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Biological pretreatment of lignocellulosic substrates for improved anaerobic digestion

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Biologische voorbehandeling van lignocelluloserijke substraten met het oog op een verbeterde anaerobe vergisting

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VOORWOORD

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LIST OF ABBREVIATIONS

4-HBA	4-hydroxybenzoic acid
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ADM1	anaerobic digestion model 1
AFEX	ammonia fiber explosion
ATPase	adenylpyrophosphatase
BMP	biomethane potential
BOD	biochemical oxygen demand
CH_4	methane
C_L	lignin concentration
CO_2	carbon dioxide
COD	chemical oxygen demand
C_P	concentration of phenolic compounds
CSTR	completely stirred tank reactor
EU	European union
FA	ferulic acid
FME	flexible modeling environment
G	coniferyl alcohol
GAE	gallic acid equivalent
GHG	greenhouse gasses
GW	gigawatts
H	*p*-coumaryl alcohol
H_2O_2	hydrogen peroxide
HMF	hydroxymethylfurfural
IAC	initially added concentration
LA	laccase
LCFA	long-chain fatty acids
LiP	lignin peroxidases
MC	measured concentration
MCS	Monte Carlo simulation
MnP	manganese peroxidases
p-CA	*p*-coumaric acid
P. eryngii	*Pleurotus eryngii*
RC	relative concentration
S	sinapyl alcohol
SP	setpoint
TPC	total phenolic compounds
TIC	Theil's inequality coefficient
TS	total solids
TSS	total suspended solids
UN	united nations
VA	vanillic acid
VDS	volatile desolved solids
VFA	volatile fatty acid

LIST OF ABBREVIATIONS

VP	versatile peroxidase
VS	volatile solids
VSS	volatile suspended solids

SUMMARY

Lignocellulosic substrates, comprising cellulose, hemicellulose and lignin, can be found as agricultural waste streams. To valorize these waste streams, the lignocellulosic substrates can be anaerobically digested in order to produce biogas. In order to improve anaerobic digestion, hydrolysis of the lignocellulosic matrix should be facilitated by a pretreatment. In this dissertation enzymatic and fungal pretreatments of various lignocellulosic substrates are studied in order to obtain an improved hydrolysis in view of biogas production. The hydrolysis step is influenced by the type of substrate, the used pretreatment and the generation of unfavorable degradation products due to pretreatment. The substrate can be pretreated by different methods, mechanical, chemical, physical and physico-chemical pretreatments are known as the traditional pretreatment techniques. All these pretreatments have advantages and disadvantages, an important disadvantage of the classic pretreatments is the release of unwanted by-products. These formed by-products, such as organic acids, furan derivatives and phenolic compounds, will inhibit further process steps in obtaining biogas or bioethanol. Biological pretreatments make use of lignin degrading enzymes or white rot fungi, and balance between partly breaking down lignocellulose and preventing production of an excess of inhibiting compounds. The smaller amount of unwanted by-products can increase the rate of hydrolysis and the final biomethane potentials (BMP) of agricultural waste streams. Based on literature a number of substrates were selected to be biologically pretreated in order to increase the hydrolysis rate.

In a first step an enzymatic pretreatment with a combination of laccase and versatile peroxidase is performed on 7 different substrates. The substrates were chosen specifically to have a broad range in lignin content. In this study, a large impact of the lignin concentration of the substrate on the BMP was noted. Ensilaged maize, containing only 0.8 g lignin/100 g DM, had a significant higher BMP than corn stover, wheat straw, flax straw and hemp straw. Miscanthus and willow, two substrates with a higher lignin content of 12 g/100 g DM and 17 g/100 g DM respectively, had a significant lower BMP. The relation of lignin content and BMP showed how important lignin degradation is to improve final

biomethane production as well as the production rate. The enzymatic pretreatment did not lead to an increased BMP, although degradation of the matrix was observed as a significant increased concentration of phenolic compounds was released, without reaching their inhibitory concentrations.

Corn stover is one of the most available agricultural waste streams in Flanders, and was therefore chosen to pretreat with a greater variety of enzymes. Corn stover was incubated with laccase, two peroxidases (manganese peroxidase and versatile peroxidase), and a combination of laccase and the two peroxidases for 6, 12 and 24 hours. The impact of the various treatments was evaluated by studying the degradation of the lignocellulosic matrix, as well as measuring the BMP after anaerobic digestion of 30 days. The concentration of released phenolic compounds increased due to a treatment with peroxidases, indicating the degradation of the matrix, resulting in an increased BMP of 17 % after 6 hours of incubation. A pretreatment of 24 hours of laccase however showed to be promising as it increased the BMP by 25 %, while the concentration of phenolic compounds did not exceed the concentrations found in the not pretreated samples.

The phenolic compounds released during the enzymatic pretreatment of the different substrates were analyzed individually by HPLC. Vanillic acid, *p*-coumaric acid, 4-hydroxybenzoic acid and ferulic acid were the most common released phenolic compounds after pretreatment of miscanthus, hemp straw, willow and wheat straw. Therefore the interactions were studied of the individual phenolic compounds with laccase and versatile peroxidase in the presence of miscanthus and hemp straw on the one hand, and their impact on the BMP of the two substrates on the other hand. Laccase indicated a diminishing effect of the concentration of phenolic compounds added to either of the two substrates, while versatile peroxidase was inhibited by the added phenolic compounds. An addition of the phenolic compounds to miscanthus and to a lesser extent to hemp straw, resulted in a decreased hydrolysis rate during anaerobic digestion. Combining both studies showed that laccase

could detoxify hydrolysates by decreasing the concentration of phenolic compounds, and removing the inhibitory effect on the hydrolysis and anaerobic digestion of the substrate.

As of now the use of enzymes as such is costly, a fungal pretreatment could be a low-cost alternative. *Pleurotus eryngii* is a white rot fungi, producing most lignin degrading enzymes, was used to incubate hemp straw and miscanthus for a duration of 6 weeks. Degradation of the matrix was seen, as an increase in release of phenolic compounds and sugar compounds was measured over time. The free sugars initially present were consumed by the micro-organism, resulting in no increase of BMP. Although the rate of hydrolysis dropped due to the initial decrease of available sugars if the incubation period was lengthened, the final biogas production was unaffected. Further studies on fungal pretreatment should be done to gain knowledge and improve the effect for an increased biogas production.

Data provided during these studies were used to develop a lignin based model to predict the BMP and hydrolysis rate of different substrates with varying lignin concentrations. The model was further extended with knowledge of the impact of inhibiting phenolic compounds released during pretreatment on the anaerobic digestion. The proposed model enables us to interpret the BMP and hydrolysis rate if the lignin concentrations is known. Sensitivity analysis and identifiability of the parameters was executed in order to assess the model further to a greater extent.

In conclusion this dissertation studied the importance of the degradation of lignin without the production of unwanted by-products. As a result different opportunities during pretreatment of lignocellulose by lignin degrading and detoxifying enzymes such as laccase, and white rot fungi in general were suggested, in order to obtain higher biogas yields during anaerobic digestion. Laccase for instance could be used as a detoxifying agent after a classic pretreatment, diminishing the concentration of inhibiting compounds. A pretreatment with white rot fungi could be combined with a classic pretreatment performed under less harsh conditions, which could yield fewer inhibiting compounds while increasing biogas production.

SAMENVATTING

Lignocelluloserijke substraten, bestaande uit cellulose, hemicellulose en lignine, worden onder andere gevonden in landbouwafvalstromen. Om deze afvalstromen op te waarderen, kunnen de lignocelluloserijke substraat een anaerobe vergisting ondergaan om zo biogas te produceren. Om de anaerobe vergisting te verbeteren, moet de hydrolyse van de lignocellulose matrix vergemakkelijkt worden door middel van een voorbehandeling. In deze thesis worden enzymatische en schimmel voorbehandelingen van verscheidene lignocelluloserijke substraten bestudeerd om een verbeterde hydrolyse en biogasproductie te bekomen. De hydrolyse stap wordt onder andere beïnvloed door het type substraat, de uitgevoerde voorbehandeling en de productie van ongewenste degradatie producten te wijte aan de voorbehandeling. Het substraat kan voorbehandeld worden door verschillende technieken: mechanische, chemische, fysische en fysico-chemische voorbehandelingen staan bekend als de traditionele voorbehandelingstechnieken. Al deze voorbehandelingsmethodes hebben voor- en nadelen, een belangrijk nadeel van de klassieke voorbehandelingen is de vrijzetting van ongewenste producten. Deze gevormde bijproducten, zoals organische zuren, furaan derivaten, en fenolische componenten, zullen de verdere stappen in het bekomen van biogas of bioethanol inhiberen. Biologische voorbehandelingen maken gebruik van lignine degraderende enzymen of witrot schimmels, ze breken de lignocellulose deels af terwijl ze een overmaat productie aan inhiberende componenten voorkomen. De kleinere hoeveelheid aan ongewenste bijproducten kan de hydrolysesnelheid en zo ook het totale biomethaan potentiaal (BMP) van de landbouwafvalstromen verhogen. Gebaseerd op deze literatuurstudie werden een aantal substraten geselecteerd om een biologische voorbehandeling te ondergaan, en zo de hydrolysesnelheid te verbeteren.

In een eerste fase werd een enzymatische voorbehandeling door middel van een combinatie van laccase en versatile peroxidase uitgevoerd op 7 verschillende substraten. De substraten zijn specifiek gekozen zodat een brede range in lignine concentratie werd bekomen. In deze studie werd een grote impact van de lignine concentratie van het substraat op het BMP vast gesteld. Geënsileerde maïs,

met 0.8 g lignine/100 g DS, had een significant hoger BMP dan maïs stro, tarwe stro, vlas stro en hennep stro. Miscanthus en wilg, twee substraten met een hogere lignine concentratie, respectievelijk 12g/100 g DS en 17 g/ 100 g DS, hadden een significant lagere BMP. De relatie tussen lignine concentratie en BMP toont hoe belangrijk de degradatie van lignine is om totale methaanproductie en productiesnelheid te verbeteren. De enzymatische voorbehandeling leidde niet tot een verbeterd BMP, hoewel de degradatie van de matrix werd vastgesteld door een significante stijging in de concentratie aan vrijgestelde fenolische componenten, zonder inhibitie concentraties te bereiken.

Maïs stover is een van de meest voorkomende landbouw afvalstromen in Vlaanderen, en was daarom gekozen om het voor te behandelen met verschillende lignine degraderende enzymen. Maïs stover werd geïncubeerd met laccase, twee peroxidases (mangaan peroxidase en versatile peroxidase), en een combinatie van laccase en de twee peroxidases gedurende 6, 12 en 24 uur. De invloed van de verschillende behandelingen werden geëvalueerd door zowel de degradatie van de lignocellulose matrix als het BMP na een anaerobe vergisting van 30 dagen na te gaan. De concentratie van vrijgestelde fenolische componenten steeg door de behandeling met peroxidases wat een degradatie van de matrix impliceert, resulterend in een verhogen van het BMP met 17 % na een incubatie van 6 uur. Een voorbehandeling van 24 uur met laccase was veelbelovend, aangezien het BMP met 25 % werd verhoogd, terwijl de concentratie aan fenolische componenten niet steeg ten opzichte van de onbehandelde stalen.

De fenolische componenten die vrijgesteld werden door de verschillende substraten werden individueel geanalyseerd via HPLC. Vannilinezuur, *p*-coumarinezuur, 4-hydroxybenzoëzuur en ferulazuur waren de meest voorkomen fenolische componenten die werden bekomen na voorbehandeling van misanthus, hennep stro, wilg en tarwe stro. Hierdoor werd de interactie onderzocht van de individuele fenolische componenten met laccase en versatile peroxidase in aanwezigheid van miscanthus en hennep stro enerzijds, en hun impact op het BMP van de twee

substraten anderzijds. Laccase verminderde de concentratie aan fenolische componenten toegevoegd aan eender van de twee substraten, terwijl versatile peroxidase werd geïnhibeerd door de toegevoegde fenolische componenten. De additie van de fenolische componten aan miscanthus, en in mindere mate aan hennep stro, resulteerde in een vermindering van de hydrolyse snelheid gedurende de anaerobe vergisting. De combinatie van beide studies gaf aan dat laccase een detoxifiërend effect op het hydrolysaat heeft door de concentratie aan fenolische componenten te verlagen, en dus het inhiberende effect op de hydrolyse tijdens de anaerobe vergisting van het substraat wegneemt.

Het gebruik van zuivere enzymen is op dit moment erg duur, een schimmelvoorbehandeling kan een goedkoper alternatief zijn. *Pleurotus eryngii* is een wit rot schimmel die de meeste lignine degraderende enzymes produceert. *Pleurotus eryngii* werd gebruikt om hennep stro en miscanthus gedurende 6 weken te behandelen. Degradatie van de lignocellulose matrix werd vastgesteld door de stijging van vrijgestelde fenolische en suiker componenten doorheen de behandeling. De initiële vrije suikers werden verbruikt door het micro-organisme, dit leidde tot een onveranderd BMP. Hoewel de hydrolyse snelheid verlaagt werd door de initiële daling van de vrije suikers als de incubatieperiode langer duurde, werd de totale biogasproductie hierdoor niet beïnvloed. Verdere studies op schimmelvoorbehandelingen zijn nodig om meer kennis op te doen om zo een verbeterde voorbehandeling te bekomen met een stijging van biogasproductie tot gevolg.

Met de data verkregen van deze studies kon een model gebaseerd op lignine opgesteld worden om het BMP en hydrolysesnelheid van verschillende substraten met verschillende lignine concentraties te voorspellen. Het model werd uitgebreid met de kennis van de impact van de fenolische componenten, vrijgesteld tijdens voorbehandeling, op de anaerobe vergisting. Het voorgestelde model geeft ons de kans het BMP en de hydrolyse snelheid te interpreteren indien de lignine concentratie bekend is. Sensitiviteit analyses en de identificeerbaarheid van de parameters werden nagegaan om de mogelijkheid tot het verder optimaliseren van het model aan te tonen.

Tot slot kan besloten worden dat deze doctoraatsthesis de belangrijkheid van lignine degradatie zonder de vorming van ongewenste bijproducten onderzocht heeft. Als resultaat worden de verschillende opportuniteiten tijdens voorbehandelingen van lignocellulose door ligninedegraderde enzymes zoals laccase en witrot schimmels in het algemeen als suggesties gegeven om een verhoogde biogasproductie te bekomen tijdens de anaerobe vergisting. Laccase bijvoorbeeld, kan als een detoxifiërende mediator gebruikt worden na een klassieke voorbehandeling om de concentratie aan inhiberende componenten te verminderen. Een voorbehandeling met witrot schimmels zou kunnen worden gecombineerd met een mildere versie van de klassieke voorbehandeling, zodat er minder inhiberende componenten vrijkomen terwijl de biogasproductie wordt verbeterd.

INTRODUCTION

Global energy demand has been increasing over the last decades and will keep rising over the following decades. Fossil fuels are as of today the majority of energy sources to fulfill this demand. The combustion of these fossil fuels however leads to the release of greenhouse gasses, resulting in global warming and climate change. Together with the depletion of fossil fuel reserves, the possible increase in oil price and the surge for energy independency has intensified the interest in renewable energy sources. The European Union (EU) has set different goals to achieve a more sustainable environment. By 2020 the EU wants to decrease greenhouse gas emissions by 20 %, 40 % by 2030, while by 2050 the EU committed to reduce greenhouse gas emissions further by 80-95 % compared to levels measured in 1990. These goals can be achieved by increasing energy efficiency, by diminishing the existing energy demand, as well as by replacing fossil energy with renewable energy such as wind energy, solar energy or biogas. Converting energy consumption towards the use of sustainable, renewable energy sources has been initiated in 1990, however there is still a long way to go (Figure 1).

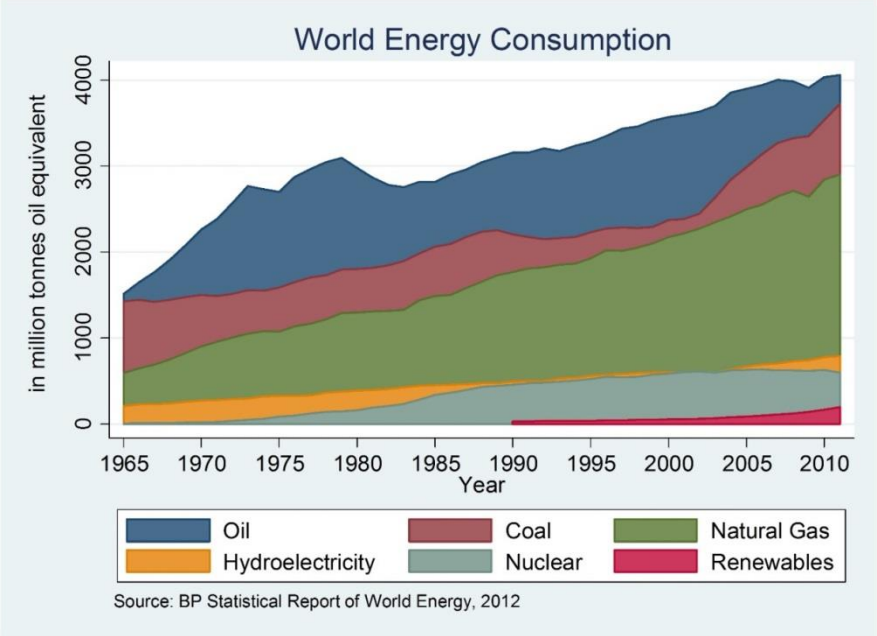

Figure 1: Sources and amount of world energy consumption in million tonnes oil equivalent since 1965 (BP statistical report of world energy, 2012)

Biogas is a mixture of methane (CH_4) and carbon dioxide (CO_2), and can be used as a heat and power source, after purification and upgrading it is able to replace natural gas or can be used as an alternative for natural gas in a variety of industrial and municipal applications as well as for vehicle fuel. As such, biogas is influencing different pathways of energy consumption.

Lignocellulosic biomass is available in great abundance as agricultural waste, forestry residues or industrial waste. An additional advantage is that lignocellulose is a source of second generation biofuels as there is no food or feed competition. The lignocellulose substrate consists of sugar-rich polymers, cellulose and hemicellulose, which are concealed within a recalcitrant lignin seal. The lignin polymer is comprised of a complex network of aromatic alcohols and its rigid structure impedes the hydrolysis of the substrate. Hydrolysis is the first and rate limiting step of the anaerobic digestion process, followed by acidogenesis, acetogenesis and methanogenesis. To facilitate the hydrolysis of lignocellulosic biomass, and thus improving the overall biogas production process, a pretreatment step is necessary.

A good pretreatment step must break down the lignin structure without causing sugar loss and unwanted by-product formation, such as phenolic compounds or furan derivatives, while having a low cost and being environmentally friendly. There are many different type of pretreatments, mechanical, chemical, biological etc., with each their specific advantages and disadvantages, which will be described further in chapter 1. Since a few years there is more interest in biological pretreatments as it has several advantages, releasing no inhibitors and requiring no additional chemicals, although the duration of incubation and the low-rate hydrolysis need to be improved to compete with the traditional pretreatments. Biological pretreatments can be executed by ensiling the substrate or using a fungal pretreatment. White rot fungi are Basidiomycota from which some are known to selectively degrade lignin. The white rot fungi use lignin degrading enzymes such as laccase, lignin peroxidase, versatile peroxidase and manganese peroxidase during the degradation process.

In this dissertation, a special interest was paid to the possibilities of biological pretreatments of lignocellulosic material in relation to an increased biogas potential. With regard to biological pretreatment, the focus was on gaining knowledge of the effectiveness and impact of the individual lignin degrading enzymes, which are produced by white rot fungi. So within the general hypothesis of the PhD, i.e. possible increased biogas production from lignocellulosic waste streams by the use of lignin degrading enzymes, several research questions were formulated: (i) what is the impact of the content of lignin on the degradation potential by lignin degrading enzymes and the biogas production, (ii) can the use of lignin degrading enzymes on lignocellulosic material reduce the release of phenolic compounds, as they are well-known biogas inhibitory compounds and (iii) what is the interaction between the lignin degrading enzymes, the inhibiting phenolic compounds formed during degradation, and biogas production. To answer these questions, several experiments were conducted as outlined briefly below, and the relation between the different experiments and chapters is presented in Figure 2.

In chapter 2 the impact on various lignocellulosic substrates different in lignin content is researched. The biomethane potential (BMP) of the substrates is evaluated as well as the degradation process as well as the chemical oxygen demand (COD) and the release of individual inhibiting phenolic compounds is measured. A more in depth study is described in chapter 3 where corn stover is degraded enzymatically by different cocktails of lignin degrading enzymes for different incubation periods. Corn stover was chosen as it is one of the most available lignocellulosic substrates in Flanders, while its lower lignin content than the other substrates showed a good opportunity for an increased biomethane potential.

Traditional chemical pretreatments release high concentrations of phenolic compounds, known to inhibit the anaerobic digestion process. The interaction of 4 different phenolic compounds, which were detected in chapter 2 and 3, with the lignin degrading enzymes laccase and versatile peroxidase

is studied in chapter 4. The inhibiting effect of the different phenolic compounds on the biogas production of two substrates itself are analyzed as well in chapter 4.

The data obtained from chapter 2 gave the opportunity to create a lignin based model predicting the biomethane production of different substrates. Inhibition caused by phenolic compounds researched in chapter 4 were implemented in the model for the two substrates, hemp straw and miscanthus as well. This model will be presented in chapter 6.

Since the lignin degradation by enzymes as such is an expensive pretreatment, *Pleurotus eryngii,* a white rot fungi was obtained to explore a fungal pretreatment of hemp straw and miscanthus. *P. eryngii* is a selective degrader, preferentially degrading lignin over cellulose, while producing all lignin degrading enzymes which were studied in chapter 2, 3 and 4. The results of a 6 week incubation with the white rot fungus are shown in chapter 5.

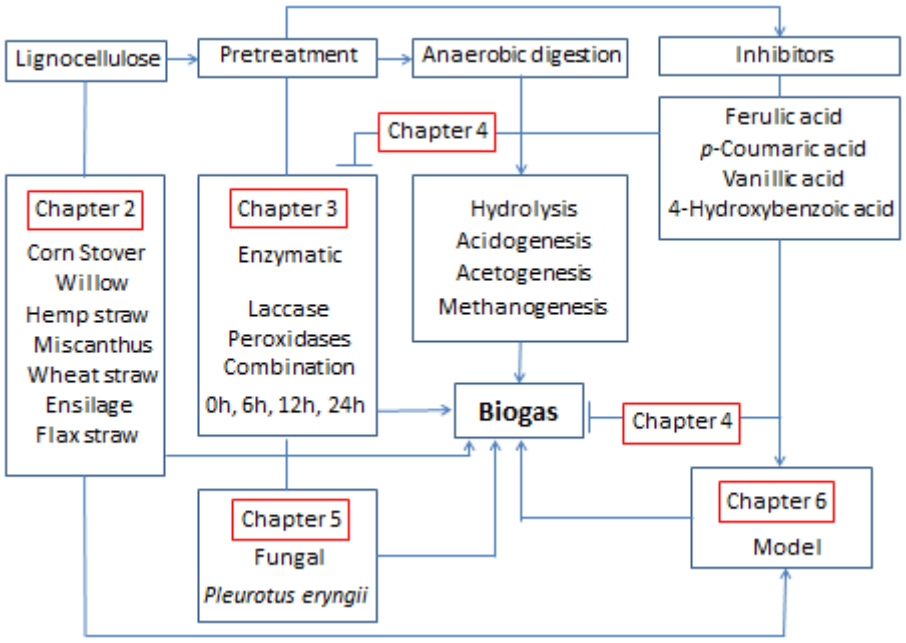

Figure 2: Outline and interactions of the various chapters in this PhD dissertation

In chapter 7 the general conclusions from the study will be discussed while various future perspectives will be explored. The proposed biological pretreatments will be evaluated and possible

alternative pretreatment pathways will be given to improve the pretreatment of lignocellulose and increase the hydrolysis rate during anaerobic digestion.

LITERATURE REVIEW: PRETREATMENT OF LIGNOCELLULOSE, A NECESSARY STEP TO IMPROVE BIOGAS PRODUCTION

1. LITERATURE REVIEW: PRETREATMENT OF LIGNOCELLULOSE, A NECESSARY STEP TO IMPROVE BIOGAS PRODUCTION

1.1. INTRODUCTION

In view of future sustainable energy supply nowadays renewables are established as a mainstream energy source. In fact, 2015 was an extraordinary year resulting in the largest global addition in renewable power capacity (hydropower, bio-power, geothermal power, wind power and solar photovoltaic power) so far. An increase of 147 Gigawatts (GW), an increment of 8.6 % in comparison to 2014 was established. This increase was driven by improvements to the cost competitiveness of renewable energy, the need for energy security and the growing demand for energy in emerging economies. Not only production itself was improved, also commodities such as smart grid technologies required to input and balance the supply and demand of the green energy into the network, as well as the energy storage developments creating a more constant energy supply, were enhanced. The increase of capacity is even more promising if the drop of the oil price since July 2014 is taken into account (Ren21 Global status report, 2015). Next to feeding upgraded biogas with a methane content of up to 98 % into the natural gas grid, it is also possible to use biogas as fuel, by mixing biomethane with methane from the natural gas. Use of biomethane as a fuel will lead to a further reduction of emissions by 20 % compared to natural gas (DENA, 2014). A comparative study of biofuels showed that biomass obtained from one hectare would provide biomethane for 67700 km, while biodiesel and bioethanol for only 23300 and 22400 km respectively (FNR, 2014).

Besides technical improvements, progress has been made on a political level. At the end of 2015 the United Nations (UN) held a climate change conference in Paris. The key results were an agreement on limiting global warming to less than 2 °C, and a zero net anthropogenic emission of greenhouse gasses (GHG) during the second half of this century. Still many efforts must be done to reach the challenging but necessary goals. In a ten year strategy the European Union (EU) launched several 20 % targets, which will help in achieving the set goals. By 2020 the EU wants to diminish the emission of GHG with 20 %. This would be done by increasing the share of renewable energy to 20 %

in the final energy consumption, and increasing the energy efficiency by 20 %. In a more recent agreement, the EU strives to decrease GHG emissions by 40 % and increase the share of renewable energy to 27 % by 2030. Next to decreasing various global problems this would also increase safety of energy supply and a greater independence of energy supply within the EU. At the moment the share of renewable energy in the EU is at 16 %, while in Belgium it is 8 %, with a target of 13 % by 2020 (Eurostat, 2014; Ren21 Global status report, 2015).

Apart from global natural effects, fossil fuel reserves are diminishing and alternatives are required. In Belgium solar and wind power are commonly used as alternative energy sources, representing 5 % and 46 % respectively of the renewable energy sources. Renewable energy formed through anaerobic digestion and burning biomass, representing respectively 6 % and 40 % of renewable energy sources, should not be ignored as it has different advantages discussed in this chapter. However, in order to avoid solving problems by creating new ones, pitfalls must be tackled in the use of biomass, and to protect biodiversity and soil fertility as well (SWD, European Commission 2014). Nonetheless a combination of sources of renewable energy leading to a sustainable world must be the future goal, in which biomass is and stays an important resource. To improve biomass conversion and to increase its potential, the use and pretreatment of lignocellulosic biomass will be studied in this dissertation, as the facilitation of the conversion and hydrolysis of the biomass will lead to an increased biomethane yield (Figure 1.1).

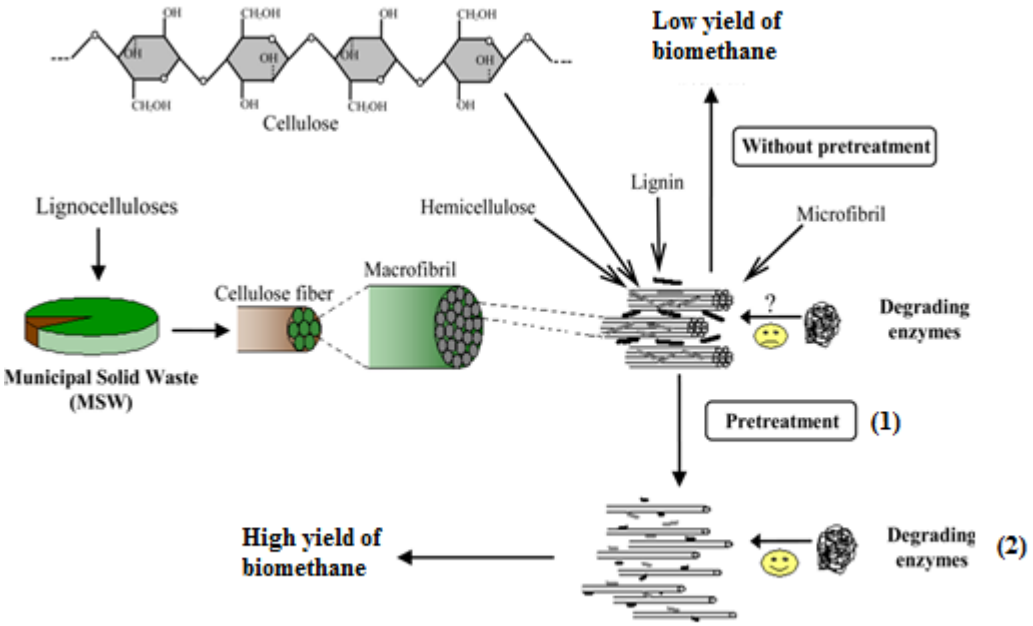

Figure 1.1: The effect of pretreatment (1) of lignocellulosic biomass, to improve conversion and hydrolysis by degrading enzymes (2) (Adapted from Taherzadeh and Karimi, 2008)

The first part of this chapter will focus on the lignocellulosic biomass itself, the second part will discuss the different types of pretreatments available and their advantages and disadvantages. Furthermore inhibition during the biogas production and how to overcome it, as well as the biogas production process itself will be handled.

1.2. LIGNOCELLULOSE

1.2.1. Introduction

For the EU to achieve its 20 % renewable energy target by 2020, biomass can play an important role. Biomass has several advantages compared to solar and wind power, as it can be stored and thus used as a buffer to balance the variability of solar and wind power sources in moments of high demand or lack of sun or wind. Use of biomass can also lead to a more active forestry management, reducing fire risks. Farmers can use manure and agricultural waste as a source to perform anaerobic digestion for local heating or inject upgraded biogas as biomethane into the natural gas network, resulting in a decrease of the release of GHG, as the amount of manure storage in open air is

reduced (SWD European commission, 2014). Biomass varies widely in type, source and composition (Table 1.1), and is mostly obtained from forestry residues or agricultural waste, but they all contain lignocellulose.

Lignocellulose is a source for the production of value added products, i.e. ethanol, food additives, organic acids, energy, and others (Iqbal et al. 2013). Therefore lignocellulose often seen in the form of mere waste, should be seen as a product with potential.

Lignocellulose is composed out of three groups of polymers, namely cellulose, hemicellulose and lignin (Figure 1.2). These three polymers are linked in a cellular complex, where long cellulose chains form the center intertwined with shorter branched hemicellulose chains concealed by a lignin seal (Rubin, 2008). The cellulose microfibril contains hydrophobic and hydrophilic surfaces which are thought to bind to xyloglucan, xylan and lignin (Cosgrove, 2014). Xyloglucans are very important hemicelluloses together with xylans, manans and glucomannans as they tether the cellulose microfibrils, strengthening the cell wall. The cross-linking of microfibrils by the hemicelluloses is known as the sticky-model, which is the most influential model to date (Figure 1.3) (Cosgrove, 2000). These polymers will be discussed individually later in this dissertation (1.2.3-1.2.5).

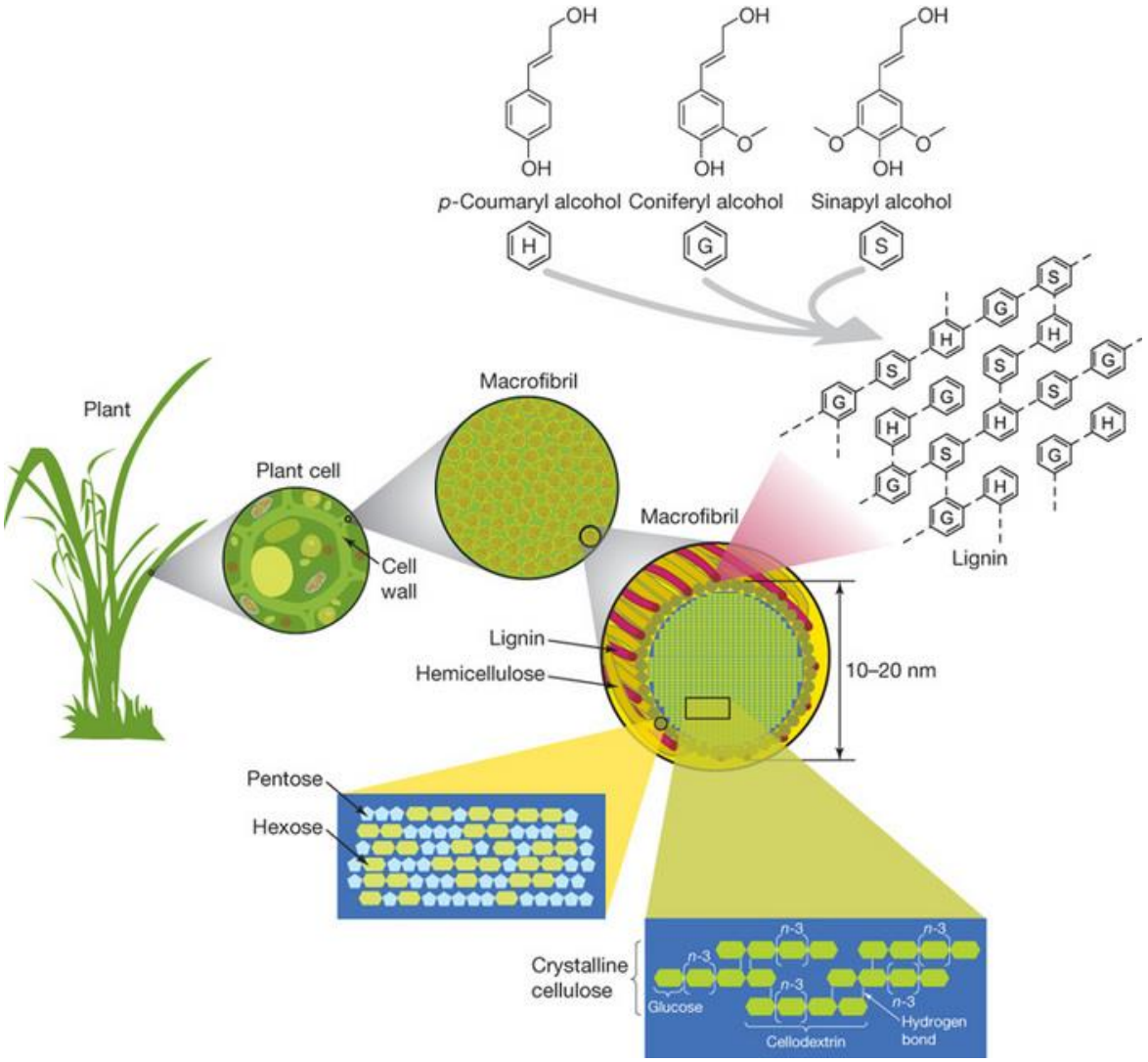

Figure 1.2: The structure of lignocellulose, containing cellulose a linked chain of glucose molecules, intertwined with hemicellulose built from different pentoses and hexoses i.e. arabinose, galactose, glucose, mannose and xylose. The two main components are concealed within a lignin barrier, composed of mostly three phenolic components, *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S) (Rubin, 2008)

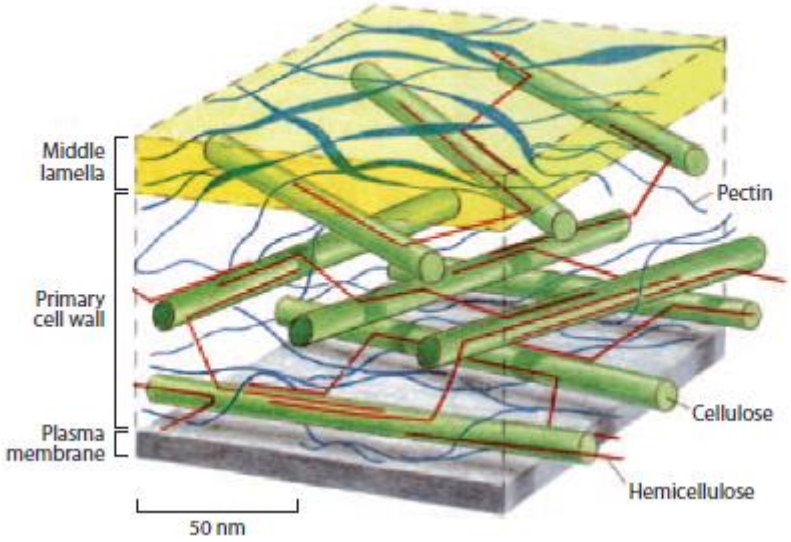

Figure 1.3: Sticky model of the primary cell wall (McCann & Roberst, 1991)

1.2.2. Agricultural waste

There is a broad range of lignocellulosic sources, with a variety in the composition of the three polymers, cellulose (35-50 %), hemicellulose (20-35 %) and lignin (10-25 %) (Table 1.1) (Liu et al. 2008). As the composition changes during the life time of a plant, there is a variation in composition within one biomass source, and it varies depending on the part of the plant sampled. Other growth conditions can have an effect as well on the lignocellulosic composition, resulting in broad ranges for the different polymers within one substrate (Table 1.1).

Table 1.1: The composition of cellulose, hemicellulose and lignin of various lignocellulosic substrates, the values are measured on a dry matter basis (g/100gDM)

Lignocellulose substrate	Cellulose (g/100g DM)	Hemicellulose (g/100g DM)	Lignin (g/100g DM)	References
Aspen	45	21	21	Xu and Tschirner, 2012
Barley hull	34	38	19	Kim et al. 2008
Barley straw	34-39	22-26	14-16	Nigam et al. 2009; Duque et al. 2011
Big bluestem	34-37	17-19	19-21	Karunanithy and Muthukumarappan 2012b
Birch	40	27	25	Goshadrou et al. 2013
Corn cobs	34	31.9	6	Nigam et al. 2009
Corn stalks	35	17	7	Nigam et al. 2009
Corn stover	33-38	19-26	15-21	Zhu et al. 2005; Kim and Lee 2006; Li et al. 2010c; Karunanithy and Muthukumarappan 2012b; Zhang et al. 2012
Cotton stalks	14	14	22	Nigam et al. 2009
Douglas fir	47	29	25	Lee et al. 2010
Eucalyptus	42	10-19	29-30	Lee et al. 2010; Park and Kim 2012
Giant reedleaves	21	18	25.4	Monlau et al. 2012
Giant reedstalk	33	19	24.5	Monlau et al. 2012
Miscanthus	34-38	24-37	25	de Vrije et al. 2002; Jurisic 2012
Oak	45	24	24	Shafiei et al. 2010
Oat straw	39	27	18	Nigam et al. 2009
Olive tree pruning	25	16	18	Cara et al. 2007
Pine	34-45	22-28	27-35	Karunanithy et al. 2012a
Pitch Pine	43	24	29	Park and Kim 2012
Poplar	44-45	20-37	18-29	N'Diaye et al. 1996; Wymen et al. 2009
Prairie cord grass	33-34	13-18	21-22	Karunanithy and Muthukumarappan 2012b
Rice straw	31-36	19-24	10-13	Nigam et al. 2009; Chen et al. 2011
Rye straw	31-38	22-37	18-22	Garcia-Cubero et al. 2009; Nigam et al. 2009
Salix	43	21	26	Sassner et al. 2006
Sorghum	22-36	19-20	18-21	Li et al. 2010a; Monlau et al. 2012
Soya stalks	35	25	20	Nigam et al. 2009
Soybean hull	31-36	17-19	2-14	Lamsal et al. 2010; Yoo et al. 2011
Spruce	44	21	29	Shafiei et al. 2010
Sugarcane bagasse	40	27	10	Nigam et al. 2009; Kim and Day 2011; Rabelo et al. 2011
Sunflower stalks	34-42	19-30	13-18	Nigam et al. 2009; Monlau et al. 2012
Sweet sorghum	45	27	21	Kim and Day 2011
Sweet sorghum bagasse	27	14	14	Li et al. 2010a
Switch grass	26-45	20-31	12-27	Li et al. 2010b; Kim et al. 2011; Brown et al. 2012; Karunanithy and Muthukumarappan 2012b
Wheat bran	10	37	7	Lamsal et al. 2010
Wheat straw	30-38	21-24	9-23	Ballesteros et al. 2006; Nigam et al. 2009; Petersen et al. 2009; Brown et al. 2012

1.2.3. Cellulose

Cellulose is the most abundant polysaccharide in lignocellulose, accounting for 15 to 30 % of the dry mass of the primary cell walls according to Carpita and McCann (2000), while the secondary cell walls contain even more cellulose. Liu et al. (2008) states that lignocellulose is comprised of primarily cellulose, containing 35 to 50 % of cellulose. The variance in cellulose is highly dependent on the lignocellulosic biomass, which is also shown in Table 1.1. It is a sugar-rich fraction and of great interest in fermentation processes, since the sugars can be converted in many types of value-added products (Iqbal et al. 2013). Cellulose is polymerized by a cellulose synthase complex, composed of a catalytic and a transmembrane domain. An intra-protein tunnel provides a low-energy pathway translocating the growing glucan chain to the external membrane surface of the cytoplasma. Here the catalytic domain transfers a glucose residue to the reducing end of the glucan (Cosgrove, 2014). The formed polymer cellulose is a linear homopolymer of repeated units of cellobiose, which are two glucose rings linked by a β-1,4 glycosidic bond (Figure 1.4). The cellulose polymers themself are packed by van der Waals interactions and hydrogen bonds into microfibrils (Parthasarathi et al. 2011). The microfibrils can contain about 250 glucose chains and is about 36 glucose chains thick in cross-section. These microfibrils are parallel arranged for the greater part (2/3) of the cellulose, forming a sturdy crystalline structure. The crystalline regions are alternated with more degradable amorphous regions (Himmel et al. 2007). As a sugar-rich fraction cellulose is an important source for the anaerobic digestion, and the availability and accessability should be increased. Meanwhile the crystallinity index should be decreased, facilitating the breakdown of cellulose and rendering the substrate easily hydrolyzable by cellulase or microbial organims (Sannigrahi et al. 2009).

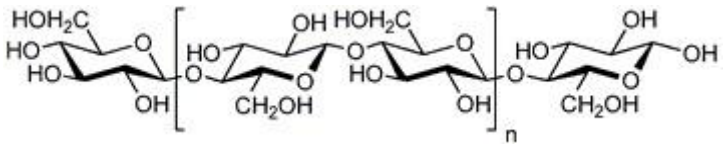

Figure 1.4: The chemical structure of cellulose, comprised of repeating untis of cellobiose, two glucose molecules linked by a β-1,4 glycosidic bond (Kobayashi et al. 2012)

1.2.4. Hemicellullose

Hemicelluloses are cross-linking glycans containing sugars as well, however this polymer is far more heterogeneous. It comprises of L-arabinose, D-galactose, D-glucose, D-mannose, and D-xylose as sugar components (Scheller and Ulvskov, 2010). The most common glycans of primary cell walls are xyloglucans and glucuronoarabinoxylans, less common are the glucomannans, galactoglucomannans and galactomannans. Grasses and cereals have a third major cross-linking glycan, namely a mix of linkages of the (1→3) and (1→4)-β-D-glucans (Carpita and McCann, 2000) (Figure 1.5). Glycosyltransferases are Golgi localized enzymes producing xyloglucan precursor molecules to be transported to the cell walls. The molecules are modified after the initial synthesis by apoplastic glycosidases, who are capable of trimming the nascent xyloglycan chain, and determining the heterogenity of the polymer (Scheller & Ulvskov, 2010). Hemicellulose is a multi-branched polymer, the branching gives the polymer a more amorphous design, making it easier to hydrolyze than cellulose. Ferulic acid is found in hemicellulose and crosslinks the polysaccharides, but can also form a link with the aromatic lignin polymer increasing rigidity of the cell wall, which decreases biodegradability (Mussatto and Teixeira, 2010). Degradation of hemicellulose allows the solubilization of pentoses and hexoses, usable as a source for second generation fuels (Baêta et al. 2016). Released hydroxymethylfurfural (HMF) and furfural will have a negative impact on anaerobic digestion, and will be described further in section 1.4.3. (Santucci et al. 2015).

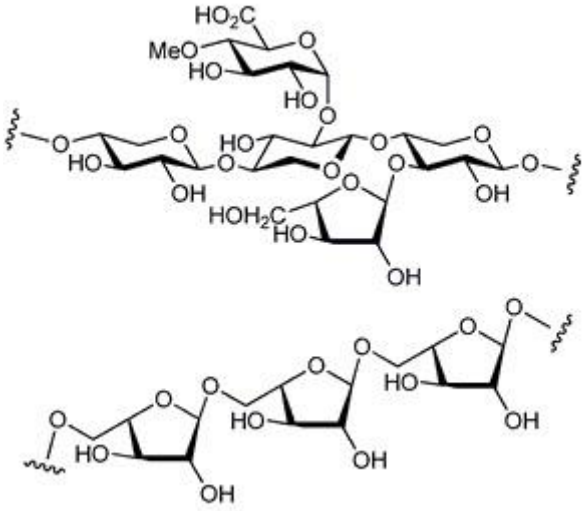

Figure 1.5: The chemical structure of hemicellulose, a branched polymer containing different types of sugars (Kobayashi et al. 2012)

1.2.5. Lignin

Lignin is a structure from the secondary cell wall, it is a heterogeneous polymer of aromatic alcohols. These aromatic alcohols are the base units also known as monolignols or phenylpropane units. More specifically the complex network is comprised of *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S) (Figure 1.2) (Faik, 2013). The corresponding aromatic constituents are called 4-hydroxyphenyl, guaiacyl (4-hydroxy-3-methoxphenyl) and syringyl (4-hydroxy-3,5-dimethoxyphenyl) units. The proportion of these units varies as softwood lignin is dominated by G-units, while hardwood contains more a mixture of G- and S-units and grasses are composed of all 3 units (Boerjan et al. 2003; Ralph et al. 2004). The S-monomer is believed to increase the recalcitrance of the plant biomass more compared to the G-monomer (Li et al. 2001). The phenylpropane units are linked by ester, ether and carbon-carbon bonds to form a large three-dimensional phenylpropanoid structure (Figure 1.6). The synthesis of monolignols is well documented, as they are synthesized in the cytoplasm and transported through ABC transporters to the apolast (Miao and Liu, 2010). Here they are polymerized via ether and C-C bonds, however the formation of lignin is less clear, due to the fact that there is no specific arrangement of the monomeric units indicated (Guerriero et al. 2014; Guerriero et al. 2016). The function of lignin is to

protect the plant by giving it strength due to its rigid structure, forming a barrier against pathogens and holding the water within the plant (Himmel et al. 2007).

The rigidity of the lignin structure is a reason why lignin is a problem in industrial application as breaking down and/or removal of lignin is required to make the desired sugar rich fractions available. However, the structure is very resistant to pretreatment (Kallioninen, 2014). How to increase the hydrolysis rate of lignocellulose by pretreating the biomass, will be addressed in the following section.

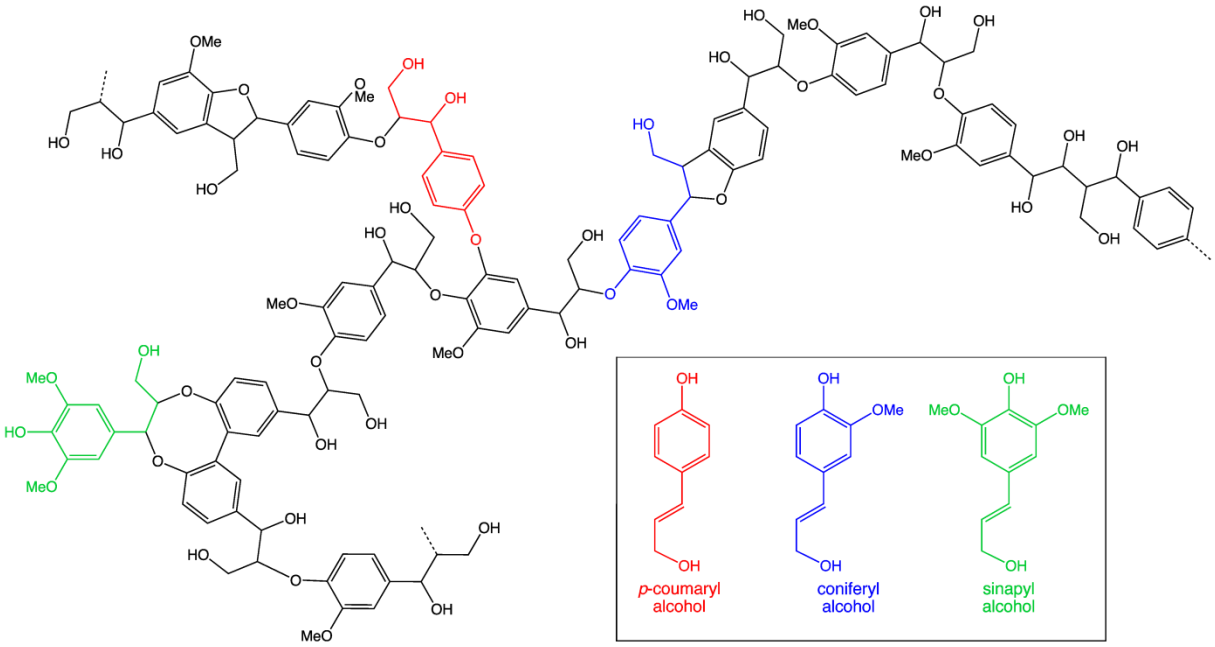

Figure 1.6: The chemical structure of lignin, a complex network of the 3 main building blocks: *p*-coumaryl alcohol (red), coniferyl alcohol (blue) and sinapyl alcohol (green)

1.3. PRETREATMENT OF LIGNOCELLULOSE

1.3.1. Introduction

Recalcitrant biomass material makes pretreatment a necessary step in the process of transforming lignocellulosic biomass to energy through the four-step anaerobic digestion: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Indeed, hydrolysis can be seen as the key bottleneck step in bioprocessing of lignocellulose to biofuels and must be facilitated by a pretreatment step (Sun & Cheng, 2002; Appels et al. 2008). The main goal of pretreating the lignocellulosic material is to open

the complex structure and facilitate the access of hydrolytic enzymes to cellulose and hemicellulose and increase the processability (Figure 1.1) (Li et al. 2010b). This is done by increasing the surface area, reducing the cellulose crystallinity and providing a greater porosity of the material, without decreasing the sugar concentration, and avoiding loss of organic material (Karunanithy and Muthukumarappan, 2012b).

Other factors play also a role in defining a good pretreatment method, inhibition should be taken into account as unfavorable products, like potential inhibiting phenolic compounds or acids, can be produced. These inhibitors can have a negative effect on fermentation to obtain bio-ethanol or biogas, and therefore biomethane (Palmqvist and Hanh-Hägerdahl, 2000). These are problems which should be addressed in evaluating the different pretreatment methods.

There are many pretreatment methods, all having advantages and disadvantages (Table 1.2). Choosing which pretreatment and which conditions to use is also dependent on the final objective, and on the feedstock, more particularly the chemical composition, lignin content, moisture content and particle size. Evaluating these techniques can be done by measuring the aforementioned goals, surface area, crystallinity index and pore sizes of the substrate, as well as the quantification of inhibiting by-products and more importantly glucose and xylose concentration. Apart from feedstock analysis, the energy requirements, total costs and environmental impact should be taken into account. However due to the dependence on feedstock, pretreatment and the final objective, different parameters are of importance, which makes it difficult to compare pretreatment techniques quantitatively. Basically the methods can be divided into physical, chemical and biological methods which will be discussed further.

1.3.2. Physical pretreatment

Milling, extrusion, freezing and microwaving are the most known physical pretreatments. The objective of these types of pretreatment is size reduction, decreasing the polymerization of the material and lowering the crystallinity of the cellulose polymer. This is done by applying stress on the

substrate and so increasing the surface area available for the hydrolytic enzymes (Shirkavand et al. 2016). An increase of glucose and xylose yield was reported due to wet disk milling and ball milling, respectively glucose yield improved with 49 % and 79 % while the xylose yield improved by 37 % and 72 % when applied to sugarcane bagasse (da Silva et al. 2010). Both techniques were also studied by Hideno et al. (2009) and they reported increased glucose yields of 79 % and 42 % while xylose yield improved by 89 % and 54 % respectively for wet disk milling and ball milling of rice straw. These studies indicate a substrate dependence as the type of milling shows very different yields for the two different substrates. Extrusion increases the digestability and thus improving the methane yield by 18 - 70 % and 9 - 28 % after digestion of 28 and 90 days, as reported by Hjorth et al. (2011). Yoo et al. (2011) studied the extrusion at 350 rpm and 80 °C of soybean hulls, and achieved 95 % glucose conversion. During the physical pretreatments no inhibitors or toxic by-products are formed and it is easily combined with other types of pretreatment. However there is a major drawback as a high energy input is required, defeating the purpose of the pretreatment (Shirkavand et al. 2016).

Thermal pretreatments at increased temperatures alter the structure of the substrate, making it easier to biodegrade. Viscosity is decreased, while soluble chemical oxygen demand, and soluble sugar concentrations are increased. The temperature used during pretreatment differs from study to study. Kitchen waste treated at 60 °C showed an increase of hydrolytic efficiency of 27 %. Kitchen waste treated at 175 °C improved hydrolysis but overall methane production was reduced by 7.9 %. Often thermal pretreatments are coupled with chemical pretreatments to have a similar effect but with a reduced temperature requirement and chemical reagens usage (Cesaro and Belgiorno, 2014).

1.3.3. Chemical pretreatment

Chemical pretreatments all have a common advantage as the pretreatments have an extremely efficient degradation of the hemicellulose and lignin fractions, which lead to higher glucose and methane yields than the physical pretreatments. However they all share a disadvantage as well, as they are very expensive and most of them form unwanted by-products and a considerable waste production, making it less environmentally sustainable (Table 1.2) (Shirkavand et al. 2016).

The two most common chemical pretreatments are acid and alkaline pretreatments. Acid pretreatments are known for primarily breaking down the hemicellulose. The most commonly used acids are H_2SO_4 and HCl. These acids are effective at degradation, yielding high sugar concentrations. Pretreating rice straw with a 1 % (w/w) sulphuric acid for 5 minutes at 160 - 180 °C resulted in a higher sugar yield of 83 % (Hsu et al. 2010), while the same pretreatment for 10 minutes at 180 °C of rape seed straw led to a xylose and glucose conversion of respectively 75 % and 63 % (Lu et al. 2009). In another study a mixture of acetic acid and sulphuric acid removed up to 90 % of hemicellulose in sugarcane bagasse (Rocha et al. 2011). An extra disadvantage of the acidic pretreatment however, is the requirement of permits and special materials due to the corrosive nature of the strong acids. This will increase the investment of the already costly pretreatment. Alternatively dilute acid pretreatment can be applied if the original substrate has a low lignin content. By mixing diluted acid into the mixture at 160 - 220 °C, hemicellulose can be completely removed. This pretreatment can reduce the amount of acid required and the recycling costs (Harmsen et al. 2010).

Alkaline pretreatments degrade hemicellulose as well as lignin, however alkaline pretreatments focus more on the lignin fraction. This is mainly done by disrupting the ester and glycosidic chains. NaOH is mostly used in the lime pretreatments, and is highly effective. Soybean treated with NaOH at room temperature removed 46 % of the hemicellulose, resulting in a glucose yield of 65 % (Wan et al. 2011). The conversion rate of glucan and xylan of coastal Bermuda grass achieved by a 0.75 % NaOH pretreatment of 15 minutes was respectively increased to 90 % and 65 % compared to the control (Wang et al. 2010b). $Ca(OH)_2$ is a better low cost alternative for NaOH, and is almost just as effective (Khor et al. 2015). Next to the general disadvantages of chemical pretreatments, alkaline pretreatments take a longer process time and sometimes a formation of unwanted salt is noticed as well (Brodeur et al. 2011).

Other chemical pretreatments are liquid hot water processes, organic solvent pretreatment (organosolv), ionic liquid pretreatment and ozonolysis. The organosolv method breaks down

hemicellulose, lignin and the bonds between hemicellulose and lignin, and is one of the most efficient pretreatments. However solvents, like ethanol or methanol, can be an inhibitor for the biological conversion, the costs are extremely high and the solvents have a high flammability, contributing to the risks of the process (Mood et al. 2013).

The ionic liquids are organic salts which are liquid at temperatures below 100 °C. They break down the bonds between cellulose, hemicellulose and lignin. Li et al. (2010) pretreated switchgrass with 1-ethyl-3-methylimidazolium-acetate for 3 hours at 160 °C and so removed 69 % of the lignin. The known disadvantages are the expenses of the liquids, the fact they become more viscous during the process, and they are toxic for most hydrolytic enzymes. This requires an extra step in the process chain to remove the ionic liquids. Advantages compared to regular volatile organic solvents are a low hydrophobicity, initially low viscosity and the thermal stability (Zheng et al. 2014).

Ozonolysis provides the good degradation of a chemical pretreatment, resulting in a better enzymatic hydrolysis as seen by Garcia-Cubero et al. (2009) where an increase of 300 and 350 % was noted respectively with wheat straw and rye straw. Ozone reacts with polysaccharides, proteins and lipids to transform them into smaller molecular-weights compounds, rupturing cell membranes and releasing cell cytoplasm. Other major advantages are that there is no production of toxic by-products and its solubility in water. On the downside ozone is a not-specific degradation agent and a high amount of ozone is required, which will increase the overall costs and safety regulations (Balat, 2011).

1.3.4. Physico-chemical pretreatment

Physico-chemical pretreatments are known for removal of hemicellulose and lignin, as well as disruption of the cellulose fraction. This is mostly done by pressuring the substrate, followed by a fast decompression at a moderate to high temperature. These parameters are also increasing the costs of the pretreatment.

Steam explosion however is cost-effective and breaks down hemicellulose and lignin partially by using pressurized steam (20 - 50 bar) at high temperatures (160 - 270 °C) in a first step and rapid decompressing to atmospheric pressure in a second step. The lignin however is not totally removed, toxic by-products are formed and a part of the cellulose fraction is disrupted as well. In a combination with an alkaline treatment afterwards the lignin fraction could be removed totally however there would be an increase of inhibiting compounds and cost price as well (Mood et al. 2013; Shirkavand et al. 2016). In several studies an increase of 20 to 30 % of the methane yield was reported, this was seen with the pretreatment of wheat straw (Bauer et al. 2009; Bauer et al. 2010), an increase of 29 % was noted by Bruni et al. (2010) using biofibers as substrate at 180 °C for 15 minutes. A steam explosion at 1.72 MPa for 8.14 minutes led to an increase of 24 % of the methane yield of bulrush (Wang et al. 2010a).

Ammonia fiber explosion (AFEX) pretreatment uses a high pressure (2 MPa) at a more moderate temperature (60 - 120 °C). After decompression, the lignin is removed without the production of by-products. The used substrate should be carefully selected as AFEX is known to have difficulties with higher lignin concentrations, like hard- and softwood feedstocks, resulting in a partial removal. Supercritical carbon dioxide pretreatment is a more cost effective variant of the AFEX pretreatment, however high pressures (> 2 MPa) are also required reducing the safety during batch fermentations. The surface area is increased due to the penetrating abilities of CO_2 molecules which are of the same size as water molecules. This way the cellulose and hemicellulose can be disrupted more, obtaining a greater accessibility for the hydrolytic enzymes (Mood et al. 2013; Shirkavand et al. 2016).

1.3.5. General drawbacks of traditional pretreatments

As of now it is hard to quantify results due to different pretreatments, or compare the different pretreatment types. Firstly this is due to the impact of the substrate, which influences results greatly as indicated in chapter 2. Secondly the literature reports their results often differently in the form of glucose yield, xylose yield, biomethane production or loss of lignin. Thirdly the experiments are often

performed under different conditions such as a different temperature, a different concentration of solvent, or even a different solvent or a shorter or longer incubation time etc. In order to fill the lack of this knowledge more studies should combine compositional analysis of the substrate with a parameter indicating the impact on the final result. Evaluating the pretreatment by using the same parameter, as for instance BMP or glucose yield, should improve the capability to compare different pretreatment techniques better.

Due to lack of information to quantify pretreatment techniques, comparing different pretreatments can be done by listing the different advatages and disadvantages of each technique (Table 1.2).

Table 1.2: Advantages and disadvantages of the various pretreatment techniques of lignocellulose, the different effects are indicated: very positive (++); positive (+); neutral (+/-) and negative (-). N/A = not available in present literature. (Harmsen et al. 2010; Chandra et al. 2012; Mood et al. 2013; Cesaro and Belgiorno, 2014; Zheng et al. 2014; Sun et al. 2016; Jönsson and Martin, 2015; Rouches et al. 2016)

Pretreatment Type	Sugar yield	Inhibitor formation	Residue formation	Need for recycling chemicals	Low investment costs	Low operational costs	Applicable to various biomass	Proven at pilot scale	Increase specific surface	Cellulose decrystallization	Hemicellulose removal and solubilization	Lignin removal
Physical												
Mechanical	--	++	++	++	+	-	+	++	++	++	-	-
Irradiation	N/A	+/-	N/A	N/A	N/A	N/A	N/A	N/A	++	+/-	+/-	-
Chemical												
Liquid hot water	++	-	++	++	+	N/A	N/A	++	++	N/A	++	+/-
Weak acid	++	-	-	-	+/-	+	+	++	++	-	++	+
Strong acid	++	-	-	-	-	-	++	++	++	-	++	+
Alkaline	++	+	-	-	++	+/-	+/-	+/-	++	-	+	++
Organosolv	++	++	+	-	-	-	+	++	++	N/A	+	++
Wet oxidation	+/-	+	+	++	+	-	N/A	-	++	+	++	++
Ionic liquid	N/A	++	N/A	N/A	N/A	-	N/A	N/A	++	++	+/-	+
Ozonolysis	N/A	-	N/A	++	N/A	-	N/A	N/A	++	N/A	-	++
Physico-chemical												
Steam explosion	+	-	+	++	+	+	+/-	++	++	-	++	+/-
AFEX	++	+	N/A	-	-	-	-	N/A	++	++	+	++
CO_2 explosion	+	+	++	++	-	N/A	N/A	-	++	-	+	-
Biological												
Fungal	N/A	++	N/A	++	++	++	N/A	N/A	++	+	+/-	++

1.3.6. Fungal pretreatment

In comparison to the pretreatments mentioned above, the great advantage of fungal pretreatments is the low energy demand and costs attached, and it is seen as environmentally friendly, as mild and clean strategies can be used as pretreatment techniques (Mood et al. 2013). The drawback of these pretreatments is the time required to get an efficient reduction of lignin and hemicellulose. The long incubation time will be disadvantageous as the cellulose polymer will be used by the fungi as a source for growth (Table 1.2) (Cesaro and Belgiorno, 2014). In the first weeks of pretreating with white rot fungi an increase in cellulose crystallinity is even noticed (Shirkavand et al. 2016), indicating the consumption of the easier hydrolyzable amorphous regions of cellulose by the fungi. The growth conditions need to be carefully monitored, and due to the long duration space is needed as well to perform the pretreatment, or store the biomass. These disadvantages make a fungal pretreatment as of now less interesting at industrial scale, and should be improved to make it competitive with the traditional pretreatments i.e. mechanical or chemical pretreatments. Suggestions have been made to combine biological pretreatment step as a primary pretreatment step with other pretreatments like organosolv pretreatment (Rouches et al. 2016). Fungal pretreatments can also be seen as a second step in a combination pretreatment, since studies show a decrease in the concentration of inhibiting phenolic compounds when treated with laccase produced by most white rot fungi (Table 1.3) (Jönsson et al. 1998).

1.3.6.1. White rot fungi

White rot fungi belong to the asomycete or basidiomycete groups, but in contrast to brown and soft rot fungi they can degrade lignin completely to CO_2 and H_2O. White rot fungi can be divided into two groups based on the preference of degradation. Some fungi selectively delignify the biomass, degrading preferably the lignin fraction over the sugar-rich celluse and hemicellulos fraction. These selective fungi are therefore more interesting to be used for the increase of bioenergy production. Examples studied are i.e. *Ceriporiopsis subvermispora, Pleurotus eryngii* and *Lentinula edodes* (Martinez et al. 2005). The degradation process starts by the release of lignin degrading enzymes such as lignin peroxidases, manganese peroxidases, versatile peroxidases and laccases. Not all these

enzymes are necessary to degrade lignin, and not all enzymes are produced by the different white rot fungi (Table 1.3). The working mechanism of the various enzymes will be discussed further (see 1.3.6). A general decay can be seen as the biomass becomes light, soft and spongy, this is due to side-chain oxidation, propyl side-chain cleavage and demethylation (Wan and Li, 2014).

Other, non-selective fungi break down cellulose, hemicellulose and lignin simultaneously, i.e. *Phanerochaete chrysosporium*, *Trametes versicolor* and *Irpex lacteus* (Martinez et al. 2005). Genetic engineering has been used to modify fast-growing non-selective white rot fungi to obtain cellulase-deficient strains. This has been unsuccessful as the performance and degradation efficiency were not as good as expected.

Other basidiomycetes are the aforementioned brown rot fungi, however as they primarily attack cellulose, and only degrade lignin to a limited extent, they are studied far less (Agbor et al. 2011). Modification of the lignin structure happens through demethylation or hydroxylation, but brown rot fungi are incapable of fragmenting aromatic rings.

The fermentation itself can be submerged in a liquid or performed in a solid-state. Solid-state fermentation has the advantage of better mimicking the natural environment of the fungi during the pretreatment, requiring smaller fermenters, lower sterilization costs, and having less risk of contamination as the environment is less suitable for bacteria (Wan and Li, 2013).

1.3.7. Enzymatic pretreatment

1.3.7.1. Laccase

Laccases (LAs) (EC. 1.10.3.2) are blue multicopper oxidases, causing oxidation of phenolic compounds by one-electron abstraction. The laccase molecule is a dimeric glycoprotein, containing four copper atoms per monomer, distributed in three redox sites, T1, T2 and T3. Oxidation is initiated by an electron transfer between the reducing substrate and copper (Cu^{2+}) at the T1 site, as primary electron acceptor. The electron is then transferred from the T1 to the T2/T3 site fully reducing the enzyme after 4 electron oxidations (Figure 1.7) (Coyne et al. 2013). During the oxidation radicals are

formed which can re- or depolymerize. Through oxidation laccases are able to degrade a phenolic model compound into a mixture of different products (Majumdar et al. 2014).

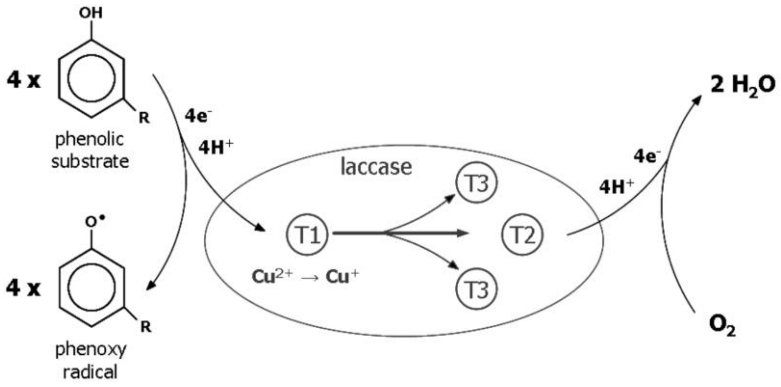

Figure 1.7: The mechanism of laccase, oxidizing phenolic compounds (Baldrian, 2006)

Oxidation of non-phenolic compounds has been seen in the presence of a mediator i.e. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Baratto et al. 2006). The mediators act as redox shuttles between laccase and the lignin structure. Most effective mediators are *N*-heterocycles, as for instance *N*-hydroxybenzotriazole (Figure 1.8) (Gonzalo et al. 2016).

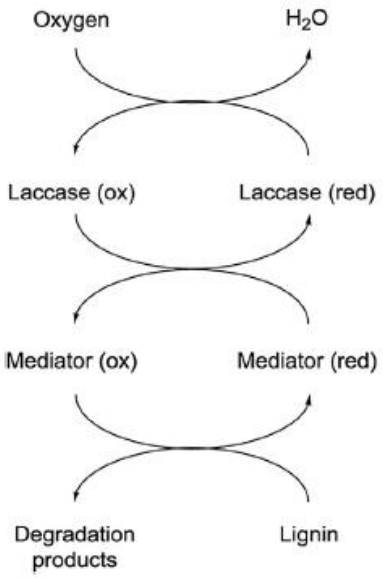

Figure 1.8: The laccase catalyzed redox cycle for lignin degradation in the presence of chemical mediators (adapted from Gonzalo et al. 2016)

Laccases can industrially be used to degrade or remove phenolic compounds, which is advantageous during a pretreatment to obtain biofuels or biogas. Other industrial applications can be found in the

paper, textile or food industry, removing phenolic compounds responsible for browning or assisting in the decolorization of dyes. Laccases are synthesized in multiple isoforms, and are also more readily available and easier to manipulate than peroxidases, raising their desirability to use on industrial scale (Strong and Claus, 2011).

1.3.7.2. Lignin peroxidase

Lignin peroxidases (LiPs) (EC. 1.11.1.14) are heme-containing proteins, with iron (Fe^{3+}) in a porphyrin ring. LiPs are oxidized by H_2O_2, produced by white rot fungi or added to the mixture, to form an intermediate, deficient in two electrons. The intermediate extracts one, producing two cation radicals. The cation radicals can break into smaller fragments as the LiP intermediate in resting state can return to a native state in the presence of H_2O_2 and veratryl alcohol. Veratryl alcohol is required to protect the LiPs, as excessive H_2O_2 will inactivate LiP (Figure 1.9) (Narayanaswamy et al. 2013). More even, purified LiPs without the presence of veratryl alcohol did not react with lignin, the addition of veratryl alcohol was required to observe depolymerization (Hammel & Moen, 1991). Further studies show the LiP as molecule is too large, and diffusible radicals are induced, which are able to diffuse through cell walls and initiate a further decay of biomass from within, facilitating penetration and thus improving the hydrolysis of the lignocellulosic biomass. Veratryl alcohol functions as a redox mediator, and the oxidezed veratryl alcohol molecule, a radical cation VA^{+*} is the actual oxidant of the lignin polymer (Schoemaker & Piontek, 1996). However the strong oxidizing power of LiP is highly effective in breaking down lignin, by cleaving C_α-C_β bonds in lignin related compounds.

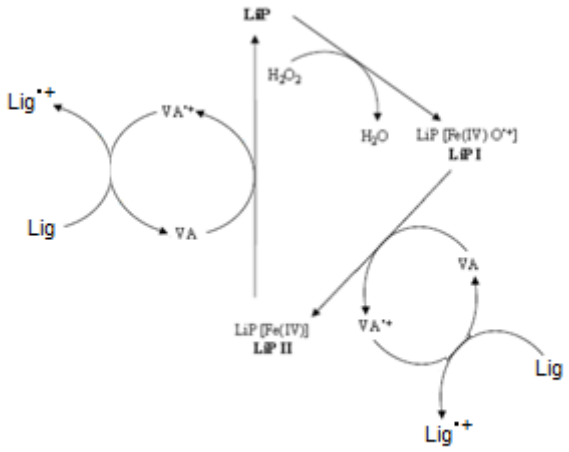

Figure 1.9: The catalytic mechanism of lignin peroxidase (LiP), with veratryl alcohol (VA) and H_2O_2 as intermediates in the degradation of lignin (adapted from Narayanaswamy et al. 2013)

1.3.7.3. Manganese peroxidase

Manganese peroxidases (MnPs) (EC. 1.11.1.13) are like LiP heme-containing glycoproteins, oxidizing lignin related compounds thanks to H_2O_2, produced by white rot fungi or added to the mixture. The main difference with LiP is the production of diffusible oxidizing agents, capable of oxidizing lignin indirectly (Coyne et al. 2013). Mn^{2+} is oxidized to Mn^{3+}, generating other oxidants like peroxyl and acyl radicals, and so extensively increase the ability of degrading lignin, as peroxyl radicals are even capable of cleaving non-phenolic lignin structures. Phenolic structures are beinig degraded as well, as C_α-C_β-bonds and alkyl-aryl bonds are being cleaved in the presence ofsodium malonate, Mn^{2+} and H_2O_2 (Figure 1.10) (Hofrichter, 2002). Mn^{3+} can be stabilized by a chelator like oxalate produced by white rot fungi as a secondary metabolite, decreasing its oxidizing power (Figure 1.11) (Narayanaswamy et al. 2013).

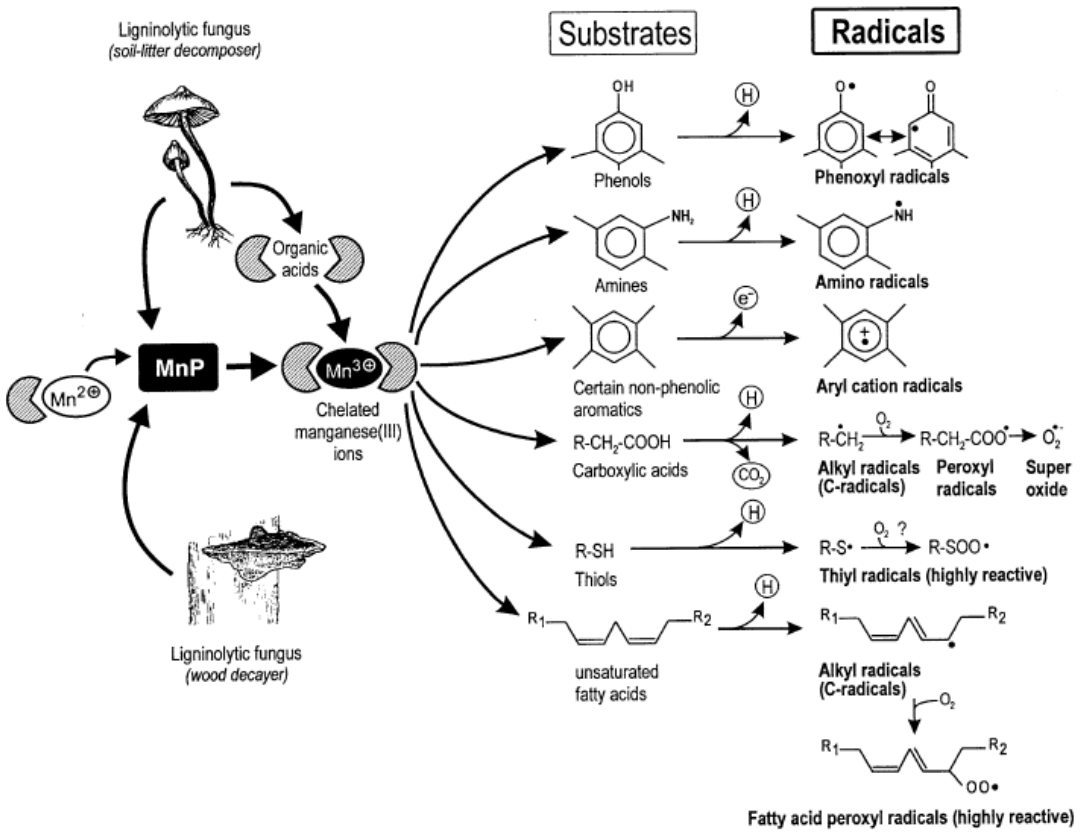

Figure 1.10: The formation of radicals by MnP in the presence of different substrates (adapted from Hatakka, 2001)

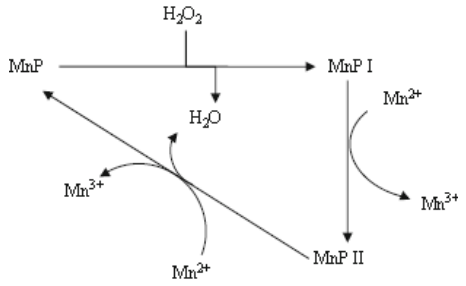

Figure 1.11: The catalytic mechanism of manganese peroxidase (MnP) (Narayanaswamy et al. 2013)

1.3.7.4. Versatile peroxidase

Versatile peroxidases (VPs) (EC. 1.11.1.16) are a hybrid form between LiP and MnP as the two oxidation pathways are available for VP. Mn^{2+} binding sites as well as the tryptophan residues for direct one-electron oxidation are present. The tryptophan residue was recognized as the amino acid radical in moderate distance from the ferryl heme. This way VPs are also able to degrade phenolic as well as non-phenolic lignin associated compounds (Moreira et al. 2005; Pogni et al. 2006).

Table 1.3: Enzymes produced by various white rot fungi and their effect on lignin content

Fungus	Laccase	LiP	MnP	References
Bjerkandura adusta		x	x	Levin et al. 2004; Dinis et al. 2009
Ceriporiopsis subvermispora	x		x	Fernandez-Fueyo et al. 2012
Daedalea favida	x	x		Arora et al. 2002
Dichomitus squalens	x		x	Arora et al. 2002
Euc-1	x	x		Dias et al. 2010
Irpex lacteus		x	x	Dias et al. 2010
Phanerochaete chrysosporium		x	x	Arora et al. 2002; Levin et al. 2004
Phlebia fascicularia	x	x	x	Arora et al. 2002
Phlebia floridensis	x	x	x	Arora et al. 2002
Phlebia radiata	x	x	x	Arora et al. 2002; Chen et al. 2010
Phlebia rufa	x		x	Dinis et al. 2009
Pleurotus eryngii	x	x	x	Fernandez-Fueyo et al. 2012; Akpinar and Urek, 2014
Pleurotus ostreatus	x		x	Qi-He et al. 2011
Trametes versicolor	x	x	x	Arora et al. 2002; Dinis et al. 2009

1.4. FORMATION OF INHIBITORS DURING PRETREATMENT

1.4.1. Introduction

Evaluating pretreatments can be done by looking at the final result, a possible increase in bioethanol or biogas production. However the goal is influenced by several other measurable parameters such as the degradation efficiency of lignin, production of free sugars and the release of unfavorable by-products. Most of the inhibiting compounds are released due to the degradation of hemicellulose and lignin during pretreatment. Pretreatment however is necessary as the lignin itself is seen as the major inhibitory factor during hydrolysis, as it forms a physical barrier as well as it induces non-productive binding of enzymes. In this part a closer look on these inhibiting compounds is taken, as there are a variety of them with a difference in impact. The three main groups are divided in weak acids, furan derivatives and phenolic compounds. These inhibitory compounds can be overcome by adding a detoxification step in the production process, just as the pretreatment step there are physical, chemical and biological methods (Mussatto and Roberto, 2004).

1.4.2. Weak acids

Most common weak acids are acetic, formic and levulinic acid, produced during the degradation of hemicellulose. Weak acids will inhibit cell growth as they diffuse through the plasma membrane,

flowing into the cytosol. Dissociation of the acid in the cytosol causes a drop in cytosolic pH, decreasing cell proliferation. The drop in intracellular pH is neutralized by plasma membrane adenylpyrophosphatase (ATPase) pumping protons out of the cell. ATP generated to maintain stability under anaerobic conditions will lead to ethanol production in favor of biomass formation. At higher acid concentrations the ATP content within the cell will be depleted, resulting in a lack of protons pumped through the plasma membrane, leading to a more acidic cytoplasm and greater inhibition (Agrimi et al. 2012; Palmqvist and Hahn-Hägerdal, 2000; Jönsson and Martin, 2015). Other theories claim that the inhibition is caused by the accumulation of anions. Lower extracellular pH due to high concentrations of weak acids, will result in higher intracellular anion accumulation as equilibrium is reached by undissociated acid diffused into the cell. Mixing glucose and acetic acid lowered pH from 6.0 to 3.5, increasing the amount of anions within cells of *Saccharomyces cerevisiae* by a factor of 10 to 1000 (Casal et al. 1996). While Bellido et al. (2011) noted a full inhibition of the growth of *Pichia stipites* due to 3.5 g/l of acetic acid.

1.4.3. Furan derivatives

Widely discussed furan derivatives are furfural and hydroxymethylfurfural (HMF). They have less or no effect on cellulase activity compared to phenolic compounds, but are well-known inhibitors of fermenting microorganisms (Kim et al. 2011). Furfural is formed from pentoses i.e. xylose, arabinose and decreases the specific growth rate. Furfural is reduced to furfuryl alcohol by NADH-dependent yeast alcohol dehydrogenase. Furfuryl alcohol inhibits anaerobic and aerobic growth of different yeasts, significantly reducing glycerol production. HMF is formed from hexoses and has a lower membrane permeability, so conversion to 5-hydroxymethyl furfuryl alcohol occurs at a lower rate. However a longer lag-phase in cell growth due to HMF was noted (Larsson et al. 1999a). When 3-5 mg/l of HMF was added to algae residue, a lag phase occurred in the CH_4 production, while a total inhibition was seen after addition of 10 mg/l (Park et al. 2012). Monlau et al. (2014) added furfural but did not see a lot of inhibition even if 4 g/l was added, however a total inhibition in methane production was seen if 6 g/l of HMF was added.

1.4.4. Phenolic compounds

Aromatic compounds are the second most abundant organic compounds in nature. Phenolic compounds, a group of aromatics, are seen as the most toxic by-products affecting further hydrolysis and fermentation by cellulases (Hernandez and Edyvean, 2008). Vanillin, ferulic acid and 4-hydroxybenzoic acid are the most common phenolics. The phenolic compounds originate from the degradation of lignin, phenol and *p*-hydroxybenzoic acid originate from hydroxyl compounds, guaicacol, vanillin and ferulic acid originate from guaiacyl compounds and syringol, syringic acid and sinapyl alcohol derive from syringyl compounds. The different origins of the phenolic compounds can be interesting when deciding for a substrate as softwoods mostly have G-units, and hardwoods contain both G- and S-units of the aromatic constituents (Klinke et al. 2004). Phenolic compounds already have a severe impact at micromolar to millimolar concentrations as cellulose conversion is inhibited. Higher concentrations will lead to inhibiting enzymatic hydrolysis and deactivation of enzymes. The oligomeric phenolic compounds like tannic acids have been reported to have an even greater inhibiting effect on the cellulases, however these phenolic compounds are less found in pretreated biomass (Kayembe et al. 2013).

The phenolic compounds are disrupting microbial membrane integrity, by inhibiting the electrochemical proton gradient. The following reduction of electron transport and energy production causes an inhibition of microbial activity and of the fermentation by micro-organisms. The low molecular weight phenolic compounds like syringic acid, vanillin and hydroxybenzaldehyde are known to be toxic when present at low concentrations in the fermentation of lignocellulose. The influence of individual phenolic compounds has been studied to evaluate the biogas production of anaerobic bacteria digesting glucose, yeast extract and nutrient broth. The concentrations required for a 50 % inhibition varied from 121 mg phenol/g $VSS_{biomass}$ to 594 mg protocatechuic acid/g $VSS_{biomass}$. The variation in inhibition level is caused by the number of OH substitutes, decreasing the toxicity due to the increase of polarity. The size of these substitutes does not affect the toxicity according to Hernandez and Edyvean (2008), however this is not in agreement with studies

previously reported by O'Connor and Young (1989), finding the type of substitution being more important than the apolarity of the inhibitor. These discrepancies indicate more research should be done to understand the mechanisms and impact of the various inhibiting compounds.

Anaerobic digestion of pig manure with addition of phenolic compounds was studied by Kayembe et al. (2013) and showed an increased toxicity of the phenolic compounds when they have a lower boiling point. Benzene, with a boiling temperature of 80 °C, added in a concentration of 3.7 mg/g VSS inhibited methanogenic activity by 50 %, while pyrogallol with a boiling temperature of 309 °C had the same toxic effect at a concentration of 56 mg/g VSS. The study of Kayembe et al. (2013) confirmed the increased inhibition due to increased OH substitutes of the toxic compound. Anaerobic digestion of mere sludge with additions of phenolic compounds or furan derivatives has been studied more widely. Chapleur et al. (2015) noted a severe inhibition of methane production if 2 g/l of phenol was added, and a total inhibition at 4 g/l. Barakat et al. (2012) reported a serious inhibition in methane yield when 12 and 24 mg/gVSS of vanillin was added to the sludge in a batch reactor. These inhibition studies have been done using glucose or sludge as a substrate, which can provide insight on the inhibition mechanisms, however it might not be useful to extrapolate these results to agricultural waste streams used in industrial applications. Natural waste streams also contain a mixture of inhibitors, and one should keep synergistic interactions between inhibitors in mind. Next to a greater understanding of the impact of the various inhibitors on more complex substrates, a standardized method to determine the impact of the inhibiting compounds should be determined. Comparing various studies is hard by the use of different parameters or substrates. The inhibition is mostly determined, based on the production of the final product, like biogas. For further application however the length of the lag phase should be reported as well, since phenolic compounds in high enough concentrations can increase the duration of the process with several weeks.

1.4.5. Inhibition of lignin degrading enzymes

Less studied is the inhibition of the enzymes produced by white rot fungi, and preventing the necessary breakdown to improve the hydrolysis rate. Oxidation of veratryl alcohol by versatile peroxidase from *Bjerkandera* sp. BOS55 is known to be severely inhibited by manganese ions at concentrations greater than 0.1 mM. VP from *Bjerkandera fumosa* was tested by studying the oxidation rate of 2,6 dimethoxyphenol. In this study VP was shown to be inhibited by sodium azide, Tween 80, antracene and fluorine, however a positive effect was seen with the addition of *p*-aminobenzoic acid. Pozdnyakova et al. (2013) found VP was inhibited irreversibly by sodium azide, in comparison to the other inhibitors where a reversible binding was noted. Thanh Mai Pham et al. (2014) studied the inhibition of lignin peroxidase from *P. chrysosporium* by phenolic unit structures released during biodegradation of lignin. Free hydroxyl phenolic groups interacted with the surface active sites and so inhibiting the oxidation rate of veratryl alcohol, indicating the enzyme activity. The presence of 5 µM of guaiacol lead to a complete inhibition if enough (2000 µM) H_2O_2 was present. A second study examined the formation of veraldehyde in the presence of 250 µM H_2O_2 and 0.1 µM LiPH8. After addition of various phenolic compounds at different concentrations complete inhibition was seen when 50 µM of guaiacol, vanillyl alcohol or coniferyl alcohol was added. Complete inhibition was also found after addition of 100 µM of 2,6-dimethoxyphenol, vanillin, vanillic acid, coniferyl aldehyde and ferulic acid. The LiP was inhibited for only 50 % after 100 µM phenol was added (Thanh Mai Pham et al. 2014). Laccase activity can be tested by investigating the oxidation reaction of ABTS. The laccase activity of *T. versicolor* after addition of 1 mM of various inhibitors was measured. No inhibition was seen by cysteine, dithiothreitol, diethyldithiocarbamic acid, thioglycolic acid, only direct inhibition was noted with the addition of sodium azide (Johannes & Majcherczyk, 2000). Jönsson et al. (1998) tested laccase in the presence of various phenolic compounds, however no inhibition of the laccase enzyme was noted. The study showed a breakdown of the phenolic compounds of 2562 mg/l to 166 mg/l, proving that laccase as enzyme could be used for the removal of various inhibitors (Sindhu et al. 2015).

1.4.6. Detoxification

The formation of inhibitors is dependent on which substrate is treated, as well as which pretreatment is used (Table 1.2). To overcome inhibition problems a less recalcitrant substrate can be chosen, requiring a less harsh pretreatment. However the used substrate is not always a choice, as it depends on the supply, or a variation of substrates is used in order to not be dependent on one substrate, then a detoxification step can be introduced to break down or remove inhibiting compounds. The detoxification of hydrolysates can be done chemically, physically or biologically (Mussatto and Roberto, 2004; Parawira and Tekere, 2011).

Physical methods based on evaporation efficiently remove volatile compounds such as acetic acid, furfural and vanillin. Several studies reported a total removal of furfural (Larsson et al. 1999b; Rodrigues et al. 2001), however the concentration of non-volatile compounds increased causing strong interference during the following fermentation (Parajo et al. 1996; Silva and Roberto, 2001). A further dilution of the non-volatile compounds is required to avoid the inhibition tresholds. Another physical detoxification method is the separation by the use of adsorptive micro porous membranes. Functional groups on the surface of the membrane eliminate inhibitors released due to acidic pretreatments. Grzenia et al. (2008) reported a 60 % removal of acetic acid as effect from various diluted organic phases, alamine 336 and aliquat 336. Alamine 336 was used as well after a pretreatment with sulphuric acid. Sulphuric, acetic, formic and levulinic acid, and the furan derivatives were removed by alamine 336, octanol and oleyl alcohol (Grzenia et al. 2010). Even though a good removal of inhibitors is seen, improvements must still be done using low cost and more effective chemicals, with a higher affinity for inhibiting compounds and less effect on the sugar content (Chandel et al. 2013).

Chemical methods are used to increase the pH after acidic pretreatments, $Ca(OH)_2$ or NaOH are used to neutralize the hydrolysate to pH 6-7. Furfural and phenolic compounds can be precipitated and removed during the process (Chandel et al. 2013). Increasing the pH further by overliming with an increased temperature was seen as a promising and very efficient method (Jönsson et al. 2013).

Furan derivatives are removed, however a loss of 10 % in sugar content was reported which leads as well to lower ethanol yields (Nilvebrant et al. 2001). Ethyl acetate extraction is effective for most inhibitors such as acetic acid, formic acid, furfural, HMF and phenolic compounds. Further treatment of the extraction with an alkali can reduce HMF and furfural concentrations. Another approach is an exchange with anions, reducing levulinic, acetic and formic acids as well as the furan derivatives but led to a considerable loss of fermentable sugars from the hydrolysate (Chandel et al. 2013).

The most promising detoxification in the long run are the biotechnical solutions (Jönsson et al. 2013). Microbial detoxification is done on different types of substrates i.e. corn stover, willow, spruce etc. by different micro-organisms i.e. *Coniochaeta ligniaria* C8, *Trichoderma reesei*, encapsulated *S. cervisiae.* All studies reported a removal of furfural and HMF, which were reviewed in Parawira and Tekere (2011). To increase the speed of detoxification crude enzyme mixes with higher catalytic activity can be used. Phenolic monomers can be removed from the hydrolysates by lignin oxidizing enzymes, such as laccase (Jönsson et al. 1998). Martin et al. (2012) noted a 80 % removal of phenolic compounds by laccase treatment. In these studies the effect of laccase is evaluated via a spectrophotometric measurement technique. This general method can give an indication, although further HPLC analysis is required to have more insight in the production or detoxification of phenolic compounds. Biological detoxification is a low cost step that could improve pretreatment efficiency of lignocellulosic biomass, but it requires extra incubation time and can result in a loss of sugars.

If an extra detoxification step is not desirable, adaptation of the used micro-organism can overcome inhibiting problems, however this also requires time and a good control of the experiment. As an example *S. cerevisiae* is known to be able to adapt to furfural concentrations in the hydrolysate (Banerjee et al. 1981). Biomass adaptation and stability after strong community shifts within an anaerobic sequencing batch reactor was seen even if 800 mg/l of phenol was added by Rosenkranz et al. (2013). Another technique which can be suggested, is the genetic engineering of fermenting

organisms to tolerate inhibitors, for example *S. cerevisiae* can be mutated to produce laccase (Larsson et al. 2001) or exhibit tolerances to furan aldehydes (Liu et al. 2008).

1.5. ANAEROBIC DIGESTION OF LIGNOCELLULOSIC BIOMASS

1.5.1. Introduction

Although bioethanol and biodiesel production have experienced more attention worldwide, production of biogas has also become of more awareness in years (Weiland, 2003; Weiland, 2010). This interest for biogas and biomethane is due to several advantages over bioethanol and biodiesel. No separation step is required since the biogas will be separated automatically from the liquid (Braun et al. 2007). Several studies show a greater potential of energy production by methanization of the substrate than production of the biofuels. The energy from biogas generated from sugarcane was 60 % higher than if the sugarcane was converted into alcohol (Van Haandel and Lubbe, 2011). The net energy yield obtained from wheat or maize was three times higher by producing biogas compared to biodiesel or bioethanol (Borjesson and Mattiasson, 2008). Apart from the higher yield, anaerobic digesters can be used more locally with a great variety of input, becoming interesting for farmers as a source of extra income (Koçar and Civas, 2013).

Anaerobic digestion is a mature technology, used for the conversion of the organic fraction found in various waste streams, like municipal solid wastes and sludge from water-waste treatment plants. Anaerobic digestion is a four step process, resulting in methane, generated through anaerobic digestion from organic wastes or energy crops. The anaerobic digestion of energy crops resulted in a high methane production, up to 450 l/kg VS (Frigon and Guiot, 2010). However the use of sugar and starch crops to produce biofuel is debatable as it is in direct competition with land and crops used for food or feed purposes. Second generation biofuels, originating from various lignocellulosic substrates evade this problem, as it is waste from agricultural or forestry industry, or can be grown on marginal lands. The yield of these crops is generally lower (Table 1.4), however having a great potential due to a good pretreatment, followed by an effective hydrolysis.

1.5.2. Biogas process

Anaerobic digestion results in biogas production through 4 steps, hydrolysis, acidogenesis (or fermentation), acetogenesis and methanogenesis (Figure 1.12) (Appels et al. 2008). These individual steps will be explained later in this paragraph.

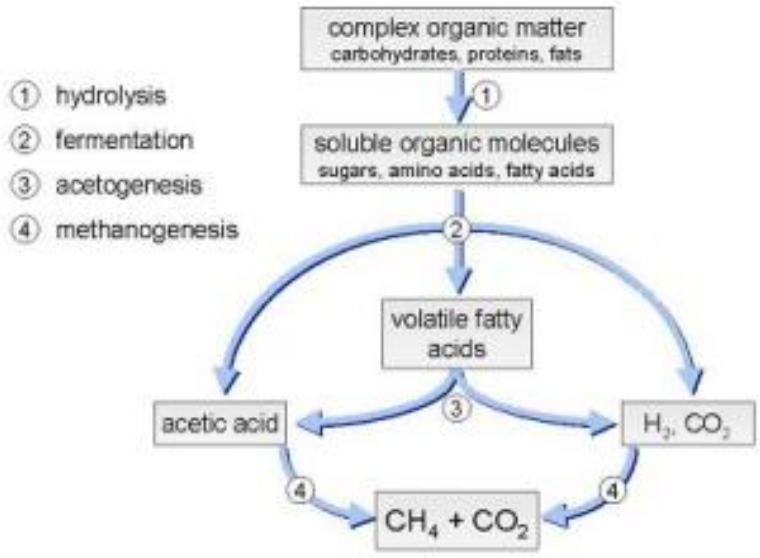

Figure 1.12: The four steps of the anaerobic digestion process (Long, 2010)

The biogas process always follows the four steps, however different methods of operation have been studied. The process can be performed at mesophilic (35 °C) or thermophilic (55 °C) temperatures, resulting in different methanogenic bacteria which will function optimally (Abbasi et al. 2012). Most experiments are performed in the mesophilic temperature range (20-40 °C) as only little gain is reported at higher temperatures, making it unfavorable as operating costs increase as well. Improved methanization has also been reported by using different crops together or mix lignocellulosic material with animal manure. The anaerobic digestion of a mixture of different substrates is called a co-digestion, is mostly used and reaches higher methane yields than mono-digestion (Parawira et al. 2004). The load of total solids (TS) in the reactor determines if wet or dry (with a cut-off value of 15 % TS) digestion is performed. An increasing trend of dry digestion occurred within the municipal solid waste in Europe, as dry fermentation systems become more efficient. In 2005 dry digestion plants represented 50 % of the total number of digestion plants, in 2010 the number has risen to 60 %,

while in 2015 65 % of the plants in Europe are dry digestion plants (De Baere and Mattheeuws, 2012; Abbasi et al. 2012; European Bioplastics, 2015). Allthough the number of dry digestion plants is higher the capacity of biogas production through wet anaerobic digestion is still greater (5800 kTon/y) compared to capacity of dry digestion plants (3000 kTon/y) Concluding that the wet digestion plants are bigger in capacity terms (Figure 1.13).

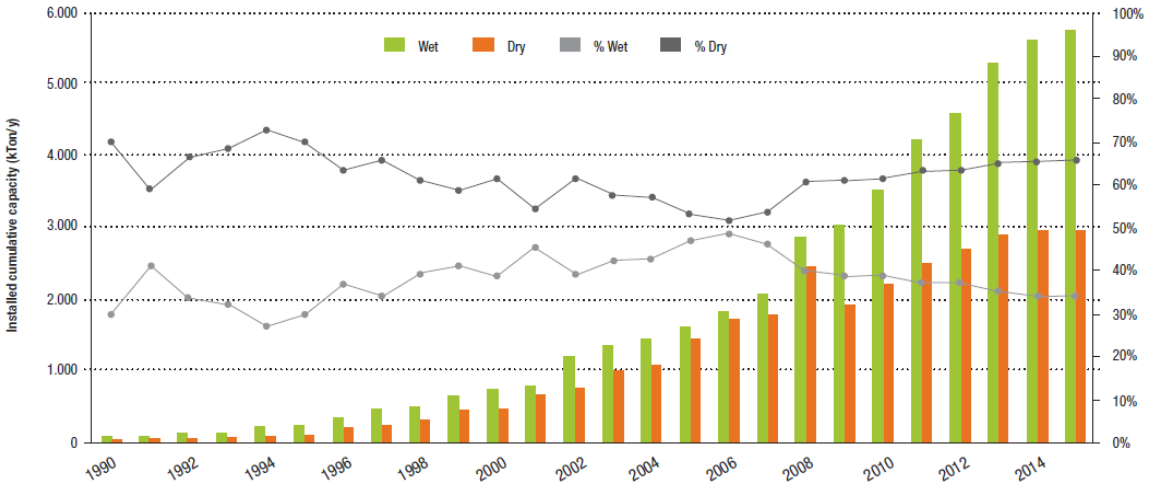

Figure 1.13: The evolution of ratio and capacity of wet and dry digestion plants (European Bioplastics, 2015)

The four-step process is mostly performed as a single phase in the same reactor. However the process can be split up into two and even three phase processes. The phase separation is done since the different steps have different optimal pH conditions. In a two phase process the methanogenesis is run separate, the end product of the first stage being volatile fatty acids (VFAs). Uncoupling these two stages is done due to the difference in reaction speed and tolerance for inhibitors. (Parawira et al. 2006). In a three phase process the methanogenesis is performed separately as well as the hydrolysis. The more phases, the more costly it becomes, but the better controllable it is.

The advantages of the separation of the steps does not measure up to the extra costs required as of now, together with advancements in efficiency of single phase, dry digestion makes the single-stage digestion the more interesting setup (Abbasi et al. 2012).

1.5.2.1. Hydrolysis

Hydrolysis is the first step in the biogas production process, as discussed above it is also the rate limiting step and can be facilitated by pretreatment of the biomass (Wang et al. 1999; Mosier et al. 2005). Initially facultative anaerobic micro-organisms take up the dissolved oxygen from the water, creating a low redox potential necessary for obligatory anaerobic micro-organisms (Chandra et al. 2012). Complex polymers, i.e. carbohydrates, lipids and proteins are broken down by hydrolytic bacteria into soluble monomers, i.e. monosaccharides, amino acids and long-chain fatty acids (LCFAs). Covalent bonds are split in a chemical reaction with water (Chandra et al. 2012). Disintegration of carbohydrates by cellulase, cellobiase, xylanase and amylase takes place within a few hours, while degrading proteins and lipids by respectively protease and lipase, take a few days during the hydrolysation (Schnürer and Jarvis, 2009; Deublein and Steinhauser, 2010). The soluble intermediates are able to pass through the cell membrane of the microorganism providing the required energy and building materials for synthesizing cellular components (Deublein and Steinhauser, 2010; Batstone et al. 2002). Lignin is not affected during the hydrolysis as it is non-degradable and should be addressed during a pretreatment step as was discussed in section 1.3.

1.5.2.2. Acidogenesis

During the acidogenesis or fermentation step the obtained monomers are degraded further. A number of VFAs are formed i.e. propionic acid and butyric acid as well as acetic acid, alcohols, H_2 and CO_2. The acidogenesis is the fastest step and in a well-balanced anaerobic digestion the most common products formed are acetate, CO_2 and H_2. A lower partial pressure of H_2 in the reactor however leads to a higher production of acetic acid. Overall the bacteria present in the hydrolytic phase are responsible for the further degradation during the acidogenesis (Deublein and Steinhauser, 2010).

1.5.2.3. Acetogenesis

During acetogenesis LCFAs and VFAs are converted by other micro-organisms. Homoacetogenic micro-organisms reduce H_2 and CO_2 to acetic acid. Acetic acid is also formed through oxidation of the LCFAs as long as the H_2 partial pressure is low enough as the reaction is endergonic. Methane

forming bacteria grow symbiotically with the acetogenic bacteria and use the hydrogen, thus preventing an overconcentration of H_2 (Chandra et al, 2012).

1.5.2.4. Methanogenesis

Methanogenesis is the final phase where methanogenic bacteria strictly anaerobically convert the fermentation products into CH_4, CO_2 and H_2O. There are several different pathways for methane to be formed. These different exergonic reactions can be divided into different groups depending on the substrate to be converted. Acetoclastic methanogesis converts acetate into CH_4 and CO_2, hydogenotrophic methanogenesis converts H_2 and CO_2 into CH_4 and methyltrophic methanogensis converts methanol into CH_4 and H_2O (Chandra et al. 2012). The methanogenesis in general is the longest phase and is influenced by pH, temperature and inhibitory substances, such as NH_3 or H_2S (Chen et al. 2008). *The optimal pH for methanogens is 7 to 8, while in operation between 6.5 and 8.5 is considered. Ionic equilibriums have a large effect on the anaerobic digestion, as undissociated forms may pass through cell membranes causing inhibition. Free ammonia is more inhibitive, so for a given ammonia-nitrogen concentration lower pH is favored. A too low pH (<5) however would cause an overload of acetic acid. Sulfur present in protein rich materials will be reduced to H_2S by sulfur reducing bacteria. These bacteria will compete with the methanogenic bacteria for the same nutrients in order to produce H_2S (Figure 1.14). This has a negative impact as it lowers the biogas quality. Even in low concentrations (50 – 10000 ppm) H_2S is known to have an unpleasant odor and can be corrosive to the biogas installation as well. Removal of H_2S can be achieved through different techniques. Absorption into a liquid, adsorption on a solid, as for instance iron oxide or activated carbon, or add oxygen to the biogas, and thus oxidizing the H_2S into sulfur (Al Mamun and Torii, 2015).*

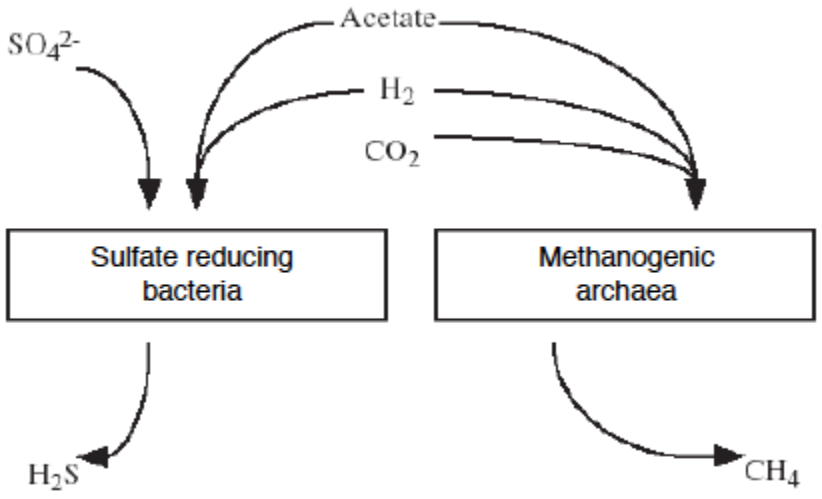

Figure 1.14: Competition of sulphur reducing bacteria and methanogens in order to produce respectively H_2S and CH_4 (Cavinato, 2011)

1.5.3. Bio-methane potential

To evaluate pretreatments, substrates and the overall biogas process, bio-methane potential (BMP) tests are widely used. It is an inexpensive and repeatable method giving significant information on the anaerobic digestion process as the cumulative methane production can be measured as well as the daily gain over 30 days. The BMP value is mostly given by the produced amount of methane per kg added VS, however some studies reported methane production per hectare of land used. This could give an important insight for countries like Belgium where the amount of available land is limited. In Table 1.4 a large variation of different lignocellulosic substrates is shown with their various BMP values. Within a substrate a large variance can be noted, as BMP and lignin are highly linked (Triolo et al. 2011). There is as well a dependence on which part of the plant was taken and when it was harvested. The lack of a standard protocol to carry out these experiments is an important problem, next to the fact that the applied temperature, or the duration of the BMP tests are different. (Esposito et al. 2012).

Table 1.4: The biomethane potential of lignocellulosic substrates

Substrate	BMP (l/kg VS)	References
Alfalfa	210-500	Zauner and Kuntzel, 1986; Plochl et al. 2009; Koçar and Civas, 2013
Animal manure	151-444	Cu et al. 2015
Barley	271-658	Bauer et al. 2010; Dinuccio et al. 2010; Nzila et al. 2010; Koçar and Civas, 2013
Chaff	229-316	Koçar and Civas, 2013
Clover	140-390	Kaparaju et al. 2002; Amon et al. 2007
Cocksfoot	308-344	Mähnert et al. 2005; Seppälä et al. 2009
Corn stover	360	Tong et al. 1990
Corn ensilage	270-298	Zauner and Kuntzel, 1986
Faba beans	440	Petersson et al. 2007
Festlolium	328-359	Seppälä et al. 2009
Flax	212	Koçar and Civas, 2013
Fodder beet	420-500	Koçar and Civas, 2013
Giant knotweed	170	Lehtomaki, 2006
Grass	128-467	Eleazer et al. 1997; Buffiere et al. 2006; Lehtomaki, 2006; Scaglione et al. 2008; Plochl et al. 2009
Hemp	230-409	Braun, 2007; Kreuger, 2012
Jerusalem artichoke	300-370	Koçar and Civas, 2013
Kale	240-334	Koçar and Civas, 2013
Leaves	417-453	Koçar and Civas, 2013
Lucerne	247-357	Badger et al. 1979; Bauer et al. 2010
Lupine	260-360	Lehtomaki, 2006
Maize	196-450	Amon et al. 2007; Pabon Pereira et al. 2009; Plochl et al. 2009; Raposo et al. 2012; Koçar and Civas, 2013
Miscanthus	179-218	Koçar and Civas, 2013
Napier grass	190-340	Lehtomaki, 2006
Nettle	120-420	Lehtomaki, 2006
Oat straw	250-320	Lehtomaki, 2006
Oilseed rape	240-340	Koçar and Civas, 2013
Peas	390	Koçar and Civas, 2013
Poplar	230-320	Turick et al. 1991; Chynoweth et al. 1993
Potatoes	276-400	Koçar and Civas, 2013
Rapeseed	240	Lehtomaki, 2006
Reed canary grass	340-430	Lehtomaki, 2006
Rice straw	195-292	Sharma et al. 1988; El-Shinnawi et al. 1989; Dinuccio et al. 2010; Lei et al. 2010
Rhubarb	320-490	Lehtomaki, 2006
Rye	250-295	Koçar and Civas, 2013
Ryegrass	390-410	Koçar and Civas, 2013
Slaughter waste	142-327	Cu et al. 2015
Sorghum	295-372	Koçar and Civas, 2013
Straw	242-324	Koçar and Civas, 2013
Sudan grass	213-303	Koçar and Civas, 2013
Sugar beet	236-450	Koçar and Civas, 2013

Sugarcane	230-300	Chynoweth et al. 1993
Sunflower	154-400	Koçar and Civas, 2013
Tall fescue	332-340	Seppälä et al. 2009
Timothy	333-385	Seppälä et al. 2009; Lehtomaki, 2006
Triticale	337-555	Koçar and Civas, 2013
Turnip	314	Koçar and Civas, 2013
Vetch	323	Zauner and Kuntzel, 1986
Wheat straw	140-333	Sharma et al. 1988; Hashimoto, 1989; Tong et al. 1990; Bauer et al. 2010
Willow	130-370	Turick et al. 1991; Chynoweth et al. 1993

1.6. CONCLUSION

From the literature study the necessity of a pretreatment in order to degrade lignin becomes clear. A major drawback of traditional pretreatments is the result of a release of various inhibiting compounds during degradation. Biological pretreatments are known to produce less unfavorable by products. However as of now knowledge on the impact of biological pretreatments on lignin degradation, release of inhibitors such as phenolic compounds, and the effect on BMP is limited. Therefore several experiments were conducted and will be discussed further in the following chapters.

CHAPTER 2: BIOMETHANE POTENTIAL OF VARIOUS LIGNOCELLULOSIC SUBSTRATES, AND THEIR RELEASE OF PHENOLIC COMPOUNDS

Redrafted from

Schroyen, M. Vervaeren H., Vandepitte H., Van Hulle S.W.H., Raes K., 2015. Effect of enzymatic pretreatment of various lignocellulosic substrates on production of phenolic compounds and biomethane potential. Bioresource Technology 192, 696-702.

2. BIOMETHANE POTENTIAL OF VARIOUS LIGNOCELLULOSIC SUBSTRATES, AND THEIR RELEASE OF PHENOLIC COMPOUNDS

2.1. ABSTRACT

Pretreatment of lignocellulosic biomass is necessary to enhance the hydrolysis, which is the rate-limiting step in biogas production. Laccase and versatile peroxidase are enzymes known to degrade lignin. Therefore, the impact of enzymatic pretreatment was studied on a variety of biomass. A significantly higher release in total phenolic compounds (TPC) was observed, never reaching the inhibiting values for anaerobic digestion. The initial concentration of TPC was higher in the substrates containing more lignin, miscanthus and willow.

The anaerobic digestion of these two substrates resulted in a significant lower biomethane production (68.8 - 141.7 Nl/kg VS). Other substrates, corn stover, flax, wheat straw and hemp reached higher BMP values, between 241 to 288 Nl/kg VS. Ensilaged maize reached 449 Nl/kg VS, due to the ensilation process, which can be seen as a biological and acid pretreatment. A significant relation ($R^2 = 0.89$) was found between lignin content and BMP.

2.2. INTRODUCTION

Alternative green energy can form a solution for global problems such as climate change and the diminishing amount of available fossil fuels (Divya et al. 2015). Biogas production via anaerobic digestion of agricultural waste streams is a cheap alternative energy source. These agricultural waste streams can be lignocellulose-rich materials which contain the polymers cellulose, hemicellulose and lignin (Theuretzbacher et al. 2015). The ratio of these polymers differs between different types of substrate. Lignin is a major natural source of phenolic compounds, containing variously linked phenylpropane units, such as H-,G- and S-units (Jung et al. 2015). Phenolic compounds are desired to produce chemicals with aromatics and their derivatives after catalytic pyrolysis of lignin (Ma et al. 2012).

A higher lignin content increases the release of phenolic compounds during degradation and decreases the availability of cellulose. Examples of lignocellulosic biomass are corn stover, wheat

straw, flax, hemp, miscanthus and willow. Table 2.1 presents the compositions of such lignocellulosic biomass according to recent literature. As lignin is the most recalcitrant polymer and as it forms a barrier around the cellulose and hemicellulose, lower BMP of these substrates are reported, as shown in Table 2.1. Although a relation between lignin content and BMP is expected, the determination of lignin concentration together with BMP values for this range of agricultural residues has not yet been described under the same experimental conditions, i.e. in one study. Moreover the large range of lignin concentration reported for the same substrate in different studies vary with the selected parts of the plant or time of harvest, thus complicating comparisons between studies, if both lignin and BMP are not characterized on the same sample.

In the biogas production process from such lignocellulosic biomass four steps occur: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The hydrolysis is the rate limiting step in which the lignin barrier is broken down (Appels et al. 2008). Known methods for improving the hydrolysis step are acidic, mechanical, thermal and biological pretreatments (Pilli et al. 2014; Li et al. 2015). Acidic, mechanical and thermal pretreatments have been shown effective on lignin degradation, however these techniques are more costly and result in a higher production of inhibitory compounds such as *p*-coumaric acid and 4-hydroxybenzoic acid (Wang et al. 2012; Kratky et al. 2015). Biological pretreatments using white rot fungi to breakdown lignin by producing enzymes such as laccase or versatile peroxidase can be a cheap alternative to improve the hydrolysis step, and prohibit the formation of high concentrations of inhibiting compounds (Dong et al. 2013). Some studies even have shown a decrease of phenolic compounds due to the use of laccase (Jönson et al. 1998; Ramirez et al. 2014; Schroyen et al. 2014).

Table 2.1: BMP and the composition of cellulose, hemicellulose and lignin of the selected substrates according to recent literature and as determined in this study (measured values).

Substrate	Measured values in this study		Literature values				References
	Lignin (g/100g)	BMP (Nl/kg VS)	Cellulose (g/100g)	Hemicellulose (g/100g)	Lignin (g/100g)	BMP (Nl/kg VS)*	
Corn stover	4.5	192 - 288	32.6 - 40.5	13.7 - 31.2	5.0 - 25.9	317 - 363	Raposa et al. 2011*; Schilling et al. 2012°; Tuyen et al. 2013°
Wheat straw	6.0	200 - 251	35.1 - 39.2	25.6 - 26.1	7.5 - 15	227 - 333	Raposa et al. 2011*; Chandra et al. 2012°; Krishania et al. 2012°
Flax	8.6	207 - 244	33.3	21.9	26.0	212	Ross and Mazzu, 2010°; Koçar et al. 2013*
Hemp	9.2	184 - 248	62.6	17.2	9.8	355 - 409	Amaducci et al. 2000°; Koçar et al. 2013*
Miscanthus	12.0	129 - 142	25.8 - 48.5	19.1 - 27.0	20.5 - 30.0	179 - 218	Xu et al. 2012°; Koçar et al. 2013*; Saleh et al. 2013°
Willow	17.0	69 - 97	37.3	17.9	25.3	130 - 370	Raposa et al. 2011*; Mante et al. 2014°

* = study determining the BMP value, ° = study determining C/H/L content.

In this chapter the effect of an enzymatic pretreatment, being a combination of laccase and versatile peroxidase, on different plant biomass with different lignin concentrations for BMP values is investigated. Doing so, a relation between lignin content and BMP is determined. Moreover, the release of inhibitors like total phenolic compounds as well as several individual phenolic compounds is determined.

2.3. MATERIALS AND METHODS

2.3.1. Substrates

Substrates were chosen based on their lignin content as reported in literature (Table 2.1). Hemp and flax were obtained from InAgro vzw (Roeselare, Belgium). Miscanthus and willow were acquired from the Institute for Agricultural and Fisheries Research (Merelbeke, Belgium), while ensilaged maize, corn stover and wheat straw were collected from a local farm (Den Hoef, Maaseik, Belgium). The lignin values were determined according to Van Soest et al. (1991).

2.3.2. Chemicals

o-Coumaric acid, *p*-coumaric acid, ferulic acid, gallic acid, vanillin, syringic acid, sinapic acid, vanillic acid, hydroxymethylfurfural (HMF), dinitrosalicylic acid, sodium chloride (NaCl), sodium malonate ($C_3H_2O_4Na_2$), hydrogen peroxide (H_2O_2), *4*-hydroxybenzoic acid ($C_7H_6O_3$), potassium dichromate ($K_2Cr_2O_7$), potassium hydrogen phthalate ($C_8H_5KO_4$) and laccase enzyme were obtained from Sigma-Aldrich (Bornem, Belgium) while versatile peroxidase was attained from Jena Bioscience (Jena, Germany). Silver sulfate (Ag_2SO_4), hydroxylamine hydrochloride (H_4ClNO), sulfuric acid (H_2SO_4), furfural and Folin-Ciocalteu's phenol reagent were acquired from ChemLab (Zedelgem, Belgium). Tween 80 was purchased from Acros Organics (New Jersey, USA), sodium carbonate (Na_2CO_3) and mercury(II) sulfate ($HgSO_4$) were obtained from Merck (Darmstadt, Germany) and *1*-hydroxybenzotriazole was purchased from Janssen Pharmaceuticals (Beerse, Belgium). HPLC-grade methanol (MeOH), HPLC-graded water, acetic acid, glucose, potassium sodium tartrate ($KNaC_4H_4O_6{\cdot}_4H_2O$) were purchased from VWR (Leuven, Belgium). All chemicals were used as provided.

2.3.3. Enzymatic pretreatment

The substrates were cut in fragments of ± 0.5 cm to achieve the average size of ensilaged maize. An acetate buffer (0.1 M, pH = 4.5) was used to submerge 9 g of the substrate, the enzymes and the accompanying additives in a total of 180 ml. The enzymes used were laccase derived from *Trametes versicolor* (2 U/g substrate; 1 U is defined as the release of 1 µmol catechol/min at pH 6 and 25 °C), and versatile peroxidase from *Bjerkandera adusta* (1.5 U/g substrate; 1 U is defined as the release of 1 µmol Mn(II)/min at pH 4.5 and 25°C). Additives were added to increase enzyme activity. Per gram biomass, 4 mg 1-hydroxbenzotriazole, 58 mg sodium-tartrate, 148 mg sodium-malonate, 156 µl H_2O_2 30% (w/w) in H_2O and 111 µl Tween 80 was added (Frigon et al. 2012, Schroyen et al. 2014). A control treatment, without the enzymes and additives, was included. An incubation at 30 °C for 0 h, 6 h and 24 h was performed for the control flasks, while an incubation at 30 °C for 6 h and 24 h was setup for the flasks containing the enzymatic pretreatment. During the incubation the flasks were continuously shaken at 60 rpm. After the incubation periods, the substrates were filtered at room temperature using a filter paper (VWR, Leuven, Belgium) with pore sizes of 5 to 13 µm. The solid residue (further denoted as solid fraction) was kept to analyze total suspended solids (TSS) and volatile suspended solids (VSS), to extract phenolic compounds and to determine the biomethane potential (BMP). The filtrate (further denoted as liquid fraction) was used to analyze the phenolic compounds, biolochemical oxygen demand (BOD) and chemical oxygen demand (COD). For each treatment, three independent replicates were performed. To measure an increase in parameters during time of incubation, the results from the control flasks at the start of the incubation are subtracted from the results of the other samples. Extra control flasks with only enzymes and additives, or only buffer solution were made to correct for their background from the enzymatic pretreated samples in order to compensate the interferences of the additives.

2.3.4. Analysis

TSS and VSS were determined on both solid and liquid fraction, by weighing difference with an accuracy of 0.001 g after 24 h at 105 °C, followed by 30 min at 550 °C for TSS and VSS respectively, according to Standard Methods (APHA, 2005).

To determine the phenolic content and profile of the solid fraction, 5 g of the solid fraction was extracted by adding 15 ml 100 % methanol and vortexed using an Ultraturrax IKA T18 Basic (30 s). The mixture was put 10 min on ice and centrifuged at 2540 x *g* for 10 min to separate supernatants from the pellet. Extraction of the pellet was repeated with 10 ml 80 % methanol, placed on ice and centrifuged again. After pooling the supernatants of both extractions, these were filtrated using filter paper with pore sizes 5-13 µm (VWR; Leuven, Belgium) and diluted to a final volume of 25 ml with methanol. Total phenolic compounds were determined on both the solid fraction and the liquid fraction using the Folin-Ciocalteu method, as described by Singleton et al. (1999). The total concentration of phenolic compounds was calculated based on a standard curve with gallic acid and expressed as mg gallic acid equivalent (GAE)/g solid in the solid fraction and mg GAE/l liquid in the liquid fraction.

The methanolic extract from the solid fraction and the centrifuged liquid fraction were analyzed using an Agilent 1100 high pressure liquid chromatography (HPLC) with a diode array detector (DAD) to determine individual phenolic compounds and furan derivatives. Separation was carried out using an Alltima^{TM} C18 5u column (150 mm x 4.6 mm; GRACE, Deerfield, USA). As mobile phase solvent A, HPLC-grade H_2O: acetic acid (99:1; v/v) and solvent B HPLC-grade methanol: acetic acid (99:1; v/v) were used. The elution program was as follows: 0–5 min, 90 % solvent A isocratic; 5–30 min, 50 % solvent A linear; 30–38 min, 50 % solvent A isocratic; 38–40 min, 90 % solvent A linear. The flow rate was 1.0 ml/min. *O*-Coumaric acid was added as internal standard. Quantification of the individual phenolic compounds was done based on an external standard curve for each compound.

The release of sugars from the matrix into the filtrate during incubation was followed by measuring the reducing sugar content with a spectrophotometric DNS method at 550 nm as described by Miller (1959). A standard curve was obtained using glucose.

Biochemical oxygen demand (BOD) was determined via BOD-5 method, by measuring the difference in O_2-content in the liquid at the start and after 5 days in a closed Winkler bottle at 20 °C, carried out according to Standard Methods (APHA, 2005). The O_2-content was measured using an LDO-probe, Impro®6860i, from Mettler-Toledo with iSenseLight software.

Chemical oxygen demand (COD) was determined using a spectrophotometric analysis (APHA, 2005). One ml of diluted sample was added to 0.5 ml potassium dichromate (0.25 N), 1.5 ml sulfuric acid – silver sulphate mixture and $HgSO_4$. After incubating 2 h at 148°C the absorbance was measured at 600 nm to determine the amount of Cr^{3+}.

The solid fraction was stored under vacuum at -20 °C for 60 days before the bio methane potential (BMP) assay was executed, using 1.5-2 g dry matter of biomass and 50-60 g of inoculum to keep a substrate to inoculum ratio of 0.5 (g VS/g VS). The inoculum was taken from a co-digestion plant treating cow manure and maize silage. The inoculating sludge was filtered, defermented and stored at 4 °C before use. The solid fraction and inoculum were put into air-tight batch reactors (250 ml) and incubated at a mesophilic temperature of 37°C. In order to measure the biogas production a water displacement system was used and the biogas production was measured daily. Room temperature and the atmospheric pressure have an influence on the volume of biogas obtained. In order to avoid errors the measured temperature and atmospheric pressure were converted to standard conditions. In a first step the pressure of the biogas (Pb) should be calculated by subtracting the vapor pressure of liquid (Pw) and the static pressure (Ps) due to the difference of the level of the liquid from the pressure of the collected gas (P).

$$P_b = P - P_w - P_s \qquad (1)$$

In order to calculate the vapor pressure of the liquid a modified equation of the empirical Arden Buck Equation is used:

$$P_w = 6.1121\, e^{\left(\left(18.678 - \frac{T_c}{234.5}\right) * \frac{T_c}{257.14 + T_c}\right)} \qquad (2)$$

While the static pressure is calculated using the difference of height between the initial level of the liquid (b1) and the bottom of the column and the height between the measured level (b2) and the surface of the barrier solution in the basin (Figure 2).

$$P_s = \rho * g * (b_1 - b_2) \qquad (3)$$

To calculate the volume (V_0) of biogas produced under standard conditions the combined gas law is used.

$$V_0 = V * \frac{T_0}{T} * \frac{P_b}{P_0} \qquad (4)$$

Which will lead to following equation, where V0 is the volume of biogas produced under standard conditions. With A the surface area of the gas container (0.0019 m²); P the atmospheric pressure; Pw the saturated water vapor pressure, describing the relation between temperature and water vapor pressure. In order to obtain the volume in liters, the value is multiplied by 10^3.

$$V_0 = \frac{T_0}{T * P_0} \left(\left((P - P_w - \rho * g * b_1) * A * a_1 \right) - \left((P - P_w - \rho * g * b_2) * A * a_2 \right) \right) * 10^3 \qquad (5)$$

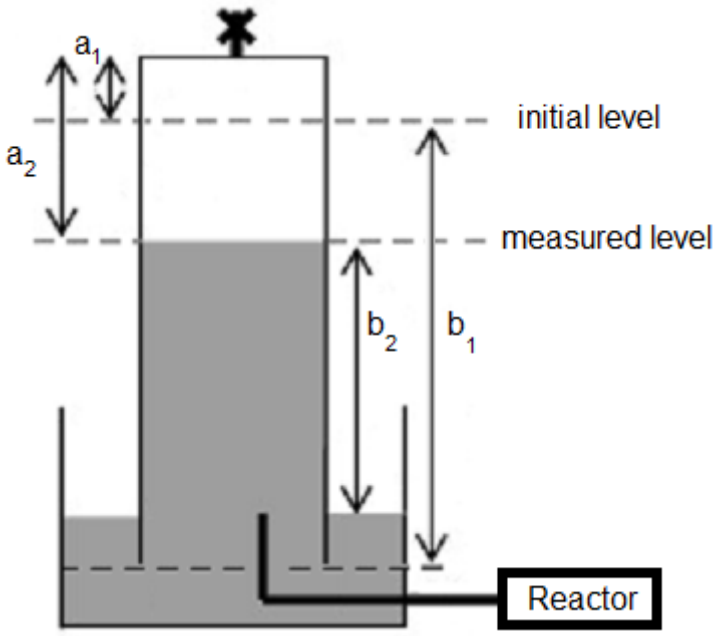

Figure 3.1: The water displacement system in order to measure the biogas production (adapted from Walker et al. 2009)

Compositional analysis of the biogas was done with the Agilent 6890 Series gas chromatograph. A gas sample of 10 µl was injected at 280 °C at the inlet, detector temperature was 60 °C, for 6 min at a flow of 46.6 ml/min under a pressure of 230 kPa, separation was carried out using an CP-PoraBOND Q (25 m x 0.53 mm, Agilent technologies, Santa Clara, USA) CH_4 and CO_2-ratios were analyzed using Chemstation software (Agilent technologies, Santa Clara, USA). The digestion process was stopped after 30 days (Gonzalez et al. 2013).

2.3.5. Statistical Analysis

For all statistical analysis IBM® SPSS® Software version 22 was used. A two-way ANOVA was executed on all data including the fixed effects of enzyme treatment and incubation time, as well as the 2-way interaction term. As the 2-way interaction term was not significant, only the main effects are reported. Comparison of mean was done using Tukey post hoc test ($p < 0.05$). Correlations were checked using a bivariate Pearson correlation.

The biogas production was measured daily for 30 days and was modeled as a function of time (t (d)) using a first-order model, $P = \gamma*(1-e^{(-\mu t)})$ (Bilgili et al. 2009). In this first-order model biogas production is assumed to have an exponential rise to a maximum biogas yield, which is given by γ (Nl/kg VS). The exponential rise is characterized by the specific production rate µ (d^{-1}). The model was fitted using Microsoft Excel's solver to minimize the sum of squares of differences between the model and the measured biogas production of three independent experiments. The model gives the opportunity to interpret and quantify the data as well as omit the effect of missing data points. For each substrate, the initial slope of the biogas production curve during the first 7 days of the experiment was calculated based on the calibrated model as a measure of the hydrolysis rate.

2.4. RESULTS AND DISCUSSION

The measured lignin contents of the different substrates used in this study are given in Table 2.1. Most of the lignin concentrations were lower than the ones reported in literature, however a good variation in lignin content between the different substrates was still achieved. The lignin content of

the ensilaged maize was very low (0.8 g/100 g) due to the ensilation process, which can be seen as a biological and prolonged acid pretreatment (Ambye-Jensen et al. 2013).

In Table 2.2, the results of total phenolic concentration (TPC) in liquid and solid phase, BOD, COD, TSS and VSS obtained after different incubation periods are shown, for both treatments with and without addition of the enzyme mix.

The duration of the incubation had no significant impact on the measured parameters (Table 2.2, Table 2.3). This indicates that the impact of enzymatic pretreatment happens in the first hours of incubation and levels off afterwards. The fast working mechanism of a combination of laccase and versatile peroxidase was also reported in other studies, where a significant removal of pharmaceuticals was already obtained after 5 hours (Touahar et al. 2014). The use of enzymes gives a significant difference in VSS (p = 0.041) of the solid fraction, total phenolic content (p < 0.001) and COD (p < 0.001) in the liquid fraction. The increase of VSS and phenolic content results from the breakdown of the matrix and the lignin barrier. These results show the importance of the choice of substrate as significant differences are found for all the different measured parameters.

Table 2.2: Experimental data of the different pretreatments on the selected substrates. C 6 and C24 are control samples with no enzymes incubated for respectively 6 and 24 hours, LP6 and LP24 are samples enzymatically pretreated for respectively 6 and 24 hours. The data shows the increase of the parameters, as the measurements of the substrates at the start of the incubation are subtracted from the original data (n=3)

Substrate	Pretreatment	Liquid fraction			Solid fraction		
		TPC (mg/l)	BOD (mg/l)	COD (mg/l O_2)	TPC (mg/g)	TSS (g/100g)	VSS (g/100g)
Corn stover	C6	36.3	746	9810	0.26	26.5	13.3
	LP6	106	1424	-814	0.20	25.0	13.1
	C24	47.7	1252	8566	0.13	12.9	9.0
	LP24	106	828	-703	0.16	34.4	13.4
Wheat straw	C6	77.7	1640	7289	0.24	20.9	20.3
	LP6	158	2342	387	0.19	21.1	20.2
	C24	92.7	1383	6756	0.24	17.1	16.7
	LP24	147	1495	3476	0.18	26.4	25.6
Flax	C6	11.9	1377	10089	0.04	26.8	25.7
	LP6	150	2763	2098	0.07	31.4	27.2
	C24	24.8	1578	8422	0.09	22.7	25.7
	LP24	65.6	820	2905	0.07	27.3	26.4
Hemp	C6	20.5	1550	9054	0.15	22.4	22.2
	LP6	112	1541	-1103	0.19	23.1	22.4
	C24	35.3	1633	9466	0.16	20.3	20.2
	LP24	110	2195	-3	0.16	20.6	20.2
Miscanthus	C6	52.0	1387	5533	0.22	25.2	24.9
	LP6	134	551	209	0.29	29.9	30.8
	C24	65.0	923	8378	0.18	21.0	20.9
	LP24	177	669	2587	0.28	25.5	24.9
Willow	C6	174	757	5622	0.47	34.9	34.6
	LP6	170	719	76	0.36	40.7	39.8
	C24	132	744	7244	0.40	32.0	32.0
	LP24	195	886	-902	0.29	36.7	36.1
Ensilaged maize	C6	48.7	913	8599	0.22	15.4	14.9
	LP6	81.3	1347	-2003	0.17	14.0	13.0
	C24	58.2	1215	8521	0.19	19.5	19.0
	LP24	82.0	1554	-637	0.19	18.3	17.4
Total average		95.4	1294	4247	0.21	24.7	22.5
Total standard deviation		62.4	815	5503	0.13	12.1	8.7
Coefficient of Variance		65.4	63.0	129.6	62.8	49.0	38.7

Table 2.3: Significance of the pretreatment, duration, selected substrate for the measured parameters in the solid and liquid fraction.

Parameter	p-value Pretreatment	p-value Duration	p-value Substrate	Substrate
Liquid fraction				
TPC	**<0.001**	0.948	**<0.001**	Willow^{A}; Wheat straw^{AB}; Miscanthus^{AC}; Hemp^{BCD}; Corn stover^{CD}; Flax^{D}, Ensilaged maize^{D}
BOD	0.41	0.446	**0.005**	Corn stover^{AB}, Miscanthus^{AB}, Willow^{AB}; Flax^{AC}, Wheat straw^{AC}; Ensilaged maize^{B}; Hemp^{C}
COD	**<0.001**	0.365	**0.002**	Corn stover^{A}, Hemp^{A}; Flax^{B}; Wheat straw^{AB}; Ensilaged maize^{AC}; Miscanthus^{ABC}, Willow^{ABC}
Solid fraction				
TPC	0.28	0.125	**0.002**	Corn stover^{A}, Miscanthus^{A}; Willow^{AB}; Wheat straw^{AC}; Hemp^{C}, Ensilaged maize^{C}; Flax^{ABC}
TSS	0.16	0.571	**<0.001**	Hemp^{A}; Flax^{B}, Wheat straw^{B}, Corn stover^{B}, Miscanthus^{B}, Willow^{B}, Ensilaged maize^{B}
VSS	**0.041**	0.319	**0.002**	Hemp^{A}, Willow^{A}; Flax^{B}, Wheat straw^{B}, Corn stover^{B}, Miscanthus^{B}, Ensilaged maize^{B}

^{ABCD} = different indices indicate significant differences in substrate ($p<0.05$).

2.4.1. The impact of lignin and enzymatic pretreatment on phenolic compounds

An increase of the TPC in the liquid fraction, as seen in Table 2.2 and 2.3, is a result of the enzymatic pretreatment, due to the destruction of the lignin barrier. Willow has the highest lignin content and provided a higher amount of TPC in the liquid phase. Overall the impact on release of phenolic compounds of the enzymatic pretreatment is higher than the initial lignin content. Figure 2.2 shows the average of total phenolic concentrations in the liquid phase are calculated for each substrate, both for the control and enzyme pretreated incubations after 0 h, and 6+24 h. The release of phenolic compounds caused by the buffer solution is clear, as seen in the non-enzymatic treated substrates. Yet a significant increase in the released phenolic concentration is observed if the substrate is enzymatically pretreated (Table 2.3). The pretreatment has a greater impact than the lignin concentration and seems to have less effect on willow. This could also be the result of an inhibition of the enzymes by the phenolic compounds leaking out of the more lignin concentrated willow (17 g/100 g) (Table 2.1).

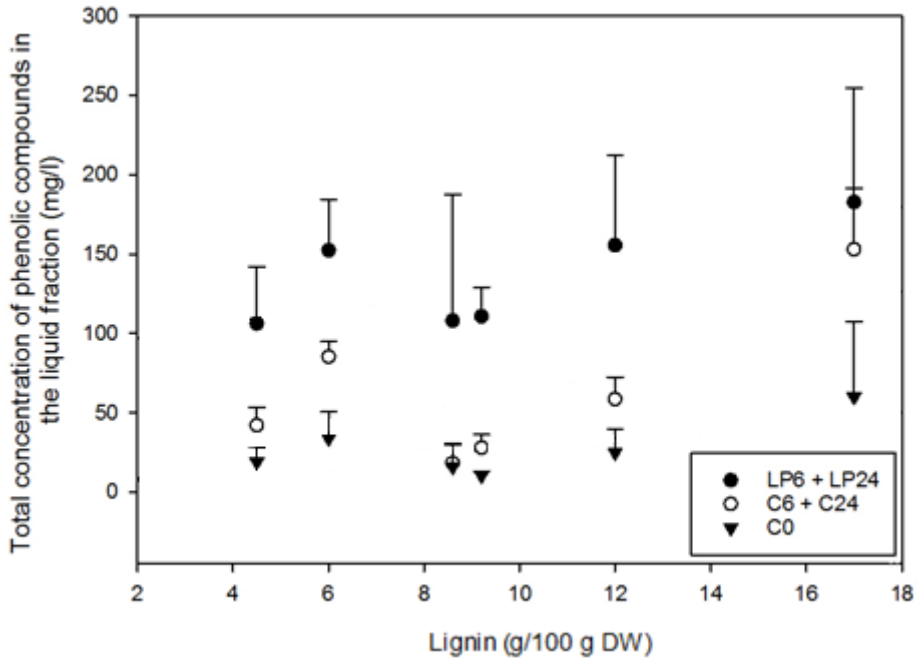

Figure 2.2: Total phenolic concentrations of the selected substrates and the different pretreatments, LP6 + LP24 = enzymatically pretreated samples; C6 + C24 = control samples incubated for 6 and 24 hours; C0 = control samples taken at the start of the incubation (n=3).

The individual phenolic compounds in the solid and liquid fraction after incubation of the different substrates with or without enzymes were analyzed. The values of the control samples at the start of the incubation are subtracted from the values obtained after 6 and 24 hours of incubation. Vanillic acid, 4-hydroxybenzoic acid and *p*-coumaric acid were the most dominant ones (Figure 2.3). Miscanthus showed a significantly higher release of *p*-coumaric acid compared to the other substrates (p = 0.003) (Table 2.4). No significant differences between the substrates were observed for vanillic acid and 4-hydroxybenzoic acid, neither in the solid, or in the liquid fraction (Table 2.4). Also no significant effect of the pretreatment nor of the duration of the incubation was observed on the release of the individual phenolic compounds (Table 2.4).

An increase of the individual phenolic compounds in the liquid fraction was observed, except for wheat straw. Degradation of the lignin barrier is seen in the decrease of phenolic compounds extracted in the solid phase from the miscanthus, willow and wheat straw matrix (Figure 2.3). This indicates that the matrix was substantially impacted and the phenolic compounds were leaked to the liquid fraction where an increase was established. In the liquid fraction of the willow samples a rise in gallic acid (0.6-1.2 mg/l) and decline in furfural (1-5.1 mg/l) was also noted. In the samples containing the wheat straw residue, an increase of ferulic acid (0.6-3.9 mg/l) was seen in the liquid phase, while it decreased (0.003-0.011 mg/g) in the solid phase compared to the initial value (0 h). In the other substrates i.e. ensilaged maize, flax and corn stover, no noteworthy concentration shifts were detected apart from an increase of vanillic acid (21.6 mg/l) and of 4-hydroxybenzoic acid (6.8 mg/l) in the liquid phase after 24 hours of enzymatic pretreatment of corn stover. In hemp an increase of *p*-coumaric acid can be the result from matrix breakdown making the compound more available for extraction. Although the reported inhibition levels of individual phenolic compounds (Mussatto and Roberto, 2004; Hernandez and Edyvean, 2008) are not reached by the enzymatic pretreatment (Figure 2.3), the higher lignin concentrations can cause problems during the anaerobic digestion due to the inhibiting compounds, acting as toxins, together with the remaining lignin seal around the cellulose structure (Quéméneur et al. 2012). Furthermore, increased phenolic compound

concentrations give an indication of what is happening to the substrate matrix. Enzymatic pretreatment led to a higher release of total phenolic compounds in corn stover, 36.3 mg/l to 106 mg/l, and miscanthus, 52 mg/l to 134 mg/l, after six hours of pretreatment, as well as a non-significantly higher release of vanillic acid and 4-hydroxybenzoic acid in hemp and wheat straw. The high variation in types of phenolic compounds released can be related to the different substrates used. Lignin is composed of H, G and S units, and it is known that in willow, a hardwood, there is less of H units present compared to crop plants (Pinto et al. 2012), resulting in a small amount of *p*-coumaric acid in the willow samples. Also Wang et al. (2012) reported this variation on a different set of substrates: only a negative exponential relation between lignin and ferulic acid was observed. In this study, ferulic acid was only found as an individual compound in wheat straw, containing 6 g/100 g of lignin.

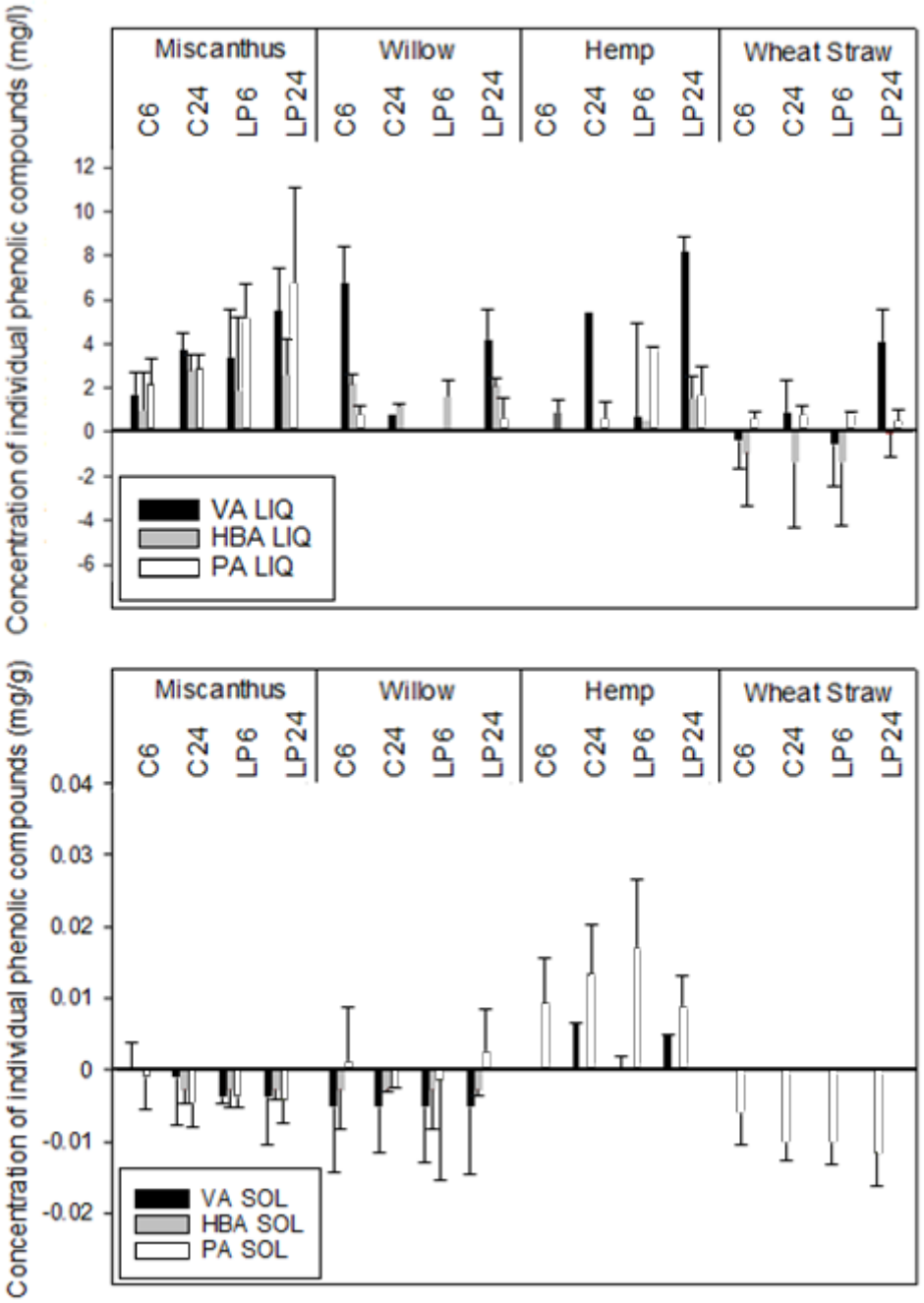

Figure 2.3: HPLC results of the common phenolic compounds in lignin degradation in miscanthus, willow, hemp and wheat straw. VA = vanillic acid; HBA = 4-hydroxybenzoic acid; PA = *p*-coumaric acid; LIQ = liquid fraction; SOL = solid fraction. (n=3). The measured HPLC values of the control at the start of the incubation are subtracted to show the decrease and increase of the individual phenolic compounds.

Table 2.4: Significance of the HPLC results of the more common phenolic compounds in the liquid and solid fraction.

Parameter	p-value Pretreatment	p-value Duration	p-value Substrate	Substrate
Liquid fraction				
Vanillic Acid	0.301	0.788	0.77	
4-Hydroxybenzoic Acid	0.217	0.252	0.32	
***p*-Coumaric Acid**	0.787	0.162	***0.003***	Miscanthus^{A}; Wheat straw^{B}, Corn stover^{B}, Hemp^{B}, Willow^{B}
Solid fraction				
Vanillic Acid	0.201	*0.632*	***0.036***	Corn Stover^{A}; Ensilaged Maize^{B}; Hemp^{AB}; Willow^{C}; Miscanthus ^{BD}; Flax^{ABD}, Straw^{ABD}
4-Hydroxybenzoic Acid	0.809	0.404	0.828	
***p*-Coumaric Acid**	0.615	0.762	***0.002***	Hemp^{A}; Corn Stover^{AB}; Ensilaged Maize^{ABC}; Miscanthus^{BC}, Willow^{BC}; Straw^{C}

^{ABCD} = different indices indicate significant differences in substrate ($p<0.05$).

2.4.2. The impact of lignin on BMP

The measured biomethane potential values after 30 days anaerobic digestion, as well as the results of the fit using a first-order model are shown in Table 2.5. The biomethane maximum value calculated by the model has a good relation with the biomethane production measured after 30 days, however some values of the miscanthus substrate are off due to the fact the digestion was not finished yet after 30 days, resulting in an overestimation of the final biomethane production. The initial slope of the first seven days was calculated as well. This results in the rate of the anaerobic digestion process during the first week. A difference between the substrates can be noted, showing a relation (R^2 = 0.75) with lignin concentration. Ensilaged maize has the fastest digestion rate as result of the acidic pretreatment. Miscanthus and willow are significantly slower due to the lignin concentration (Figure 2.4). This leads to a dual disadvantage for high lignin concentrated substrates where as they have a lower maximum biomethane production and need more time to obtain it, it requires a larger reactor. However more phenolic compounds can be extracted, which could be interesting for other industrial purposes, e.g. production of aromatic chemicals.

The average BMP values per substrate are shown in relation to the lignin concentration of the different substrates in Figure 2.5. The graph shows a clear trend (R^2 = 0.87), because the lignin content is a very important parameter in the biomethane production.

Dandikas et al. (2014) and Triolo et al. (2011) determined the biomethane potentials as well as the lignin concentration of a range of substrates with lower lignin concentrations, i.e. grasses, manure, barley, triticale etc. These results are combined with the data of the samples obtained in this work in Figure 2.5. The impact of the lignin concentration is very clear. This indicates that the choice of substrate as well as the pretreatment is very important when focusing on lignin degradation for increasing BMP.

Table 2.5: Values calculated by an exponential model and the measured biomethane values after 30 days of anaerobic digestion.

Substrate	Pretreatment	Initial slope (Nl/kg VS / d)	μ (d^{-1})	γ (Nl/kg VS)	Measured BMP (Nl/kg VS)
Corn stover	C0	15.4	0.114	210.6	207.0
	C6	14.3	0.115	193.2	191.7
	C24	19.4	0.081	328.3	288.4
	LP6	17.6	0.104	254.3	238.4
	LP24	17.6	0.124	228.6	223.6
Wheat straw	C0	16.0	0.077	281.2	248.2
	C6	15.4	0.089	244.0	223.0
	C24	16.3	0.095	249.5	234.3
	LP6	15.6	0.071	292.0	250.5
	LP24	12.0	0.062	244.8	199.6
Flax	C0	21.1	0.214	219.8	214.9
	C6	20.8	0.225	214.5	207.0
	C24	20.5	0.205	215.8	214.9
	LP6	20.6	0.207	216.6	220.4
	LP24	23.1	0.200	245.3	244.1
Hemp	C0	18.2	0.093	280.7	248.0
	C6	12.9	0.092	200.8	184.1
	C24	17.2	0.100	253.0	226.0
	LP6	17.8	0.097	268.5	241.0
	LP24	18.4	0.100	271.0	237.5
Miscanthus	C0	6.5	0.035	212.8	135.7
	C6	7.0	0.045	184.8	139.0
	C24	6.9	0.054	160.0	129.4
	LP6	5.4	0.015	370.4	138.1
	LP24	6.3	0.024	285.0	141.7
Willow	C0	4.7	0.084	77.7	73.6
	C6	5.4	0.093	83.0	82.7
	C24	4.4	0.087	70.7	68.8
	LP6	5.2	0.074	94.0	87.0
	LP24	5.4	0.059	116.4	97.2
Ensilaged maize	C0	32.3	0.296	322.6	333.3
	C6	37.0	0.239	377.0	393.3
	C24	43.1	0.346	433.8	448.7
	LP6	34.4	0.293	343.6	354.8
	LP24	24.9	0.257	251.3	259.5

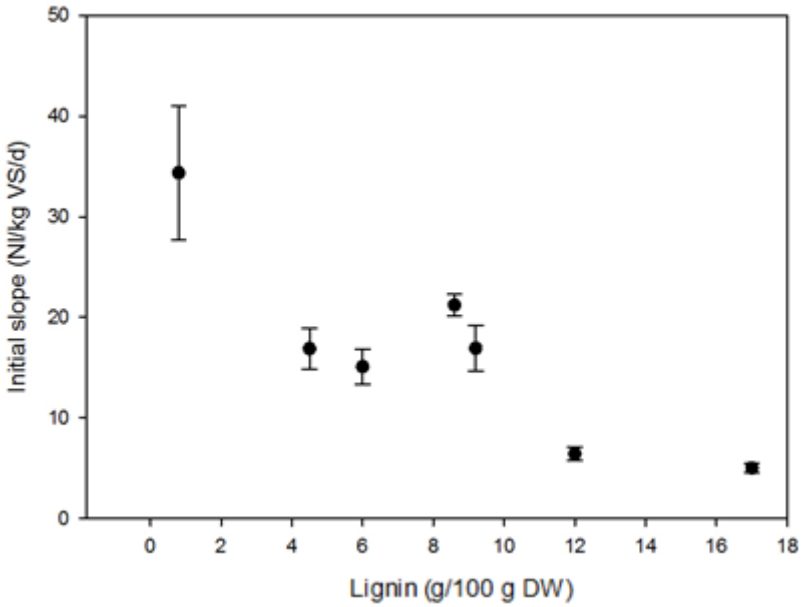

Figure 2.4: Relation between lignin concentration and the initial slope of the biogas curve after 7 days of anaerobic digestion.

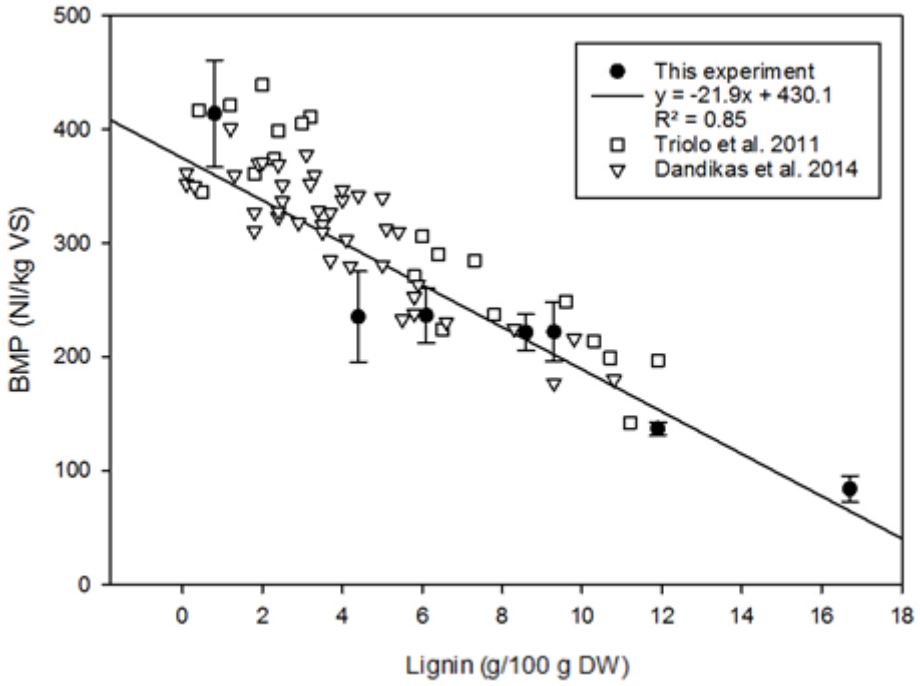

Figure 2.5: Biomethane potential values of recent studies compared to experimental results from this study in relation with the lignin values of the selected substrates. (Dandikas et al. 2014; Triolo et al. 2011).

2.5. CONCLUSION

Lignin concentration has a great impact on BMP, released content and type of inhibiting phenolic compounds. The results show that it is of utmost importance to break down the lignin barrier and diminish the lignin concentration to improve BMP and production rate. Enzymatic pretreatment can help to degrade the matrix, as observed by the significant increase of phenolic compounds leaking out of the matrix. The pretreatment should be optimized to have a greater impact on lignin degradation and BMP. The substrate is an important factor to take into account as it shows a different pattern of individual phenolic compounds released. Corn stover has a low concentration of lignin and is a very common cultivated in flanders. In chapter 3 different enzymatic pretreatments with laccase and peroxidases of corn stover will be investigated.

CHAPTER 3: IMPACT OF AN ENZYMATIC PRETREATMENT ON CORN STOVER DEGRADATION AND BIOGAS PRODUCTION

Redrafted from

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3. IMPACT OF AN ENZYMATIC PRETREATMENT ON CORN STOVER DEGRADATION AND BIOGAS PRODUCTION

3.1. ABSTRACT

Corn stover is an agricultural residue consisting of lignocellulose, cellulose and hemicellulose polymers, sheeted in a lignin barrier. Corn stover can be used as feedstock for biogas production. Previous studies have shown biological pretreatment of lignocellulose materials can increase digestibility of the substrate improving hydrolysis, the rate-limiting step in biogas production.

The impact of pretreating with different enzymes (laccase, manganese peroxidase and versatile peroxidase) and different incubation times, (0, 6 and 24 hours) was studied. The effect on the matrix and biomethane production was determined. Pretreatments did not yield high concentrations of phenolic compounds, inhibitors of biogas production. The laccase enzyme showed an increase in biomethane production of 25 % after 24 hours of incubation. Pretreatment with peroxidase enzymes increased biomethane production with 17 % after 6 hours of incubation. As such it can be concluded that by introducing the different enzymes at different stages during pretreatment an increased biomethane production can be obtained.

3.2. INTRODUCTION

In 2008, 17 % of the consumed renewable energy in Europe was originating from biomass (Eurostat, 2010). In 2012, about 13 % of the generated green electricity in Flanders, the northern part of Belgium, was acquired from biomass (VREG, 2013). This biomass can be obtained as byproduct from forestry industry and agriculture.

In Flanders, the most common agricultural crop is corn. It is planted on over 190000 ha, 47 % of the land used for agricultural crops, excluding grasslands (Danckaert, 2013). Corn stover is a lignocellulosic waste product from this corn production, present in great abundance. In general, it contains 33-40 % cellulose, 13-31 % hemicellulose and 5-26 % lignin (Krishania et al., 2012; Schilling et al., 2012; Tuyen et al., 2013; Wan and Li, 2010).

To transform biomass into energy without excessive energy losses, dry biomass is directly incinerated while semi-dry or wet biomass is converted into biogas. Biogas production is a four-steps anaerobic process: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Appels et al., 2008). The first step (hydrolysis) is considered as a rate-limiting process step and much research efforts have been done to improve this step by different biomass pretreatments (Wang et al., 1999; Tiehm et al., 2001; Mosier et al., 2005).

Pretreatment techniques aim at reducing the cellulose crystallinity and promote the removal or disruption of the lignin barrier. As such, higher accessibility to the cellulose polymers is obtained which results in an increased bio-energy production (Li et al., 2010). Mechanical and chemical pretreatments have proven to be effective, though several disadvantages are associated with it. Mechanical processes such as milling, grinding or the use of irradiation require high energy input and are expensive. Alkali or acidic pretreatments are mostly done under harsh conditions, using extreme pH and high temperature, and are rather expensive pretreatments (Chandra et al., 2012). The extreme conditions have a large impact on hemicellulose degradation, resulting in the formation of inhibiting compounds, such as furfural and 5-hydroxy-2-methyl furfural (HMF) (Karp et al., 2013).

Biological pretreatments using white rot fungi are performed under mild environmental conditions, with low energy input and low chemical requirements (Chandra et al., 2012; Cesaro et al. 2014). These white rot fungi have the unique ability to delignify lignocellulosic biomass producing specialized enzymes i.e. laccase, lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP) (Kuhad et al., 1997). Additionally, some studies demonstrate that treatment with white rot fungi reduces the amount of phenolic compounds. Phenolic compounds and furan derivatives, like furfural and HMF, are released due to lignin and hemicellulose degradation and are proven to have an inhibitory effect on the biogas production (Palmqvist et al., 2000b). Biogas production can be reduced with 20 % by 25 mg phenolic compounds/g volatile suspended solids (VSS) (Hernandez and Edyvean, 2008). The impact of biological pretreatment on degradation of

lignocellulose is widely researched (Sanchez, 2009), though the subsequent impact on biogas production is rarely studied (Frigon et al., 2012).

Therefore, in this paper the effect of biological pretreatments with pure enzymes derived from white rot fungi on the resulting biogas production was investigated in detail. The impact of different enzymes and different pretreatment durations on a range of parameters, such as total soluble solids, volatile soluble solids, phenolic compounds, non-reducing sugars, BOD, COD and biogas production was investigated. This study should provide a better understanding of the effect of enzymatic pretreatment on the release of potential inhibitory compounds and on biogas production of corn stover, chosen from a set of substrates investigated in chapter 2.

3.3. MATERIAL AND METHODS

3.3.1. Chemicals

HPLC-grade methanol (MeOH), HPLC-graded water, acetic acid, glucose, potassium sodium tartrate ($KNaC_4H_4O_6{\cdot}4H_2O$) were obtained from VWR (Leuven, Belgium). Chemical standards from *o*-coumaric acid, *p*-coumaric acid, ferulic acid, gallic acid, vanillin, syringic acid, sinapic acid, vanillic acid, hydroxymethylfurfural (HMF), dinitrosalicylic acid, sodium chloride (NaCl), sodium malonate ($C_3H_2O_4Na_2$), hydrogen peroxide (H_2O_2), *4*-hydroxybenzoic ($C_7H_6O_3$) acid, potassium dichromate ($K_2Cr_2O_7$), potassium hydrogen phthalate ($C_8H_5KO_4$) and the laccase enzyme were purchased from Sigma-Aldrich (Bornem, Belgium) while manganese peroxidase and versatile peroxidase were purchased from Jena Bioscience (Jena, Germany). Tween 80 was obtained from Acros Organics (New Jersey, USA), silver sulfate (Ag_2SO_4), hydroxylamine hydrochloride (H_4ClNO), sulfuric acid (H_2SO_4), furfural and Folin-Ciocalteu's phenol reagent were purchased from ChemLab (Zedelgem, Belgium), manganese chloride ($MnCl_2$), sodium carbonate (Na_2CO_3) and mercury(II)sulfate ($HgSO_4$) were purchased from Merck (Darmstadt, Germany) and *1*- hydroxybenzotriazole was obtained from Janssen Pharmaceuticals (Beerse, Belgium).

3.3.2. Enzymatic pretreatment

Corn stover obtained from InAgro vzw (www.inagro.be, Roeselare, Belgium), was cut into pieces of ± 0.5 cm². After placing 9 g of corn stover in 140 ml acetate buffer (0.1 M, pH = 4.5), the enzymes and additives were dissolved in 40 ml buffer solution and added to the biomass, as described by Frigon et al. (2012) (Table 3.1). The enzymes used were laccase (LA, 2 U/g biomass; 1 U is defined as the release of 1 μmol Catechol/min at pH 6 and 25 °C), peroxidases as a combination of manganese peroxidase (MnP, 5 U/g biomass; 1 U is defined as the release of 1 μmol Mn(II)/min at pH 4.5 and 25°C) and versatile peroxidase (VP, 1.5 U/g biomass; 1 U is defined as the release of 1 μmol Mn(II)/min at pH 4.5 and 25°C), or the combination (COMB) of the 3 enzymes. A control treatment, without enzymes and additives, was also included. All flasks were incubated at 30 °C under continuous shaking for 0 h, 6 h, 12 h and 24 h. After incubation, the flasks were taken to room temperature, and the mixture was immediately filtrated using a filter paper (VWR; Leuven, Belgium) with pore size between 5 and 13 μm. The solid residue (further denoted as solid fraction) was used for the determination of total suspended solids (TSS) and volatile suspended solids (VSS), for determining the phenolic compounds and for the biomethane potential (after vacuum storage at -20 °C for 60 days) as described in chapter 2. The filtrate (further denoted as liquid fraction) was used for TSS, VSS, phenolic compounds, sugar content, biochemical oxygen demand (BOD) and chemical oxygen demand (COD) as described in chapter 2. For each treatment, three independent replicates were performed.

Table 3.1: Additives added for the different enzyme pretreatments

Pretreatment	Additive	Quantity per g biomass
LA	1-Hydroxybenzotriazole	4 mg
MnP + VP	Sodium-Tartrate	58 mg
	H_2O_2 30% (w/w) in H_2O	156 μl
	Sodium-Malonate	148 mg
	$MnCl_2$	5 mg
	Tween 80	111 μl

LA = Laccase, MnP+VP = Manganese peroxidase and Versatile peroxidase

3.3.3. Statistical Analysis

For all statistical analysis IBM® SPSS® Software version 22 was used. A two-way ANOVA was executed on all data including the fixed effects of enzyme treatment and incubation time, as well as the 2-way interaction term. Comparison of mean was done using Tukey post hoc test ($p < 0.05$). Correlations were checked using a bivariate Pearson correlation.

The biogas production was measured daily for 30 days and was modeled as a function of the time (t (d)) using a first-order model, $P = \gamma*(1-e^{(-\mu t)})$ as described in chapter 2.

3.4. RESULTS AND DISCUSSION

3.4.1. Impact of enzymatic pretreatment on the liquid fraction

In Table 3.2 the measured values of different parameters in the liquid fraction after different incubation times are presented. The amount of reducing sugars is not shown as for all samples the values were below 0.3 mg/l. This is because sugars, when released in a polymerized form, are not measured by the DNS-method (Miller, 1959). Further treatment with cellulases or cellulase producing fungi could give a better idea of a possible increase in cellulose availability.

Pretreatments including the peroxidase enzymes, VP and MnP, have a greater impact than laccase treatment (Table 3.2) as higher values of pH, TSS, BOD and COD were measured, as well as an increase in release of phenolic compounds was noted.

A two-way ANOVA, as described in the materials and methods, was executed on the data presented in Table 3.2, after subtracting the corresponding value at the beginning of the incubation (0 h) for each individual experiment, e.g.: the values used for TSS of the control treatment at 6 h, 12 h and 24 h were 0.07, 0.10 and 0.15 respectively (Table 3.4). This subtraction was necessary to take into account the interfering effect of the buffer and the additives used in the enzymatic pretreatments in the different analysis. From the raw data from Table 3.2 and the statistical analysis of Table 3.4, it can be seen that the amount of TSS is significantly greater for samples treated with peroxidase enzymes or the combination of enzymes than the laccase treated samples ($p < 0.05$). Therefore, these results could suggest that the corn stover matrix is broken down by attack of the lignin concealment,

releasing small solid fractions into the liquid. This observation is confirmed as the total amount of phenolic compounds transferred to the liquid fraction, had the highest values for VP+MnP and COMB treatment (Table 3.2).

Samples treated with the peroxidase enzymes or the combination of enzymes showed a greater release of phenolic compounds than the laccase treated samples as phenolic compounds are a main degradation product of the lignin barrier (Wu et al. 2013; Mood et al. 2013). Laccase however has been known to induce an oxidation reaction, resulting in unstable, reactive phenoxy radicals which can lead to a polymerization reaction (Ahmad et al. 2010; Ibrahim et al. 2011). This lowers the total phenolic compounds as measured by the Folin-Ciocalteu method, and explains why the concentration of phenolic compounds broken down from the matrix is lower than for the control samples. However only the samples treated with laccase resulted in detectable concentrations of vanillic acid (Table 3.2). Other individual phenolic compounds were not released in the liquid phase in detectable amounts. Which might indicate vanillic acid is less prone to polymerization by the laccase enzyme. Effects of enzymatic pretreatment are also observed in the obtained BOD and COD values (Table 3.2). The increase in BOD after 6 hours of incubation is significantly higher in the control samples compared to enzymatic treated samples, while effects between the different enzymatic pretreatments are minor. On the other hand, COD measurements show a significant impact of treatments with the peroxidase enzymes, alone and in combination with the laccase enzyme due to the increased release of components like phenolic compounds, broken down from the biomass matrix.

Table 3.2: Parameter data of the enzymatic treatment over different incubation times for the liquid fraction (n = 3)

Pretreatment	Duration (hours)	Final pH	TSS (g/100g)	VSS (g/100g)	Total phenolic compounds(mg/l)	Vanillic acid (mg/l)	BOD (mg/l)	COD (mg/l O_2)
Control	0	4.75	0.55	99.7	11.0	BD	150	2700
	6	4.77	0.62	99.4	30.3	BD	494	3389
	12	4.90	0.65	99.8	36.3	BD	657	1944
	24	5.22	0.70	99.5	41.5	BD	605	4056
LA	0	4.73	0.92	99.7	10.4	3.68	343	4633
	6	4.78	0.65	99.5	20.0	6.68	390	2944
	12	5.06	0.59	99.8	28.6	3.98	449	5989
	24	5.22	0.80	99.5	35.7	5.09	486	7367
MnP + VP	0	5.02	1.53	99.4	31.9	BD	532	9611
	6	5.01	1.92	99.1	53.8	BD	614	14367
	12	5.14	1.78	99.4	54.4	BD	663	14389
	24	5.33	2.24	99.2	69.6	BD	593	13922
COMB	0	5.02	1.59	99.4	29.3	4.43	480	9278
	6	5.03	1.90	99.2	56.8	7.41	462	13011
	12	5.16	1.88	99.4	53.3	5.39	764	14900
	24	5.36	2.13	99.1	66.0	6.82	477	14033
Overal mean		5.09	1.28	99.5	39.3	5.44	510	8533
Overal standard deviation		0.20	0.67	0.31	18.99	2.36	226.5	5094
Coefficient of Variance		3.93	52.3	0.31	48.3	43.4	44.4	59.7

BD = below detection limit, LA = Laccase, MnP+VP = Manganese peroxidase and Versatile peroxidase, COMB = Laccase, Manganese peroxidase and Versatile peroxidase

3.4.2. Impact of enzymatic pretreatment on the solid fraction

In Table 3.3 the measured values of different parameters in the solid fraction of the corn stover matrix after different incubation times are presented. Similar to the parameters measured in the liquid phase, the corresponding value from the start of the incubation (0 h) was substracted from the other time points per pretreatment and per experiment. On these values, a two-way ANOVA was executed (table 3.4). Although significant differences by pretreatments were noticed for several parameters in the liquid fraction when treated with peroxidases, no significant differences in pretreatments were found in the solid fraction for any of the parameters ($p > 0.05$), except for the concentration of vanillic acid (Table 3.4). Since most phenolic compounds are originating from the lignin degradation process (Wu et al. 2013; Mood et al. 2013), this indicates that lignin degradation resulted more in soluble phenolic compounds, rather than recondensation to the solid biomass.

It is known that the concentrations of 100 mg/l of phenolic compounds or higher has a negative impact on micro-organisms. Biogas production can be decreased with 20 % if phenolic compounds reach concentrations of 25 mg/g (Mussatto and Roberto 2004; Hernandez and Edyvean 2008). The measured concentrations of phenolic compounds in this study is lower than these inhibiting concentrations found in literature. However inhibition concentrations can vary and are dependent on the individual phenolic compounds and possible synergisms between different inhibitors. The shift of phenolic compounds from the solid fraction to the liquid fraction is not effective enough to have a negative impact on biogas production. In case an increase in biogas production is observed, this could possibly be the result of the degradation of lignin, the decrease in cellulose crystallinity and the increase in availability of the cellulose.

Table 3.3: Impact of the enzymatic treatment over various incubation times on the solid fraction (n=3)

Pretreatment	Duration (hours)	TSS (g/100g)	VSS (g/100g)	Total phenolic compounds (mg/g)	Vanillic acid (mg/g)	Biomethane (Nl/kg VS)
Control	0	36.0	34.7	0.28	BD	275
	6	36.3	33.7	0.26	BD	276
	12	39.5	36.8	0.48	BD	283
	24	33.1	31.3	0.42	BD	293
LA	0	34.6	32.3	0.38	0.11	277
	6	34.0	32.4	0.42	0.10	271
	12	36.7	34.1	0.44	0.14	285
	24	33.4	30.6	0.50	0.14	344
MnP + VP	0	36.8	34.5	0.34	BD	263
	6	41.9	38.5	0.33	BD	309
	12	39.1	34.9	0.33	BD	302
	24	48.5	43.0	0.44	BD	263
COMB	0	34.0	31.4	0.32	0.47	275
	6	41.2	38.1	0.42	0.41	318
	12	36.8	32.4	0.44	0.31	283
	24	32.1	26.5	0.51	0.44	314
Overal Mean		37.1	34.1	0.39	0.28	289
Overal standard deviation		5.55	5.04	0.19	0.24	40
Coefficient of Variance		15.0	14.8	48.7	85.7	13.8

BD = below detection limit, LA = Laccase, MnP+VP = Manganese peroxidase and Versatile peroxidase, COMB = Laccase, Manganese peroxidase and Versatile peroxidase

Table 3.4: Significance of the enzyme effect, the effect of duration and the interaction effect (pretreatment x duration) for several parameters measured in the solid and liquid fraction

Parameter	p-value Pretreatment	p-value Duration	p-value Interaction	Pretreatment	Duration
Solid fraction					
Biomethane production	0.340	0.335	0.012		
TSS	**0.091**	0.767	0.159		
VSS	0.239	0.448	0.137		
Total phenolic compounds	0.745	0.461	0.975		
Vanillic acid	***0.035***	0.759	0.675	LA^{a}, COMB^{b}	
Liquid fraction					
Final pH	***<0.001***	***<0.001***	0.070	Control^{a}, LA^{c} ,MnP+VP^{b}, COMB^{ab}	6h^{a}, 12h^{b}, 24h^{c}
TSS	***0.037***	0.488	0.997	Control^{ab}, LA^{a}, MnP+VP^{b}, COMB^{b}	
VSS	0.951	**0.078**	1.000		
Total phenolic compounds	0.162	***0.023***	0.955		6h^{a}, 12h^{a}, 24h^{b}
Vanillic acid	0.651	0.296	0.939		
BOD	***0.005***	0.284	0.881	Control^{a}, LA^{b}, MnP+VP^{b}, COMB^{b}	
COD	***<0.001***	0.210	0.250	Control^{a}, LA^{a}, MnP+VP^{b}, COMB^{b}	

LA = Laccase, MnP+VP = Manganese peroxidase and Versatile peroxidase, COMB = Laccase, Manganese peroxidase and Versatile peroxidase, ^{abc} = different indices indicate significant differences in pretreatment or duration ($p < 0.05$)

3.4.3. Impact of enzymatic pretreatment on methane production

Regarding methane production after anaerobic digestion for 30 days, treatment with the laccase enzyme gives the best results after 24 hours incubation, resulting in an increase of 25 % compared to the control (Table 3.5). Treatment with the peroxidase enzymes gave a maximum increase of 17 % after 6 hours, and no increase after 24 hours of incubation. Thus, different enzymes clearly show different impacts on the methane production and moreover, the importance of duration regarding enzyme treatment is crucial when reporting over its effect. As enzyme effects regarding methane production have maximal values at different incubation times, the p-value of treatment and of duration was not significant. However effects between treatments per incubation period were detected. The combination of enzymes gives an increase in biomethane production of 16 % after 6 hours and of 14 % after 24 hours. Therefore, no symbiotic effect between the two enzyme groups is determined, although increased biomethane production after both 6 and 24 hours treatment might be explained by the individual effects of peroxidase (6 h) and laccase (24 h). A possible maximum effect could be acquired if the enzymes were introduced to the biomass at different stages, where the peroxidase enzymes would be added 18 hours after a start with laccase treatment. To obtain larger effects, higher enzyme loading rates could be used, however this is not feasible in large-scale installations. Chen et al. (2012) have done a study using different laccase loadings of 0 to 4400 U/g to determine cellulose degradation and determined the optimal loading rate to be 2200 U/g. In an experiment with switch grass, Frigon et al. (2012) used LiP and MnP with loadings of 20 U/g and 40 U/g respectively, showing an increase of 29 % and 42 % in biomethane production.

In this study an increase of up to 25 % in biomethane production was reached using lower loading concentrations. A combination of 5 U/g MnP and 1.5 U/g VP gave an increase of 17 %, while a laccase loading of 2 U/g resulted in an increase of 25 %. The composition of the produced biogas was analyzed by gas chromatography, showing that the biogas consisted of 70±2.2 % CH_4, 30±2.2 % CO_2 and trace amounts of N_2. The volume of methane produced per kg volatile solids varied from 262 l CH_4/kg VS to 344 l CH_4/kg VS. Sugar and starch crops reach yields up to 450 l CH_4/kg VS (Frigon and

Guiot, 2010), but are in competition as food and feed crops. As corn stover is a lignocellulosic crop, the biomethane production in these types of crops varies from 170 l CH_4/kg VS to 390 l CH_4/kg VS, indicating that the presented results with corn stover are located in the high end of these crops (Frigon and Guiot, 2010). The increase of biomethane production is a result of lignin degradation (Figure 2.5) which would increase the concentration of phenolic compounds in the liquid phase as phenolic compounds would be released from the matrix. The increase however is not seen in samples treated with laccase, and possible countered by the detoxification ability of laccase, polymerizing the phenolic compounds (Jönsson et al. 1998).

From the measured biomethane production versus time, parameters γ and μ of the exponential model were determined. Although biological pretreatment increases the accessibility of cellulose and reduces crystallinity, facilitating the hydrolysis step, similar values for μ were found. This indicates that the enzymatic pretreatment had no or little effect on the hydrolysis rate and possibly the lignin barrier was not broken down entirely after pretreatment. Furthermore the final biomethane production had a good correlation with the actual biomethane production after anaerobic digestion of 30 days (Table 3.5, Figure 3.1). The average biomethane production of the control resulted in 290 Nl CH_4 / kg VS, which compared to the biomethane production of corn stover, 229 Nl CH_4 / kg VS (chapter 2) is an increase of 27 %. This distinctive difference can be explained due to the different origin from the corn stover. Corn stover used in this study was provided by InAgro and was already stored for some time, while the corn stover used in the experiment described in chapter 2 was taken directly of the field. The increased biomethane production could be the result of a preliminary degradation of the corn stover during storage at InAgro (Roeselare, Belgium).

Figure 3.2 shows the biomethane production calculated with the first order model when applying different enzymatic pretreatments. It can be seen that peroxidase treatments have an impact after a duration of 6 hours of incubation (figure 3.2B), while the effect is reduced after 24 hours of incubation (figure 3.2C). The best result is seen after 24 hours of incubation with the laccase enzyme, showing an increase of 25 % of biomethane production (figure 3.2C), resulting in 344 Nl CH_4/kg VS

(Table 3.5). However the cost of laccase as a commercial enzyme ranges from € 0.004 – 0.076 per unit, and thus is too high to warrant the increase of 25 % of BMP to be economically feasible (Osma et al. 2011). The main goal of the study described in this chapter was to gain knowledge of the different impact of lignin degrading enzymes over different incubation times. Lowering the overall pretreatment costs can be achieved by using white rot fungi instead of commercially available enzymes, which shall be studied in chapter 5.

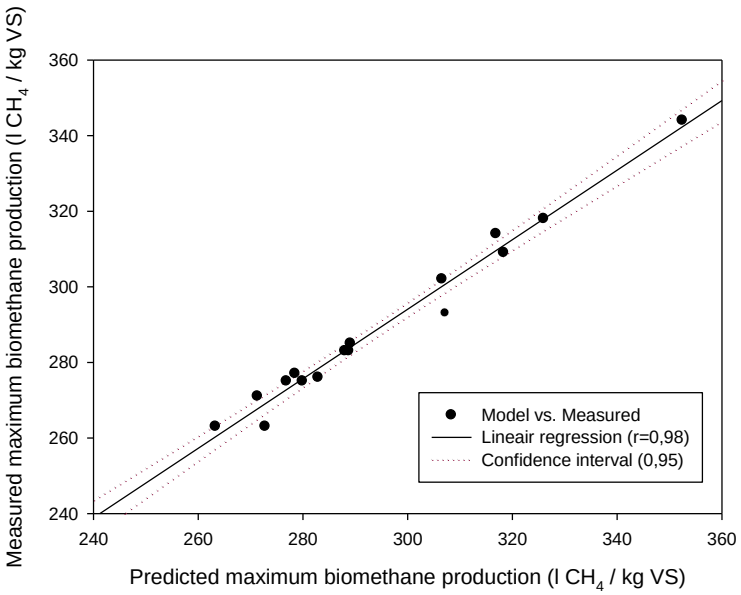

Figure 3.1: Lineair correlation between the measured biomethane potential after anaerobic digestion for 30 days and the predicted maxium biomethane potential (γ)

Table 5: Parameters for the applied first order model: Biomethane production = γ * (1-$e^{(-\mu t)}$) with γ the maximal biomethane value after anaerobic digestion for 30 days, μ the specific growth rate and t the amount of days (n = 3)

Pretreatment	Duration (hours)	Measured Biomethane (Nl Biomethane/kg VS)	γ (Nl Biomethane/kg VS)	μ (d^{-1})	Pearson Coefficient
Control	0	275	280	0.12	0.95
	6	276	283	0.11	0.91
	12	283	289	0.12	0.90
	24	293	307	0.11	0.96
LA	0	277	279	0.13	0.90
	6	271	271	0.14	0.87
	12	285	289	0.14	0.87
	24	344	352	0.11	0.93
MnP + VP	0	263	263	0.13	0.85
	6	309	318	0.11	0.97
	12	302	307	0.11	0.84
	24	263	273	0.10	0.74
COMB	0	275	277	0.12	0.94
	6	318	326	0.11	0.96
	12	283	288	0.11	0.90
	24	314	317	0.10	0.83

LA = Laccase, MnP+VP = Manganese peroxidase and Versatile peroxidase, COMB = Laccase, Manganese peroxidase and Versatile peroxidase

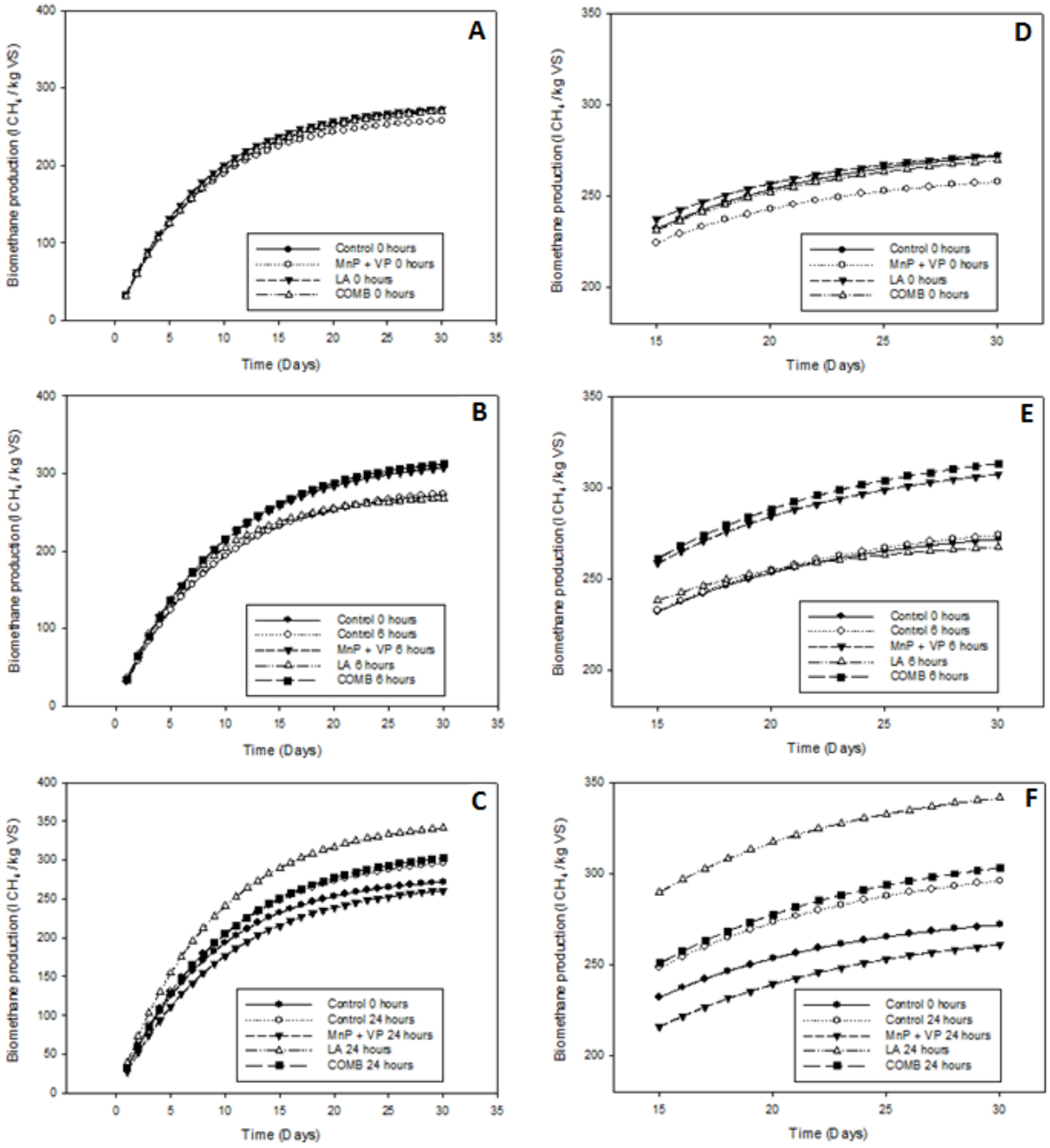

Figure 3.2: First-order models of biomethane production during 30 days of anaerobic digestion after different enzymatic treatments of 0, 6, 24 hours enzymatic incubation based on triplicate results. Graphs A, B and C show the models after respectively 0, 6 and 24 hours of the various enzymatic incubations compared to the control after 0 hours of incubation. Graphs D, E and F give a more detailed look of the last 15 days of anaerobic digestion of respectively graphs A, B and C. LA = Laccase, MnP+VP = Manganese peroxidase and Versatile peroxidase, COMB = Laccase, Manganese peroxidase and Versatile peroxidase.

3.4.4. Comparison of enzymatic pretreatment with other pretreatment techniques

The effect of enzymes on the production of biomethane is less studied than the effect of chemical or mechanical pretreatments. Chemical and mechanical pretreatments have a greater impact in general, with reported increases of over 50% in biomethane production after alkaline pretreatment (Frigon et al. 2012), 80 % after an oxidation pretreatment (Uellendahl et al. 2008) and an increase of 88% after thermal pretreatment (Zhang et al. 2014). Although the enzymes resulted in an increased biogas production, it is still an inefficient process due to the high cost of the enzymes. Therefore, the potential and feasibility of the use of enzymatic, and in general biological, pretreatments should be studied further (Zheng et al. 2014). An increase in enzyme loading rates might give conversion rates that can compete with the traditional pretreatment methods. To make the process feasible a low cost source of enzymes can be found in various white rot fungi. Studies have demonstrated the lignocellulose degradation using white rot fungi as discussed in the review of Sanchez (Sanchez, 2009). Liu et al. confirms this degradation, but suggests research should be done on the optimal experimental conditions to increase biogas production (Liu et al. 2014). White rot fungi are already used as a pretreatment step before applying traditional expensive pretreatments, trying to overcome individual process disadvantages (Mood et al. 2013). Future research should focus on a step further in the process and see what impact enzymes produced by the white rot fungi itself can have on biomethane production. As well as obtaining more information on the working mechanism of the enzymes on different non-food/feed competitive matrices to improve the efficiency of biological pretreatment by e.g varying enzyme/substrate loadings, pH, temperature, incubation time.

3.5. CONCLUSION

Effects of different enzymatic pretreatments of corn stover were compared by measuring the degradation products and the biomethane production. Pretreatments with enzymes gave a higher release of phenolic compounds indicating higher lignin degradation, but inhibition levels were never reached. The degradation of lignin by laccase pretreatment led to an increased biomethane production of 25 %, while peroxidase pretreatment gave an increased biomethane production of 17 %. No symbiotic effect between the laccase and the two peroxidases was noticed. Treatment with both enzyme groups increased biomethane production with 16 % and 14 % after respectively 6 and 24 hours of treatment. This increase after 6 hours was seen as well in chapter 2, however the treatment with a combination of the enzymes for an incubation of 24 hours did not yield a higher BMP than the control sample incubated for 24 hours in chapter 2.

CHAPTER 4: IMPACT OF PHENOLIC COMPOUNDS ON LIGNIN DEGRADING ENZYME ACTIVITIES AND ON BIOMETHANE POTENTIAL

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4. IMPACT OF PHENOLIC COMPOUNDS ON LIGNIN DEGRADING ENZYME ACTIVITY AND BIOMETHANE POTENTIAL

4.1. ABSTRACT

The impact of various phenolic compounds, vanillic acid, ferulic acid, *p*-coumaric acid and 4-hydroxybenzoic acid on anaerobic digestion of lignocellulosic biomass (hemp straw and miscanthus) was studied. Such phenolic compounds have been known to inhibit biogas production during anaerobic digestion. The different phenolic compounds were added in various concentrations: 0, 100, 500, 1000 and 2000 mg/l. A difference in inhibition of biomethane production between the phenolic compounds was noted. Hydrolysis rate, during anaerobic digestion of miscanthus was inhibited up to 50 % by vanillic acid, while vanillic acid had no influence on the initial rate of biogas production during the anaerobic digestion of hemp straw. Miscanthus has a higher lignin concentration (12-30 g/100g DM) making it less accessible for degradation, and in combination with phenolic compounds released after harsh pretreatments, it can cause severe inhibition levels during the anaerobic digestion, lowering biogas production.

To counter the inhibition, lignin degrading enzymes can be used to remove or degrade the inhibitory phenolic compounds. The interaction of laccase and versatile peroxidase individually with the different phenolic compounds was studied to have insight in the polymerization of inhibitory compounds or breakdown of lignocellulose. Hemp straw and miscanthus were incubated with 0, 100 and 500 mg/l of the different phenolic compounds for 0, 6 and 24 hours and pretreated with the lignin degrading enzymes. A laccase pretreatment successfully detoxified the substrate, while versatile peroxidase however was inhibited by 100 mg/l of each of the individual phenolic compounds. Finally a combination of enzymatic detoxification and subsequent biogas production showed that a decrease in phenolic compounds by laccase treatment can considerably lower the inhibition of the biogas production.

4.2. INTRODUCTION

Anaerobic digestion of lignocellulosic biomass is a four step production process, commencing with hydrolysis of biomass/waste, followed by acidogenesis, acetogenesis and methanogenesis. The

hydrolysation is the rate-limiting step, mainly because of the recalcitrant lignin seal in lignocellulosic material (Appels et al. 2008). The hydrolysation rate can be improved greatly by a pretreatment, which removes or degrades this lignin (Li et al. 2015). Indeed, it has been demonstrated in chapter 2 that lower lignin concentrations increase the initial rate of biogas production during the first 7 days of the anaerobic digestion.

As discussed in chapter 1 there are many pretreatment techniques available aiming to degrade the lignin content, including physical, chemical, physicochemical, and biological methods. Ideally a pretreatment facilitates hydrolysation, avoids formation of sugar degradation products and fermentation inhibitors (Agbor et al. 2011). Some physical pretreatments, like milling, do not increase the concentrations of inhibiting compounds i.e. phenolic compounds, furan deratives and organic acids (Zheng et al. 2014). However the energy demand is too high to get an economically feasible process (Hendriks and Zeeman, 2009). During acid-based chemical pretreatment acetic acid and uronic acids are released from hemicellulose, further degradation may lead to furfural and 5-hydroxymethyl-2-furaldehyde production, while formic and levulinic acids are formed from sugar degradation (Jönsson and Martin, 2015). Mitchell et al. (2014) determined the formation of most common phenolic compounds (i.e. 4-hydroxybenzoic acid, vanillin, syringic acid) during acid pretreatment. Oxidative methods lead to fragmentation of lignin and the production of carboxylic acids and phenolic compounds (Martin et al. 2007). An ionic liquid pretreatment does not result in high inhibitory levels, however remaining ionic liquids are possible toxic to enzymes and microorganisms in the further biogas production (Yang and Wyman, 2008). The different techniques have advantages and disadvantages (Table 1.2), however biological pretreatment with white rot fungi have the benefit of producing only small amounts of inhibiting compounds and provide a low cost alternative to more harsh pretreatment methods. In chapter 2 several individual phenolic compounds which were obtained after laccase and versatile peroxidase pretreatment on a set of various substrates, i.e. wheat straw, hemp straw, etc. were described. Vanillic acid, *p*-coumaric acid and 4-hydroxybenzoic acid were the most prominent ones released. For experiments with wheat

straw also a significant amount of ferulic acid was released. However the concentrations reached after enzymatic treatment were not close to inhibition levels (>1000 mg/l) of anaerobic digestion obtained for artificial media i.e. glucose and yeast extract (Hernandez and Edyvean, 2008; Kayembe et al. 2013).

As discussed in chapter 1 pretreatments such as, thermal, acidic or alkaline pretreatments, release higher amounts of these phenolic compounds which could inhibit the following biodegradation steps. Such inhibition can be solved by introducing a detoxification step, in which inhibitory compounds are degraded or removed from the hydrolysate (Sindhu et al. 2015). White rot fungi can detoxify the hydrolysate biologically by producing lignin degrading enzymes. However the fungal pretreatment requires a longer duration of incubation in order to have the same impact of lignin degradation as other pretreatment techniques . A reduction in duration can be achieved by combining a harsher pretreatment followed by a shorter detoxifying biological pretreatment (Sindhu et al. 2015). This detoxifying effect of laccase has been noted by Jönson et al. (1998). The effect of phenolic compounds on laccase and versatile peroxidase is less studied compared to the enzymatic influence on inhibiting compounds (Madhavi and Lele, 2009; Pozdnyakova et al. 2013). Bollag and Leonowicz (1984) studied the inhibition of laccase but did not report on phenolic compounds. So the impact of individual phenolic compounds on the lignin degrading enzymes is unknown but is nonetheless an important factor in creating an efficient enzymatic pretreatment step.

In this chapter, the main focus is to study the effect of higher concentrations (up to 2000 mg/l) of selected phenolic compounds on the activity of lignin degrading enzymes, and on the biogas production by anaerobic digestion. Therefore, the interaction between lignin degrading enzymes, more specific versatile peroxidase (VP) and laccase (LA), and various phenolic compounds, *p*-coumaric acid (p-CA), ferulic acid (FA), 4-hydroxybenzoic acid (4-HBA) and vanillic acid (VA) was tested at several concentration levels. Besides that the impact of different concentrations of those phenolic compounds on the anaerobic digestion of hemp straw and miscanthus was studied, and so

first insights on inhibition of lignocellulosic substrates was achieved. Finally the detoxification effect of laccase on different concentrations of p-CA was evaluated in a biomethane potential (BMP) test.

4.3. MATERIALS AND METHODS

4.3.1. Chemicals

o-Coumaric acid, *p*-coumaric acid, ferulic acid, vanillic acid, sodium chloride (NaCl), sodium malonate ($C_3h_2O_4Na_2$), hydrogen peroxide (H_2O_2), 2,2'-azinobis(3-ethylbenzthiazoline)-6-sulphonate, sodium citrate, 4-hydroxybenzoic acid ($C_7H_6O_3$), veratryl alcohol and laccase enzyme were acquired from Sigma-Aldrich (Bornem, Belgium), while versatile peroxidase was obtained from Jena Bioscience (Jena, Germany). Sulfuric acid (H_2SO_4), ethanol and Folin-Ciocalteu's phenol reagent were attained from ChemLab (Zedelgem, Belgium). Sodium carbonate (Na_2CO_3) was purchased from Merck (Darmstadt, Germany), and 1-hydroxybenzotriazole was obtained from Janssen Pharmaceuticals (Beerse, Belgium). HPLC-grade methanol (MeOH), HPLC-grade water, acetic acid, citric acid and potassium sodium tartrate ($KNaC_4H_4O_6$-$4H_2O$) were acquired from VWR (Leuven, Belgium). All chemicals were used as provided.

4.3.2. Substrates

Two substrates were chosen based on their release of individual phenolic compounds when treated with laccase and VP, as shown by Schroyen et al. [3]. Miscanthus (*Miscanthus giganteus*) was acquired from the Institute of Agricultural and Fisheries Research (Merelbeke, Belgium), while hemp straw (*Cannabis sativa L.*) was obtained from InAgro vzw (Roeselare, Belgium).

4.3.3. Experimental Setups

4.3.3.1. Effect of lignin degrading enzymes on phenolic compounds

Phenolic compounds, i.e. p-CA, FA, 4-HBA and VA, were added individually to an acetate buffer (0.1 M, pH = 4.5) in a concentration of 0, 100 and 500 mg/l. Two grams of substrate, miscanthus or hemp straw, cut into pieces of 1-2 cm length, were incubated in 40 ml buffer with different concentrations of these individual phenolic compounds for 24 hours of incubation. Laccase (LA, 2 U/g biomass; 1 U is defined as the release of 1 µmol catechol/min at pH 6 and 25°C) was added to one series, versatile peroxidase (VP, 1.5 U/g biomass; 1 U is defined as the release of 1 µmol Mn(II)/min at pH 4.5 and 25°C) was added to a second series. Samples of the liquid fraction were taken after 10 minutes, 6 hours and 24 hours of incubation. Blank incubations were included i.e. buffer with the various concentrations of phenolic compounds, without substrate, but with LA or VP. The concentration of the various phenolic compounds of the different samples and the laccase and versatile peroxidase activity was determined in the laccase and versatile peroxidase treated samples respectively. After 24 hours of incubation a methanolic extraction was done on the solid fraction, and the total phenolic compounds were analyzed. Three independent replicates were performed.

4.3.3.2. Impact of phenolic compounds on anaerobic digestion

The various phenolic compounds, p-CA, FA, 4-HBA and VA were added individually at concentrations of 0, 100, 500, 1000 and 2000 mg/l to the anaerobic digestion inoculum, taken from a co-digestion plant treating cow manure and maize silage. Two grams of hemp straw and miscanthus were added to 40 gram of sludge in order to keep a substrate to inoculum ratio of 0.5 g VS/ g VS. The reactors (250 ml) were incubated at 37 °C, connected to a water-displacement system and shaken daily. The volume of displacement was used to calculate the biogas production in norm liter under standard temperature and pressure. Next to this, the inoculum with the different concentrations of phenolic compounds without substrate and without added enzymes, were started up simultaneously as control. On a daily basis biogas production was measured over a 30 day period. Compositional analysis of the biogas to determine the methane concentration was done with an Agilent 6890 Series

gas chromatograph as described in chapter 2. For each treatment three independent replicates were done.

4.3.3.3. Impact of LA-treated p-CA on anaerobic digestion

p-CA was added to 100 ml of acetate buffer (0.1 M, pH=4.5) in concentrations of 0, 500 and 2000 mg/l. In these buffers two grams of substrate, miscanthus or hemp straw, was added. One series of samples was added to the aforementioned inoculum immediately, and the biogas production was determined for over 30 days. Another series was first incubated with laccase enzyme for 24 hours at 30°C, and the biogas production was determined the following 30 days. As a reference blank 100 ml of the acetate buffer with a concentration of 0, 500 and 2000 mg/l of p-CA were also anaerobically digested for 30 days without addition of any substrate as a base. All incubations were performed independently and in triplicate.

4.3.4. Analysis

4.3.4.1. Phenolic Compounds

To determine the total phenolic compounds in the liquid fraction, the Folin-Ciocalteau method was used as described by Singleton et al. (1999), and executed as described in Schroyen et al. (2014). However for each setup a specific standard curve was used based on the added phenolic component. This was done since the various phenolic compounds have a different reaction with the Folin-Ciocalteau reagens, resulting in different absorbance values. The concentration of the phenolic compounds in the liquid were measured at the start, after 6 and 24 hours. To quantify the results the measured concentration was calculated relatively to the initially added concentration of the phenolic compound at the start. RC = 100 * MC / IAC (RC = relative concentration (%); MC = measured concentration (mg/l); IAC = initially added concentration (mg/l)). Individual phenolic compounds of one of the three repetitions were measured with the HPLC as a confirmation of the results obtained by the Folin analysis. The HPLC analysis was performed as described in chapter 2.

4.3.4.2. Enzymatic Assays

In the assay to determine the laccase activity 2,2'-azino-bis-di-[3-ethylbenzthiazoline sulfonate (6)] (ABTS) was oxidized by laccase at 35 °C. The laccase activity assay was performed in a 1.5 ml mixture consisting of 0.5 ml of 1 mM ABTS, 0.5 ml of 0.1 M acetate buffer (pH = 4.5) and 0.5 ml enzyme solution or sample. The LA activity was measured spectrophotometrically at 420 nm (ε_{420} = 36000 M cm^{-1}) after 5 minutes reaction time (Irshad et al. 2011). The enzymatic activity in the samples was reported relatively to the laccase activity determined in the solution with only buffer, laccase and ABTS.

VP activity was measured in a mixture of 1 ml 30mM veratryl alcohol, 1 ml 0.1 M citrate buffer (pH =3), with or without a phenolic compound at a concentration of 100 mg/l and 0.5 ml 20mM H_2O_2. The difference in absorbance was measured over 150 seconds at 310 nm (ε_{310} = 4600 M cm^{-1}) [23].

4.3.4.3. Statistical Analysis

The biogas production was measured daily for 30 days. A first-order model $P = \gamma * (1-e^{(-\mu t)})$, as described in chapter 2 was created and showed the biogas production as a function of time (t(d)) (Bilgili et al. 2009). An exponential rise, characterized by the specific production rate μ (d^{-1}) was assumed to a maximum biogas yield given by γ (Nl/kg VS). The model was fitted using Microsoft Excels solver to minimize the sum of squares of differences between the model and the measured biogas production. The initial slope was based on the first 7 days of the fitted model and used as a measure of hydrolysation rate.

For all statistical analysis IBM® SPSS® Software version 23 was used. A two-way ANOVA was executed on all data. The effect of pretreatment duration, type of phenolic compound and the starting concentration of the phenolic compound on the relative concentration of phenolic compounds after a treatment with LA or VP was studied. In a second analysis the effect of the initial concentration of phenolic compounds, the pretreatment duration and the substrate on the enzyme activity was determined. The *p*-values are given with a significance level of 0.05.

4.4. RESULTS AND DISCUSSION

4.4.1. Impact of enzymatic pretreatment on phenolic compounds

Lignin-degrading enzymes include a variety of enzymes, with the most important ones laccase and peroxidases (manganese and versatile). Laccases are blue copper oxidases, which catalyze one-electron oxidation of phenolic compounds, and thus in absence of the other enzymes it should have the ability to polymerize the phenolic units (Jönsson et al. 1998). Lignin peroxidase degrades non-phenolic units, while manganese peroxidase acts as a diffusible oxidizer on phenolic and non-phenolic units. Versatile peroxidase is able to perform both oxidations (Irshad et al. 2011).

In a first step, the impact of an enzymatic pretreatment (LA or VP) on phenolic compounds in the presence of different lignocellulose substrates was investigated. In Figure 4.1 the results for laccase treated samples are presented. The concentration of the phenolic compound is given relatively to the initially added concentration of the phenolic compound. A diminishing of phenolic compounds is noticed after incubation with LA, however this effect is different for the different phenolic compounds. Initially a decrease of all phenolic compounds tested was observed as a significant difference was found between the concentrations at the start and after 6 and 24 hours of incubation (p = 0.003). VA was removed to a significant higher extent than the other compounds, and this decrease took place in the first 6 hours (p = 0.03). Initially there was a difference between the highly concentrated (500 mg/l) and the lower concentrated (100 mg/l) VA-samples. At the start of the incubation with 500 mg/l of VA, still 35-40 % VA was present, compared to maximum 20 % of VA if initially 100 mg/l was added (Figure 4.1A).. The high VA concentrations affected the activity of the laccase enzyme at the start. This has been overcome during longer incubation times as can be seen in Figure 4.3A, where laccase activity of samples when 500 mg/l of VA was added had an increased laccase activity after 6 and 24 hours compared to the start. Detoxification of FA was also very effective, but contrary to VA this effect took place immediately (Figure 4.1B). p-CA was found in relatively low amounts (20 % of the original amount added) after an incubation of 24 hours with laccase, but here the process was more gradual over time compared to the other phenolic

compounds (Figure 4.1C). Laccase seemed to have a minor effect on 4-HBA as the samples showed only a small decrease in 4-HBA concentration. The samples containing only buffer show the smallest decrease as well without interference of substrate, making the ineffectiveness of laccase on 4-HBA plausible (Figure 4.1D). As a confirmation a single repetition was measured using HPLC, the results showed a very strong correlation ($R = 0.91$; $p < 0.001$) with the total phenolic concentration obtained from the Folin method, indicating that results obtained with the Folin method are correctly estimating the concentration of the individual phenolic compounds, without interference of substrate or other compounds. The removal of phenolic compounds by laccase was previously investigated, and laccase pretreatment was seen as highly effective (Jönsson et al. 1998; Martin et al. 2002). Martin et al. (2002) noted approximately 80 % of the phenolic compounds were removed, however no identification of the phenolic compounds was done. Jönsson et al. (1998) studied a laccase treatment of wood hydrolysates and estimated after derivatization of the phenolic compounds to pentafluorobenzoyl ethers, a laccase treatment removed 94 % of the phenols. The method however did not include the quantification of phenolic acids. Measuring the total amount of phenolic compounds can give a good indication, but as results in Figure 4.1 show the different effect of laccase on the different individual phenolic compounds, identification of the type of inhibitory component can be interesting to determine the best detoxifying step.

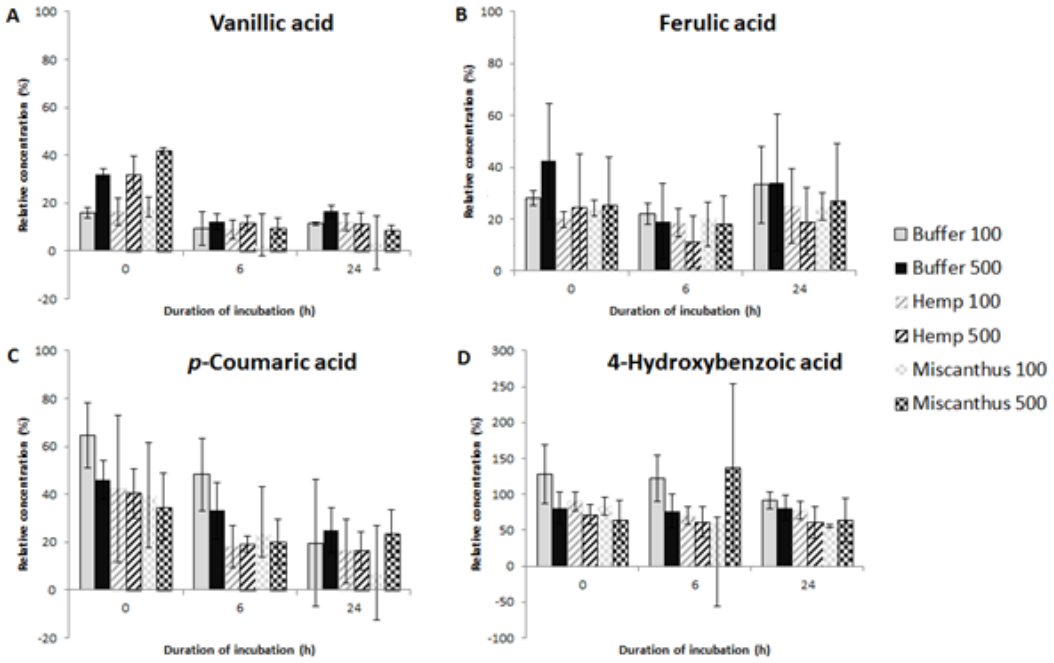

Figure 4.1: The relative concentration of phenolic compounds found in the liquid fraction of the initial added concentration of 100 and 500 mg/l of the individual phenolic component after various incubation durations with laccase. (n=3)

Versatile peroxidase, contrary to laccase, did not result in any significant (p > 0.05) change in concentration of phenolic compounds over time (Figure 4.2). p-CA decreased the most and 4-HBA had an increase, however not in the buffer. This increase can be dedicated to the overestimation of other phenolic compounds in a standard curve provided by 4-HBA. The correlation with the HPLC data obtained from one single repeat showed a positive correlation (R = 0.71; p < 0.001).

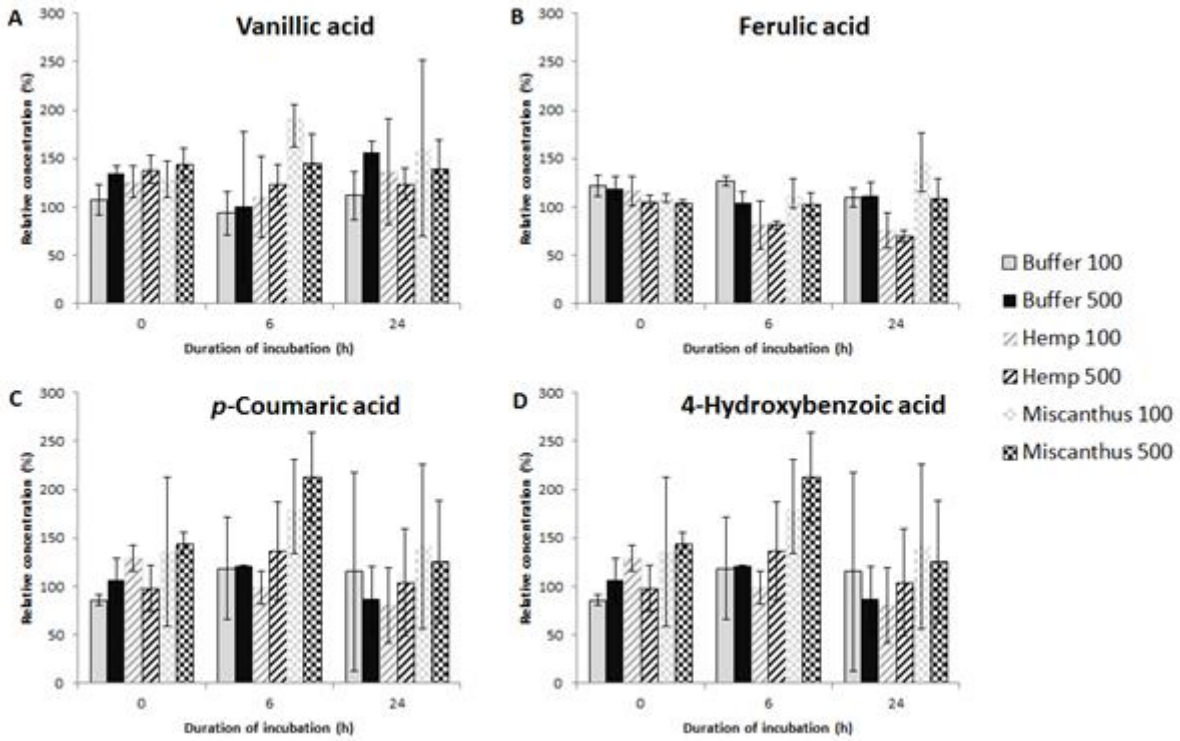

Figure 4.2: The relative concentration of phenolic compounds found in the liquid fraction of the initial added concentration of 100 and 500 mg/l of the individual phenolic component after various incubation durations with versatile peroxidase. (n=3)

4.4.2. Impact of phenolic compounds on lignin degrading enzymes

The enzyme activity of laccase on hemp straw and miscanthus (Figure 4.3) was measured relative to the laccase enzyme activity in the buffer with neither substrate, nor phenolic compounds. The samples of the liquid fraction were not always transparent, which interfered with the absorbance measurements as can be seen by the high values produced by the VA en FA samples. For VA (Figure 4.3A) a higher activity in the 500 mg/l samples after 6 and 24 hours but not at the start was observed. The initial laccase inactivity in samples with 500 mg/l of VA can be linked to the milder decrease of VA compared to incubation after the first hours (Figure 4.1A).

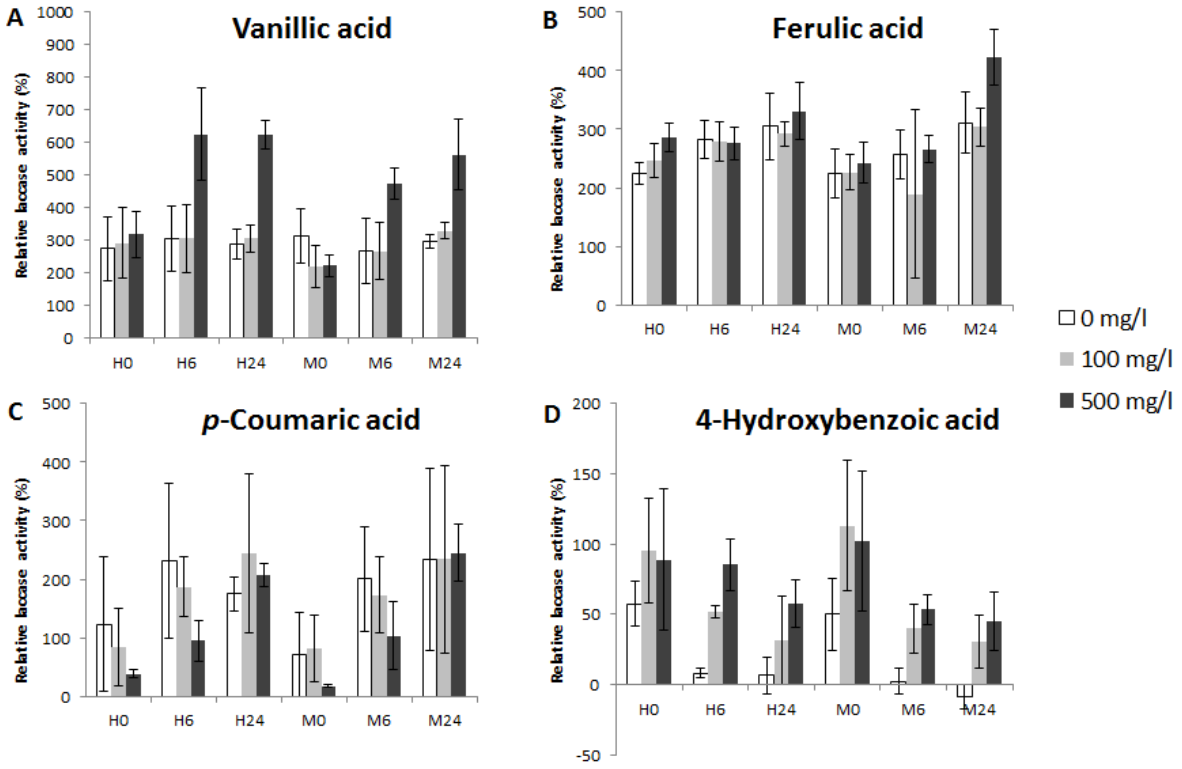

Figure 4.3: The laccase activity of the various samples if 0 (white), 100 (grey) or 500 mg/l (black) of the different phenolic compounds were added, relative to laccase activity measured in a buffer without inhibiting compounds. (n=3)

A combination of results shown in Figure 4.1 and Figure 4.3 indicates a clear association between the results of a decrease in concentration of a phenolic compound due to laccase and the activity of laccase. This link was seen as well in the p-CA samples and to a lesser extent in the 4-HBA samples. In these samples a gradual decrease of phenolic inhibitors is reached after 24 hours of incubation (Figure 4.1C/D) meaning the difference in enzyme activity between the different concentrations has disappeared. The activity of laccase at the start and after 6 hours of incubation is increased gradually over time (Figure 4.3). A significant difference in laccase activity was found in the samples of 0 and 100 mg/l of phenolic compounds compared to the samples with a concentration of 500 mg/l of phenolic compounds (p = 0.014). The relation between laccase activity and the removal of phenolic compounds is presented in Figure 4.4. Figure 4.4A shows the samples with initially 100 mg/l added phenolic compounds. A higher laccase activity than in the buffer was reached if 60 mg/l or more of the phenolic compounds was removed. In Figure 4.4B the impact of adding 500 mg/l of phenolic

compounds is shown. Here a higher laccase activity was reached after only 100 mg/l of the initial added compounds was left. Generally the activity at the start of the incubation was lower.

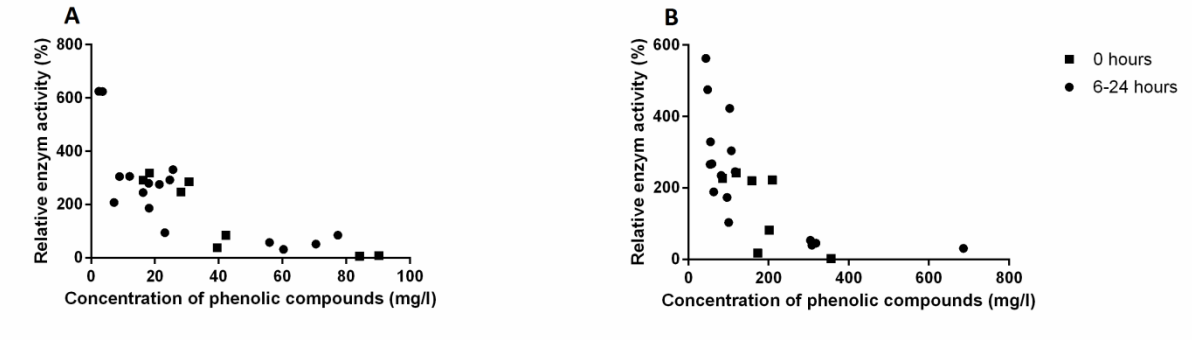

Figure 4.4: The relation between laccase activity relative to the activity in buffer, and the concentration of phenolic compounds (mg/l) in the liquid fraction. A distinction has been made between initial activity and after an incubation of 6 and 24 hours. The samples with initial concentrations of 100 mg/l of the phenolic compounds (A); The samples with initial concentrations of 500 mg/l of the phenolic compounds (B).

The activity of versatile peroxidase could not be measured. Due to the non-transparency of the samples, there was simply too much interference. However versatile peroxidase activity was measured in the buffer and after addition of 100 mg/l of the different phenolic compounds to the buffer. An increased absorbance was measured over time when activity was measured if versatile peroxidase was added to the buffer, as represented in Figure 4.5. The enzyme however was inhibited entirely after addition of every individual phenolic compound that was investigated. This agrees with the results presented in Figure 4.2, where no decrease of phenolic compounds was detected after a treatment with versatile peroxidase.

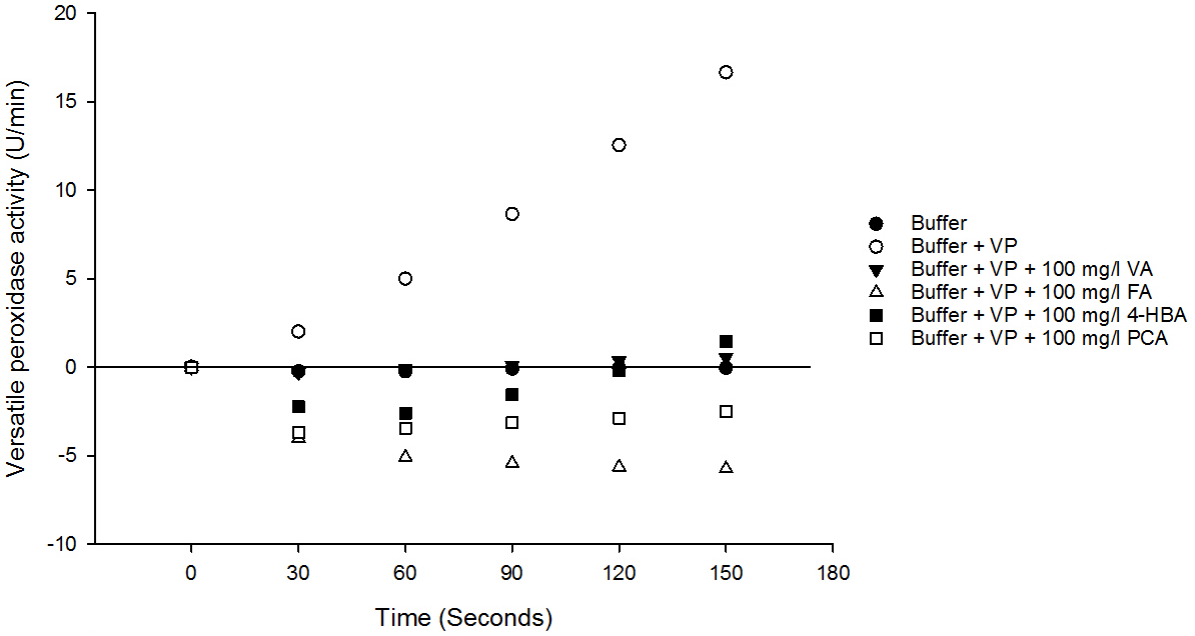

Figure 4.5: Average of the versatile peroxidase enzyme activity of the first 150 seconds measured at 310nm in buffer and in buffer with addition of VP (0.075 U/ml) and the various phenolic compounds in a concentration of 100 mg/l (n=3). VP=Versatile peroxidase; VA=Vanillic acid; FA=Ferulic acid; 4-HBA=4-Hydroxybenzoic acid; PCA=*p*-Coumaric acid

4.4.3. Impact of phenolic compounds on the biogas production

Phenolic compounds in high enough amounts are toxic for bacteria performing the anaerobic digestion in the biogas production process. Hernandez and Edyvean (2008) have tested seven phenolic compounds inhibiting the anaerobic biogas production from the digestion of D-glucose, yeast extract and nutrient broth. To reach an inhibition of 50 %, an addition of 120 mg phenol, 328 mg catechol or 271 mg 4-HBA per gram VSS biomass was required, corresponding with 2.1 g phenol, 5.8 g catechol or 4.8 g 4-hydroxybenzoic acid per liter sludge. Kayembe et al. (2013) tested the anaerobic digestion of acetic acid neutralized to pH 7, enriched with macro-nutrients, i.e. NH_4Cl (170 g/l), KH_2PO_4 (37 g/l). Addition of 1249 mg/l phenol resulted in a 50 % inhibition of methanogenic activity. This inhibition is caused by the loss of integrity of biological membranes and thus reducing cell growth and sugar assimilation (Musatto and Roberto, 2004). The toxicity of phenolic compounds is, even in smaller concentrations, more severe than furfural and hydroxymethylfurfural (Parajo et al. 1998). These reports emphasize the need to have a detoxifying step if there is an accumulation of inhibiting phenolic compounds in the (pretreated) substrate. Oliva-Taravilla et al. (2016) investigated

the impact of phenolic compounds and lignin on the enzymatic hydrolysis of steam-pretreated biomass and noted that in absence of lignin, a same concentration of phenolic compounds did not yield lower glucose hydrolysis of Sigmacell cellulose. Therefore, the aim of this experiment was to study the impact of individual phenolic compounds on the anaerobic digestion of lignocellulose rich substrates and agricultural residues.

The effect of inhibition on the rate of hydrolysation was determined by calculating the slope of the biogas curve in the first 7 days. The slopes of the anaerobic digestion of the samples with miscanthus are given in Figure 4.6, and of the samples with hemp straw are given in Figure 4.7. Miscanthus has a higher lignin concentration than hemp straw (Schroyen et al. 2015), and due to the recalcitrance of lignin, it is less accessible for degradation and thus has a much lower slope in biogas production compared to hemp. From the slopes (Figure 4.6) an inhibition is noted initially at 100 mg/l of the added phenolic compound, and increasing the concentration of the phenolic compounds resulted in an increased inhibition level. Hemp straw is inhibited less, a decrease of rate in biogas production was only seen in samples where 2000 mg/l of p-CA and VA was added (Figure 4.7).

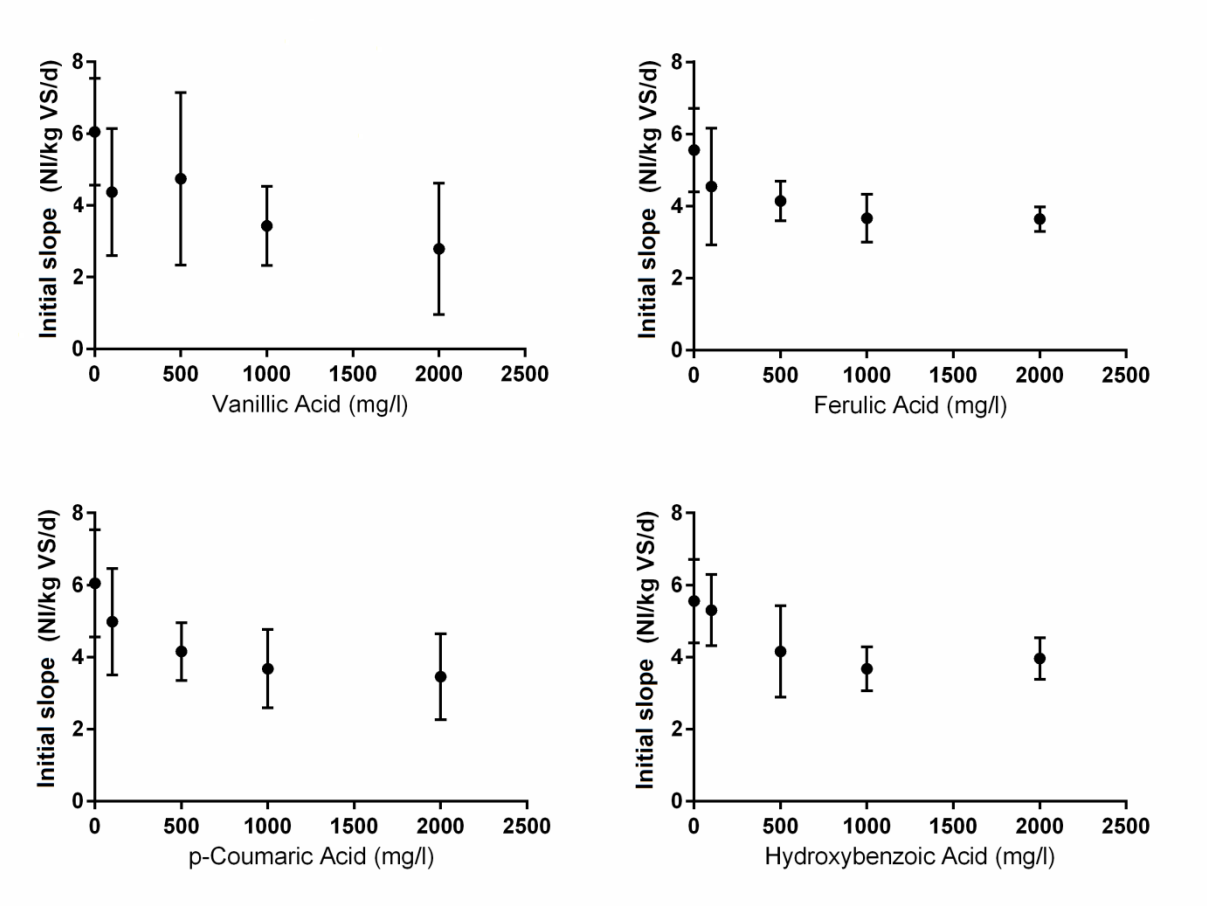

Figure 4.6: The initial slope of biogas production of miscanthus as substrate with various added concentrations of the different phenolic components. (n=3).

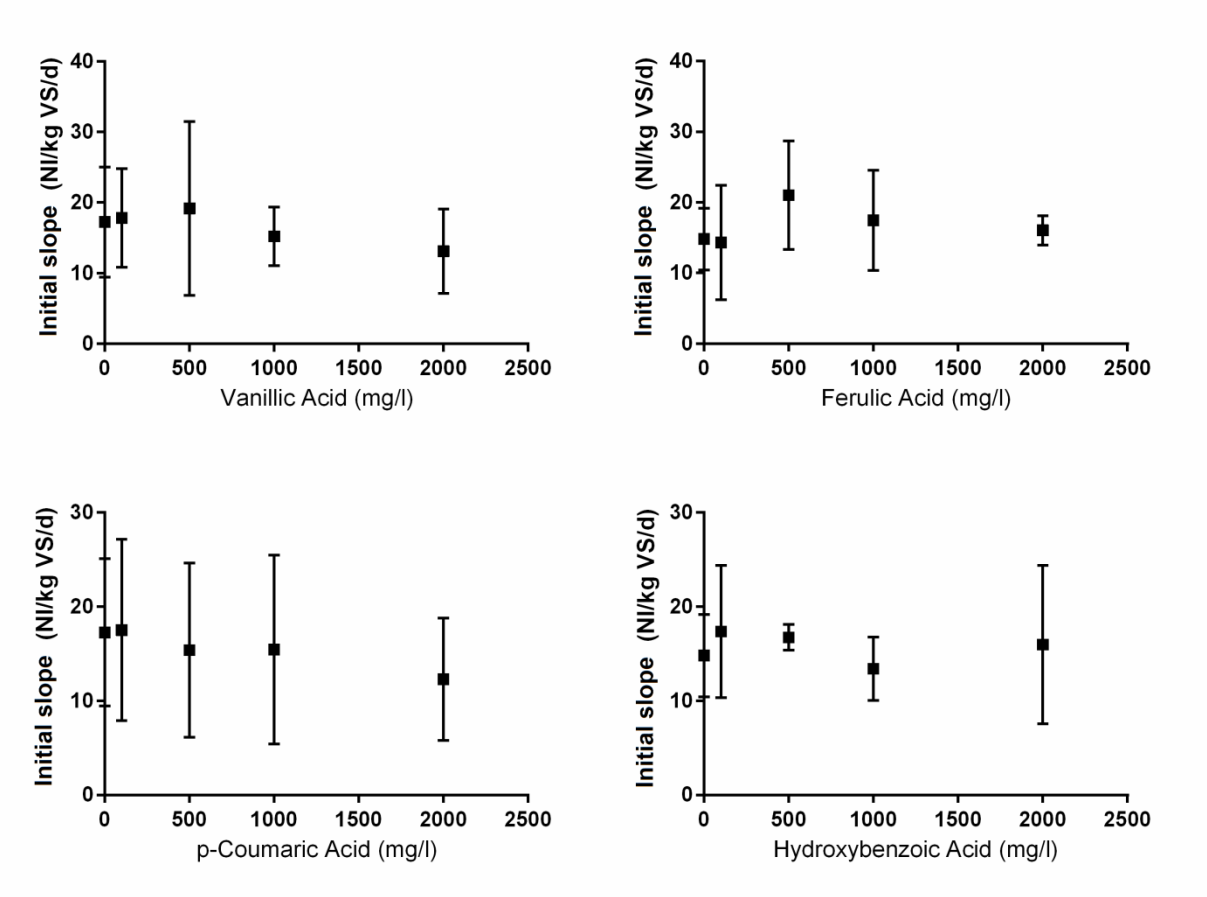

Figure 4.7: The initial slope of biogas production of hemp straw as substrate with various added concentrations of the different phenolic components. (n=3).

4.4.4. Detoxification effect on biogas production

Figure 4.1 shows a detoxification effect of laccase, removing up to 80 % of the concentration of added p-CA. In Figure 4.6 inhibition of anaerobic digestion of miscanthus was observed due to p-CA. At 100 mg/l a drop in the hydrolysation rate (Nl/kg VS/d) of 20 % was noticed, while at 1000 mg/l the biogas production dropped to 60 %. Laccase treatment of 24 hours reduced greatly the added p-CA (Figure 4.1). After 24 hours of incubation it can be assumed that the added concentrations of p-CA of 500 and 2000 mg/l would be reduced to respectively 100 and 400 mg/l concentrations. To estimate the detoxifying potential of laccase, biogas production of samples supplemented with p-CA (500 and 2000 mg/l) with and without a 24 hours laccase treatment were measured (Figure 4.8). In general, the more lignin containing miscanthus had a lower rate of biogas production than hemp straw. The rate of hydrolysis of the untreated samples declined with addition of p-CA, while laccase pretreated

miscanthus showed no inhibition of the initial biogas production by the phenolic compound. Treatment of the hemp straw shows a small increase in rate of biogas production, which was expected in a sample with a concentration of 2000 mg/l of p-CA.

The initial slopes of the biomethane production if no p-CA is added are 12 and 4.5 Nl CH4/kg VS/d for hemp straw and miscanthus respectively. These values are distinctively lower, when comparing them to the initial slopes shown in Figure 4.6 and 4.7 (i.e. 17 and 6 Nl CH4/kg VS/d for hemp straw and miscanthus respectively. In the experiment described in chapter 2 the same biomass source was used to pretreat enzymatically and digest. The initial biogas production of the control groups were 16.1 and 6.8 Nl CH4/kg VS/d for hemp straw and miscanthus (chapter 2), which is comparable with the initial slopes presented in Figure 4.6 and 4.7. This decrease in initial biogas production is however due to the addition of the acetate buffer at the start of the anaerobic digestion, prolonging the adjustment time of the bacteria.

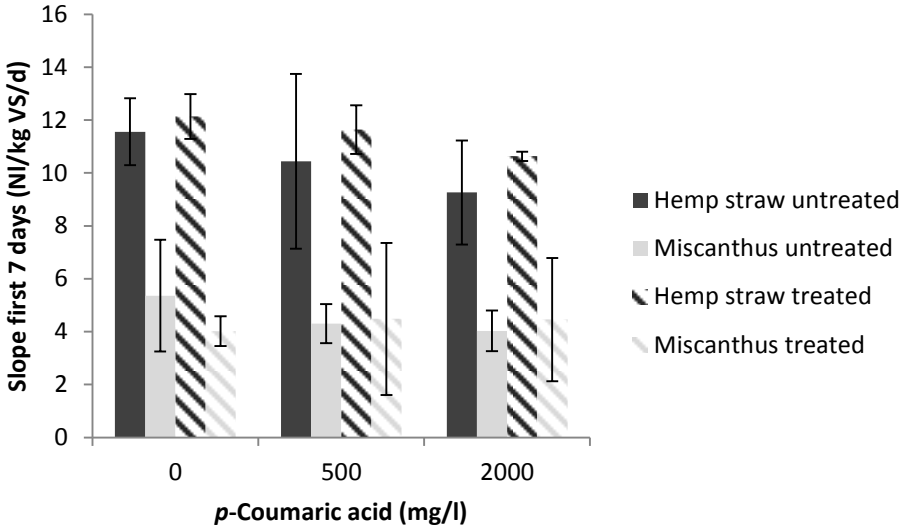

Figure 4.8: The initial slopes of the biogas curves with added phenolic inhibiting concentrations with and without a 24 hour treatment with the laccase enzyme. (n=3).

Looking at the BMP a decrease of total biogas produced was seen in the untreated samples, if the phenolic compound is added (Figure 4.9). Pretreatment reduced the concentration of p-CA, which is observed in the treated samples with 500 mg/l p-CA, where there is no decrease in BMP, while the

laccase treated samples with 2000 mg/l p-CA still show a decrease in BMP. However this decrease is similar as what was observed for the biogas production of the non-treated samples with 500 mg/l of phenolic component. This is 25 % of the initial added concentration (2000 mg/l), which is the same relative amount found of p-CA after pretreatment in the experiment shown in Figure 4.1.

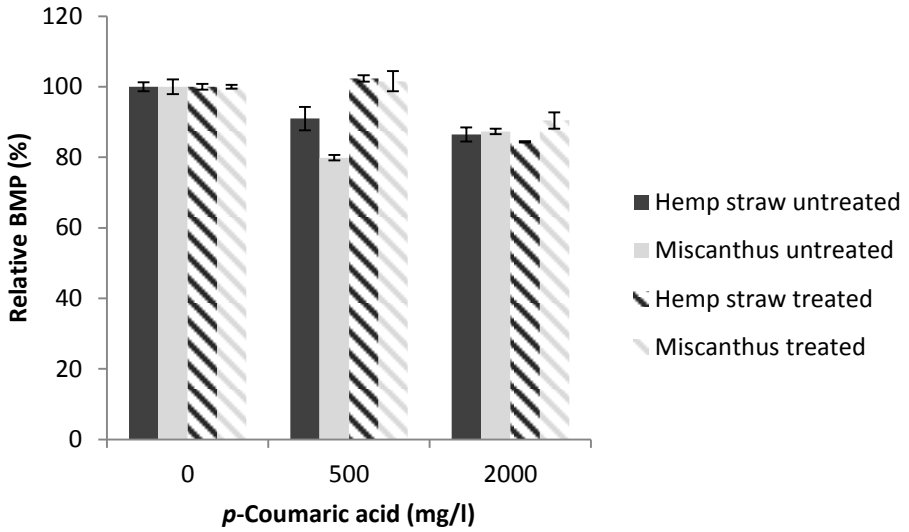

Figure 4.9: The bio methane potential (BMP) of the substrates with added phenolic inhibiting concentrations with and without a 24 hour treatment with the laccase enzyme. (n=3).

4.5. CONCLUSION

Phenolic compounds are inhibiting the activity of versatile peroxidase and also have an impact on the initial hydrolysation rate during anaerobic digestion. The effect is clearly seen for lignin rich substrates. The microbial community can overcome the inhibition after 30 days and reach a similar BMP over time. To remove the toxic effect of the phenolic compounds an incubation with laccase can be proposed. This incubation can be an important detoxifying step in a multi-step pretreatment process, removing produced inhibitors after a faster and harsher pretreatment. In chapter 6 data obtained from the inhibition of the anaerobic digestion will be used to create a lignin based model taking the inhibition of the individual phenolic compounds into account.

CHAPTER 5: PRETREATING HEMP STRAW AND MISCANTHUS WITH *PLEUROTUS ERYNGII:* THE EFFECT ON PHENOLIC COMPOUNDS, HYDROLYSIS AND BIOGAS PRODUCTION

5. PRETREATING HEMP STRAW AND MISCANTHUS WITH *PLEUROTUS ERYNGII:* THE EFFECT ON PHENOLIC COMPOUNDS, HYDROLYSIS AND BIOGAS PRODUCTION

5.1. ABSTRACT

Pretreatments are required to facilitate the hydrolysis of lignocellulosic substrates during anaerobic digestion. Classic pretreatments however produce an excess of undesirable by-products, which may inhibit the further biogas production. In this study a low cost alternative is researched as fungal pretreatments yield lower amounts of inhibitory degradation products. Hemp straw and miscanthus are inoculated with *Pleurotus eryngii,* a white rot fungi, for a duration of 6 weeks. Every week the impact on degradation and on the biogas production was evaluated. Indication of breakdown of the lignin barrier was noticed as the release of phenolic and sugar compounds increased. However the incubation with *P. eryngii* did not influence the total biogas production. The laccase enzyme produced by the white rot fungus did show the opportunity to diminish inhibitory phenolic compounds leaking from the lignocellulosic matrix.

5.2. INTRODUCTION

There is a search for alternative, renewable energy sources to obtain a higher security of energy supply, and to fulfill goals towards a more sustainable world, set by the global climate deal in Paris in 2015. Anaerobic digestion of lignocellulosic waste could be such an alternative. Lignocellulosic biomass, composed of cellulose, hemicellulose and lignin, is a highly abundant available waste source from agricultural and forestry industry (Shirkavand et al. 2016). Lignocellulosic biomass is a promising feedstock for emerging bio-refinery concepts and can be converted into a broad range of value-added products, i.e. chemicals, ruminant feed and fuels. The production of lignocellulosic based fuels is centered on the conversion of the sugar rich polymers cellulose and hemicellulose, concealed within the lignin polymer (Isroy et al. 2011).

To facilitate this conversion, lignin degradation is paramount and achieved by pretreating the lignocellulosic substrate. Classic pretreatment techniques i.e. acidic, alkaline, steam explosion etc. have been reviewed in chapter 1. The classic pretreatments require harsh conditions which will

induce the release of inhibiting compounds e.g. phenolic compounds and furan derivatives. Phenolic compounds have been proven to have a negative effect on the anaerobic digestion process. For example, various phenolic compounds dosed at a concentration of 1000 mg/l inhibited the biogas production of artificial substrate, e.g. glucose and yeast extract (Hernandez and Edyvean, 2008).

A pretreatment by incubation with white rot fungi produces less inhibiting compounds during degradation. White rot fungi are known to produce many types of enzymes, including the lignin degrading enzymes, laccase, lignin peroxidase and manganese peroxidase (Knezevic et al. 2013). Enzymatic pretreatments with a 24 hour incubation with laccase resulted in an increased biomethane production of 25 % (Schroyen et al. 2014), due to the ability of laccase to decrease the amount of inhibiting phenolic compounds in the hydrolysate and breakdown the lignin barrier (Jönsson et al. 1998). Incubating with peroxidases lead to an increase of total biomethane production after only 6 hours of incubation (Schroyen et al. 2014). However a major drawback of the use of pure enzymes during a pretreatment is their cost. A low cost fungal pretreatment with white rot fungi producing the lignin degrading enzymes should be a good alternative to be considered. Hence interest for a fungal pretreatment is growing over recent years (Rouches et al. 2016). As of now however, this pretreatment is commercially unsuitable due to the long duration and low hydrolysis rate.

To increase the low hydrolysis rate after fungal pretreatment, more knowledge should be acquired about the impact of the type of white rot fungi, the substrate, the duration of incubation and other process factors i.e. temperature, pH etc.

White rot fungi can be categorized into two groups, selective and non-selective degraders. The non-selective degraders, e.g. *P. chrysosporium*, simultaneously degrade lignin, cellulose and hemicellulose, which is less desirable (Sanchez et al. 2009). Selective degraders preferentially breakdown lignin, although hemicellulose and even cellulose can be degraded as well. Decay by e.g. *C. subvermispora* and *Pleurotus ostreatus*, initially does not affect the cellulose polymer, and only lignin and hemicellulose losses are noted (Martinez et al. 2005). The results of degradation, such as

lignin loss, have been reported in several studies. Wan and Li (2011) reported a 26 % lignin loss compared to raw material of corn stover and switchgrass after an incubation of 18 days with *C. subvermispora.* An incubation of rubberwood for 90 days with *C. subvermispora* led to a 45 % lignin loss on dry matter basis, and an increased sugar yield of 28 %. *Pleurotus eryngii* was used to pretreat wheat straw and degraded 14 % DM of the lignin after 14 days of incubation. However the increase in biogas production of BMP are more seldom evaluated. A pretreatment with *P. ostreatus* revealed an increase of 17 % in biogas production from wheat straw (Zhong et al. 2011a). After 15 days of biological pretreatment with a complex microbial agent, corn straw biogas production was increased with 33 % (Zhong et al. 2011b). Liu et al. (2014) pretreated corn stover with *P. chrysosporium* but no significant difference in biogas production was demonstrated due to the relatively higher dry mass loss. The varying results are due to the many influencing factors, i.e. biomass type, incubation time, moisture content, aeration, pH, etc. (Sindhu et al. 2016), and show again the need to gain more knowledge on fungal pretreatment process to increase biogas production. This chapter therefore investigates the degradation and related biogas production of fungal pretreatment of hemp straw and miscanthus. The enzymatic pretreatment of these substrates was studied before in chapter 2 and 4. *P. eryngii,* a selective degrader, breaking down lignin primarily, was used to inoculate the substrates for an incubation period lasting 42 days.

5.3. MATERIALS AND METHODS

5.3.1. Substrates

Two substrates were chosen with a different lignin concentration. Hemp straw (*Cannabis sativa L.*) was obtained from PetsPlace (Netherlands). Miscanthus (*Miscanthus giganteus*) was obtained from the Institute of Agricultural and Fisheries Research (Merelbeke, Belgium).

5.3.2. Chemicals

Sodium carbonate (Na_2CO_3) was obtained from Merck (Darmstadt, Germany). Chemical standards from o-coumaric acid, p-coumaric acid, ferulic acid, gallic acid, vanillic acid, dinitrosalicylic acid, Tween 80, sodium chloride (NaCl), 4-hydroxybenzoic ($C_7H_6O_3$) acid, 2,2'-azino-bis-di-[3-

ethylbenzthiazoline sulfonate (6)], and laccase was obtained from Sigma-Aldrich (Bornem, Belgium). HPLC-grade methanol (MeOH), HPLC-graded water, acetic acid, glucose, potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) were acquired from VWR (Leuven, Belgium), while Folin-Ciocalteu's phenol reagent was purchased from ChemLab (Zedelgem, Belgium). Agar No. 1, malt extract agar and buffered peptone water were acquired from Oxoid (Hampshire, United Kingdom).

5.3.3. Fungal pretreatment

The white rot fungus *P. eryngii* was collected from Mycelia (Nevele, Belgium). The fungus was cultured on malt extract agar slants for 7 days at 30°C. Spore suspensions were obtained by adding 2 ml 0.05 % Tween 80-solution and scraping the agar surface with a sterile inoculating loop. The suspension was centrifuged for 20 minutes at 2540 g after which the supernatant was removed. The pellet was resuspended with 2 ml of a 0.9 % tryptone salt solution, and centrifuged for 20 minutes at 4000 rpm after which the supernatant was removed. This last step was repeated until no mycelia was seen and the pellet was suspended in 2 ml buffered peptone water. The suspension of 2 ml was added to 10 grams of substrate with a moisture level of 85 %.

Seven different samples of hemp straw and miscanthus were inoculated. Analysis were done at the start of the experiment, and after different incubation times of 7, 14, 21, 28, 35 and 42 days at 30°C. As a control seven samples of 10 grams of hemp straw and miscanthus were incubated for a same duration at 30°C, without inoculation. As the substrate was not autoclaved or treated in another way, natural flora was still present on the substrate. For each substrate and incubation period three independent replicates were executed.

5.3.4. Analysis

After incubation the substrates were filtered at room temperature using filter paper (VWR, Leuven, Belgium) with pore sizes of 5-13 µm. A quarter of the solid residue was used to extract phenolic compounds from as described in chapter 2, the concentration of phenolic compounds in the extract were determined by the Folin-Ciocalteau method as described by Singleton et al. (1999). Half of the solid residue was anaerobically digested at 37 °C to determine the BMP over 30 days through a water

displacement system as described in (Gonzalez et al. 2013). The filtrate was used to determine the concentration of phenolic compounds, released by lignin breakdown, by the Folin-Ciocalteau method. Individual phenolic compounds of one of the three repetitions of both solid part as of the filtrate were measured with HPLC as a confirmation of the results obtained by the Folin-Ciocalteau analysis. HPLC analysis was performed as described in chapter 2. To determine the concentration of sugars in the filtrate, occuring due to the degradation of the lignocellulosic matrix, the reducing sugar content was measured spectrophotometrically at 550nm with the DNS-method as described by Miller et al. (1959). To determine the laccase activity in the samples, ABTS was oxidized by laccase at 35 °C. The laccase activity assay was performed in a 1.5 ml mixture consisting of 0.5 ml of 1 mM ABTS, 0.5 ml of 0.1 M acetate buffer (pH = 4.5) and 0.5 ml enzyme solution or sample. The laccase activity was measured spectrophotometrically at 420 nm ($\varepsilon 420$ = 36000 M cm^{-1}) after 5 minutes reaction time (Irshad et al. 2011).

5.3.5. Statistical analysis

The biogas production was measured daily for 30 days and was modeled as a function of time (t (d)) using a first-order model, $P = \gamma*(1-e^{(-\mu t)})$ according to Schroyen et al. (2014). In this first-order model biogas production is assumed to have an exponential rise to a maximum biogas yield, which is given by γ (Nl/kg VS). The exponential rise is characterized by the specific production rate μ (d^{-1}). The model was fitted using Microsoft Excel's solver to minimize the sum of squares of differences between the model and the measured biogas production. The first order model described the experimental values accurately, as the minimum Pearson correlation found was 0.89. For each experiment, the initial slope of the biogas production curve during the first 7 days of the experiment was calculated based on the calibrated model as a measure of the hydrolysis rate.

For all statistical analysis IBM® SPSS® Software version 23 was used. A two-way ANOVA was executed on all data including the fixed effects of enzyme treatment and incubation time, as well as the 2-way interaction term. As the 2-way interaction term was not significant, only the main effects are

reported. Comparison of mean was done using Tukey post hoc test ($p < 0.05$). Correlations were checked using a bivariate Pearson correlation.

5.4. RESULTS AND DISCUSSION

5.4.1. Impact of pretreatment on the lignocellulosic matrix

The hemp straw and miscanthus were biologically pretreated by an incubation with *P. eryngii* over several weeks. To study the effect of the white rot fungi, the substrate was not sterilized before, as high temperature and pressure would affect the lignocellulose structure. As a white rot fungus the *Pleurotus* species was chosen as they are selective degraders, causing lignin loss during pretreatment (Dong et al. 2013). The selectivity of degradation is an important factor, selectivity increases as lignin is broken down without loss of cellulose (Zhong et al. 2011; Mustafa et al. 2016). The degradation of lignin will cause a release of phenolic compounds. The increase of phenolic compounds released in the liquid fraction during incubation can be seen in Figure 5.1, a significant (p<0.05) difference was found between incubation periods of 14 and 21 days compared to longer incubations of 35 and 42 days. A longer incubation of more than 35 days is required to show the increase of release of phenolic compounds in the treated and untreated samples (Figure 5.1). Micro-organisms growing on the non-treated substrate degraded the substrate as well showing this significant increase of phenolic compounds after an incubation of 35 days. A more immediate effect is seen in the solid fraction as a reduction of extracted phenolic compounds is noted, a significant (p<0.05) decrease was noted after 14 days of incubation for the treated and untreated samples (Figure 5.2). The diminishing content of the phenolic compounds in the solid fraction, should give more rise to the concentration noted in the liquid fraction. *P. eryngii* is known to produce many different enzymes (Yang et al. 2016). In a study by Knezevic et al. (2013) *P. eryngii* and *P. ostreatus* are the only two fungi researched to produce laccase at high levels (2500-3000 U/L). Laccase can reduce the phenolic compounds and decrease the amount of the inhibiting compounds. Laccase activity was measured in this study, and an increased activity was determined after incubation of 7 and 14 days with hemp straw and after incubation of 21 days with miscanthus. The activity however was lower than reported in Knezevic et

al. (2013) and Dong et al. (2011), since maximally 1000±600 U/L were determined in the liquid fraction of hemp straw after 14 days of incubation and a laccase activity of 333±136 U/l was measured in the liquid fraction after 21 days of incubating miscanthus. The lower enzyme activity in the samples of miscanthus could be due to the higher lignin content of the substrate compared to hemp straw. Knezevic et al. (2013) reported laccase activity after 7 days of incubation and an increase at 14 days while pretreating wheat straw with *P. eryngii* and *P. ostreatus.* The composition of the substrate has an important role in the production of the various enzymes, as sugarcane bagasse biologically pretreated with *P. ostreatus* showed laccase production of over 10000 U/L after specifically 1, 6 and 9 weeks of incubation (Dong et al. 2013). The high production of enzymes was very effective during the pretreatment of sugarcane bagasse, as a lignin degradation of 85 % by *P. ostreatus* over an incubation of 12 weeks was noted, which is greater than ever reported before (Dong et al. 2013). The compositon of sugarcane bagasse compared to miscanthus shows a much lower lignin content as Rabelo et al. (2011) reported sugarcane bagasse had a lignin content of 10 g/100 g DM, while miscanthus has a higher lignin content of 25 g / 100 g DM (de Vrije et al. 2002).

Most studies do not report on the concentration of phenolic compounds, but Martinez et al. (2001) did note that *P. eryngii* reduced the amount of *p*-hydroxyphenyl and guaiacyl lignin units in wheat straw. The laccase produced after 7 and 14 days during the incubation of hemp straw and after 21 days during the incubation of miscanthus did result in the minimum concentration of phenolic compounds measured of 46 mg/l and 77 mg/l in the liquid fraction of respectively hemp straw and miscanthus during this experiment (Figure 5.1). This indicates the possibility that the laccase produced by *P. eryngii* decreased the amount of phenolic compounds.

Concentrations of several individual phenolic compounds, vanillic acid, 4-hyrdoxybenzoic acid, *p*-coumaric acid and ferulic acid were analyzed individually in the solid and liquid fraction during the incubation. Although an increase of the total phenolic content was seen in the liquid fraction concentrations of these individual phenolic compounds were below detection limit. This was

unexpected as enzymatic degradation of hemp straw and miscanthus with laccase and versatile peroxidase showed an increase of vanillic acid, 4-hydroxybenzoic acid and *p*-coumaric acid in chapter 3. *P. eryngii* produces many other enzymes for lignin degradation, including 13 copper radical oxidases, 11 multicopperoxidases and 6 class II peroxidases, which could degrade the phenolic compounds as well (Yang et al. 2016). As the phenolic compounds were not also found in the non-treated samples it could mean the individual phenolic compounds were not released or were degraded as well by micro-organisms present naturally.

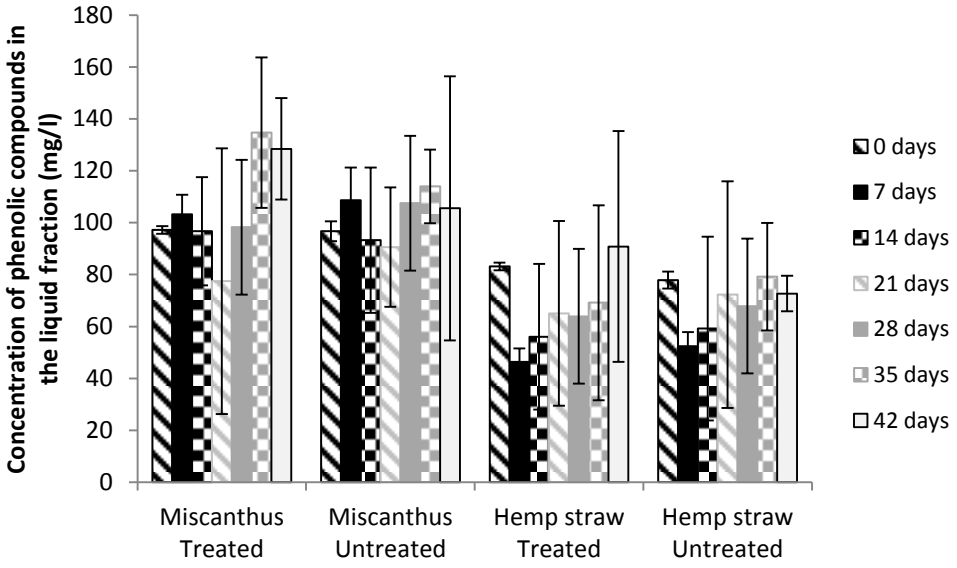

Figure 5.1: Concentration of the total phenolic compounds in the liquid fraction of the treated and untreated samples of hemp straw and miscanthus taken weekly for 42 days (n=3)

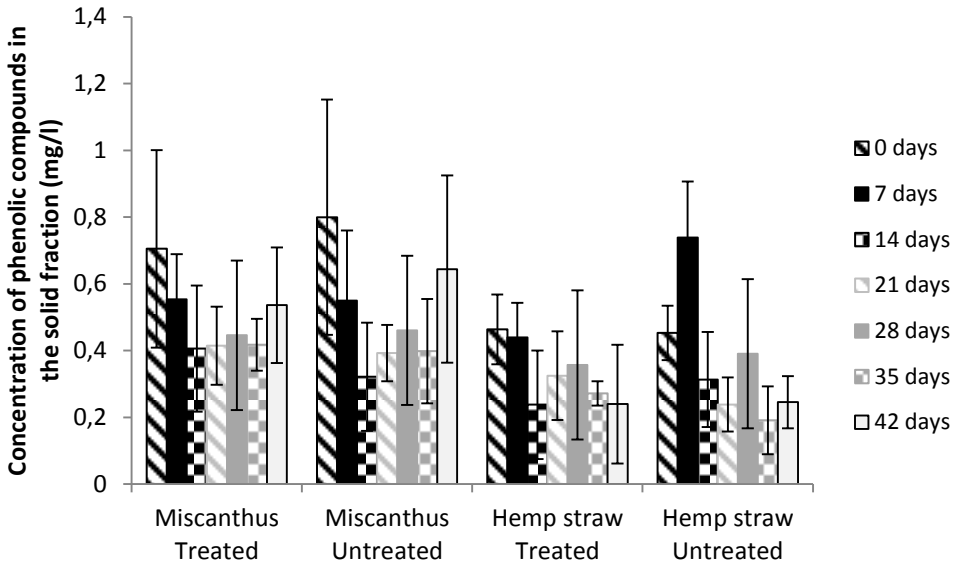

Figure 5.2: Concentration of the total phenolic compounds in the solid fraction of the treated and untreated samples of hemp straw and miscanthus taken weekly for 42 days (n=3)

To increase the hydrolysis rate during biogas production the removal of lignin must coincide with an increase of the available surface area of the cellulose fraction. Micro-organisms convert the cellulose to sugars which are converted to biogas during anaerobic digestion. During pretreatment it is important to reduce the sugar loss due to consumption by the fungi. Figure 5.3 shows the concentration of reduced sugar in the liquid fraction. Easy accessible sugars present at the start of the incubation were directly used by the fungi as a drop occurs after 1 week. Due to further pretreatment more sugars are available as the incubation proceeds. An increase of cellulose crystallinity as indicated by Shirkavand et al. (2016) occurs often during a biological pretreatment with white rot fungi as amorphous regions of the cellulose polymer are degraded. The initial loss of sugar however should be avoided as it indicates a loss of cellulose and a drop in selectivity resulting in a lower BMP.

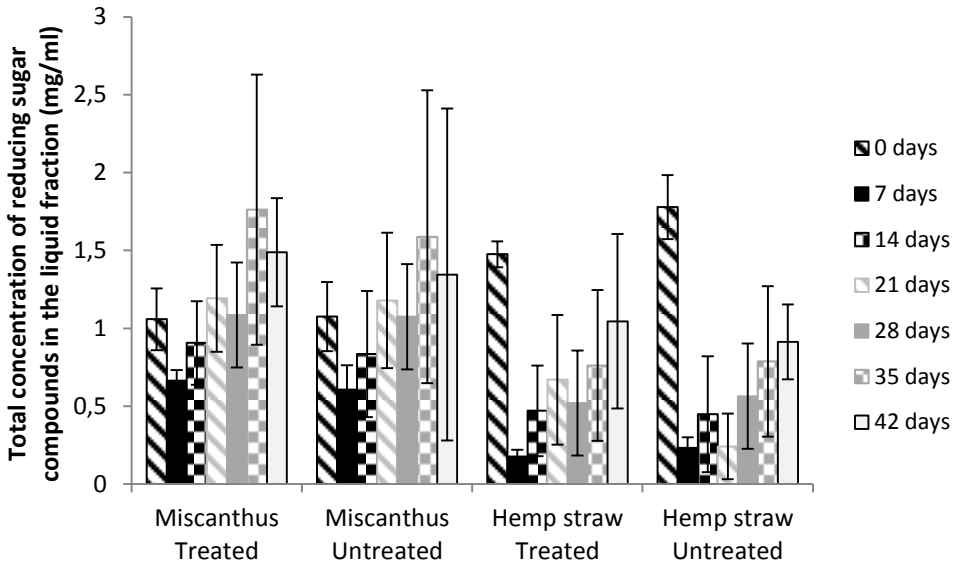

Figure 5.3: Total concentration of the total reducing sugars in the liquid fraction of the treated and untreated samples of hemp straw and miscanthus taken weekly for 42 days (n=3)

5.4.2. BMP-assay

The substrate was incubated for maximal 6 weeks and BMP tests were performed on the treated and untreated samples after each week. The increase in release of reducing sugars and phenolic compounds, indicated that the matrix was being degraded. However no increase in the biogas production was seen, as observed in the final biogas production after 30 days of anaerobic digestion as calculated by the first order model (Figure 5.4). Nonetheless the final biogas production was not affected by the white rot fungi, however a difference was noted if the hydrolysis rate was considered (Figure 5.5). As micro-organisms consumed the easy available sugars and amorphous regions, the rate of hydrolysis dropped with longer incubations. As the total biogas production was not decreased, the degradation caused during the incubation must have loosened the recalcitrant parts of the matrix, making it easier accessible later during the anaerobic digestion.

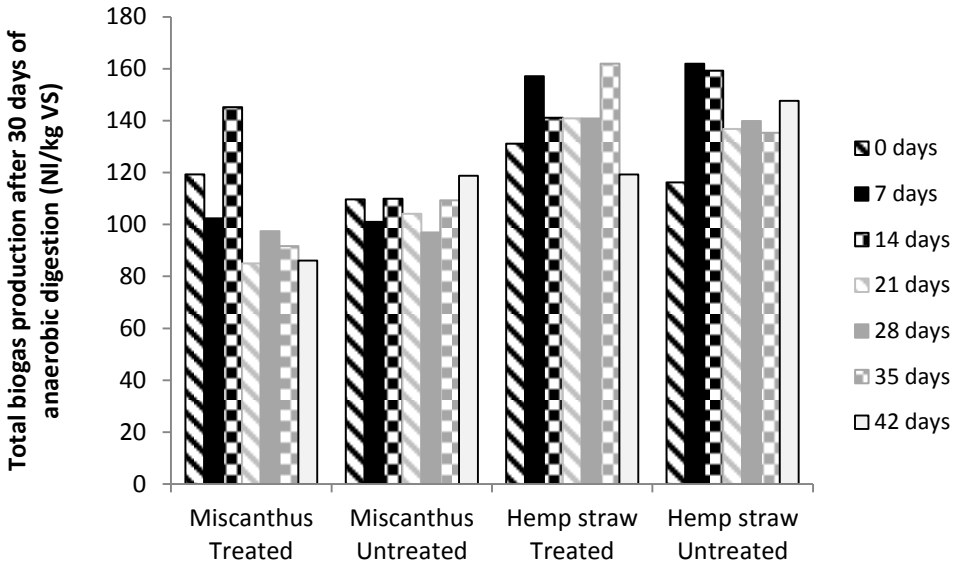

Figure 5.4: Total biogas production after 30 days of anaerobic digestion of the treated and untreated substrates, hemp straw and miscanthus, according to the exponential model calculated based on three independent replicates

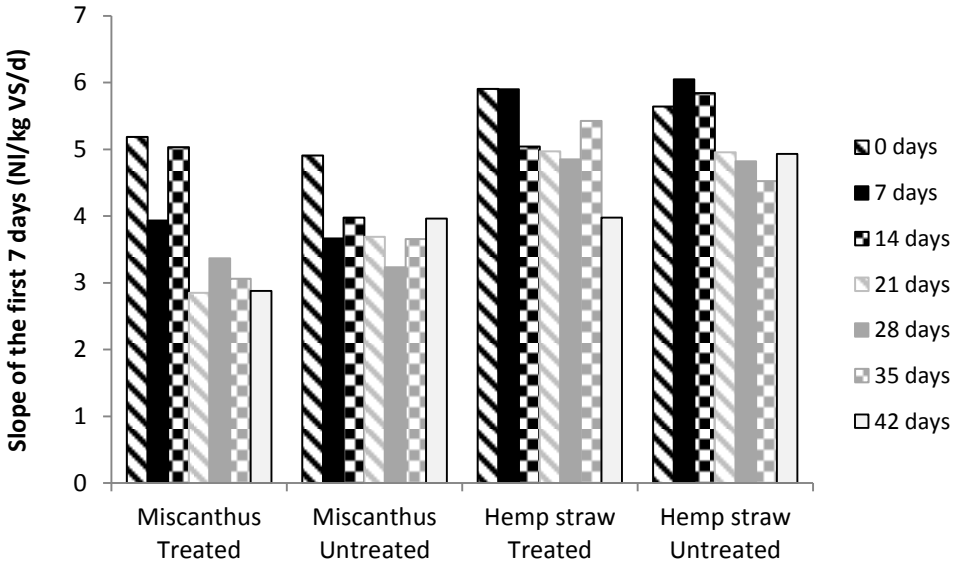

Figure 5.5: The initial slopes of the first 7 days of the biogas curves from the exponential model calculated from three independent anaerobic digestions of treated and untreated substrates hemp straw and miscanthus

A decrease in biogas production was seen by Tuyen et al. (2013) after 3 weeks of incubating maize stover and rice straw with *P. eryngii*, however an increased production was seen after 6 weeks of incubation. Significantly more biogas production was found after incubating sugarcane bagasse with *P. eryngii* for 95 days, while incubating 35 and 65 days did not show a significant difference (Okano et

al. 2007). The various results show the interrelation between the substrate and incubation times required vary greatly. A difference between substrates was even noted between harvest periods. Miscanthus that was harvested in fall and spring yielded 170 l CH_4/kg VS after 60 days of anaerobic digestion for both harvests when no pretreatment was applied. Pretreatment with *C. subvermispora* increased the biogas production of the spring harvest miscanthus by 25 %, while a pretreatment of the fall harvest miscanthus decreased the biogas production after 60 days (Vasco-Correa and Li, 2015). Such a drop of methane production was not seen in the experiments presented in this study after lengthening the anaerobic digestion to 60 days (Figure 5.6).

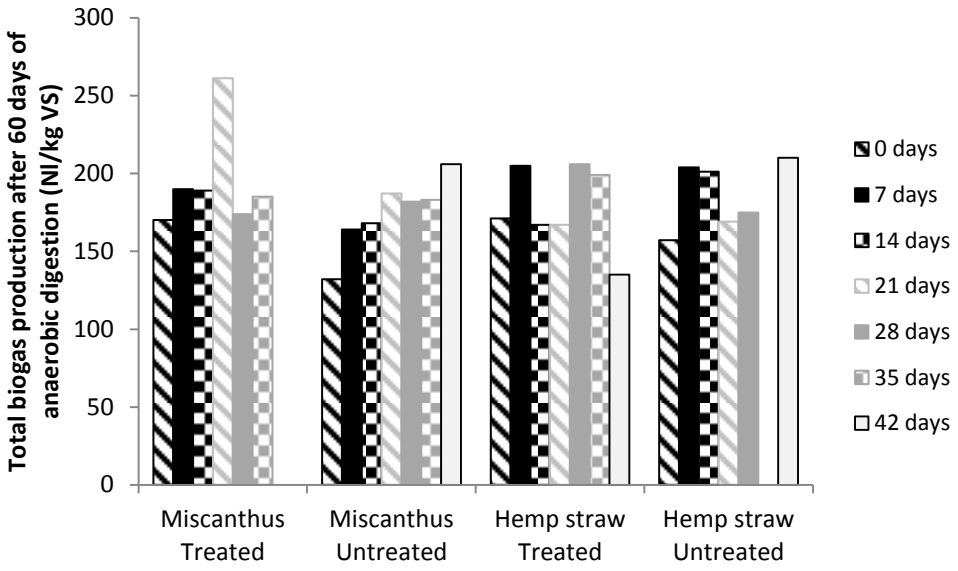

Figure 5.6: Total biogas production after 60 days of anaerobic digestion of the treated and untreated substrates, hemp straw and miscanthus (n=1)

The biomethane production of the untreated miscanthus is comparable to the BMP obtained in a previous experiment described in chapter 2, however the biomethane production of hemp straw obtained for this experiment was significantly lower compared to hemp straw acquired from InAgro (Roeselare, Belgium) which was digested during the study described in chapter 2. This shows that the choice of substrate is important, and even within one biomass source great variation occurs as was indicated in chapter 1 (Table 1.1). The significantly lower BMP of hemp straw in this experiment resulted in a much lower initial biomethane production as well as the initial slope of

hemp straw was distinctively decreased when compared to results in previous chapters (Figure 5.6). In order to have an economically feasible pretreatment, more research must be performed to increase the control during the degradation process and to optimize conditions during the pretreatment to increase the BMP. To improve hydrolysis as well as biogas production, and reduce incubation time, fungal pretreatments can be combined with traditional pretreatments under milder conditions, reducing environmental impact and cost (Shirkavand et al. 2016). Indeed, a combination of pretreatments revealed positive results of lignin and hemicellulose removal (Wan & Li, 2012; Yang et al. 2013), although an extra step in the production process is required.

5.5. CONCLUSION

A pretreatment of 6 weeks of hemp straw and miscanthus with *P. eryngii* did not improve the biogas production. *P. eryngii* however broke down the lignin barrier partly, releasing phenolic compounds which were broken down further by the production of laccase. The decrease of phenolic compounds in the liquid fraction due to the produced laccase shows opportunities for further research as white rot fungi can have a detoxifying effect of hydrolysates by diminishing inhibiting compounds. Sugars were made more accessible during the pretreatment, however these sugars were consumed by micro-organisms resulting in a lower hydrolysis rate during biogas production. A selective white rot fungi alone degrading the lignocellulose structure, should increase the cellulose/lignin ratio and decrease the sugar loss. The total biogas production after an anaerobic digestion of 30 or 60 days was unaffected by the loss of sugars.

CHAPTER 6: MODELLING AND SIMULATION OF ANAEROBIC DIGESTION OF LIGNOCELLULOSIC BIOMASS: INFLUENCE OF SUBSTRATE AND PHENOLIC COMPOUNDS

Redrafted from

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6. MODELLING AND SIMULATION OF ANAEROBIC DIGESTION OF LIGNOCELLULOSIC BIOMASS: INFLUENCE OF SUBSTRATE AND PHENOLIC COMPOUNDS

6.1. ABSTRACT

Hydrolysis of lignocellulosic substrates is impeded by the lignin polymer, acting as a seal around the cellulose and hemicellulose polymer. To facilitate hydrolysis and improve biomethane production pretreatment of the substrate is required, however harsh pretreatments can cause a release of inhibitory phenolic compounds i.e. vanillic acid, *p*-coumaric acid, ferulic acid and hydroxybenzoic acid. In this study the developed anaerobic digestion model takes the lignin concentration as well as the concentration of such phenolic compounds into account. The biomethane production and hydrolysis rate of seven different substrates were simulated. A good agreement between simulations and measurements was obtained, as the maximum Theil's inequality coefficient for the different substrates was 0.14. The impact of higher concentrations of the phenolic compounds, up to 2000 mg/l, was simulated for two of the substrates namely, hemp straw and miscanthus. As significant inhibition only occurred for the anaerobic digestion of miscanthus, a global sensitivity analysis and parameter identifiability analysis (assessing all the processes in the model) was done for this substrate. The global sensitivity analysis showed the great importance of the hydrolysis rate and the need to research factors, i.e. inhibitors and substrate types, influencing this hydrolysis step.

6.2. INTRODUCTION

Fossil fuel reserves keep diminishing, which is one of the reasons why the need for different energy sources is increasing (Divya et al. 2015). Bioenergy obtained from biomass is as such a promising alternative. Agricultural waste is an abundant source of lignocellulosic material, which is not in competition for food, nor feed. This low cost material can be used to produce biomethane via anaerobic digestion. Several studies have shown a negative correlation between lignin concentration of a substrate and its biomethane potential (BMP) (Triolo et al. 2011; Dandikas et al. 2014; Schroyen et al. 2015). Furthermore the hydrolysis process step in the anaerobic digestion is the rate limiting

step, this is due to the recalcitrant lignin polymer surrounding the cellulose and hemicellulose chains (Appels et al. 2008).

There is a great variety of agricultural waste with different concentrations of lignin. Estimation and/or enhancement of the hydrolysis rate is of great importance for assessing the overall digestion performance. To facilitate the hydrolysis, pretreatment of the biomass can be performed as it will degrade the lignin, and thus can improve the BMP (Li et al. 2010). However during harsh pretreatments a release of weak acids, furan derivatives and phenolic compounds occurs. These compounds are known to inhibit the initial rate of biogas production and more importantly the hydrolysis rate (Kayembe et al. 2013). Harsher pretreatments like alkaline or thermal pretreatments can increase the released concentration of phenolic compounds, i.e. *p*-coumaric acid, ferulic acid, vanillic acid and 4-hydroxybenzoic acid, to inhibitory levels. These concentrations (>1000 mg/l) have to be taken into account when performing an anaerobic digestion, and play an important role in estimating the hydrolysis step (Hernandez & Edyvean, 2008).

Modelling is an efficient tool to gain knowledge on the potential of the used substrate and the prevailing concentrations of phenolic compounds. The Anaerobic Digestion Model 1 (ADM1) is a commonly used anaerobic digestion model, however it focuses only on sewage sludge (Batstone et al. 2002). The use of different types of substrates has since then resulted in modifying the model. A co-digestion of manure and energy crops was studied and modeled by Lubken et al. (2007). Gali et al. (2009) proposed a modified version for agro-waste application, emphasizing the need to characterize the substrate. Appropriate modifications allowed a simulation of the anaerobic digestion of microalgae (Mairet et al. 2011). Klimiuk et al. (2015) included fractionation of maize silage and cattle manure mixture in the ADM1.

The main disadvantage of the ADM1 model (and its modifications) is that it requires the calibration of a large number of parameters and the determination of several variables, which is as a consequence difficult for implementation in plant operation (Yang et al. 2016). Therefore in this

study a simplified anaerobic digestion model is proposed based on the model presented in Van Hulle et al. (2014). This model describes the degradation of solid waste to biogas via a 3 step process. The model assumes the insoluble organic matter or volatile suspended solids (VSS) is hydrolysed to volatile dissolved solids (VDS) respectively through first-order kinetics. Acetogenic bacteria transform the VDS to volatile fatty acids (VFA), which were transformed by methanogenic bacteria to methane according to Monod kinetics. In this study, the model developed by Van Hulle et al. (2014) was extended in order to take the lignin content of the substrate into account as well as the impact of phenolic compounds. The first order hydrolysis kinetics were adapted to account for the lignin content as well as for the inhibition due to the phenolic compounds. The model is used to assess the biogas production of 7 substrates with different lignin content, while the impact of phenolic compounds is simulated for 2 of these substrates with data obtained from chapters 2 and 4. A global sensitivity analysis is performed to identify the model parameters with the most influence on the predicted biogas concentration. The identifiability of these parameters is also assessed.

6.3. MATERIALS AND METHODS

6.3.1. Experimental data collection

The experimental data used in this study was obtained in a previous study (Schroyen et al. 2015). Seven different lignocellulosic substrates were characterized in Schroyen et al. (2015). Lignin concentration, release of phenolic compounds and BMP over 30 days were determined. Anaerobic digestion was done in a lab-scale completely stirred tank reactor (CSTR) with an overall volume of 0.25 l operated in batch mode (Antonopoulou et al. 2015) at 37 °C with a substrate to inoculum ratio of 0.5 (g VS/g VS). The substrates, corn stover, ensilaged maize, wheat straw, flax straw, hemp straw, miscanthus and willow, were mixed with inoculum which was collected from a co-digestion plant treating cow manure and maize silage. The inoculum was stored at 4 °C until 3 days before use, when it was placed at 37 °C. The biogas production was measured daily via a water displacement system and samples were taken 3 times during the anaerobic digestion to determine the methane (±70%) and carbon dioxide content by gas chromatography (GC). The BMP analysis was repeated 3 times,

however averages of the daily biomethane production were taken of at least 2, in case of missing data, and maximum 3 repetitions. The inhibition of the anaerobic digestion was examined while adding 0, 100, 500, 1000 and 2000 mg/l of the individual phenolic components, vanillic acid (VA), *p*-coumaric acid (PCA), 4-hydroxybenzoic acid (HBA) and ferulic acid (FA) to the inoculum with hemp straw or miscanthus.

6.3.2. Reaction and reactor model

The simplified model following Monod kinetics proposed by Van Hulle et al. (2014) was extended with an inhibition term for phenolic compounds and a term for the lignin content (Table 6.1). Thus the effect of lignin content and the concentration of phenolic compounds on hydrolysis were taken into account. The VSS are transformed to VDS according a first-order kinetics (in terms of VSS). This hydrolysis is influenced by the lignin and total phenolic content (as indicated by the (adapted) Monod kinetics). An increased content of lignin (C_L) will slow down the hydrolysis and stop the process if the lignin content becomes too high (Schroyen et al. 2014). The concentration of the inhibitory phenolic compounds is dependent of the substrate type and phenolic compound, and will inhibit the hydrolysis until a minimum is reached defined by a setpoint (SP), which presents a value between 0, possible maximum inhibition, and 1, no inhibition by the phenolic compounds. In a next step, VDS was converted to VFA, which was transformed further to methane by respectively acetogenic and methanogenic bacteria following Monod kinetics (Van Hulle et al. 2014). Acetogenic and methanogenic bacteria were also assumed to undergo a decay process, modelled with first order kinetics (Table 6.1) (Silva et al. 2009). Similar to ADM1, this model used Chemical oxygen demand (COD) as a base unit (Batstone et al. 2004).

Table 6.1: Gujer Matrix of the anaerobic digestion model, with inhibition of lignin and phenolic compounds, used in this work

Process	VSS	VDS	VFA	CH_4	X_1	X_2	Process rate
Hydrolysis	*-1*	*1*					$k_1[VSS]\frac{K_L}{K_L+C_L}\frac{SP*C_P+K_I}{C_P+K_I}$
VFA formation		*-1*	*1-Y_1*		*Y_1*		$k_2\ \frac{[VDS]}{k_3+[VDS]}\ [X_1]$
CH_4 formation			*-1*	*1-Y_2*		*Y_2*	$k_4\ \frac{[VFA]}{k_5+[VFA]}\ [X_2]$
Decay acetogenic bacteria					*-1*		$b_1\ [X_1]$
Decay methanogenic bacteria						*-1*	$b_2\ [X_2]$

To determine the initial value of the VSS, data from chapter 2 was taken. The VSS value for ensilaged maize with a small amount of lignin and a BMP of 414 Nl/kg VS was measured to be 40 g COD/l, as 30 g VSS/l was introduced to the reactor and multiplied with the conversion factor of 1.33 g COD/g VSS (Rittmann and Mccarty, 2000). The VSS values of the other substrates were obtained from the BMP values in Table 2.2, relatively to the VSS value of ensilaged maize.

Most parameter values presented in Table 6.3 were derived from Van Hulle et al. (2014), while the affinity constants K_I and K_L were manually calibrated based on the insights of performed experiments (Table 6.3). Lignin concentration (C_L) was substrate dependent, taken from chapter 2 and ranged from 0.8 to 17 g/100g DM. Also the concentration of phenolic compounds (C_P) varied in every experiment from 17 to 74 mg/l, as the increase in concentration of total phenolic components was used from chapter 2 (Table 2.2). The parameter SP was chosen as 1 for hemp straw as no inhibition by the phenolic compounds was noted, while for miscanthus a value of 0.4 was taken when FA or HBA was added and a value of 0.8 when, PCA or VA was added. As an increased inhibitory effect was seen with an increased concentration of phenolic compounds during the anaerobic digestion of miscanthus. The parameter SP was required as biomethane production is not reduced to zero if great concentrations of phenolic compounds are added. The anaerobic digestions of hemp straw and miscanthus with the addition of the various phenolic compounds were performed with different sludges, resulting in different BMP values if no phenolic compounds were added. Hence different

initial VSS values were used in the simulations. The VSS value of hemp straw in the experiments with FA and HBA was 12.33 g/l and 18.86 g/l with VA and PCA. The VSS value of miscanthus in the experiments with FA and HBA was 8.2 g/l and 11.06 g/l with VA and PCA.

Table 6.2: The substrates and BMP values used to calibrate the model based on the different lignin concentrations and initial VSS values without the addition of phenolic compounds

Substrate	BMP (Nl/kg VS)	VSS (g COD/l)	C_L (g/100 g)	C_P (mg/g)
Ensilaged maize	413.9	40.0	0.8	48
Corn stover	242.4	22.8	4.5	35
Wheat straw	247.1	22.8	6.0	47
Flax straw	233.1	21.6	8.6	17
Hemp straw	237.8	21.6	9.2	42
Miscanthus	144.5	13.2	12.0	46
Willow	88.6	8.0	17.0	74

Table 6.3: Parameter values for the kinetic model used in this study

Parameter	Value
k_1	0.2 d^{-1}
k_2	8 d^{-1}
k_3	10 g COD/l
k_4	8 d^{-1}
k_5	1.2 g COD/l
b_1	0.16 d^{-1}
b_2	0.16 d^{-1}
K_I	150-3000 mg/l
K_L	35 g/100 g DM

The model was simulated using *R*, while the Flexible Modeling Environment (FME) package allowed sensitivity analysis and parameter estimations (Soetaert and Petzoldt, 2014). The initial active biomass concentrations of the simulation were X_1 = 0.7 g COD/l and X_2 = 0.7 g COD/l while initial concentrations of VDS, VFA and CH_4 were assumed to be 0 g COD/l. Biomethane production was measured over 30 days giving a final BMP value at the end of the digestion. The slope, k_7, of the first 7 days was determined and used as a rate of hydrolysis (Schroyen et al. 2015). These experimental

biomethane curves as well as the simulated curves are displayed in Figure 6.1 and will be discussed further.

6.3.3. Model performance analysis

The goodness of fit of the model was validated by determining the Theil's inequality coefficient (TIC) as follows:

$$TIC = \frac{\sqrt{\sum_i (y_i^2 - y_{i,m}^2)}}{\sqrt{\sum_i y_i^2} + \sqrt{\sum_i y_{i,m}^2}}$$

y_i, $y_{i,m}$ representing respectively the simulated and the measured data points of the bio-methane concentration at each day. A good fit is indicated by a TIC value lower than 0.3 (Hvala et al. 2005; Vandekerckhove et al. 2008).

A global sensitivity analysis was performed using the Monte Carlo simulation (MCS) technique, assuming a uniform distribution of the parameters. Sampling was done through Latin hypercube sampling and a total of 1500 simulations were run at 7 days in the anaerobic digestion. To analyze the MCS a linear regression between biogas production and the different input parameters (k_1, k_2, k_3, k_4, k_5, b_1, b_2 and K_L) was used to obtain the regression coefficients. As parameter space minimum and maximum values were respectively 50 % and 150 % of the used values (Neumann et al. 2009), K_I was set to 150 mg/g for miscanthus and an SP value of 0.4 was used if VA or PCA were added (Table 6.3). The effect of the parameters were evaluated by the t-statistic value calculated from the standard errors of the regression coefficients. The impact of a parameter is significant when the t-statistic value exceeds 1.96 (Saltelli et al. 2005).

To check the identifiability of the parameters the simulation was done over the complete duration of the anaerobic digestion of miscanthus(30 d). The sum of squared errors was calculated for all simulations and were plotted for all parameters, indicating identifiability if a clear minimum is found for a parameter (Decostere et al. 2016).

6.4. RESULTS AND DISCUSSION

6.4.1. BMP and hydrolysis rate predictions of various lignocellulosic substrates

Seven different substrates were anaerobically digested and the experimental biomethane production was measured. With the model described in this work, the biomethane production was also simulated. For these simulations the effect of the phenolic compounds was not taken in to account as the concentrations of phenolic compounds were too low to have a significant inhibitory effect (Table 6.2). Overall the predictive power of the lignin driven model shows a good fit as TIC values vary from 0.03 to 0.14. A difference between the experimental data and the simulated data can be noted at 30 days, as the experimental curves still show a small increase of biomethane production at the end of the anaerobic digestion, while the model assumes that all material is digested and converted into biogas after 30 days. Therefore the model reaches a maximum in biomethane production after 30 days (Figure 6.1). Figure 6.1C and 6.1G show the measured and simulated biomethane production of respectively flax straw and ensilaged maize. These two substrates showed a higher hydrolysis rate than was used in the model. Ensilaged maize underwent an acid pretreatment during the ensilation which could explain the faster hydrolysis process. Flax straw showed the smallest amount of total phenolic compounds in the liquid as well as the solid fraction in previous experiments (Schroyen et al. 2015), providing a possible larger hydrolysis rate.

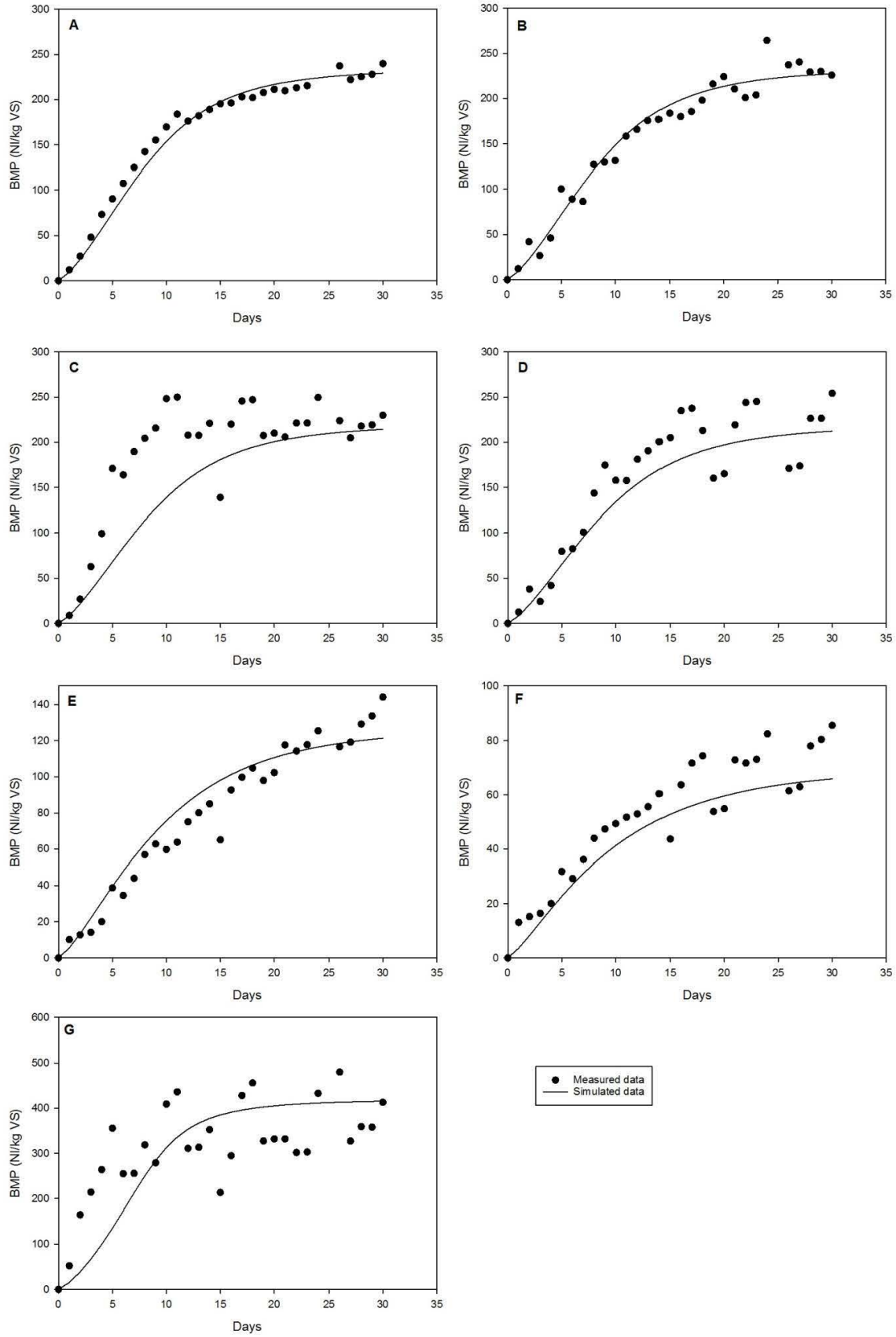

Figure 6.1: Measured and simulated biomethane curves of the various substrates, corn stover (A), wheat straw (B), flax straw (C), hemp straw (D), miscanthus (E), willow (F) and ensilaged maize (G). Averages of measured data taken from at least 2 and maximum 3 reactors.

The resulting simulated and measured BMP values, based on the methane content, after 30 days of anaerobic digestion show a high agreement (Figure 6.2A). Figure 6.2B represents the simulated and measured hydrolysis rates, defined by $k_{7,}$ the slope of the biomethane production in the first 7 days. To have an even better estimation of the hydrolysis rate for each substrate the k_1 value could be estimated for every substrate individually, however a good fit was obtained with k_1 = 0.2 d^{-1} and individual fitting is therefore not necessary.

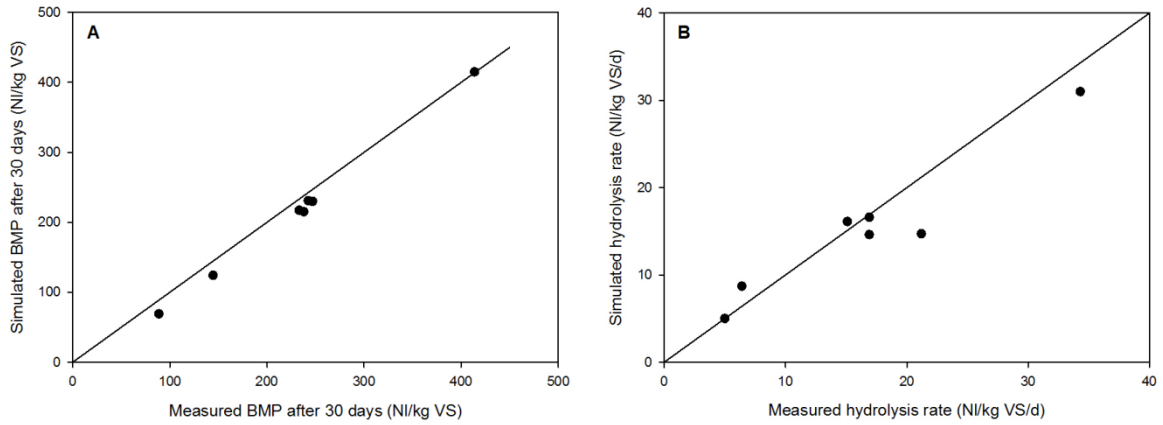

Figure 6.2: Experimental (n=3) and simulated BMP values of the different substrates (R^2=0.988) (A), experimental (n=3) and simulated hydrolysis rate of the various substrates (R^2=0.897) (B), represented with the bisector shown

6.4.2. Influence of inhibiting phenolic components

The lignin concentration of the various substrates plays an important role in the total biogas production as well as in the initial hydrolysis rate during anaerobic digestion. Degrading the lignin through harsh pretreatments could resolve this, however lignin degradation will also lead to the release of phenolic compounds in higher concentrations. These phenolic compounds have a negative impact on the hydrolysis of a substrate. In previous studies inhibition by phenolic compounds was seen in the anaerobic digestion of miscanthus. Hemp straw contains less lignin and thus the cellulose and hemicellulose have a higher availability. As such, only high concentrations (2000 mg/l) of phenolic compounds led to a slight inhibition in the hydrolysis rate. To implement the inhibition the SP value was calibrated manually for each substrate and each phenolic component. No significant inhibition by any of the phenolic compounds on the anaerobic digestion of hemp straw was seen. As

such SP was set to 1 for hemp straw. However the addition of 2000 mg/l FA and 4-HBA to miscanthus caused an average inhibition of the hydrolysis rate of 22 %. VA and p-CA acid inhibited the hydrolysis rate up to 50 % at a concentration of 2000 mg/l. The difference in inhibition resulted in two different SP-values for the individual phenolic compounds: SP = 0.4 was used for VA and p-CA, while SP = 0.8 was used for FA and 4-HBA.

Table 6.4 shows the measured and simulated BMP and hydrolysis rates of hemp straw. As there was no significant inhibition the simulated values are the same for every concentration (0, 100, 500, 1000 and 2000 mg/l) of added phenolic compounds. The addition of FA and 4-HBA had even a non-significant positive effect on the experimental hydrolysis rate. The model did give a good prediction of total BMP after 30 days of anaerobic digestion, and every individual experiment had a TIC value smaller than 0.3.

Table 6.3: Measured and simulated hydrolysis rate (k_7), and measured and simulated BMP of the anaerobic digestion of hemp straw with the addition of the different phenolic compounds at concentrations of 100, 500, 1000 and 2000 mg/l (VA = vanillic acid, FA = ferulic acid, PCA = *p*-coumaric acid and HBA = 4-hydroxybenzoic acid) (n=20)

Phenolic Compound	Measured k_7 (Nl/kg VS/d)	Simulated k_7 (Nl/kg VS/d)	Measured BMP (Nl/kg VS)	Simulated BMP (Nl/kg VS)
VA	16.5 ± 7.0	13.0	178.3 ± 68.9	185.2
FA	16.7 ± 5.9	8.5	143.8 ± 52.3	115.7
PCA	15.5 ± 7.6	13.0	176.0 ± 73.6	185.2
HBA	15.7 ± 4.9	8.5	119.6 ± 52.9	115.7

Table 6.4 and Figure 6.3 present the measured and simulated values of BMP and hydrolysis rate of miscanthus. As the inhibition due to the phenolic compounds is included in the model, the hydrolysis rate can be predicted better. However the model underestimates the BMP, as the model assumes that the anaerobic digestion is finished after 30 days. Nonetheless a good fit has been achieved as the average TIC-value of all experiments per phenolic component was smaller than 0.15 and overall was smaller than 0.3.

Table 6.4: Measured and simulated hydrolysis rate (k_7), and measured and simulated BMP of the anaerobic digestion of hemp straw with the addition of the different phenolic compounds at concentrations of 100, 500, 1000 and 2000 mg/l (VA = vanillic acid, FA = ferulic acid, PCA = *p*-coumaric acid and HBA = 4-hydroxybenzoic acid) (n=20)

Phenolic compound	Initially added concentration (mg/l)	Measured BMP (Nl/kg VS)	Simulated BMP (Nl/kg VS)	Measured hydrolysis rate (Nl/kg VS/d)	Simulated hydrolysis rate (Nl/kg VS/d)
VA	0	114	102	5.8	7.3
	100	81	93	3.2	6.0
	500	87	79	3.3	4.6
	1000	81	73	3.2	4.2
	2000	74	70	1.0	4.0
FA	0	87	73	5.6	5.5
	100	107	72	6.5	5.3
	500	86	70	5.6	5.1
	1000	81	69	4.7	5.0
	2000	92	68	4.3	4.8
PCA	0	114	102	5.8	7.3
	100	89	93	5.2	6.0
	500	92	79	3.8	4.6
	1000	96	73	3.3	4.2
	2000	81	70	3.5	4.0
HBA	0	87	73	5.6	5.5
	100	90	72	6.5	5.3
	500	82	70	2.2	5.1
	1000	84	69	5.0	5.0
	2000	96	68	4.9	4.8

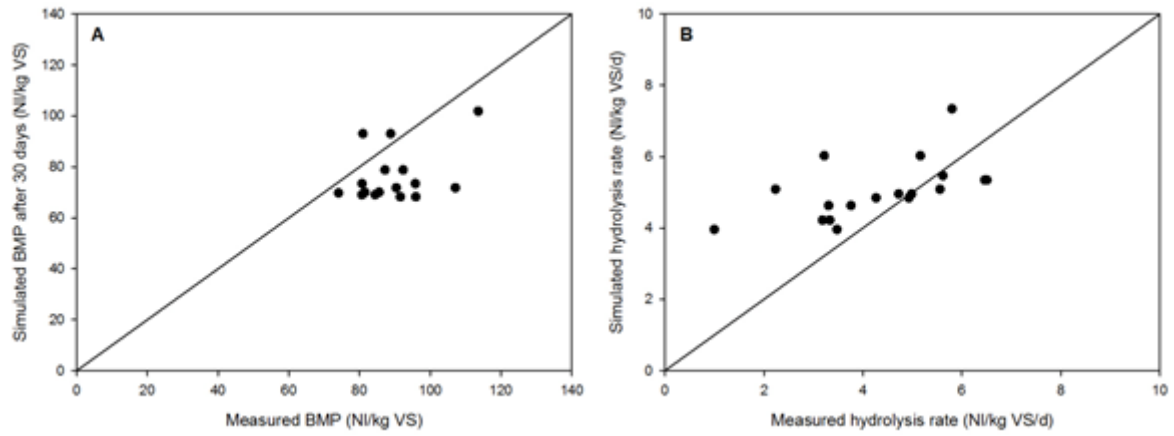

Figure 6.3: Experimental (n=15) and simulated BMP values (A) and the experimental (n=20) and simulated hydrolysis rate (B) of the anaerobic digestion of miscanthus with the addition of phenolic compounds, represented together with the bisector

6.4.3. Global sensitivity analysis of the miscanthus model

Inhibition was only included in the simulations of the anaerobic digestion of miscanthus. The full model capability is therefore only exploited when simulating the anaerobic digestion of miscanthus and therefore only data for this substrate is considered for further model analysis.

A global sensitivity analysis was done with the starting values of miscanthus with 0 and 500 mg/l of VA or PCA added. A Monte Carlo run with 1500 simulations was performed for the different experiments. Figure 6.4A shows the tornado plots summarizing the sensitivities of all model parameters with respect to biomethane production after 7 days of digestion without the addition of a phenolic compound. Figure 6.4B shows the results for the experiment with 500 mg/l VA or PCA (SP=0.4).

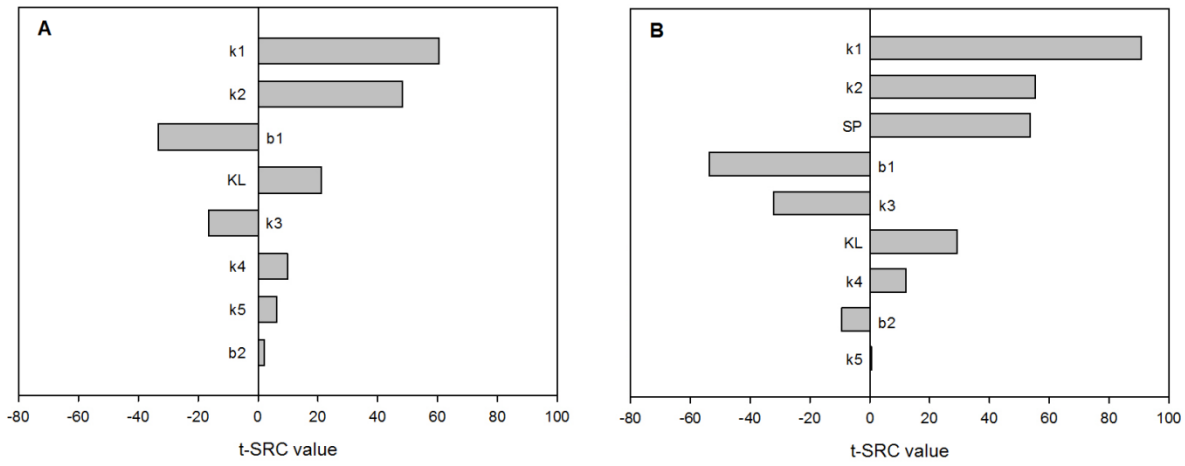

Figure 6.4: Tornado plot of a Monte Carlo run (n=1500), showing the sensitivity of the biomethane production during anaerobic digestion without the addition of phenolic compounds (A) and with the addition of 500 mg/l vanillic acid or *p*-coumaric acid

In figure 6.4A and 6.4B it can be seen that k_1 and k_2 are the most sensitive parameters. Parameter k_1 emphasizes the importance of the hydrolysis step during anaerobic digestion. The maximum hydrolysis rate was noted as the most sensitive parameter by Myint et al. (2007), which is in agreement with previous reports of hydrolysis being the rate limiting step in the digestion (Higuchi et al. 2005). From figure 4B it can be deduced that the parameter SP, specifying the level of inhibition at larger concentrations of the phenolic compounds have a sensitive impact on the biogas production.

The positive values of the sensitivities for k_1, k_2 and SP indicate that increasing the parameter will cause a higher biogas production. On the other hand, the negative value of the sensitivity for b_1 signifies that the decay of the acetogenic bacteria has a decreasing effect on the biogas production.

Since there are many significant ($t > 1.96$) parameters, the identifiability of the 3 most significant parameters was examined (Figure 6.5). In Figure 6.5B and 6.5F minima for sum of squared errors can be found for respectively k_1 and b_1, given the potential of identifying the optimal value in the range of 0.1 to 0.2 $d^{-1}$for these two parameters. The values used in the simulations in this study are within this range, showing a good performance of the proposed model.

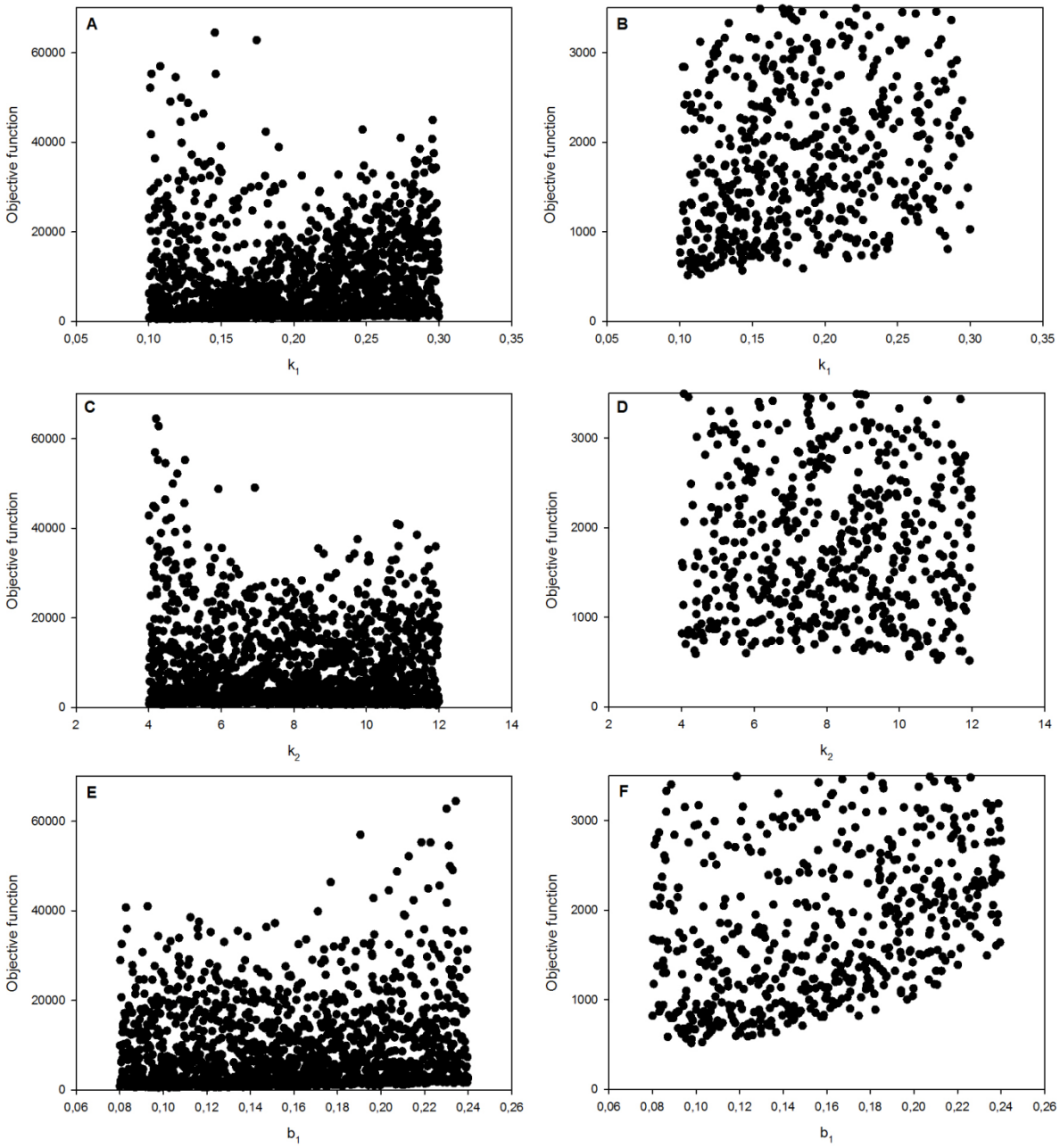

Figure 6.5: Scatter plots on the left for k_1 (A), k_2 (C) and b_1 (E) to investigate the parameter identifiability by calculating the sum of errors. The minimal of the scatter plots on the right for k_1 (B), k_2 (D) and b_1 (F) to detect the minimum of the sum of errors

6.5. CONCLUSION

In this study a model was designed for substrates in a large range of lignin content, showing that a good interpretation of the BMP can be achieved without extensive substrate characterization. An implementation of inhibiting phenolic compounds in the model enables to study the impact of a release of phenolic compounds during harsh pretreatments. This gives the opportunity to gain insights to determine if a lignin degrading pretreatment outweighs the release of phenolic compounds, when a maximal BMP of the selected substrates wants to be achieved. A sensitivity analysis showed the importance of the hydrolysis rate and the need to determine factors influencing the hydrolysis rate. Two important parameters, k_1 and b_1, were found to be identifiable for further optimizing the model. Overall the proposed model is a simplified model enabling understanding of the BMP and hydrolysis rate if lignin content is known.

CHAPTER 7: GENERAL DISCUSSIONS AND FUTURE PERSPECTIVES

7. GENERAL DISCUSSIONS AND FUTURE PERSPECTIVES

7.1. INTRODUCTION

Lignocellulosic biomass is an abundant resource that can be used for its conversion to second generation biofuels. The sugar rich polymers cellulose and hemicellulose are however concealed within a recalcitrant lignin structure, which acts as a rigid barrier. Biogas can be obtained from the lignocellulose as a result of a four step process which starts with the hydrolysis of the biomass followed by acidogenesis, acetogenesis and methanogenesis. The hydrolysis step forms the bottleneck in the biogas production process, and therefore the hydrolysis rate must be increased to improve biogas production. Pretreating the lignocellulose matrix will help to disrupt the lignin seal, increase the surface area of the sugar rich areas and facilitate the following hydrolysis.

There are many types of pretreatments as reviewed in chapter 1: mechanical, chemical, physical, physicochemical, biological pretreatments. A good pretreatment is a cost effective, environmental friendly technique, breaking down the lignin without sugar loss and formation of inhibiting degradation products. Pretreatments used as of now however comply to only a part of these requirements. Throughout the last decades a lot of research has been done on improving the various pretreatment techniques, but more recent there is a greater interest in fungal pretreatments.

White rot fungi are a wood decaying type of fungi producing lignin degrading enzymes such as laccase, manganese peroxidase and versatile peroxidase. The white rot fungi can be divided into two classes, selective and non-selective degraders. The selective degraders are the most interesting as they will preferentially degrade lignin before cellulose and hemicellulose. To enhance fungal pretreatment however a lot of research still must be done as there are several parameters to take into account. The length of incubation, temperature, moisture content, pH etc. all these parameters should be optimized specifically for the type of white rot fungi and type of biomass used in the process. Also a comprehensive understanding of the interaction of lignin degrading enzymes produced by these white rot fungi with the lignin containing substrate is needed, as well as the

interaction of these enzymes and the lignin derived degradation products such as vanillic acid, *p*-coumaric acid, ferulic acid and hydroxybenzoic acid.

7.2. LIGNIN DEGRADATION, THE FIRST REQUIREMENT FOR A FACILITATED HYDROLYSIS

Lignin content is important for anaerobic digestion as it forms an important barrier during the degradation process, a correlation between lignin content and biomethane production has been noted in chapter 2 and 6 . Many studies related to the effect of different pretreatments describe only the impact on the degradation of lignin, hemicellulose and cellulose (e.g. Chandra et al. 2007; Su et al. 2015; Karimi and Taherzadeh, 2016). Other studies focus on the BMP of a substrate or the increase in biogas/biomethane production that is noted due to pretreatment (e.g. Kaur and Phutela et al. 2016; Li et al. 2016; Rodriguez et al. 2016). Seldomly however studies have combined the determination of lignin content or composition of the substrate, and the BMP of the substrate. Combining the different studies for one substrate is difficult since other factors play a key role in biogas production, such as the formation of inhibiting compounds during pretreatment, or the part of the plant used in the study. Several studies have measured both parameters and a clear link can be seen, this was as well noted in chapter 2 (figure 7.1) (Triolo et al. 2011; Dandikas et al. 2014; Schroyen et al. 2015). The link between lignin and BMP shows the need to degrade lignin, and so opening the material resulting in a higher biogas production. Lignin degradation will improve the hydrolysis step, increasing the rate of biogas production so smaller biogas installations can be used.

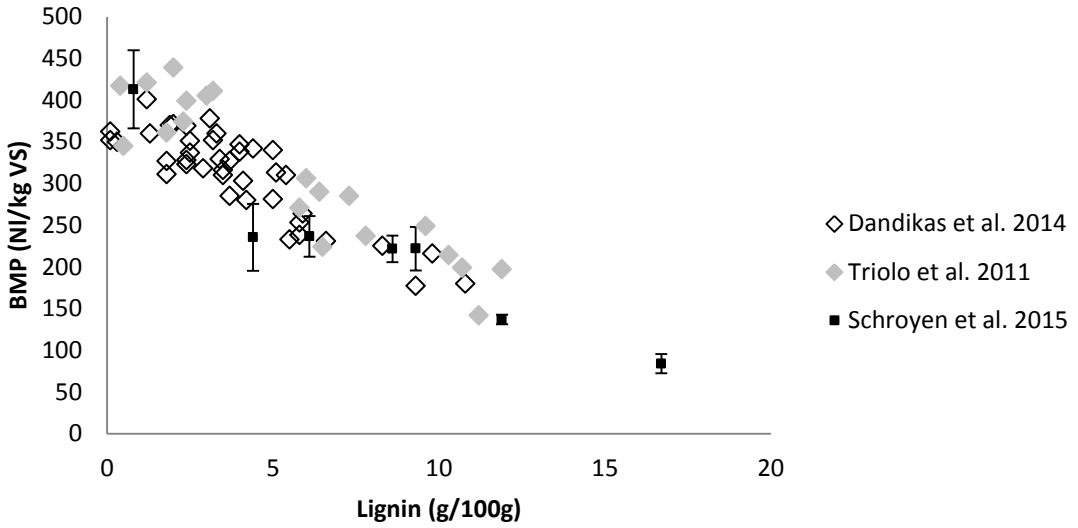

Figure 7.1: Biomethane values in relation with the lignin values of the selected substrates (Dandikas et al. 2013; Triolo et al. 2011; Schroyen et al. 2015)

Although lignin degradation is required to successfully improve the hydrolysis, the process of lignin degradation goes hand in hand with several disadvantages. A fungal pretreatment must be monitored carefully as even selective delignifiers will at one point start to consume sugars at a high rate. Dong et al. (2013) incubated sugarcane bagasse with 3 white rot fungi separately and noted an efficient lignin degradation of 85 to 93 % based on dry mass weight. The incubation with the non-selective degrader *P. chrysosporium* resulted also in a degradation of 88 % of the hemicellulose fraction and a 67 % loss of the cellulose fraction. The selective delignifiers *L. edodes* and *P. ostreatus* degraded 64 % and 15 % of respectively the hemicellulose and cellulose fractions (Dong et al. 2013). An enzymatic pretreatment with laccase and peroxidases is shown to break down the matrix of corn stover (chapter 3) and other lignocellulosic substrates such as hemp straw and miscanthus (chapter 2), resulting in an increase of released lignin derived phenolic compounds. Hemp straw and miscanthus were incubated as well with *P. eryngii* (chapter 5) which resulted in a significant (p<0.05) increase of release of phenolic compounds after 35 days of incubation, while the concentration of phenolic compounds able to be extracted from the substrate itself lowered during a longer incubation time. The selective white rot fungi are interesting to use as a pretreatment, however even the minor loss of sugars during a pretreatment is highly undesired and must be prevented at all

cause. A loss of sugars was seen at the start of the incubation with *P. eryngii,* consequently no increase in biogas production was seen due to the fungal pretreatment. The more traditional acidic and alkaline pretreatments are known for their great efficacy of degrading lignin. An alkaline pretreatment of giant reed during 24 hours with 1 g/l NaOH leads to a lignin removal of 32 % and a cellulose and xylan recovery of respectively 86 % and 81 %. Pretreating the giant reed with 20 g/l NaOH for 24 hours resulted in a 55 % lignin removal and a 85 % and 67 % recovery of cellulose and xylan respectively (Jiang et al. 2016). Although the lignin degradation results in a higher biogas production, the cost for chemical input makes the process economically unfeasible (Jiang et al. 2016). Dilute acid pretreatment at 170 °C maximized the total sugar yield, however lignin was not fully removed. This lignin caused inhibition during the cellulose conversion process by binding cellulase enzymes, blocking the glucose conversion process (Singh et al. 2015). The severe degradation process due to the acidic conditions yields high concentrations of unwanted by-products as well, which will lead to inhibition of biogas production (Shirkavand et al. 2016).

7.3. FORMATION OF PHENOLIC COMPOUNDS DURING LIGNIN DEGRADATION NEEDS TO BE AVOIDED

Furan derivatives, organic acids and phenolic compounds are released during the degradation of lignin. Pretreatment under harsh conditions will result in an excess of these unwanted by-products. The undesired products can inhibit anaerobic digestion, and therefore need to be removed. As a future perspective these phenolic compounds could be extracted. Pretreatment studies however tend to neglect the concentrations of inhibitory compounds released during the degradation process. Inhibition studies reveal that the addition of phenolic compounds to artificial media such as yeast extract and glucose, lead to a drop in biogas production. At high enough concentrations even total inhibition of biogas production is noticed. Phenolic compounds in presence of lignocellulosic material show a decrease in hydrolysis rate and final biogas production (chapter 4). Next to complicating the anaerobic digestion process, phenolic compounds can cause problems during the pretreatment step. During an enzymatic or biological pretreatment, lignin degrading enzymes produced by white rot fungi are responsible for degrading the rigid lignin structure. Versatile peroxidase is such a lignin

degrading enzyme, however in presence of 100 mg/l of phenolic compounds a deactivation was noted of the versatile peroxidase activity, the laccase enzyme on the other hand is able to decrease the amount of phenolic compounds. The activity of laccase was still affected at higher concentrations, however the enzymes were not deactivated (Chapter 4).

The effects of phenolic compounds in hydrolysates is also a known problem for the activity of cellulolytic and hemicellulolytic enzymes. A removal or mitigation of the inhibitor effects was suggested by Michelin et al. (2016) to improve cellulose hydrolysis of bagasse. A removal of the phenolic compounds would reduce the inhibition of cellulase, resulting in higher saccharification yields due to an enhanced enzymatic hydrolysis of rice straw (Lee et al. 2012). The elimination of phenolic compounds and furan derivatives by deacetylation and oxalic acid hydrolysis of olive pruning biomass lead to an improved ethanol production as a consequence of facilitated saccharification (Moya et al. 2016).

Phenolic compounds have been studied in food research extensively as an advantageous byproduct as it has antimicrobial and antioxidant properties. The release of phenolic compounds during a biological pretreatment can present an opportunity if an extraction or separation step of the phenolic compounds can be implemented. Dey and Kuhad (2014) researched the extraction of phenolic compounds during the solid-state fermentation of wheat by *Rhizopus oryzae.* Strong antioxidant properties were found in the water extract and the fermented extract could serve as a source of natural antioxidants to replace synthetic antioxidants used in the food and pharmaceutical industry (Dey and Kuhad, 2014). To extract bound phenolic compounds environmental-friendly methods were suggested by Huynh et al. (2015). In that study a 2.8 fold increase of released phenolic compounds from incubation of cauliflower leaves with *Aspergillus sojae* was noted. Separation of the phenolic compounds from hydrolysates of lignocellulose could be performed by using a cation exchange resin as medium. Indeed 81 % of the initially added phenolic compounds was successfully recovered from a synthetic hydrolysate (Chen et al. 2017). A separation of a hydrolysate of rice

straw, obtained after liquefying the rice straw due to a high temperature of 300 °C could recover 70 % of the phenolic compounds from the hydrolysate (Chen et al. 2017). Extraction and separation however require additional costs, and extra process steps. Extraction of the phenolic compounds needs to be optimized as well, as there is no standardized method of extraction and many process parameters depend on the handled substrate. The removal of phenolic compounds from the hydrolysate would improve the biogas production (chapter 4), and extraction of phenolic compounds should be considered if a value-added product can be separated. The cost price of implementing an extraction or separation step should be weight against the expected market price of the product as extracts prices vary greatly. Plant extracts with antimicrobial properties to be added in animal feeds are sold at 2 euro per kg, while functional extracts can be sold at 100 - 750 euro per kg. Concentrations of phenolic compounds obtained from delignifications however are low and upconcentration can be difficult.

7.4. A DETOXIFICATION STEP, A LONGER ROUTE TO A BETTER RESULT

To improve the anaerobic digestion process and to overcome the inhibition problem caused by phenolic compounds, a detoxification step can be introduced as suggested by the green arrows in figure 7.2. Laccase, one of the lignin degrading enzymes produced by white rot fungi, is known to polymerize phenolic compounds. In chapter 4 it is shown that laccase decreases the amount of several phenolic compounds, such as vanillic acid, *p*-coumaric acid, ferulic acid and hydroxybenzoic acid, after an incubation of 24 hours. Lignocellulosic substrates with an addition of 500 mg/l of *p*-coumaric acid were treated for 24 hours with laccase, the detoxifying effect increased the biomethane potential of both hemp straw and miscanthus. Versatile peroxidase on the other hand was inhibited by the presence of 100 mg/l of the different phenolic compounds, and was unable to remove the phenolic compounds from the hydrolysate. An incubation with laccase from *T. versicolor* for 12 hours at 30 °C reduced the amount of total phenolic compounds to 6 % of the initial amount in the wood hydrolysates (Jönsson et al. 1998). Laccase produced by the yeast *Yarrowia lipolytica* showed a higher catalytic efficiency of phenolic and non-phenolic compounds than laccases

produced by other micro-organisms. Rice straw was pretreated with the purified laccase from *Y. lipolytica,* which resulted in a higher saccharification yield due to reduced inhibition of cellulase activity of the added enzyme Celluclast 1.5 L (Lee et al. 2012). Detoxification by laccase from *Pycnoporus cinnabarinus* and *Trametes villosa* of steam-exploded wheat straw, resulted in both an enhanced enzymatic hydrolysis, and a cost reduction of the lignocellulosic ethanol process (Moreno et al. 2012). The suggested detoxification takes place after a classic pretreatment to decrease the concentration of unfavorable degradation products caused by this pretreatment. The two steps should be attuned, as laccase can degrade the lignin further during the detoxification step and thus the classic pretreatment can be performed under milder conditions. Milder conditions can decrease the costs related to the use of chemicals or energy required to reach higher temperatures. Increasing laccase production, the catalytic effect of the enzyme or improving the purification of the enzyme could further enhance the efficiency of the detoxification step.

7.5. FURTHER RESEARCH IS REQUIRED TO EXTENT OUR KNOWLEDGE ON THE LIGNIN DEGRADATION PROGRESS BY FUNGAL PRETREATMENT, AS WELL AS TO ENHANCE THE BIOGAS PRODUCTION PROCESS

Biological pretreatment of lignocellulose by white rot fungi is an interesting approach to improve the degradation and hydrolysis of the substrate. However more knowledge on how the fungi work, and what conditions are required must be obtained. In current studies a great variety in results is seen as different types of fungi, different substrates and a different length of incubation is used. Most studies look at the rate of lignin degradation caused by the white rot fungi, however following BMP studies are rarely executed in the same studies. Lignin degradation can be an indication of a higher BMP, but as the fungi consume the free sugars an increase in BMP cannot be guaranteed. Therefore it is advised to perform a BMP test, or use the pretreated material to enzymatically convert the sugars in order to obtain bio-ethanol. By the production of a second generation biofuel it is easier to evaluate the impact of the fungal pretreatment on the various substrates.

A classical approach by optimizing all parameters during the pretreatment could increase the efficiency of the white rot fungi. An extensive study with many different types of white rot fungi and

substrates with a variation in lignin, hemicellulose and cellulose content could give more insight in which combination of fungi and substrate could be efficient. Next to the choice of micro-organism and substrate, temperature, length of incubation, pH, humidity etc. should be investigated to further optimize the fungal pretreatment. If the improvements of the pretreatment could shorten the incubation time, increase the hydrolysis rate and finally the BMP, the gap with the efficiency of traditional pretreatments could be closed.

7.6. A COMBINATION OF PRETREATMENTS WORKING SYNERGISTICALLY, REDUCING DISADVANTAGES WHILE EXPECTING GREATER RESULTS

Several papers have suggested the possibility of combining a traditional pretreatment with a biological pretreatment. First results have shown a positive result as synergistic effects are noted. Wang et al. (2012) reported a combination of a liquid hot water treatment with a biological pretreatment of poplar wood lead to a 2.66 increase of glucose yield compared to a pretreatment using only the liquid hot water technique. A combined treatment where 2% H_2O_2 was added for 48 hours to rice hulls, followed by an 18 days incubation with *P. ostreatus*, yielded the same results in lignin removal as a pretreatment of 60 days with *P. ostreatus.* This approach of a fungal pretreatment after a classic pretreatment is shown in Figure 7.2 by the red arrows. The fungal pretreatment step can be seen as a detoxification step, with an increased degradation potential. However the possible consumption of the sugars and cellulose made available by the preceding pretreatment should be prohibited. Shortening the duration of the fungal pretreatment and genetically modifying the fungi are possibilities that could be addressed. If sugar loss is unavoidable, the detoxification pathway (green arrows) should be considered.

Swapping the two types of pretreatments, pretreating the material enzymatically before a traditional pretreatment is performed, is suggested by the orange arrows in Figure 7.2. By performing an initial degradation by lignin degrading enzymes, the conditions used by the traditional pretreatment could be milder, while yielding the same or an improved result and less risk of losing valuable sugars. Milder conditions reduces the amount of energy or chemicals required during the pretreatment,

lowering the costs as well. Decreasing the severity factor of the traditional pretreatment diminishes the formation of inhibitors making a detoxification step in this pathway unnecessary. In general a biological pretreatment can make the substrate last longer and could be used in a combination of storage with functional degradation.

7.7. MODELING AND DATA ANALYSIS, TO GAIN KNOWLEDGE

The model suggested in chapter 6 can give a good prediction on BMP based on the lignin content of the substrate. More research should be done where lignin degradation, formation of inhibitors and BMP-assays are investigated. The data obtained from such complete studies could help extending the model, so the effect of various pretreatments and the resulting lignin degradation could be taken into account. A more detailed modeling of the anaerobic digestion itself, meaning modeling the production of the other end products, H_2, VFA and CO_2 should be done in order to optimize the reactor for different purposes, as for now only CH_4 is foreseen.

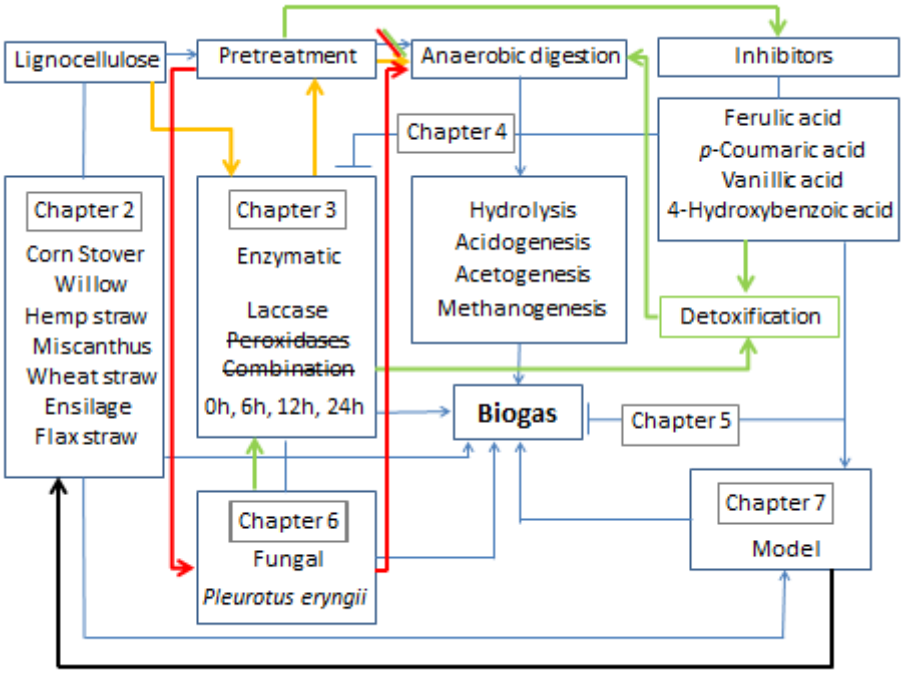

Figure 7.2: Outline and interactions of the various chapters as well as the new proposed pathways. Detoxification pathway (Green), combination of a classic pretreatment followed by a fungal pretreatment (Red) and a combination of an enzymatic pretreatment followed by a classic pretreatment (Orange)

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