Working volume was 20mL with a 10% DM suspension. Prior to addition of enzyme, buffer, base and water was mixed with the biomass. The buffer used was a 500mM sodium acetate buffer with a pH of 5. To adjust the final pH of the mix, 1M NaOH was added according to the calculations made by titration of the biomass. Amounts of the different components added are shown in Table 2.2.

Figure 2.5: 50ml bottles with substrate, buffer, dH2O and NaOH. In this picture, all bottles have been preheated and mixed from 15 minutes at 50°C and are ready for enzyme addition to start the hydrolysis reaction.

During this project, several runs of EH has been carried out. The fist EH was screening of all samples under three conditions, aerobic, anaerobic and anaerobic with addition of hydrogen peroxide (H_2O_2).

2.2.7 EH screening experiments

2.2.7.1 Aerobic EH screening

Biomass, buffer, water and base was mixed in the 50mL bottles as seen in Figure 2.5. All samples were hydrolysed in triplicates with a blank (no enzyme added) as control. The amounts of the different ingredients are listed in Table 2.3. When all ingredients (except enzyme) were mixed, a rubber cap was added to seal the bottles. All bottles were left in an incubator for 15 minutes at 50°C and shaking at 200rpm to ensure correct starting temperature. During the heating, the rubber caps were lifted to release pressure to maintain normal pressure. After 15 minutes an aluminium cap was mounted ensuring an air tight seal. Then the reactions were started by adding 0.39mL of diluted enzyme cocktail using a 1mL syringe (Table 2.3). The amount of enzyme used in this screening was 4mg protein/ g DM substrate. The enzyme solution was prepared by diluting the commercial stock enzyme cocktail four times. Time was recorded at the moment enzyme was added and the incubator was set to a shaking speed of 200rpm. The EH was carried out for 48 hours with sampling points at 0h, 24h and 48h. A 300µL sample was collected in Eppendorf tubes using a syringe with a thick needle to prevent clogging. The enzyme in the samples inactivated by boiling for 15 minutes in a heat block at 100°C before being stored at -20°C until HPLC analysis.

2.2.7.2 Anaerobic EH screening

For this experiment, when mixing biomass with the ingredients the same approach was used as in the aerobic screening (see description above). However, in this experiment all bottles were sparged with N_2 for 2 minutes at a flow rate of 200mL/min before pre-heating (Figure 2.6). After sparging any over-pressure was removed by inserting a syringe filled with water until no bubbles were formed. Temperature, shake speed and sampling points were the same as the aerobic screening.

Figure 2.6: Sparging setup.

2.2.7.3 Anaerobic EH screening with addition of hydrogen peroxide (H_2O_2)

The last screening was with the addition of hydrogen peroxide. The preparations for this screening was the same as for the anaerobic screening experiment (Table 2.3).

Hydrogen peroxide (H_2O_2) was added manually every hour through the rubber caps with a Hamilton syringe 20µl of 90mM H_2O_2 was added every hour to achieve H_2O_2 concentration of 90µM/hr. Temperature, shake speed and sampling points were the same as the aerobic and anaerobic screening experiments.

2.2.8 Aerobic EH of select samples with different enzyme doses

Based on the results from the screening experiments, the samples used for further experiments were narrowed down to the best performing samples with and without 2-naphhol, namely 210U, 210N, 220U and 220N (Table 2.2). This time samples were hydrolysed without triplicates. Before adding the enzymes, pH was adjusted to 5 in each bottle using 1M NaOH and a pH-meter. The correct amount of NaOH to achieve a successful EH is listed in Table 2.3.

The enzyme doses used for this hydrolysis were 2, 4 and 8mg/g DM. The enzyme cocktail was diluted just before it was added. To make 2,4 and 8mg/g DM dilutions, the enzyme stock was diluted (with dH_2O) 8, 4 and 2 times, respectively. After enzyme was added, sampling was carried out at 0, 4, 12, 24 and 48 hours using syringes. The samples were then boiled for 15 minutes. Samples were stored in -20°C before analysis with HPLC.

Table 2.3: Listing of the different components needed in the enzymatic hydrolysis.

Sample ID	Substrate (wet weight(g))	Ctec2 (Concentration?) (ml)	SodiumAcetate Buffer 500mM (mL)	1M NaOH (mL)	dH2O (mL)
190U	8,26	0,39	2	0,1	9,25
190U	8,94	0,39	2	0,15	8,52
190U	6,14	0,39	2	0,2	11,26
190N	6,02	0,39	2	0,5	11,13
200U	6,25	0,39	2	0,3	11,06
200N	6,35	0,39	2	0,4	10,86
200N5	5,39	0,39	2	0,45	11,97
210U	6,57	0,39	2	0,6	10,44
210N	7,07	0,39	2	0,7	9,84
210A	13,25	0,39	2	3	1,16
220U	7,89	0,39	2	0,8	8,91
220N	7,28	0,39	2	0,95	8,45

2.2.9 Compositional analysis

Determination of cellulose, hemicellulose and lignin was carried out based on the standard operating procedure by NREL (Sluiter A., 2008). The first step in the analysis was drying the steam exploded materials.

Approximately 150mg of dry sample was added to 50ml pressure tubes. The exact weight of added substrate was recorded for future calculations. H_2SO_4 hydrolysis was used to dissolve the biomass. 1.5mL of the acid was added to the pressure tube. It was crucial that all the substrate was completely covered by the acid (72% sulfuric acid). Right after acid is added, the tube was incubated in a water bath at 30°C for one hour.

After one hour of incubation, the solution was diluted with 42ml dH2O. In addition, sugar recovery standards (SRS) were prepared by mixing a 5mL SRS-stock (1g/L L-arabinose, D-mannose, D-galactose, D-glucose and D-xylose from Sigma Aldrich, Missouri, USA) with 174uL of 72% H2SO4. The next step was to autoclave the tubes for 1 hour.

The timer was started when the autoclave reached 121C. After autoclaving and cooling, the solution in the tubes was filtered through pre dried and pre-weighed filter crucibles (one filter per tube) A small amount of the filtrate (5mL) was sampled for analysis. The residual solids in the crucibles was dried over night at 105°C and then weighed again before burning in a furnace at 550°C for two hours. Weight of the burned crucible was recorded to calculate residual solids.

The filtrate was diluted 20 times using a 15mM NaOH solution. The applied standards were diluted from the stock solution of 1g/L arabinose, maltose, galactose, xylose and glucose. Five standards were made: 0.1g/L, 0.07g/L, 0.05g/L, 0.03g/L and 0.01g/L. All diluted samples and standards were added to HPLC-vials for analysis.

2.2.10 Analysis by High performance liquid chromatography (HPLC)

The equipment used for the HPLC analysis was a Dionex Ultimate 3000 (Dionex, Sunnyvale, USA) connected to a refractive index (RI) detector 101 (Shodex, Japan). For analysis of glucose release a Rezex ROA-organic acid H^+ (8%) 300x7.8mm analytical column (Rezex) (Phenomenex, Torrance, USA) was used. To analyse the filtrate from the compositional analysis and detect C4-oxidized products from the EH a Dionex ICS 3000 connected to a pulsed amperometric detector (ICS 3000) (PAD, Dionex) was used (Müller et al., 2017)

Before samples can be analysed by HPLC, they must first be diluted and filtered. All samples were prepared the same way, but with different dilution depending on which system was being used. The setup and equipment used in this process can be seen in Figure 2.6. When running compositional analysis, samples must be diluted 20 times to be in the right range for quantification, while for determination of glucose and C4-oxidised products concentrations, hydrolysis samples were diluted 5 times.

When handling samples for analysis of oxidised products, everything was done on ice since this C4-oxidised cellobiose is unstable and will degrade over time. Samples for compositional analysis was diluted 20 times by adding 50µL of sample to 950µL dH_2O.

Figure 2.6: Setup for sample preparation prior to HPLC.

The 5 times dilutions made for both the ICS 3000 and Rezex were made by mixing 40µL of sample with 160µL of dH_2O. Wide pipette tips were used to get both solids and liquid in the dilution without clogging. After dilution, the samples were transferred to a 96 well filter plate and filtered into a Microtiter® 96 well microplate. When filtered and diluted, the samples were transferred to 0.3mL snap ring micro-vials sealed with ring caps ready for analysis (Figure 2.7).

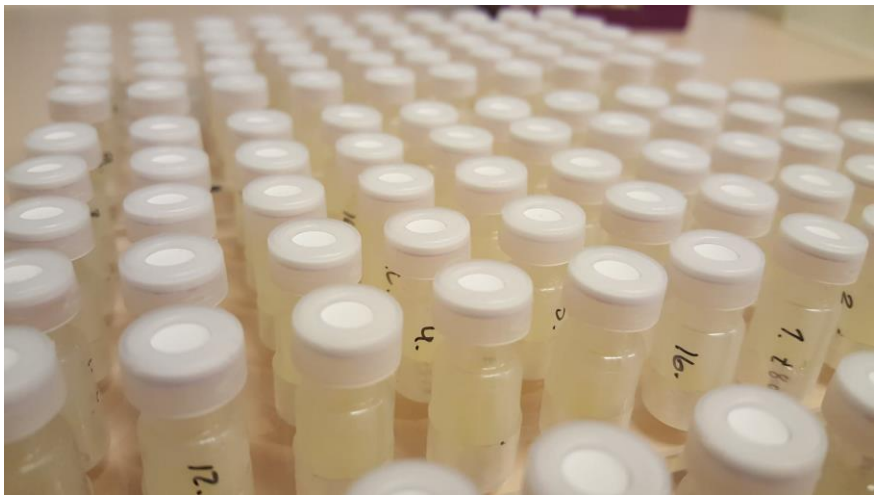

Figure 2.7: Picture of vials used for analysis with high performance liquid chromatography.

3. Results and discussion

3.1 Dry-matter analysis pH measurement

As mentioned in the introduction, steam explosion pre-treatment of lignocellulosic biomass will result in release of acidic compounds from the hemicellulose. In a set of experiments milled spruce was steam exploded at temperatures ranging from 190C to 220C with and without impregnation of 2-naphthol. Dry matter content and pH were measured for all samples after the pre-treatment. Figure 3.1 illustrates the relationship between severity and pH in addition to the dry matter content of the samples after SE (Figure 3.1).

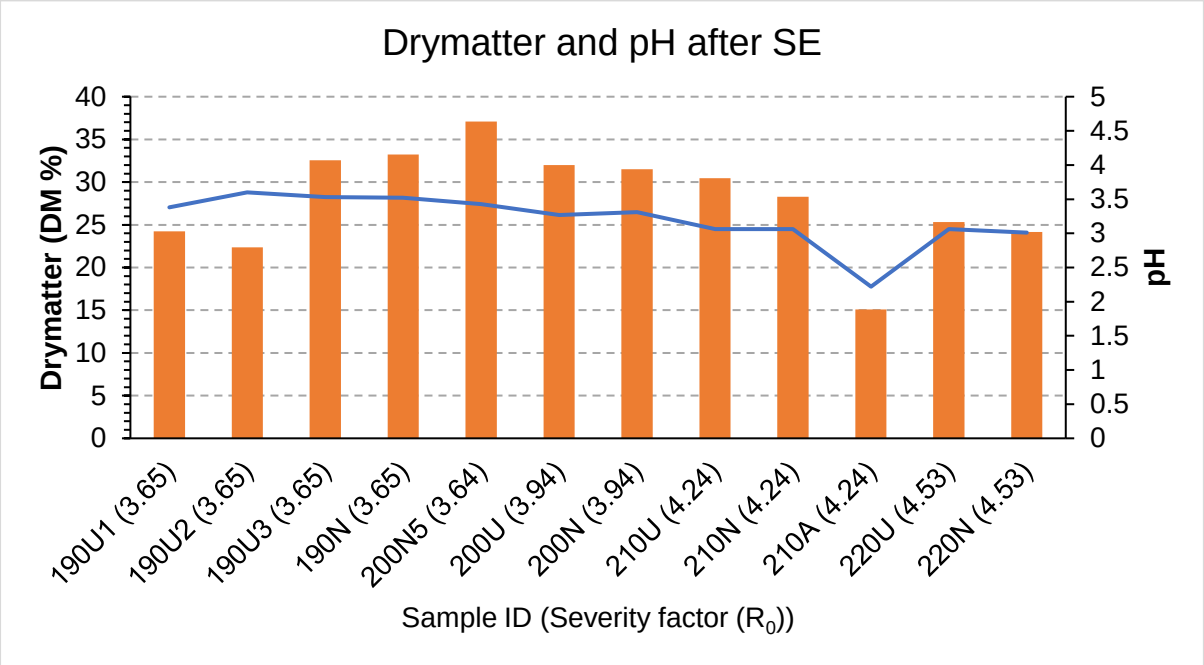

Figure 3.1: Combination graph that shows how the severity factor affects dry matter content and pH in the biomass (blue line). The sample ID explains the treatment conditions. The number represents the temperature of the treatment and the three letters U, N and A. U refer to unimpregnated, impregnated with 2-naphthol and acid treated respectively. 200N5 is impregnated spruce with residence time of 5 minutes. All other samples had a residence time of 10 minutes.

The blue pH line in Figure 3.1 shows that pH decreases when the severity of the treatment increases. In general, the pH of the samples was in the range 3-3.5, except the sample treated with sulfuric acid (210A) where the pH was added acid prior to the steam explosion treatment. Such low pH after pre-treatment makes it necessary to add NaOH to adjust the pH to the optimum of the enzyme cocktail used prior to the saccharification experiments

The bars in Figure 3.1 shows the dry matter content (DM%) in each sample after SE. By looking at the three initial triplicate samples (190U1, 2 and 3) it seems like the first two steam explosion treatments resulted in higher content of water in the samples. The reason could

be that the pre-treatment reaction initially was not fully heated up so more steam needed to be added to reach the set point temperature. Aside from that, the bars in Figure 3.1 indicates that samples treated with the same severity had approximately the same dry matter content (excluding 210A). Samples treated at temperatures above 200°C had higher water content. Sample 210A had a very high moisture content due to extensive acid degradation (Figure 3.2).

Figure 3.2: Picture of steam exploded spruce. The different samples were added to cups to show how the appearance of treated samples after steam explosion. Note how the high moisture content in the acid-hydrolysed sample (210A).

In Figure 3.2, clear differences in the appearance of the steam exploded samples can be seen. The biomass turns darker with higher severity. This is due to the higher amounts of exposed lignin in the samples (Vivekanand V., 2013). The acid treated sample has the highest water content and appears more liquid, while impregnation with 2-naphthol did not have a visible effect on the samples.

3.2 Compositional analysis

Initially compositional analysis of all samples was carried out but gave unreliable results with large standard deviations. After optimizing the procedure and minimizing user errors however, a new set of samples were analysed. Due to time constrains, only select samples were re-run in order to calculate glucose-yields for the best performing samples during

enzymatic hydrolysis. The samples selected were 210U, 210N, 220U, 220N in addition to the untreated sawdust. Pre-treatment severity, dry-matter, pH and results of the compositional analysis are listed in Table 3.1. The amounts of components in the samples are too low to use in calculation of glucose yield. A possible reason for the low values obtained could be user error such as leaving the pressure tubes in the autoclave for too long. Another reason can be that the acid hydrolysis is too weak to completely degrade the sugars in the samples.

Even though the compositional analysis was partially unsuccessful, it is still possible to see how the pre-treatment affects the substrate. Table 3.1 shows that spruce consists mainly of glucan (cellulose) and lignin. Glucan content in the four samples was around 350 g/kg DM. Spruce steam exploded at the same conditions as sample 210U is supposed to have around 45% glucan according to a study done by Kalyani et al. (Kalyani et al., 2017).

When softwood is exposed to hydro thermal treatments at high severities the hemicellulose fraction will partially degrade. Comparing the steam exploded samples (210U, 210N, 220U and 220N) with the untreated biomass (Table 3.1, Untreated) confirms that the hemicellulose is partially degraded. Arabinan is completely degraded while there are still small amounts of galactan, xylan and mannan present in the treated biomass. Pentoses like arabinose and xylose are known to be more labile to temperature than hexoses (Kabel et al., 2007).

Table 3.1: An overview of the different components of the pre-treated biomass as well as the untreated biomss.

Compositional analysis of selected samples

Sample ID	Severity (R_0)	DM(%)	pH	Arabinan (g/kg DM)	Galactan (g/kg DM)	Glucan (g/kg DM)	Xylan (g/kg DM)	Mannan (g/kg DM)	Lignin (g/kg DM)
190U	3.65	24.22	3.38	n.a	n.a	n.a	n.a	n.a	n.a
190U	3.65	22.37	3.6	n.a	n.a	n.a	n.a	n.a	n.a
190U	3.65	32.55	3.53	n.a	n.a	n.a	n.a	n.a	n.a
190N	3.65	33.25	3.52	n.a	n.a	n.a	n.a	n.a	n.a
200N5	3.64	32.00	3.27	n.a	n.a	n.a	n.a	n.a	n.a
200U	3.94	31.52	3.31	n.a	n.a	n.a	n.a	n.a	n.a
200N	3.94	37.11	3.43	n.a	n.a	n.a	n.a	n.a	n.a
210U	4.24	30.46	3.06	0.00	10.21	320.39	16.46	45.52	336.71
210N	4.24	28.30	3.06	0.38	6.36	372.67	23.51	56.10	356.73
210A	4.24	15.09	2.22	n.a	n.a	n.a	n.a	n.a	n.a
220U	4.53	25.32	3.06	0.00	4.17	356.57	7.61	24.30	426.30
220N	4.53	24.15	3.01	0.00	2.38	375.06	10.28	21.84	433.88
Untreated	-	95.00	-	8.44	10.73	313.79	41.76	84.77	295.77

3.3 Screening of samples by enzymatic hydrolysis

The goal of this initial screening was to assess the effects of the different pre-treatments on the enzymatic hydrolysis step and identify the best pre-treatment conditions. To evaluate this, glucose release was measured to identify pre-treatments were the cellulose was readily available for the enzymes.

Three screening conditions were carried out, aerobic, anaerobic and anaerobic with additions of hydrogen peroxide (H_2O_2). LPMOs in the enzyme cocktails can be activated under aerobic conditions (in situ production of H_2O_2) or by H_2O_2, but not under anaerobic conditions (Bissaro et al., 2018). Thus, the anaerobic hydrolysis represented control reactions were only the hydrolytic enzymes of the enzyme cocktail were active. The release of glucose in g/L for the screening reactions are seen in Figure 3.3, 3.4 and 3.5. The sample treated with acid (210A) resulted in the highest amount of glucose release (30g/L under aerobic conditions after 24

hours). This sample however, had a lot of the glucose already in solution before the enzymatic hydrolysis (see time zero) since most of the glucan had already been hydrolysed by the acid during the SE, making it more of an acid hydrolysis than an enzymatic hydrolysis.

The remaining samples had relatively low glucose release under all conditions. It was expected that the hydrolysis under aerobic conditions would perform better than the anaerobic, but in these screening reactions, there were no significant differences. In addition, it is believed that the reactions with added H_2O_2 should perform better than aerobic and the anaerobic based on a study done by Müller et al (Muller et al., 2018). Müller et al. were able to achieve a glucan conversion of 76.5% when adding 90µM H_2O_2 per hour in a 10% DM enzymatic hydrolysis. For sample 220N in the aerobic, anaerobic and the H_2O_2 screenings glucose release after 48 hours of hydrolysis were 19.05g/L, 16.83g/L and 18.83g/L respectively. This indicates that addition of an oxidant was important for sugar release, where the oxygen or H_2O_2 seemed to have similar positive effect on the hydrolysis. pH of the hydrolysates was measured and found to be around 6.0-6.5. Thus, the pH had been adjusted to a too high level compared to the optimal pH of the enzyme cocktail (in the range of 4.5-5.0) and can explain the relatively low glucose release. For the remaining experiments the pH was correctly adjusted. As seen in Table 3.2, the amount of added NaOH in the screening was quite high compared to the adjusted amount that resulted in higher yields in the later experiments (see section 3.4 and 3.5).

The environment in the bottles was too basic for the enzyme cocktail resulting in low glucose release. The reason for this was that the pH-meter did not measure accurately in the biomass slurry (see Table 3.2).

Table 3.1: Showing the amount of NaOH added to adjust pH to 5. Initial NaOH is the same amount used in the screening. Adjusted is the amount added to further experiments.

Sample	210U	210N	220U	220N
initial NaOH	1.0mL	1.2mL	1.6mL	1.8mL
Adjusted	0.6mL	0.7mL	0.8mL	0.95mL

Even though the glucose yields were low in these screenings, clear differences could still be seen between the samples. Narrowing down to fewer samples was done based on the best performing samples not including the acid hydrolysed sample. This resulted in selecting sample 210U, 210N, 220U and 220N for further experiments. This set of samples clearly gave the highest release of glucose, indicating that high severity in the pre-treatment is important. There also seems to be a trend where samples impregnated with 2-naphthol gave a higher release of glucose.

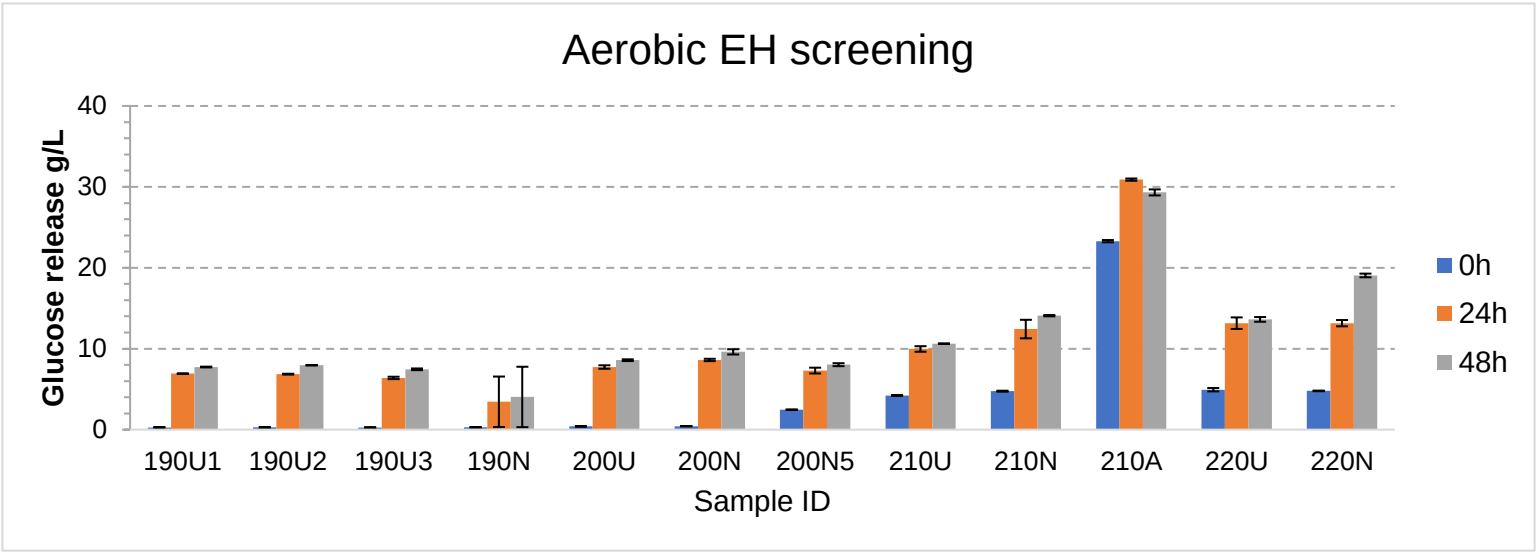

Figure 3.3: Bar graph showing glucose release during 48 hours of aerobic enzymatic hydrolysis.

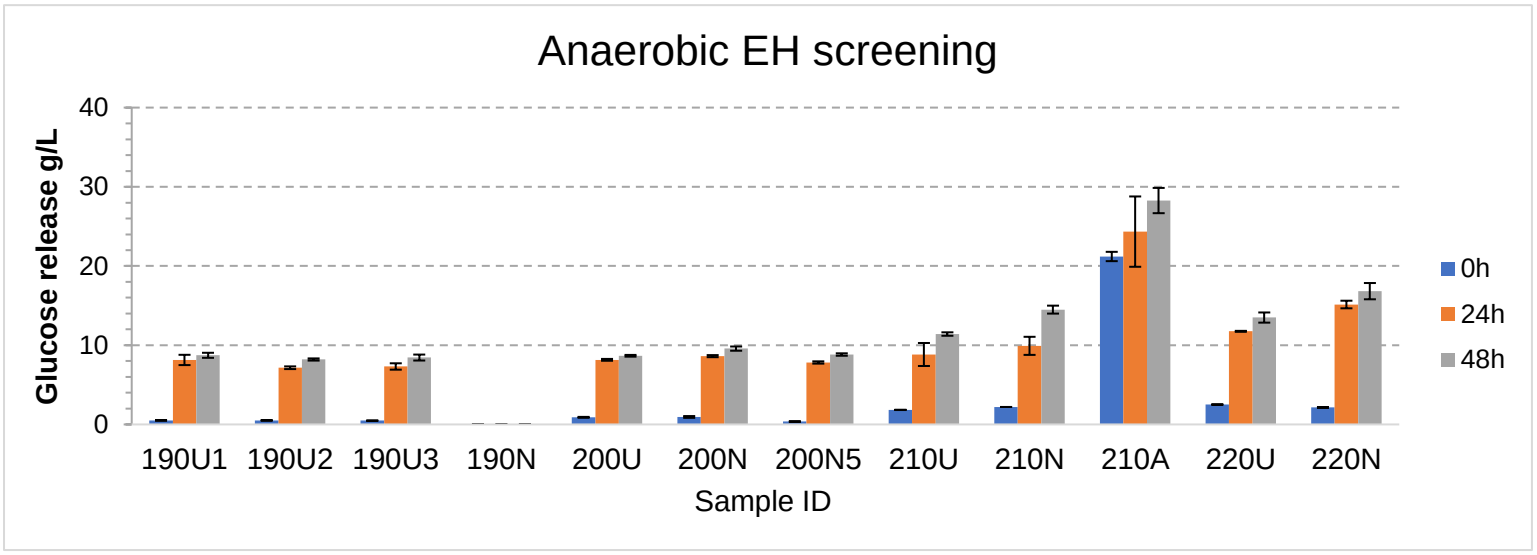

Figure 3.4: Bar graph showing glucose release during 48 hours of anaerobic enzymatic hydrolysis. 190N excluded because of malfunction during analysis with HPLC.

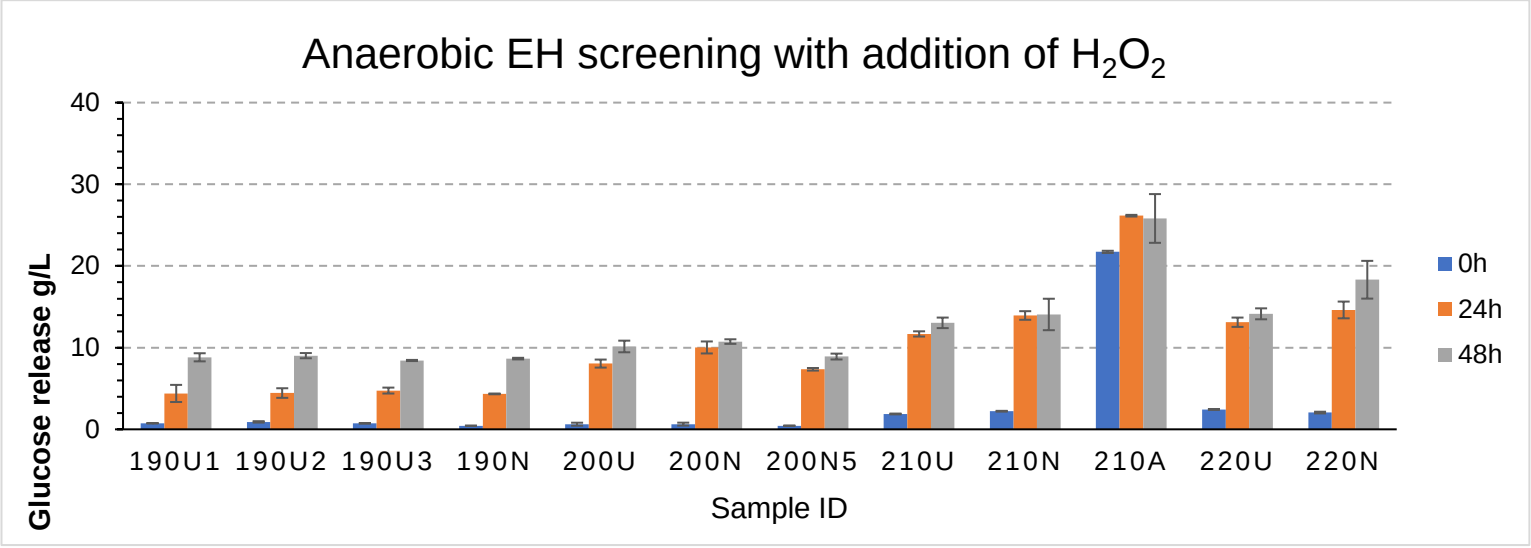

Figure 3.5: Bar graph showing glucose release during 48 hours of anaerobic enzymatic hydroysis with addition of H_2O_2 every hour.

3.4 Aerobic EH of selected samples with different enzyme doses

After narrowing down the samples to 210U, 210N, 220U and 220N and correctly adjusting the pH, a new set of experiment were carried out aerobically with different enzyme doses. The experiment was carried out without replicates to save substrate and time to identify the best pre-treatment conditions for maximum glucose release. The screening experiment had also showed that the standard deviation of these types of experiments were rather low.

Based on the results shown Figures 3.6 and 3.8 it is apparent that the addition of 2-naphthol influences enzymatic saccharification in a positive way, similar to what has been observed by Pielhop et al. (Pielhop et al., 2017). When comparting 210U and 210N there is a significant difference between the saccharification of unimpregnated and the impregnated biomass. After 48 hours sample 210N had a glucose release of 29 g/L while 210U had around 20 g/L glucose (Figure 3.6 and Figure 3.7). These amounts of glucose were achieved with the highest concentration of enzyme, 8mg/g DM.

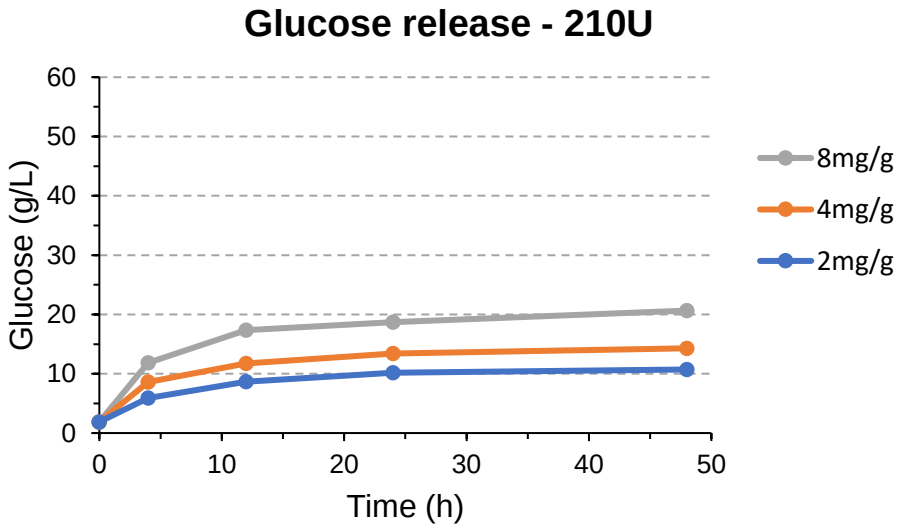

Figure 3.6: Line plot showing glucose release during 48 hours of aerobic EH of sample 210U, unimpregnated spruce steam exploded at 210°C

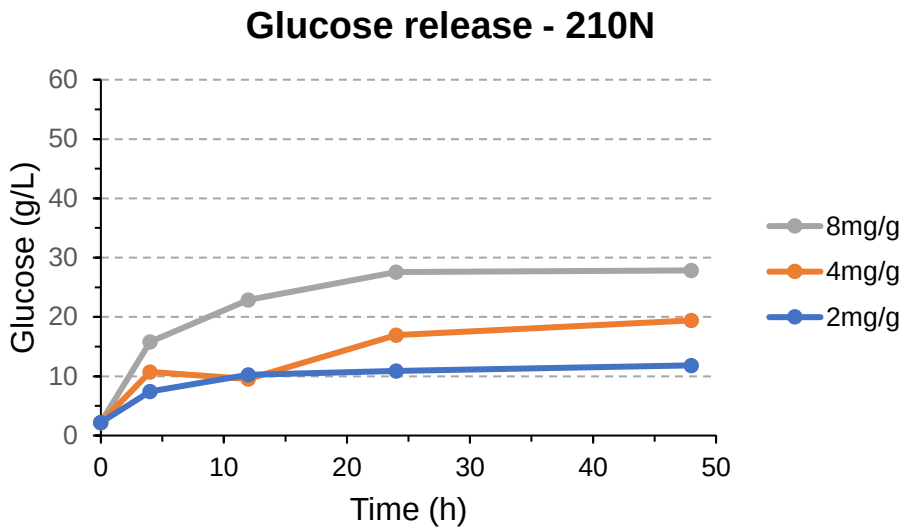

Figure 3.7: Line plot showing glucose release during 48 hours of aerobic EH of sample 210N, 2-naphthol impregnated spruce steam exploded at 210°C.

Increasing the treatment temperature by 10°C resulted in a significant difference in saccharification. Spruce steam exploded at 220°C without 2-naphthol impregnation (220U) had similar glucose yields as the unimpregnated spruce exploded at 210°C (210N) (Figure 3.7 and 3.8).

When the same experiment was done with spruce treated at 220°C much higher glucose yields were achieved (Figure 3.8 and 3.9). The sample that stood out for the 220°C treatments was 220N. The use of 2-naphthol increased the glucose release by 54% compared to the non-impregnated sample (220U) when using an enzyme dose of 8mg/g DM. Thus, the positive effect of 2-naphthol impregnation seems to be larger at higher pre-treatment severities. The amount of saccharification increased with increased amount of enzyme added to the reaction. Overall, the results show that the enzymes performed better in the reactions with 2-naphthol, possibly due to a change in the lignin structure that is favourable for the enzymes as suggested by Pielhop et al (Pielhop et al., 2015).

Woody biomass exposed to treatments with high severities will have more depolymerised lignin, causing higher concentrations of lignin carbocations which will react with 2-naphthol. This means less formation of inhibitory compounds, making saccharification more effective. Thus, it is reasonable that the largest effect of 2-naphthol impregnation is found for the highest pre-treatment condition were most carbocations are formed. As seen in Figure 3.9, 2-naphthol impregnation had a great effect on the saccharification of pre-treated spruce,

reaching a final glucose concentration of 56 g/L glucose after 48 hours. Based on the results, further work was limited to the 220U and 220N samples, since they gave superior sugar release.

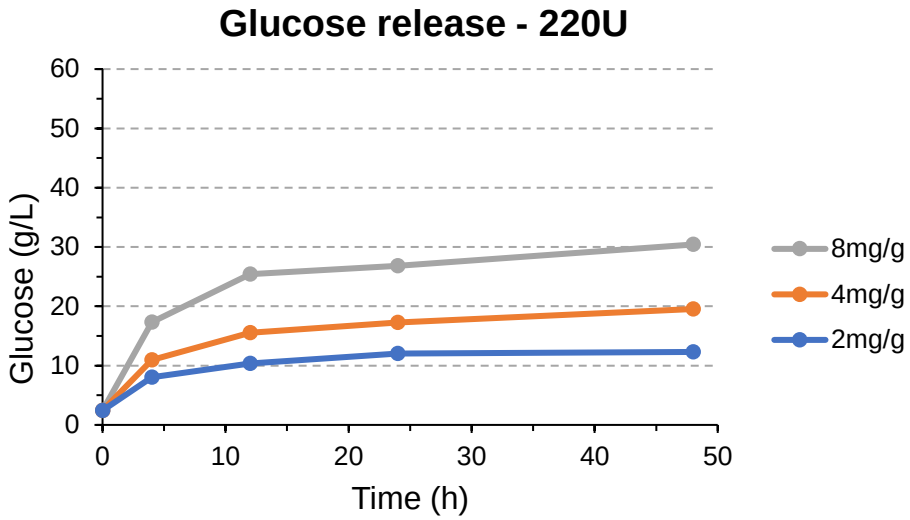

Figure 3. 8: Line plot showing glucose release during 48 hours of aerobic EH of sample 210U, unimpregnated spruce steam exploded at 220°C

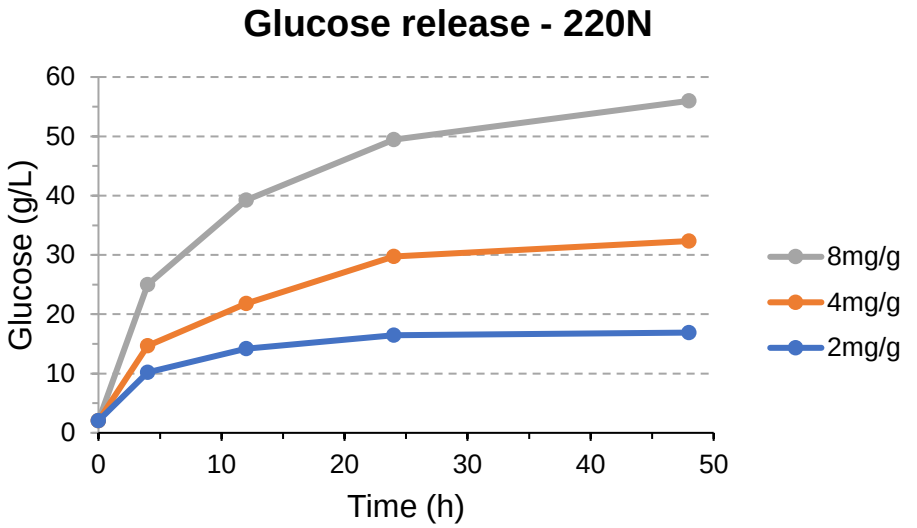

Figure 3.9: Line plot showing glucose release during 48 hours of aerobic EH of sample 210N, 2-naphthol impregnated spruce steam exploded at 210°C.

3.5 Aerobic EH of 220U and 220N in triplicates

Saccharification of the 220-samples was further studied in triplicate reactions. Figure 3.10 and 3.11 shows the results from the experiment. Hydrolysis of the 220U sample resulted in a glucose release of 30.60 g/L after 48 hours of hydrolysis with addition of 8mg/g DM at 0 hours and again at 24 hours (8mg/g+). After 12 hours, several of the reactions showed a decreasing trend in glucose yields, indicating some microbial contamination in the bottles since the amount of glucose decreased over time. Figure 3.11 shows the concentration of C4 oxidized

LPMO products in the reactions. There seems to be a clear correlation between LPMO activity and glucose release. Decrease in the concentration of LPMO products at the end of the reaction as seen in Figure 3.11 has been observed before and is probably due to instability of this compound (Müller, 2017).

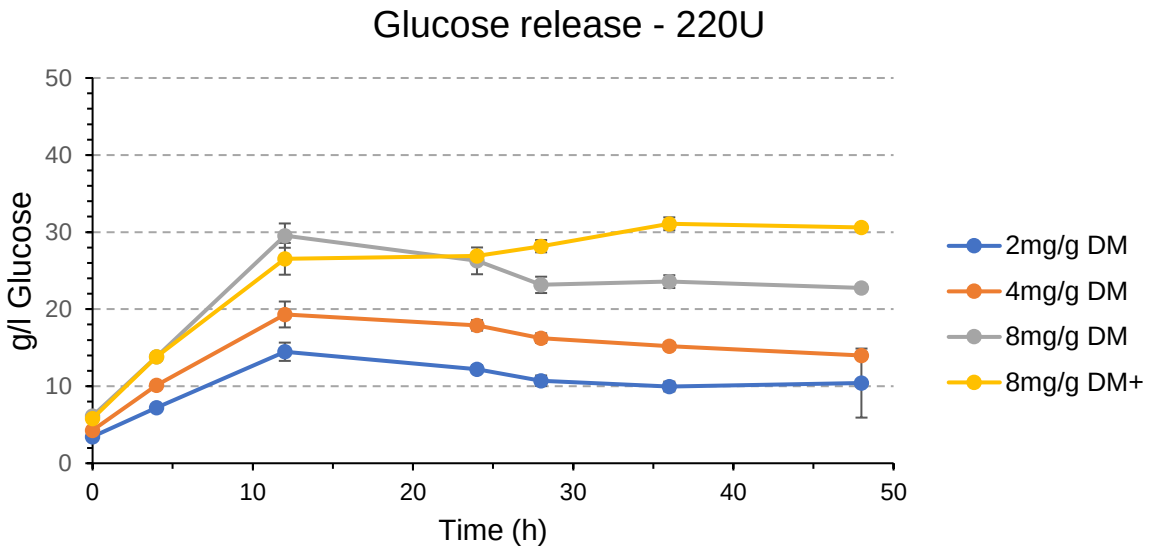

Figure 3.10: Line plot illustrating glucose release during 48 hours of aerobic EH of sample 220U.

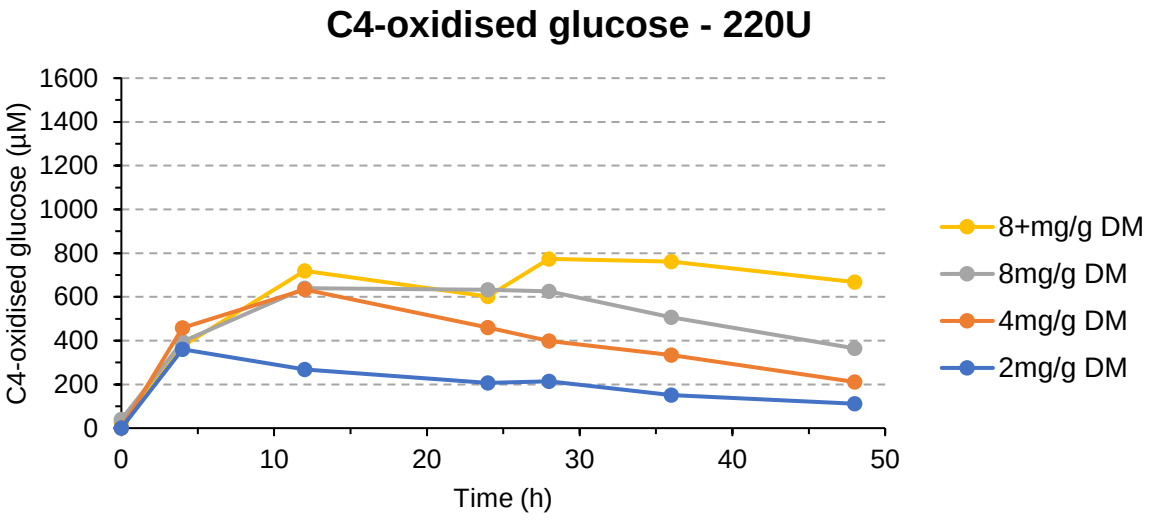

Figure 3.11: Figure showing the amont of C4-oxydised products (LPMO activity) during 48 hours of aerobic EH.

There was a significant difference between 220U and 220N regarding glucose release (Figure 3.10 vs 3.11) and LPMO activity (Figure 3.11 vs 3.13). By adding 8mg/g DM enzyme at 0 hours and again at 24 hours, final glucose concentration reached 46g/L (Figure 3.12). Again, it is visible that the 2-naphthol impregnation had a significant positive effect on glucose

release. As seen in Figure 3.12, the amount of glucose released doubles when doubling the amount of enzyme. Figure 3.12 shows that all reactions except 8mg/g + DM stagnates after 24 hours of hydrolysis.

By adding extra enzyme after 24 hours it was possible to extract additional glucose present in the biomass, indicating that the substrate is not limiting the reaction because there are still binding sites for the enzymes. Rather, enzymes seem to be inactivated over time.

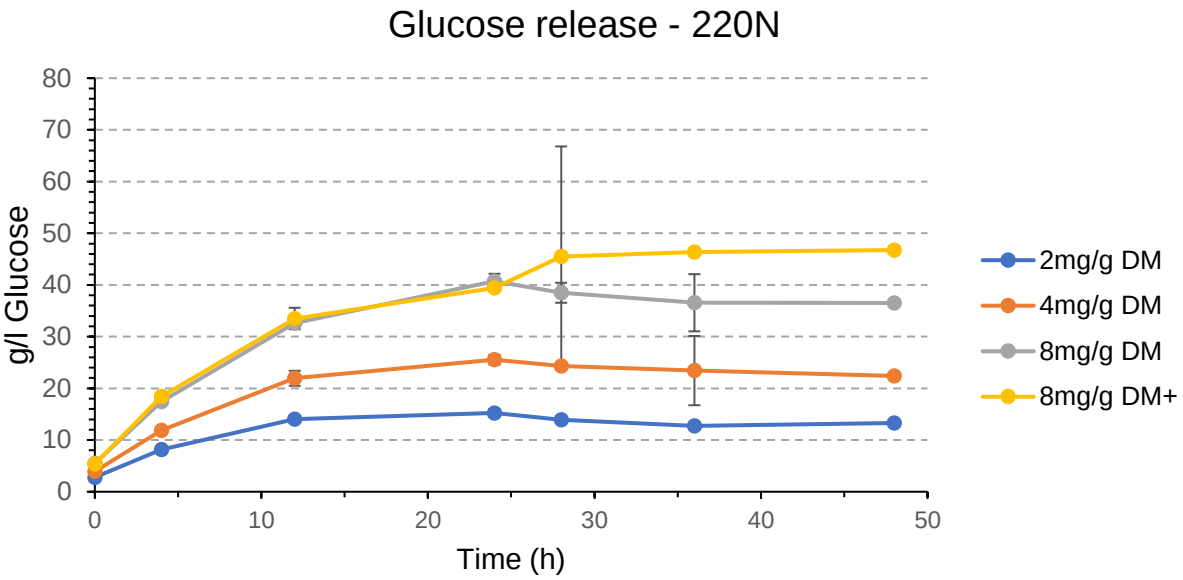

Figure 3.12: Line plot illustrating glucose release during 48 hours of aerobic EH of sample 220N.

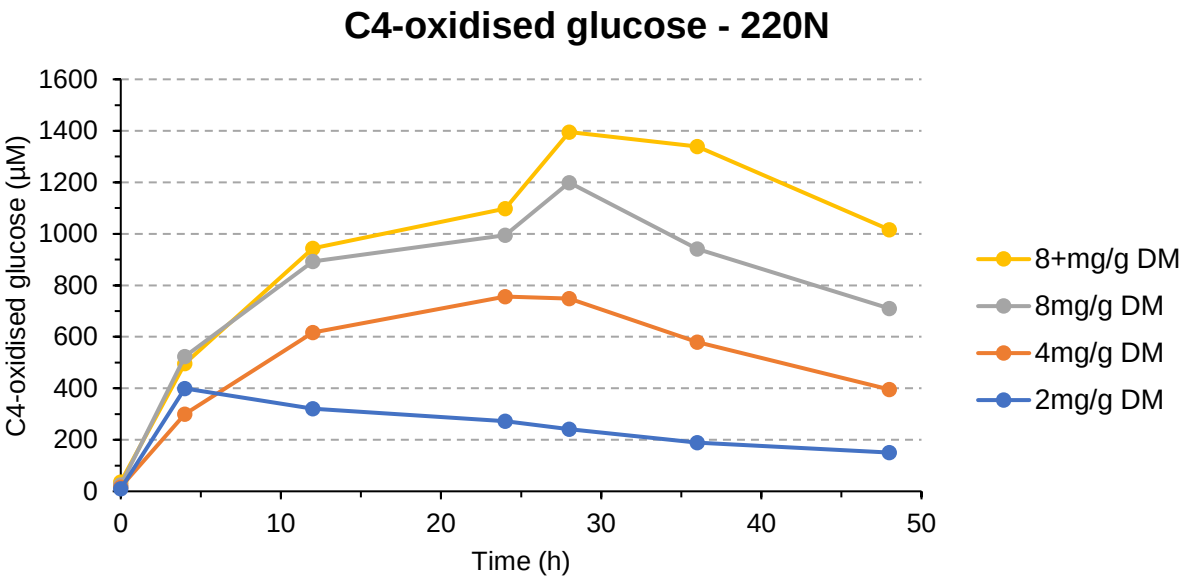

Figure3. 13: Line plot showing amounts of C4-oxidised products during 48 hours of aerobic enzymatic hydrolysis of sample 220N with different enzyme doses.

Very interestingly, there seems to be a correlation between LPMO activity and 2-naphthol impregnation. When comparing Figure 3.11 and 3.13 it is seen that the use of 2-naphthol increases the LPMO activity for all doses of enzymes applied. Since LPMO activity is linked to glucose saccharification yield this represent a novel explanation for the positive effect of 2-naphthol. Previous studies have hypothesized that this increase is due to prevention of lignin re-polymerisation (Pielhop et al., 2017, Pielhop et al., 2015), which could also explain the positive effects of 2-naphthol seen in this study as related to increased LPMO activity which in turn positively impacts saccharification yield. Thus, under these aerobic conditions 2-naphthol impregnation probably lead to more in-situ generation of H_2O_2 (

To further strengthen the hypothesis that 2-naphthol increases saccharification by increasing LPMO activity, enzymatic reactions were also carried out for the 210U and 210N samples. Analysis of C4 oxidized products were carried out. Figure 3.14 and 3.15 again show a difference between impregnated and unimpregnated substrates. According to Figures 3.14 and 3.15 impregnation with 2-napthol makes it possible to increase LPMO activity with 72% (C4-ox concentration for 210U peaking at 408µM while 210N peaking at 703µM). This difference is also seen in sample 220U and 220N (Figure 3.11 vs 3.13) with 220U peaking at 773µM and 220N at 1395µM C4-oxidised products which is an increase of 80% when using 2-naphthol. Since LPMOs need H_2O_2 as a co-substrate, and since H_2O_2 usually is the limiting factor during hydrolysis, this means that 2-naphthol impregnation leads to more in situ production of H_2O_2 (Müller et al., 2018).

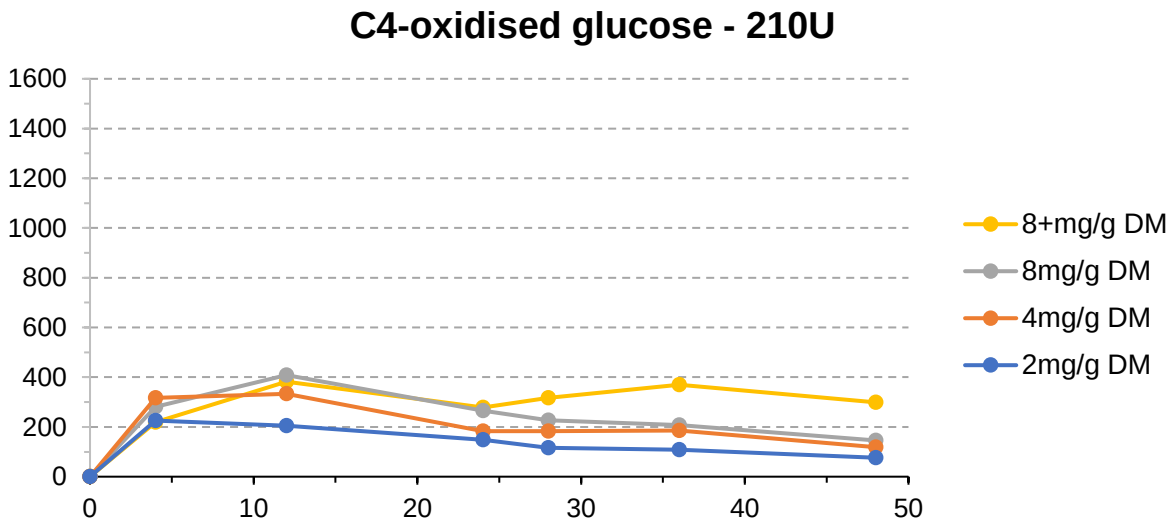

Figure 3.14: Line plot showing amounts of C4-oxidised products during 48 hours of aerobic enzymatic hydrolysis of sample 210U with different enzyme doses.

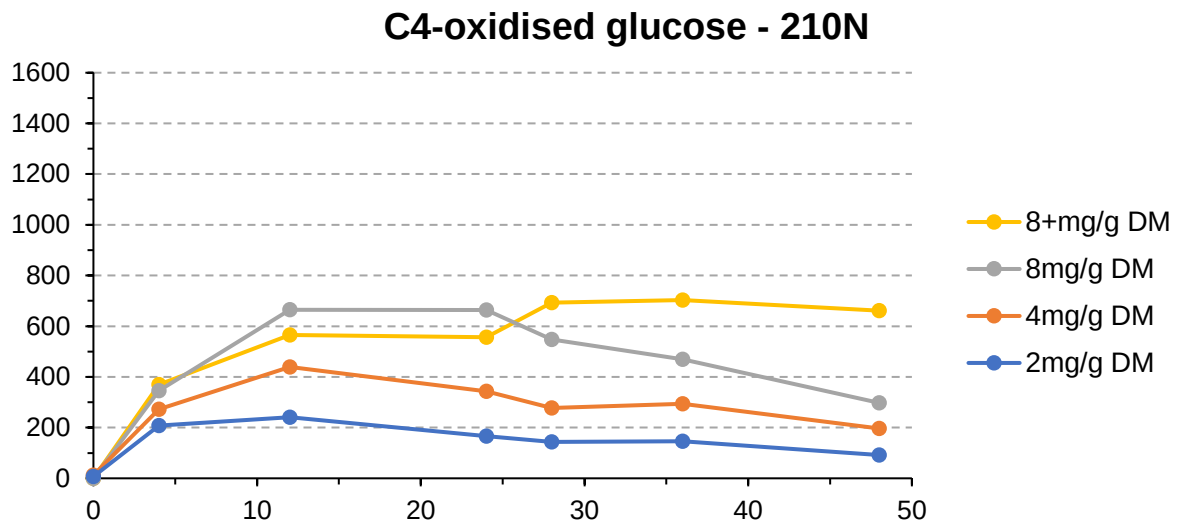

Figure 3.15: Line plot showing amounts of C4-oxidised products during 48 hours of aerobic enzymatic hydrolysis of sample 210N with different enzyme doses.

4. Conclusion and future perspectives

This study has confirmed that spruce is a challenging substrate to efficiently hydrolyse to sugars. According to Pielhop et al., spruce is called the ‘’worst case scenario’’ regarding recalcitrance (Pielhop et al., 2015) due to its lignin composition.

Even though lignocellulose from spruce has an extraordinary recalcitrance, it has been shown possible to reduce it substantially by combining steam explosion at high severity with 2-naphthol impregnation. Other studies have shown that saccharification can be increased by 2-naphthol impregnation, and the effect has been attributed to hindrance of repolymerisation of the lignin carbocations (Pielhop et al., 2017, Seidel et al., 2019. This lignin has been thought to form less non-productive bindings with enzymes and thus be less inhibitory. However, none of these studies has addressed the LPMO activity of the enzyme cocktail.In this study, for the same time, it is seen that 2-naphthol impregnation positively affects LPMO activity, probably due to higher production of H_2O_2.When LPMOs are more active, more binding cites for other cellulases are formed, resulting in higher glucose release. It is also noticeable that the effect of 2-naphthol impregnation increases with increased severity of the hydrothermal pre-treatment. Based on the results in Section 3.5 it is clear that impregnating spruce with 2-naphthol has a

positive effect on the formation of C4 oxidized product which correlates with increased amount of glucose released after 48 hours of enzymatic hydrolysis.

In the future it would be interesting to see how well the enzymatic hydrolysates would perform in different fermentations. Furthermore, a detailed chemical analysis of pre-treated substrate would be interesting to better understand why 2-naphthol impregnation is positive for LPMO activity. Such an understanding could also help identify other chemicals that may have similar effect.

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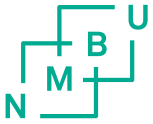

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