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Pyrolysis Based Biorefineries for the Production of Fermentable Substrates

Luis Carlos Luque-Moreno
The University of Western Ontario

Supervisor
Dr. Lars Rehmann
The University of Western Ontario

Graduate Program in Chemical and Biochemical Engineering
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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by

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The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

Fast pyrolysis is rapid thermal conversion process capable of transforming multiple feedstocks into various energy carriers, specifically pyrolysis oil, bio gas, and bio char. The oil phase is a rich mixture containing several organic molecules that could be used as platform chemicals and as fuel additives. In addition, this oil phase contains large number of anhydrous carbohydrates, which can be easily transformed into glucose via acid hydrolysis. These carbohydrates can be either biocatalyzed into fuels and chemicals by microorganisms or converted after further treatment steps. However quantities of these carbohydrates in the oil are a function of feedstock composition and process parameters. In addition, utilization of these sugars by microorganisms is hindered by the presence of inhibitors. The research presented by this thesis focuses on producing a detoxified sugar fraction for biofuels production via microbial biocatalysis.

The essential work was undertaken by utilizing two pyrolytic oils derived from demineralised and non-demineralised pinewood, as the sources of both inhibitors and anhydrous sugars. Cold water, solvent extraction, acid hydrolysis and neutralization were utilized to upgrade pyrolysis oil to procure a fermentable substrate, to produce ethanol with *Saccharomyces cerevisiae*. In order to reduce the development time for pretreatment processes, a high throughput analysis to assess fermentability was realized utilizing microtiter plates. Based on the cellulose fraction, a 41.3% ethanol yield was achieved. The inhibition on *S. cerevisiae* was correlated to quantified growth kinetic parameters allowing to connect the relevance of demineralization with the ethanol titers achieved.

To improve the upgrading strategies, and to pinpoint the main inhibitors found in pyrolysis oils, a screening for possible inhibitors was performed. This screening suggested that inhibition could possibly be explained by at least six different compounds. Analysis of the synergy of these compounds by a central composite design allowed to obtain a response surface polynomial which was utilized to analyze the inhibition observed when utilizing pyrolytic fractions. The polynomial proved a good fit when using pure sugars, however, it was not in good agreement with the observed inhibition when utilizing pyrolytic sugars.

The knowledge gained in the early chapters was then applied to develop a new quantification strategy to measure the levels of inhibitors in pyrolytic oils derived from two different biomasses, switch grass and corn cobs. The new technique was used to assess the efficiency of the upgrading steps and to identify which upgrading configuration resulted in a less toxic fermentable substrate to *S. cerevisiae*. The new configuration, enhanced the ethanol productivity as the fermentation time was reduced in 30% to 15 hours. It was shown that the approach to procure a fermentable substrate also worked with different types of biomass, which contributed to the robustness of the process proposed in the first chapter.

As a final contribution of this thesis, the biorefiney approach was used in lipid accumulation by *Rhodosporidium diobovatum* and *Chlorella vulgaris from* detoxified substrates. Utilization of complete pyrolytic fractions was observed by *R. diobovatum* reching 24.9 ± 1.3 % by total FAME analysis. However, *C. vulgaris* growth was inhibited in blends > 30 % v/v and achieving a lipid accumulation maximum of 32.2 ± 1.2 %. The results on lipid accumulations observed in both microorganisms suggests that optimization of pyrolytic fraction:nitrogen concentration could increase the overall lipid yield.

The conclusions from this research provide guidance for the utilization of inexpensive residual biomass in pyrolysis based biorefineries for the production of biofuels and chemicals as an alternative to crude oil derived products. This integration, allowed to propose a novel and robust biorefinery approach that proved to work with different biomasses. Improvement in the area of biomass selection and pretreatment prior to pyrolysis, in the upgrading strategies in addition to the use of more tolerant strains can augment the potential to compete with established biofuel processes.

Keywords

Pyrolysis, levoglucosan, biomass, upgrading, fermentation inhibition.

Co-Authorship Statement

This thesis was completed under the supervision of Dr. Lars Rehmann and Dr. Franco Berruti. The research was part of a collaboration with the Sustainable Process Technology group at the University of Twente, where I had the opportunity to spend two months to undertake the experiments with corn cobs and switchgrass. Three articles were written and coauthored, the extent of the collaboration of the co-authors is stated below.

Chapter 3.

Paper title: Pyrolysis based bio-refinery for the production of bioethanol from demineralized ligno-cellulosic biomass

Current Status Published in Bioresource Techonology, June 2014. Vol 161, Pages 20-28

Luis Luque: carried out the design and completion of upgrading and fermentation experiments, HPLC, TOC, and modelling data collection and interpretation and completed the manuscript drafting and final writing.

Roel Westerhoff: assisted with the demineralization and pinewood pyrolysis experiments. Completed the pyrolysis oil GC characterization. Collaborated with the drafting and the completing of the manuscript.

Stijn Oudenhoven: assisted with the pyrolysis experiments and the pyrolysis oil characterization. Collaborated with the collection and interpretation of the oil characterization data.

Guss Van Rossum: participated in the design of the biorefinery approach, collaborated with the data interpretation and analysis as well as providing key details and corrections in the manuscript writing.

Sascha Kersten: collaborated in the design of the pinewood pyrolysis experiments and in the interpretation and analysis of the data. In addition assisted with key insights and correction in the drafting of the manuscript.

Franco Berruti: technical and theoretical advisor, corrections in the drafting and writing of the manuscript.

Lars Rehmann: Drove the idea of the pyrolysis based biorefinery. Assisted with the modelling. As well as with the data interpretation and analysis. Also assisted in the drafting, editing and correction of the manuscript. He is also the corresponding author of this article.

Chapter 5

Paper title: Ethanol production from corn cobs and switchgrass following a pyrolysis-based biorefinery approach

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Luis Luque: carried out the design and completion of characterization, demineralization, pyrolysis, upgrading and fermentation experiments, HPLC and modelling data collection and interpretation and completed the manuscript drafting and final writing.

Roel Westerhoff: participated in the demineralization and pyrolysis experiments and assisted with corrections in the drafting of the manuscript.

Stijn Oudenhoven: participated in the demineralization and pyrolysis experiments, carried out the ICP determination and assisted in the manuscript drafting.

Guss Van Rossum: Collaborated in the design of the pyrolysis experiments, participated in the analysis and interpretation of the collected data, and assisted with corrections in the drafting of the manuscript

Sascha Kersten: collaborated in the design of the demineralization experiments, participated in the analysis of the collected data and assisted with important correction in the writing of the manuscript.

Franco Berruti: assisted in the design of the pyrolysis experiments, and in the drafting of the manuscript.

Lars Rehmann: collaborated with the design of the upgrading and fermentation experiments, participated in the analysis and interpretation of the analytical data, collaborated with the numerical analysis and helped with the drafting editing and correction of the manuscript and is the corresponding author of this article.

Chapter 6.

Paper title: Lipid accumulation from pinewood pyrolysates by *Rhodosporidium diobovatum* and *Chlorella vulgaris* for biodiesel production

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Luis Luque: carried out the upgrading of the pyrolytic fractions, lipid production with the *Rhodosporidum diobovatum* and assisted in cultivation of *Chlorella vulgaris*. Carried out lipid extractions for analysis. Collected, analyzed and interpreted the data. Writing of several drafts as well as the final manuscript.

Valerie Orr: carried out lipid production experiments with *Chlorella vulgaris* and assisted in cultivation of *Rhodosporidium diobovatum*. Carried out lipid extractions for analysis. Collected, analyzed and interpreted the data and assisted in the drafting and writing of the manuscript.

Sean Chen: assisted during the lipid production experiments in the literature review and in the drafting of the paper.

Roel Westerhoff: assisted with the demineralization and pinewood pyrolysis experiments. Completed the pyrolysis oil GC characterization. Collaborated with the drafting and the completing of the manuscript.

Stijn Oudenhoven: assisted with the pyrolysis experiments and the pyrolysis oil characterization. Collaborated with the collection and interpretation of the oil characterization data.

Guss Van Rossum: participated in the design of the biorefinery approach, collaborated with the data interpretation and analysis as well as providing key details and corrections in the manuscript writing.

Sascha Kersten: collaborated in the design of the pinewood pyrolysis units and experiments and in the interpretation and analysis of the data. In addition assisted with key insights and correction in the drafting of the manuscript.

Franco Berruti: technical and theoretical advisor, corrections in the drafting and writing of the manuscript.

Lars Rehmann: assisted with the modelling, the upgrading, characterization and fermentation experiments. As well as with the data interpretation and analysis. Also assisted in editing, and correction of the manuscript.

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Finally to my mom, Alda Moreno, my dad Luis Carlos Luque and my brother Francisco Luque Moreno for being my strength throughout my live, because no matter where we are the distance has only brought us closer to each other.

Dedication

To my parents, Alda Moreno and Luis Carlos Luque for being the best support someone could ask for. To my brother Francisco, for being my second conscience. Thanks to you three for all the love.

(A mis padres, Alda Moreno y Luis Carlos Luque por ser y dar el mejor apoyo que alguien pueda pedir. A mi hermano Francisco por ser mi segunda conciencia. A todos tres por todo su amor.)

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Acronyms list

AACC	*Acetic acid pretreated corn cobs*
AASG	*Acetic acid pretreated switch grass*
AFEX	*Ammonia fiber explosion*
BOD	*Biochemical Oxigen Demand*
BZL	*4-Hydroxybenzaldehyde*
CW	*Cold/chilled water*
CCD	*Central composite design*
CFPP	*Cold flow plugging point*
CL	*m-Cresol*
CN	*Cetane number*
DAD	*Diode array detector*
EAc	*Ethyl acetate*
FAME	*Fatty acid methyl ester*
FID	*Flame ionization detector*
FF	*Furfural*
g	*Gram*
GC	*Gas chromatography*
GHGs	*Green house gases*
GL	*Guaiacol*
H	*Hydrolysis*
h	*hour*
HMF	*Hydroxymethylfurfural*

HPLC	*High pressure liquid chromatography*
HTP	*High Throughput*
IL	*Ionic Liquid*
IV/G	*Inhibitor value normalized by sugar concentration*
L	*Liter*
LC	*Liquid chromatography*
LG	*Levoglucosan*
MS	*Mass spectroscopy*
NACC	*Nitric acid pretreated corn cobs*
NASG	*Nitric acid pretreated corn cobs*
PO_1	*Biorefined pyrolytic oil stream after cold water extraction only*
PO_2	*Biorefined pyrolytic oil stream after cold water and ethyl acetate extractions*
PO_3	*Conventional pyrolytic oil stream after cold water extraction only*
PO_4	*Conventional pyrolytic oil stream after cold water and ethyl acetate extractions*
RI	*Refractive index*
s	*Second*
SCO	*Single celled oils*
SE	*Steam Explosion*
VA	*Vanillin*
W	*Water*

WH	*Water hydrolysis route*
WEAcH	*Water Ethyl Acatate Hydrolysis route*
WHEAc	*Water Hydrolysis Ethyl Acetate route*
WO	*Wet Oxidation*
YPG	*Yeast Extract Peptone Glucose media*
N_{max}	*Maximum cell density*
λ	*Lag or adaptation time*
μ_{max}	*Maximum growth rate*
Ω	*Glucose consumption rate*

Chapter 1

1 Introduction

1.1 Background

Global ethanol output in 2008 was 66.77 billion liters (Gupta and Verma, 2015) reaching 88.69 billion liters in 2013 and projected to achieve the 90 billion liters mark in 2014 (Baker, 2014). This active bioethanol production is mainly derived from sugarcane or from starches from corn (Gupta and Verma, 2015; Wall, 2008). An alternative to utilizing food feedstocks for the production of biofuels is lignocellulosic biomass. It has been estimated that ethanol production from such sources can reach 491 billion liters per year (Kim and Dale, 2004). These lignocellulosic materials have a low cost, are available in large volumes and are renewable (Gupta and Verma, 2015). Several investigations have been devoted to ethanol production from these biomasses (Binod et al., 2010; Cadoche and López, 1989; Duff and Murray, 1996; Sarkar et al., 2012) yet pretreatments to separate the sugars from the lignin are still the main challenge for commercialization (Menon and Rao, 2012). Fast pyrolysis of lignocellulosic biomass is a potential option which could render fermentable sugars for the production of different biofuels such as ethanol or biodiesel (Chi et al., 2013; Jarboe et al., 2011; Lian et al., 2012, 2010; Luque et al., 2014; Rover et al., 2014) and could serve as a potential source for platform chemicals and fuel additives (Lian et al., 2013; Ramakrishnan et al., 2011; Westerhof et al., 2011).

1.1.1 Traditional 1st generation processes for ethanol production

First generation biofuels are derived from food sources such as starch, sugar cane, vegetable oil and animal fats (Kang et al., 2014). The majority of fuel ethanol in North America is derived from corn, based on a process following the schematic presented in Figure 1.1. In this process, corn kernels are separated from the chaff and then they are milled to coarse flour. The milled particle size has to meet certain requirements; they have to be small enough (larger surface area) to maximize mass transfer in swelling for an increased enzymatic hydrolysis but also has to be sufficiently large so that the residual solids can be separated physically from the liquid at the end of the fermentation and distillation. These solids are called distillers dry grains, and can be used as animal feed (Wall, 2008).

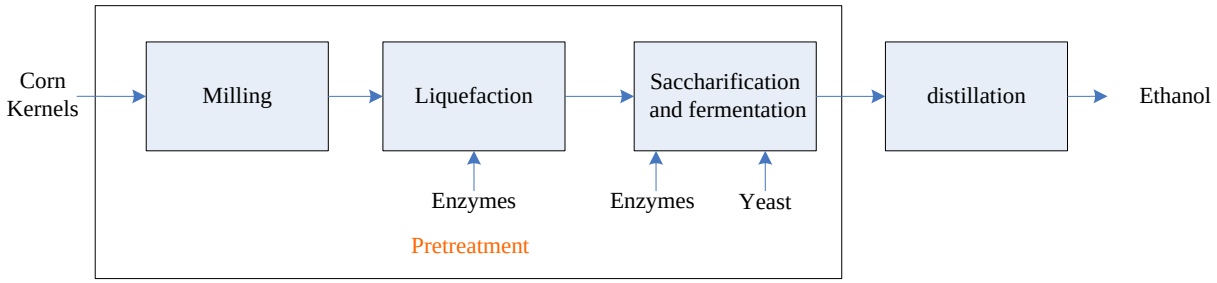

Figure 1.1 Bioethanol from corn general process flow diagram (Wall, 2008).

The sugar cane process (South America), Figure 1.2, is slightly different; biomass is washed and chopped to expose the fiber bound sugar juice to a recovery process. Through intensive processing the juice becomes a syrup, which is later diluted and fermented. Unlike the corn process there is no need for an enzymatic hydrolysis, since the juice recovered from the mill already contains water with dissolved sugars ready for concentration and a later processing (Brandes, 1952). In addition, the stillage process is not as easy as that of corn due to the high content of residuals in it. The stillage produced from the fermentation contains low concentrations of protein and lipids, and its organic fraction includes non-fermentable sugars, waxes, gums, organics acids and bagasse that are not as useful as animal feed due to its high potassium content (Wall, 2008). However, the main process result depends on the composition of the bagasse. If it is a low solid, low biochemical oxygen demand (BOD) vinasse it can be returned to the cane fields as irrigation water, returning nutrients and organics to the soils.

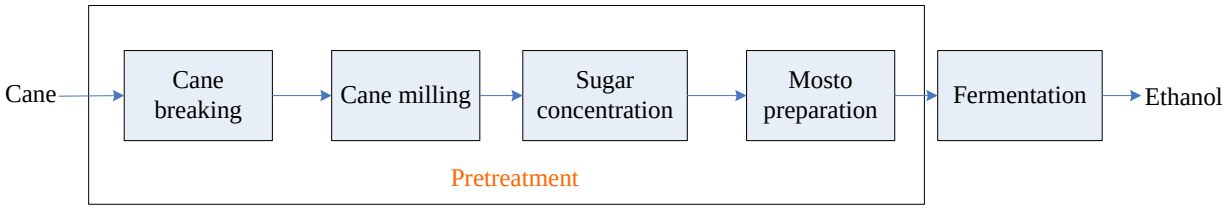

Figure 1.2 Bioethanol from sugar cane general process flow diagram (Wall, 2008)

1.1.2 Lignocellulosic and 2nd generation ethanol production

Second generation biofuels are derived from lignocellulosic biomass (non-food crops), such as agricultural residues, wood, forest residues (de Miguel Mercader et al., 2010; Menon and Rao, 2012). Agricultural residues are inexpensive feedstocks which avoid the direct competition with food production and (Fargione et al., 2008; Searchinger et al., 2008). Lignocellulos however, is a more

complex feedstock than corn kernels and sugar cane (1st generation biofuel feedstocks). It composed of a strong interwoven matrix encapsulating fermentable sugars, which on their own are organized in a compact structure highly resistant to regular enzymatic hydrolysis. Consequently, several approaches to overcome the recalcitrance displayed by these feedstocks in order to access fermentable fractions have been developed over the years (Cherubini, 2010; Eklund and Zacchi, 1995; Gollapalli et al., 2002; Ibrahim et al., 2011; Jacquet et al., 2015; Liang et al., 2010; Sluiter et al., 2004; Taherzadeh and Karimi, 2008; Xu and Huang, 2014). Due to the influence pretreatment of biomass has on downstream cost, an ideal pretreatment needs meet certain criteria to be cost effective. Firstly, an effective pretreatment needs to decouple the main biopolymers composing lignocellulose (lignin, hemicellulose and cellulose), to gain access to fermentable sugars while yielding low concentrations of inhibitors, as they would hinder either further pretreatment with enzymatic hydrolysis or compromise fermentation due to microbial growth inhibition. Secondly, it needs to be able to recover lignin derivatives (Westerhof et al., 2011) and preserve the five carbon sugar fractions (Banerjee et al., 2010). Thirdly, it needs minimal energy input, and circumvent waste treatment.

Generally pretreatment of lignocellulosic biomass (Figure 1.3) starts with a particle size reduction, in order to yield a larger surface exposing the encapsulated sugars thus improving hydrolysis (Parajuli et al., 2015). This pretreatment is usually followed by a chemical or physicochemical pretreatment both attempting to achieve the overall goal of disrupting the biomass, Figure 1.3. Chemical pretreatments, include acid pretreatment which removes hemicellulose fractions (Digman et al., 2010), alkaline pretreatment which removes lignin and improves hemicellulos and cellulose digestibility (Ibrahim et al., 2011), utilization of ionic liquids (ILs) which reduces cellulose crystallinity, while reducing lignin and hemicellulose content thus increasing surface are (Perez-Pimienta et al., 2013) and wet oxidation with fractionates lignocellulose by removing lignin and solubilizes hemicellulose. Physicochemical pretreatments like steam explosion (SE) decouples the lignocellulosic structure by exposing the biomass to high pressure saturated steam for a short period of time and then this pressure is quickly released. The sudden expansion, disrupts the matrix and improves the accessibility of cellulolytic enzymes (Jacquet et al., 2015). Ammonia fiber explosion (AFEX) is an alternate physicochemical pretreatment, with the same physical principles of the SE. In AFEX, biomass is impregnated with liquid ammonia at relatively high temperatures and pressures for a period of time after which, pressure is suddenly released. As a consequence, cellulose

crystallinity is decreased, hemicellulose is partially depolymerized and lignin is decoupled from the carbohydrate fraction, thus affecting the overall structure of the biomass and increasing surface area (Zheng et al., 2009).

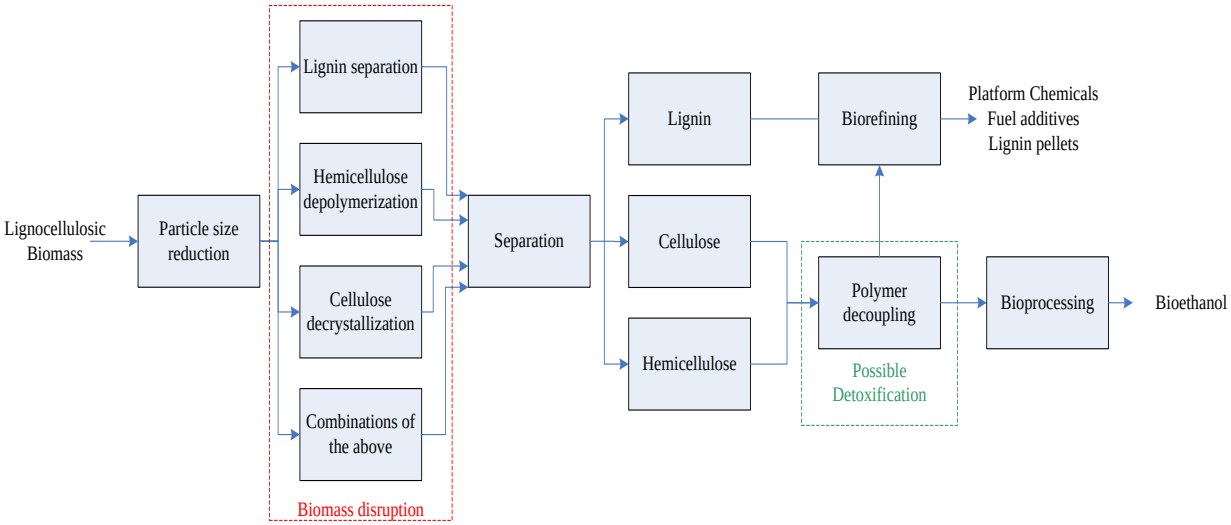

Figure 1.3 Overall biofuel production process from lignocellulosic biomass via traditional pretreatments. Adapted from Dermibas (2009) and Parajuli et al. 2014 (Demirbas, 2009; Parajuli et al., 2015)

Once pretreatment is complete, these pretreatments need to be evaluated for possible inhibitory compounds derived from sugar or lignin degradation. This removal is an important step, as some of these compounds would ultimately inhibit growth of fermentative microorganisms if present in certain concentrations. Some of the strategies include overliming (Yu and Zhang, 2004) sorption into different matrices such as activated carbons (Yu and Zhang, 2004) polymeric adsorption (Weil et al., 2002) or air stripping and solvent extraction (Wang et al., 2012)

1.1.3 Fast pyrolysis in 2nd generation biofuel production

1.1.3.1 Biomass Fast Pyrolysis

Biomass pyrolysis, occurs at high temperatures (500°C) in the absence of oxygen (nitrogen is generally used as a carrier gas) with low residence times <2s. This process. As a consequence biomass

is transformed into different energy carriers such as bio-char, gas and an organic liquid fraction, commonly referred to as pyrolysis oil (Bridgwater et al., 1999; Butler et al., 2013; Dobele et al., 2003; Lian et al., 2012; Oasmaa and Meier, 2005; Westerhof et al., 2011, 2007). Several process advances have been made over the past 20 years such as increased thermal efficiencies and new pyrolysis technologies (Westerhof et al., 2011). These new and more efficient pyrolysis technologies have been yielding higher quantities of pyrolytic oil (Westerhof et al., 2007). Pyrolysis oil is a complex mixture of numerous compounds that can be broadly classified into four different main groups: i) low molecular weight compounds, ii) furan/pyran ring derivatives, iii) phenolic compounds and iv) anhydrous sugars (Patwardhan et al., 2009). Different biomass feedstocks will yield different amounts of these compounds distributed amongst the three main resulting phases from the pyrolysis (oil, biogas and biochar), making the prediction of the resulting product composition an extremely challenging proposition. Pyrolysis oil can be combusted as a fuel, however it has properties that vastly differ from crude oil and lacks stability (Lian et al., 2010) (polymerizes, ages, corrosive, etc.).

1.1.3.2 Fast pyrolysis as a biomass pretreatment for

Fast pyrolysis has been recently studied for its ability of overcoming lignocellulose recalcitrance and transforming biomass into three main phases. One of these phases, pyrolytic oil, has been the focus of recent studies as the sugars released from biomass are found in their vast majority in this fraction (Bennett et al., 2009; Chi et al., 2013; Helle et al., 2007; Jarboe et al., 2011; Lian et al., 2012, 2010; Luque et al., 2014; Rover et al., 2014). The composition of this pyrolytic oil resembles the composition of the original biomass (Czernik and Bridgwater, 2004) making it a very complex matrix for the direct utilization of the sugars. In addition, the majority of the sugars found in this matrix are not easily assimilated by natural occurring microorganisms and it is necessary to hydrolyze them to convert them into glucose (Jarboe et al., 2011; Lian et al., 2010; Luque et al., 2014). Nevertheless, upgrading of sugars and removal different inhibitors have proven that pyrolysis can be used to procure a source for biofuels production utilizing different microorganisms (Lian et al., 2013; Liang et al., 2013; Luque et al., 2014). A general description of the biofuel production via biomass fast pyrolysis is shown on Figure 1.4 . In addition, utilization of these sugars would be beneficial for pyrolytic oil downstream processing as it reduces the oxygenated compounds in the oil, therefore making it more suitable for hydrotreatment (Lian et al., 2013).

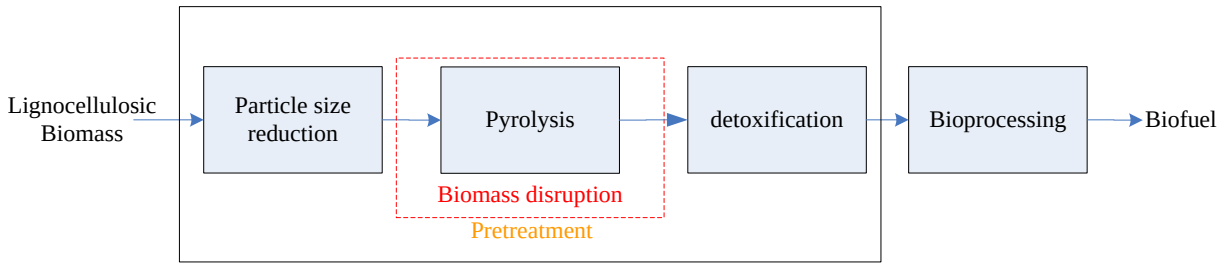

Figure 1.4 Outline of biofuel production utilizing pyrolysis as a biomass pretreatment.

There is several room for improvement in making pyrolytic oils a source of fermentable substrates. Production of anhydrous sugars depends to a great extent in the composition of the biomass used, and in the ions that it contains. In addition, a lack of understating on which compounds are exerting the inhibition of microbial growth impedes the design of detoxification techniques that could produce a higher quality fermentable stream. Moreover, it is desired to understand how the fermentable streams can be applied in the production of different biofuels other than ethanol.

1.2 Research Objectives and Contributions

1.2.1 General objective

The overall objective of this research was to demonstrate the feasibility of turning lignocellulosic biomass, e.g. pinewood, switch grass and corn cobs into a second generation biofuel via fast pyrolysis and subsequent fermentation of the sugars produced.

1.2.2 Specific Objectives

Objective 1: To develop a high throughput methodology to assess the fermentability of different pyrolytic oils.

Developing a method to screen several concentrations of different pyrolytic oils was necessary to replace time consuming experiments designed for shake flasks. By adapting fermentations to a micro scale (96- or 24 well plates), a large number of simultaneous fermentations on the same 96- could be

performed. This facilitated data collection, which allowed to correlate the effects of different concentrations of pyrolytic oils on growth kinetics and ethanol production.

Objective 2: To evaluate the impact of ion removal (leaching) of pinewood biomass on the fermentability of pyrolysis oil.

Removal of alkaline and alkaline earth metals (AAEMs) showed an increase in anhydrous sugar production, by decreasing the amount loss to degradation reactions yielding inhibitory compounds. The obtained increment eased the subsequent upgrading steps as well as enhanced the final ethanol and lipid production of the produced substrates.

Objective 3: To assess the inhibitory properties of pyrolytic oils.

The inhibitory properties of pyrolytic oils were quantified by determining the effect on different growth kinetic parameters. The high throughput methodology was applied in order to evaluate the several conditions in parallel.

Objective 4: To upgrade pyrolytic oil for mitigating inhibition properties.

Upgrading of the pyrolytic oils was achieved by analyzing three detoxification steps, cold water extraction, which allowed to precipitate the insoluble lignin and extract anhydrous sugars into an aqueous solution for easier processing. Solvent extraction with ethyl acetate removed carried over compounds which would hinder fermentative microorganism's growth. Lastly acid hydrolysis was used to convert extracted sugars in the oils to fermentable glucose. As a result of these three processes *S. cerevisiae* exhibited full tolerance achieving complete sugar depletion within 20 hours.

Objective 5: To integrate of leaching and pyrolysis with upgrading and fermentation for the production of biofuel (biorefinery).

The three different steps in the upgrading process were subject to reconfiguration to determine which order would yield the most fermentable substrate. It was found that, these steps cannot be a standalone process before the fermentation, as each one targets specific components, and has a different effect on the overall fermentation result. Water extraction proceeded by an acid hydrolysis with a further solvent extraction procured the most reliable source for a fermentable substrate.

Objective 6: To evaluate different biomass for the previously developed approach

The impact of utilizing different feedstocks was evaluated by utilizing corn cobs and switchgrass. Pyrolytic oils derived from these two biomasses were successfully upgraded and converted to ethanol. This proved the robustness of the process by using an agricultural residue and an energy crop.

Objective 7: To develop a technique to quantify or approximate the total amount of inhibitors in pyrolytic substrates.

A simple yet robust technique which allows to quantify simultaneously inhibitors and sugar levels would be beneficial for evaluating the performance of the detoxification steps, and to correlate sugars inhibitors and growth kinetics for fermentability evaluation. This step would avoid preparing

Objective 8: To verify and assess the application of the biorefinery concept in lipid accumulation.

The improved detoxification configuration was applied to pinewood pyrolytic oil to procure a fermentable substrates high in anhydrous sugars and low inhibitors. Conversion of anhydrous sugars was increased and lipid production with *Rhodosporidium diobovatum* and *Chlorella vulgaris* accomplished.

1.3 Research Structure

The first phase of the investigation evaluated how the integration of biomass leaching (ion removal), fast pyrolysis and upgrading steps, increased the fermentability of the produced oils when complemented by proceeding upgrading steps. Upgraded and non-upgraded oils from leached and unleached biomass were assessed in parallel to determine the necessary steps in order to procure a fermentable substrate. A high throughput screening methodology was design in order to evaluate and quantitate the tolerance levels and ethanol production of *Saccharomyces cerevisiae* from the pyrolytic oils. This pyrolysis based biorefinery approach led to increased production of laevoglucose to 18 wt % from 4 wt% and to a successful production of ethanol with a substrate composed solely of pyrolytic sugars rendering an ethanol yield, $Y_{gram\ ethanol/gram\ glucose}$, of 0.49 corresponding to the 96% of the theoretical maximum.

The second phase of the research investigated the possible compounds responsible for the inhibition in ethanol fermentation. After a literature review and identification of some inhibitory compounds in selected pyrolytic oils, a screening for fermentation inhibitors in upgraded oil fractions was performed. Six compounds were selected to represent the total inhibition observed when using different oils. However, compounds and concentrations found did not fully explain the growth inhibition.

For the third phase of the research, the outlined biorefinery approach was applied to two different Canadian agro-industrial wastes, corn cobs and switch grass. An additional leaching agent, nitric acid (HNO_3), was utilized to determine its effects on the levoglucosan production. In corn cobs, the new leaching agent increased the levoglucosan production 14-fold, compared to a 9-fold increase when the established technique (acetic acid as leaching agent) was used. As for the switchgrass, the new agent did not have any effect on the levoglucosan production as both, acetic and nitric acid, were responsible for an 11-fold increase. In addition, different configurations of the established upgrading steps were evaluated to enhance the ethanol productivity. A new way of correlating inhibition with the overall presence of inhibitors was elucidated and showing a strong correlation with the observed results.

The final phase of the investigation evaluated lipid production applying the proven biorefinery approach fermenting the sugars with an oleaginous yeast, *Rhodosporidium diobovatum*, which has previously shown to grow on waste glycerol streams and a microalgae, *Chlorella vulgaris*.

1.4 References

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Chapter 2

2 Literature Review: Lignocellulosic biomass pretreatment strategies for 2nd generation biofuels production

2.1 Introduction

Lignocellulose is a complex and compact matrix entrapping cellulose, a glucose polymer. Second generation biofuels take advantage of the low cost associated with lignocellulosic biomass to extract the glucose within its matrix. To release sugars however, it is necessary to overcome the recalcitrant nature of the entire structure (lignin, hemicellulose and cellulose). This resistance to degradation has been regarded as the main bottleneck in 2nd generation biofuels production and strategies to overcome it have been the focus of several studies. Consequently, several biomass pretreatment strategies have been developed to date but none of them having a competitive advantage. In addition, biomass pretreatment releases not only sugars but also fermentation inhibitors that would hamper the direct utilization of the fractions by the fermentative microorganisms. To overcome the toxicity of these product streams, several approaches have been undertaken, such as optimization of process parameters, combination of different technologies, development of detoxification techniques for inhibitor sequestration and improving the tolerance of fermentative strains.

This literature review attempts to give an overall picture of the current biofuels production status. It then proceeds to explain how lignocellulosic biomass is composed and how it translates into different pretreatment strategies. Secondly, this literature review provides a general overview of the different types of lignocellulose pretreatments, discussing their mode of action and their advantages and disadvantages. It then introduces pyrolysis as a potential option to pretreat biomass detailing different technologies that could be used to produce carbohydrates for fermentation. Moreover, it gives and overview of the microorganisms that could be used for the production of 2nd generation biofuels. Lastly it finishes explaining different methods which have been developed to upgrade and produce a cleaner and less toxic fermentable stream when dealing with pyrolytic products.

2.2 Opportunity for lignocellulosic biomasses

The most widely produced biofuels are bioethanol and biodiesel both blended with gasoline or diesel as additives (Gupta and Verma, 2015). The United States and Brazil account for 89% of the global ethanol production, with corn starch being the primary sugar source in the US and sugar cane juice and molasses in Brazil This production is based either on corn starch or sugar cane juice and molasses (Singh et al., 2016) both of which requires a costly pretreatment (Demirbas, 2005). However, production depending on simple sugars from sugarcane and corn starch have been under big scrutiny due to their food and feed value (Gupta and Verma, 2015). Given this reason, increase in biofuel output has been paralleled by a rise in crop prices from the mid-2000s, achieving historical highs in 2008 and 2011(FAO, 2013). As an example, the diversion of corn harvest to ethanol production increased gradually from less than 10% to 40% between 2000 and 2012, a period that coincides with the increase in corn price (Condon et al., 2015). However a different report shows that increase food prices are a consequence of increased energy (oil) prices, as well as potentially negatively impacting the environment through increased CO_2 emissions due to land utilization (Searchinger et al., 2008). Despite the controversy between studies, The Energy and Independence Security Act (EISA) of 2007 set a target biofuel production by 2022 of 35 billion gallons with corn ethanol capable of supplying only 43% of the desired target by 2015 (Condon et al., 2015). The major consequences of this scenario would fall principally on the feedstock market and on the global capability of the current agricultural system to sustain the biomass demand, which leads to a diversification of the feedstocks used for ethanol production (Parajuli et al., 2015).

Lignocellulosic biomass is a possible candidate due to its abundance and low cost (Balat, 2011; Menon and Rao, 2012). It is mainly composed of three different interwoven polymers, cellulose, hemicellulose and lignin. The complex and strong linkages among the polymers produce a high recalcitrant composite which results in the major technical and economic challenge to releasing the fermentable sugars in a cost-effective manner (Zhang, 2011). In the past two decades, ethanol production has been extensively studied and recorded using different lignocellulosic biomasses such as rice straw, corn stover, switchgrass, poplar and sugarcane bagasse (Binod et al., 2010; Buaban et al., 2010; Cadoche and López, 1989; Gupta and Verma, 2015; Sarkar et al., 2012). It has been projected that the liquid biofuels share will be 27% in 2050 from the 2% observed in 2010 (The

International Energy Agency, 2011) with lignocellulosic biofuels (2nd generation) projected to be dominant over starch and sugar cane base biofuels (1st generation) to their reduced environmental impact (The International Energy Agency, 2010).

2.3 Lignocellulosic biomass composition

Lignocellulosic biomass can be classified in four general groups based on the resource type: municipal solid waste, waste-paper, agro industrial residues and wood (Demirbas, 2009). As expected the composition of the biomass would depend on the type of resource, however it is generally composed of cellulose, hemicellulose, lignin and ash. The fraction of these polymers found in different plant cell walls could vary greatly and as a result cell walls have different forms and properties, Table 2.1. The complexity of this interwoven matrix found in lignocellulose is the foundation of the high resistance to biological and chemical degradation (Zhang, 2008). In a natural environment lignocellulose degradation requires the synergistic effects from several different hydrolyzing enzymes including cellulases such as endoglucanase, cellobiohydrolases and betaglucosidase, hemicellulases and lignin-degrading enzymes (Zhang et al., 2006).

Lignocellulose composition is a function of several variables such as plant species, harvest time, soil type, soil amendment techniques used, pesticides usage and environmental factors such as precipitation and sun exposure (Liu et al., 2015; Monti et al., 2008). These variables are so pronounce that different composition could be observed in plants of the same species.

Table 2.1 Typical compositions of different types of lignocellulosic biomass (% dry weight)

Biomass	Cellulose	Hemicellulose	Lignin	Ash
Corn stover	31-47	26 - 43	3 - 13	11 - 16
Wheat straw	33 - 41	20 - 32	13 - 20	4.6 - 14
Switchgrass	30 - 50	10 - 40	5 - 20	4.8
Corn Cob	32.3 - 45.6	35 - 39.8	6.7 - 13.9	0.51
Hardwoods	22 - 40	20 - 38	30 - 55	0.38 - 0.8
Softwoods	18-38	15 - 33	30 - 60	0.8

Sources: (Chandrasekaran and Hopke, 2012; Demirbas, 2005; Isahak et al., 2012; Prasad et al., 2007; Radlein, 1985)

Like starch, cellulose is a glucose polymer, however, in cellulose glucose monomers are linked via β-1-4- glycosidic bonds not α-1-4 bonds like as in stach. During the biosynthesis parallel cellulose chains produce microfibrils via inter and intra chain hydrogen bonding and Van der Waal's forces. In turn, these microfibrils are compacted into fibers, rendering a more insoluble and crystalline structure (Singhvi et al., 2014). As a consequence, this tightly compacted structure is hard to access by hydrolyzing enzymes which hinders an efficient saccharification. A complete depolymerization of cellulose would yield only glucose molecules (Singhvi et al., 2014). The structure of the plant cell wall is depicted on Figure 2.1.

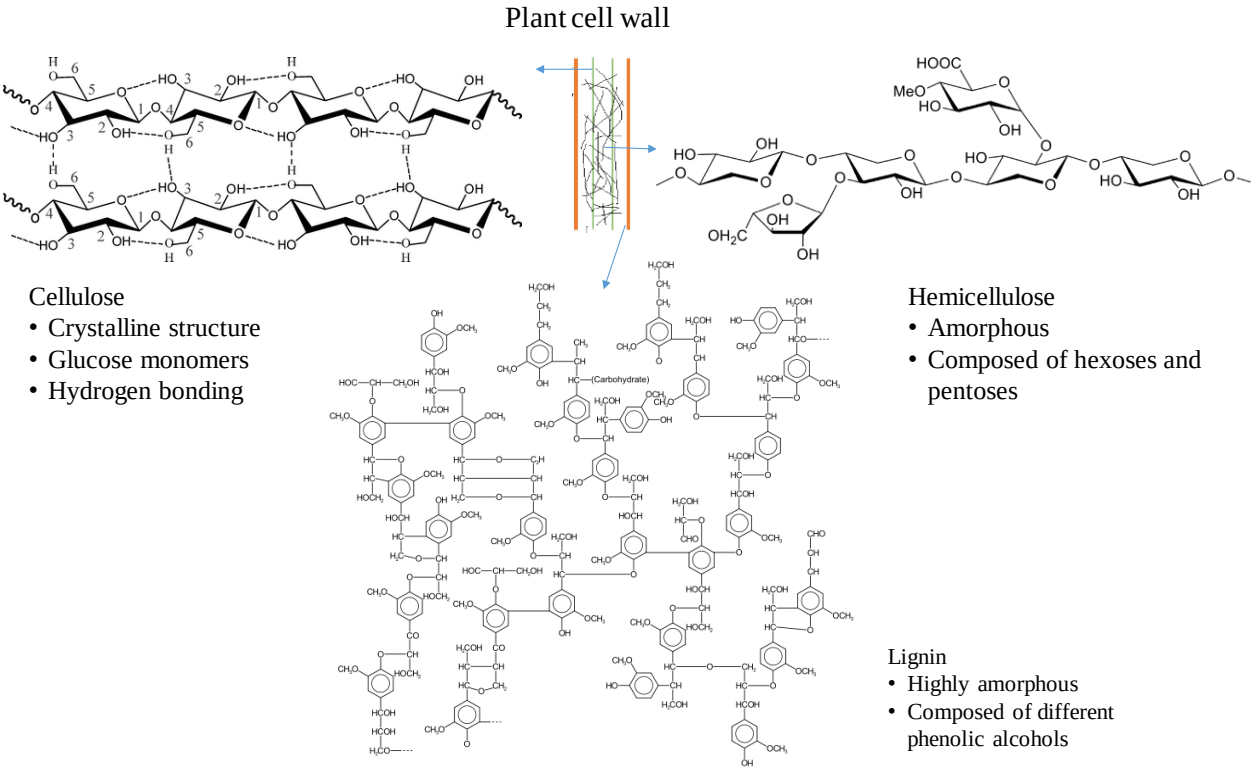

Figure 2.1 Plant cell wall composition and structure (Dusselier et al., 2014)

Hemicellulose is found around the cellulose fibers and works as the bridge between cellulose and lignin. It is a short, greatly branched polymer, composed of 5- and 6-carbon sugars along with sugar acids where pentoses and hexoses sugars are linked by 1-3, 1-4 and 1-6 glycosidic bonds. These bonds are often acetylated and as a result of their hydrolysis, acetate can be produced which is known to inhibit both enzymes and fermentative microorganism (Singhvi et al., 2014).

Lignin is a polyphenolic substance composed of phenyl, propyl, and methoxy groups. It is a non-carbohydrate polymer that encrusts the cell walls and cements the cells together. The combination of hemicelluloses and lignin provides an effective casing around the cellulose which has to be removed before efficient cellulose hydrolysis can occur. Due to the high complexity of its chemical structure and how its composition varies according to the biomass source and the recovery techniques, it has not been possible to define a unique structure of lignin. Nevertheless, general building blocks of lignin are p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Demirbas, 2005; Singhvi et al., 2014; Zhang, 2008).

The highly crystalline and complex structure, conferred by the linkages among these three polymers, makes the depolymerization into glucose, and thus a subsequent ethanol production a challenging feat (Mosier et al., 2005). To increase fermentable sugars yield, accessibility to the cellulose fraction needs to be augmented by weakening the linkages between these polymers in a pretreatment step (Singhvi et al., 2014). Overcoming lignocellulose recalcitrance efficiently, to release fermentable sugars, has been the topic of many research studies as it accounts for one of the costliest steps in cellulosic ethanol production operations (Menon and Rao, 2012; Parajuli et al., 2015; Zhang et al., 2006).

2.4 Lignocellulose pretreatment technologies

Establishment of a successful bioethanol production depends on the implementation of a cost effective pretreatment process. Due to the strong influence this step has on downstream cost (e.g. fermentation inhibition, product concentration and purification) an effective pretreatment needs to achieve decoupling of the main biopolymers composing biomass in order to ease sugar bioprocessing. It needs to avoid sugar degradation and yield low inhibitor concentrations and be able to recover lignin-derivatives for their conversion into valuable coproducts (platform chemicals and fuel additives) (Ramakrishnan, 2011; Westerhof et al., 2011). An additional factor to be pondered is the compatibility of the feedstock to be used (structural carbohydrates) (Menon and Rao, 2012).

Pretreatments found in literature could be classified in different categories depending on the used criteria (Xu and Huang, 2014). The most common grouping is based on the principal mechanism

involved in the process. Therefore lignocellulose pretreatments can be classified as chemical, physical and physicochemical methods (Xu and Huang, 2014) with the option of different combinations among them (Menon and Rao, 2012). To date several strategies for lignocellulose have been developed, but none of them have a specific edge over another due to their natural advantages and disadvantages. As described by Banerjee and collaborators (Banerjee et al., 2010), a suitable pretreatment is highlighted by avoiding size reduction, preserving five carbon sugar fractions, minimal energy input, avoiding hindering products, waste treatment, catalyst utilization and recycling in addition to cost-effectiveness. As lignocellulosic ethanol production is gaining momentum an optimal pretreatment decision should considered the current industrial relevance, needs and applications to further accommodate for its future marketing.

2.4.1 Physical pretreatment

Among all the pretreatments for biomass physical pretreatment is probably the most common since the majority of the biomass requires some sort of particle size reduction. As a result, a larger surface area and lower crystallinity improves hydrolysis results (Parajuli et al., 2015). This could be achieved by different methods such as milling, extrusion and irradiation. The energy requirements will depend on the final particle size. Due to its high energy requirement, if physical pretreatment is the only available option, the energy input will often exceed the energy available in the biomass (Menon and Rao, 2012). Therefore, physical pretreatment will not be a standalone process and is of general practice to combine it with others to increase the energy output.

2.4.2 Biological pretreatment

Biopretreatments focus in the utilization of several wood-degradation microorganisms and their enzymes to alter the composition and structure of biomass (Sun and Cheng, 2002). Brown-, white- and soft- rot fungi have the ability to degrade lignin and hemicellulose. White rots fungi attack the lignin and cellulose fractions by producing enzymes capable of degrading lignin and lignin peroxidase (Boominathan and Reddy, 1992). Some of the advantages of this pretreatment include low energy inputs, mild environmental conditions and no chemical requirements (Salvachúa et al., 2011). Despite these advantages, two main downsides of this technology are the utilization of

cellulose and hemicellulose fractions by the lignin-degradation fungi in addition to slow degradation rates (Sun and Cheng, 2002).

2.4.3 Chemical pretreatments

Chemical pretreatments are the most extensively studied biomass pretreatments. Developed and utilized by the paper industry originally, they have achieved higher quality paper products. The main goal of these pretreatments aimed to remove lignin and desired hemicellulose thus enhancing cellulose biodegradability (Menon and Rao, 2012). Some of these chemical pretreatment strategies techniques involve acid, alkali, solvent, pH controlled liquid hot water and ionic liquids (Mosier et al., 2005; Wyman et al., 2009).

2.4.3.1 Acid pretreatment

Acid pretreatment has been established as one of the main processes in lignocellulosic biomass fractionation (Zhang et al., 2007) due to its ability of removing hemicellulose fraction. It has been successfully used to pretreat biomasses such as corn stover (Digman et al., 2010), poplar (Du et al., 2010) and switchgrass (Li et al., 2010), and it is used in the industrial manufacture of furfural by converting xylose derived from hydrolyzed hemicellulose (Mosier et al., 2005). In acid pretreatment biomass is contacted with diluted or concentrated solutions of a certain acid under specified temperature and pressure conditions. Acid hydrolysis is a reaction in which an acid catalyzes cellulose breakdown releasing oligomers and monosaccharides (glucose), proceeded by degradation of the released glucose into compounds such as hydroxymethylfufrural (HMF) (Saeman, 1945). Concentration of acid is an important parameter to take into consideration since lower pHs will tend to degrade produced sugars while breaking down lignin and hemicellulose, while higher pHs will tend not to overcome the lignin recalcitrance. Ideally pH needs to be in the range of 2.0 to 2.5 to maximize sugar yields. The utilization of sulfuric acid started as a hemicellulose removal agent to increase the digestibility of cellulose of the remaining solids (Brownell and Saddler, 1984). Despite sulfuric acid being the most widely used acid in lignocellulosic biomass pretreatment (Kim et al., 2000), other acids such as phosphoric acid (H_3PO_4) (Israilides et al., 1978), nitric acid (HNO_3) (Mosier et al., 2005) and hydrochloric acid (HCl) (Israilides et al., 1978) have also been tested.

However, some of the limitations for acid pretreatment include the high costs associated with construction materials, due to the high corrosion, a required neutralization of acids prior to sugar utilization, generation of fermentation inhibitors (Mosier et al., 2005) and expensive disposal of neutralization salts (Mcmillan, 1994).

2.4.3.2 Alkaline pretreatment

Alkali pretreatment strategies resemble the Kraft paper pulping technology. It removes the lignin from biomass therefore improving the digestibility of hemicellulose and cellulose. Alkaline pretreatment acts by degrading ester and glycosidic side chains, thus altering the lignin structure, partially dissolving the hemicellulose structure (Ibrahim et al., 2011) in addition to cellulose swelling and partially decrystallizing cellulose (Cheng et al., 2010; McIntosh and Vancov, 2010). The alkaline pretreatments consists of wetting the biomass with an alkaline solution under mixing at a set temperature and time. Some of the reported types of biomass which have been pretreated with this method include corn stover, switchgrass bagasse, wheat and rice straw (Hu et al., 2008; Liang et al., 2010; Park et al., 2010; Sun and Cheng, 2002). The two most widely used strategies comprise processes with sodium hydroxide or lime. Between the two, lime has an advantage over NaOH due to associated costs. Conditions for alkaline pretreatment are usually less drastic than other pretreatments, it can be done at room temperatures although requiring longer reaction times (Sun et al., 1995). As an example the delignification efficacy of different alkaline solutions was analyzed on wheat straw, and it was found that the highest lignin removal (80%) and hemicellulose release (60%) was achieved when the biomass was pretreated at 20°C for 144 hours with a 1.5% NaOH solution (Sun et al., 1995). Nevertheless, alkaline pretreatment precedes enzymatic hydrolysis and requires a step/steps to remove enzymatic and fermentative inhibitors produced in the pretreatment, both are also present when biomass undergoes acid pretreatments.

2.4.3.3 Ionic liquids

Ionic liquids (ILs) are defined as organic salts which melt below 100°C (Ninomiya et al., 2015). Commonly regarded as green solvents, they have unique properties such as low vapor pressure, non-flammable, chemical and thermal stability (Liu et al., 2012). Preparation of ionic liquids (ILs) is

realized using different cations and anion, resulting in hydrophobic or hydrophilic types (Trinh et al., 2015). ILs are capable of breaking the chemical linkages in matrix polymers by disrupting the hydrogen bonds in the crystalline cellulose structure (Tan and Lee, 2012). These solvents are capable of improving biomass digestibility and fermentability of sugars by reducing cellulose crystallinity, lignin and hemicellulose content thus increasing surface area (Perez-Pimienta et al., 2013). These solvents have been successful pretreating yellow pine wood (Cox and Ekerdt, 2013) eucalyptus, switchgrass and bagasse (Perez-Pimienta et al., 2013; Varanasi et al., 2012). As with pretreatments mediated with acids or bases, temperature and time require to be optimized in order to increase efficiency and decrease energy consumption (Yoon et al., 2012). ILs are highly toxic to fermentative microorganisms and could potentially inhibit enzyme activity, therefore thorough rinsing of the pretreated biomass is required. A major drawback from ILs is their non-volatile nature, therefore hampering removal techniques such as distillation. Consequently, concentrating the diluted ILs and treating the residual water results in high costs (Ninomiya et al., 2015).

2.4.3.4 Wet oxidation

Wet oxidation (WO) is used to fractionate lignocellulosic biomass by removing lignin and solubilizing hemicellulose. The process of WO involves two types of reactions; a low temperature hydrolytic reaction and a high temperature oxidative reaction (McGinnis et al., 1983). During this process, lignin is decomposed to carbon dioxide, carboxylic acids and water (Banerjee et al., 2009). Depending on the process parameters, including biomass type, lignin removal ranges between 50% and 70%. Moreover the process has shown to be effective in removing the dense wax coating of straw, reed and other cereal crops, which contain silica and proteins (Schmidt et al., 2002). Other crops that have been successfully pretreated via WO to obtained glucose and xylose after enzymatic hydrolysis, include corn stover, faba beans, sugarcane bagasse, cassava, rye and canola (Ramakrishnan, 2011). Martin and collaborators used WO at 195°C for 10 min using Na_2CO_3 with oxygen at 12 bar for pretreating sugarcane bagasse, rice hulls, cassava and peanuts shells. Bagasse showed the highest xylan solubilisation with 45.2% recovered as xylose and xylo-oligosaccharides, in addition to enhanced enzymatic convertibility of cellulose to 670.2 g/kg. Nevertheless, bagasse yielded the highest amount of degradation products with acetic acid concentrations of 34 g/kg and furfural concentrations of up to 1.8 g/kg of raw material. At these same conditions cellulose

conversions did not surpass 450 g/kg for the rest of the biomasses tested (Martín and Thomsen, 2007). Some other known byproducts of this pretreatment are succinic acid, glycolic acid, formic acid, acetic acid and phenolic compounds, all of which affect downstream processing (fermentation) due to their high growth inhibition potential (Ramakrishnan et al., 2011).

2.4.4 Physico-chemical pretreatments

2.4.4.1 Steam explosion

Steam explosion (SE) is a widespread pretreatment which breaks the structure of lignocellulosic biomass by utilizing both chemical and physical pathways. During steam explosion, the material is exposed to high pressure saturated steam for a short period of time and then quickly depressurized. This sudden expansion due to the rapid depressurization disturbs the microfibrils, which improves the accessibility of the cellulolytic enzymes. The two most important factor affecting the process are retention time and pressure. Pressure (usually between 0.69 and 4.83 MPa) is correlated to temperature (160 – 260°C) (Menon and Rao, 2012) and it is associated with the hydrolysis of cellulose fractions and the kinetics of degradation products formation. It also determines the intensity of the shearing forces when the biomass undergoes explosive decompression (Jacquet et al., 2015). High residence time promotes a complete hydrolysis of the hemicellulose fraction, which enhances downstream processes (fermentation) (Jacquet et al., 2015). However, if residence times are too long, hydrolysis products can undergo dehydration, fragmentation and or condensation. As result of these reactions the formation of by-products such as hydroxymethylfurfural, furfural among other known fermentation inhibitory compounds occurs. Some studies have demonstrated an increment in sugar yield from hemicellulose fractions if H_2SO_4 is added, as it serves as a catalyst (Xu and Huang, 2014). Several biomasses have been positively pretreated with SE. It is a process capable of generating close to complete sugar recoveries, at the expense of a low capital cost. In addition, the lack of harsh chemicals during the process and the conditions at which the process is performed makes it a good candidate for efficient pretreatments (Menon and Rao, 2012).

If SE is combined with wet oxidation, the coupled process would be capable of handling larger particle sizes and of operating at higher substrate loadings (Georgieva et al., 2008). Georgieva and

collaborators found that combining these techniques resulted in a cellulose conversion of 70%, a hemicellulose conversion of 68% and an ethanol yield of 68% for simultaneous saccharification fermentation (Georgieva et al., 2008).

2.4.4.2 Ammonia fiber explosion (AFEX)

Ammonia fiber explosion is a physico-chemical pretreatment similar to steam explosion. In this process instead of using high pressure vapor, the biomass is exposed to liquid ammonia at relatively high temperatures and pressures for a period of time, and as in steam explosion, pressure is suddenly released. Typical temperatures vary between 90 - 100°C, with residence times of 30 minutes. Ammonia loadings vary between 1 and 2 kg per kg of dry biomass. This process affects each biomass fraction differently, as cellulose is decrystallized, whereas hemicellulose is partially depolymerized, and lignin is decoupled from the carbohydrate fraction and at the same time the carbon-oxygen-carbon bonds in lignin are cleaved. The overall effect of these structural disruptions is increased accessible surface area as well as enhanced wettability of the biomass (Zheng et al., 2009). As some of the other pretreatments, AFEX has been used to condition biomasses such as alfalfa, wheat straw, wheat chaff and rice straw (Gollapalli et al., 2002). Low lignin biomasses such as Bermuda grass (5% lignin) and sugarcane basses (15%) have been successfully treated with AFEX to yield cellulose and hemicellulose hydrolysis over 90%. These results suggest that the pretreatment is not suitable for pretreating biomasses with relatively high lignin contents e.g hardwoods and shells (Taherzadeh and Karimi, 2008). Some of the advantages characterizing this process include recovery of the ammonia, and the low production of fermentation inhibitors easing downstream processing of the sample.

2.5 Fast pyrolysis as an alternative for lignocellulosic biomass pretreatment

Generally fast pyrolysis is not considered as a biomass pretreatment process, rather biomass is pretreated before it enters the pyrolysis process. Biomass pyrolysis occurs at high temperatures (500°C) in the absence of oxygen (as nitrogen is usually used as a carrier gas) reaching high heating rates and with low residence times (2s). Breakdown of biomass starts with the decomposition of hemicelluloses between 200°C and 260°C, followed by cellulose decomposing between 240°C and 350°C. Finally the process is completed between 280°C and 500°C when the lignin is degraded

(Demirbas and Arin, 2002). Three main phases result once pyrolysis is completed, a solid phase called biochar, a gas phase named biogas and a liquid phase known as biocrude, bio-oil or pyrolytic oil. The quantities of these yielded phases range between 60-75 wt% of liquid pyrolytic oil, 15-25 wt% of solid char and 10-20 wt% of non-condensable gases. These yields are dependent on the process variables and the compound distribution found in the feedstock to be pyrolyzed.

Fast pyrolysis possesses four characteristics: i) controlled pyrolysis temperature, ii) short vapour residence times, iii) high heat transfer rates which requires fine biomass and iv) the vapors produced are cooled to give bio-oil. There has been a significant amount of research done on the pyrolysis processes. As a result different types of reactors have been developed to improve the yield of pyrolysis-oil by providing the essential characteristics needed to achieve the decomposition mentioned before (Bridgwater, 1999). Table 2.2 summarizes some of the methodologies employed in fast pyrolysis comparing some of their features including the particle size needed and the yield achieved with each of the technologies.

Table 2.2 Fast pyrolysis methodologies and their yields adapted from (Bridgwater et al., 1999)*.

Method	**Yield wt%**	**Advantages, disadvantages and features**
Ablative Pyrolysis	75-80%	Large feedstocks, compact design, heat transfer gas not required, high mechanical abrasion, concerns with heat supply,
Circulation fluidized bed pyrolysis	75-80%	High heat transfer rates; char abrasion and char erosion, possible catalytic activity from char, 6mm max particle size
Fluidized bed	75-80%	High heat transfer rates; heat supply to fluidising gas or to bed directly, decreased char abrasion, increase solid mixing, particle size < 2mm
Vacuum pyrolysis	60-65%	Low heat transfer rates; particle size limit <2 mm; limited gas/solid mixing. Expense.

*adapted with permission from (Bridgwater et al., 1999). Copyright 1999 Elsevier

Fast pyrolysis has some advantages over other pyrolysis processes, such as low production costs, high thermal efficiency, low fossil fuel input and potential carbon dioxide neutrality from utilizing agricultural and other biomass wastes. In addition, the liquid yielded offers the possibility of easy handling and more consistent quality compared to any solid products (Oasmaa et al., 2003). Table 2.3 offers an overview of the yields for the three phases obtained through different types of pyrolysis processes.

Table 2.3 Product yields obtained from different types of pyrolysis adapted from (Mohan et al., 2006)*

Process	Product Yield (%)		
	Liquid	**Char**	**Gas**
Fast Pyrolysis (moderate temperature and short residence time)	75	12	13
Carbonization (Low temperature and low residence time)	30	35	35
Gasification (high temperature and long residence time)	5	10	85

*adapted with permission from (Mohan et al., 2006). Copyright 2006 American Chemical Society

2.5.1 Biomass pyrolysis products & properties

2.5.1.1 Pyrolytic oil

Pyrolysis oil provides several environmental advantages over fossil fuels. The first of these advantages is the potential carbon dioxide balancing; combustion of biomass releases carbon dioxide but that carbon dioxide could be offset through photosynthetic processes during plant growth, (Mohan et al., 2006) whereas crude oil is not carbon neutral.

Pyrolysis oils are dark brown organic liquids which composition resembles the biomass composition from which they were derived, therefore possessing high oxygen content. They are formed by depolymerizing and fragmenting the main components found in biomass, cellulose, hemicelluloses and lignin with the aid of a rapid and sudden increase in temperature. This liquid can be considered a microemulsion in which the continuous phase is an aqueous solution of holocellulose decomposition products and small molecules from lignin macromolecules (Piskorz and Scott, 1987). The pyrolysis-oil has some disadvantages as it could age after it is first recovered, which manifests

itself in many cases as a viscosity increase. In addition, phase separation is possible, which is believed to occur from a breakdown in the emulsion stability and to subsequent chemical reactions occurring in the pyrolysis oil. Due to the presence of aldehydes, ketones and other compounds that can react via aldol condensation, oils from pyrolysis can undergo undesirable changes in its physical properties, for example the viscosity and water content can increase, while the volatility of the oil can decrease (Czernik et al., 1994). It is known that one of most important variables driving this change in properties is temperature.

The oxygen presence is the primary reason for the difference in the properties and behavior between regular crude oil and pyrolytic oils, Table 2.4, since the amount of oxygen in the pyrolytic oils range between 45-50 wt% (Bridgwater, 1999). The oxygen is distributed in more than 300 compounds that have been identified within the pyrolysis oil. Some of these components are organic acids, e.g. acetic and formic acids, accounting for the low pH, which results in a corrosive nature of the pyrolytic-oil and limits the use of common storage materials such as aluminum (Czernik and Bridgwater, 2004). It is immiscible with liquid hydrocarbons because of its polarity and hydrophilic nature (Mohan et al., 2006). One consequence of this high oxygen content is the resulting low energy density (heating value) 14-18 MJ kg-1, which is less than 50% of that for conventional fuels. Table 2.4 depicts a comparison between the properties of pyrolysis -oil and regular crude oil.

The single most abundant compound in pyrolysis-oil is water ranging from 15-30 wt% (Bridgwater and Boocock, 1997; Czernik and Bridgwater, 2004). This high water concentration is the result from the original moisture in the feedstock and as a product of the dehydration reactions occurring during pyrolysis.

Despite the difference in energy density, pyrolytic-oil has been used successfully for generating heat at the Red Arrow Products pyrolysis plant in Wisconsin (Bridgwater and Boocock, 1997). This swirl burner uses different fractions of the byproducts, pyrolytic lignin, char and gas from the plant producing food flavoring compounds. However there are still many challenges to tackle before pyrolysis oil becomes a substitute for conventional oil at larger scales (Czernik and Bridgwater, 2004).

Table 2.4 Properties of pyrolysis-oil and of regular crude oil adapted from (Czernik and Bridgwater, 2004)*

Physical property	Fuel	
	Pyrolysis oil	Crude oil
moisture content, wt%	15-30	0.1
pH	2.5	-
specific gravity	1.2	0.94
elemental composition wt%		
C	54 - 58	85
H	5.5 - 7.0	11
O	35 - 40	1
N	0 - 0.2	0.3
ash	0 - 0.2	0.1
HHV, MJ/kg	16 - 19	40
Viscosity @50°C, cP	40 - 100	180
solids wt%	0.2 - 1	1
distillation residue, wt%	up to 50	1

*adapted with permission from (Czernik and Bridgwater, 2004). Copyright 2004, American Chemical Society

2.5.1.2 Phase Separation of Liquid Fraction

As mentioned, pyrolysis oil is a mixture which contains up to 30% water. This water is miscible with oligomeric lignin derived components and it dissolves low molecular weight acids, alcohols, hydroxyaldehydes, aldehydes, ketones, furans, phenols, sugars and anhydrous sugars which result from the decomposition of carbohydrates (Brown, 2007). It is likely that yielding higher amounts of pyrolysis oil will yield more carbohydrates, which then can be extracted in the water. The separation of the bio-oil into organic and aqueous phases occurs during storage. It is known that the majority of the components identified in the organic phase are also present in the aqueous phase, with the ratio being empirically measured using a partitioning coefficient and the solubility in water (Xu et al., 2009).

Despite the high concentration of different compounds in the aqueous phase, it cannot be used directly as a bio-fuel due to the high water content. However, the aqueous phase can serve as a source for the extraction of valuable compounds such as: steroids (Pakdel and Roy, 1996), phenolics (Pakdel et al., 1997), formic and acetic acid, products for the food industry such as syringol responsible for the smoky smell, hydroxyacetone, furfural and small amounts of guaiacols, but most importantly sugars such as glucose, xylose, arabinose, mannose, galactose and levoglucosan 1,6-anhydro-β-D-glucopyranose, which can be hydrolyzed to produce glucose for later fermentation. It is important to note that currently there are no industrial uses for the aqueous phase pyrolysis fraction, but studies regarding the fermentation of this phase are increasing (Chan and Duff, 2010; Lian et al., 2013, 2012, 2010; Luque et al., 2014; Yu and Zhang, 2003), bringing the aqueous phase into the picture of the second generation bio fuels.

2.5.1.3 Carbohydrates from pyrolysis

Immersed in this complex pool of chemicals resulting from the pyrolysis of biomass, the main component found is Levoglucosan 1,6-anhydro-β-D-glucopyranose. Levoglucosan results mainly from the pyrolysis of the cellulose fraction found in the biomass and it has become a potential feedstock in the fermentation industry (Zhuang, 2001). Direct utilization of levoglucosan as a carbon source by microorganisms require a levoglucosan kinase enzyme that will convert it to glucose (Xie et al., 2006). Utilizing cloning one of these genes into *E. coli*, Layton and collaborators (Layton et al., 2011) were able to genetically modify a strain of *E. coli* to produce ethanol from pure levoglucosan, but inhibited when pyrolytic levoglucosan fractions were used (Chi et al., 2013). Some studies have shown that the utilization of this compound can be achieved by oleaginous yeast (Lian et al., 2013) or hydrolyzed to produce glucose for ethanol production (Luque et al., 2014). As a consequence, strategies aiming to increase the carbohydrate fraction in pyrolytic oils have been developed. These strategies have focused on increasing the yield of levoglucosan by leaching the biomass prior to pyrolysis (Oudenhoven et al., 2015, 2013) or by increasing the collected oil utilizing a fractional condensation approach (Westerhof et al., 2011) or a combination of both (Oudenhoven et al., 2013). During biomass leaching, removal of alkaline and alkali earth ions (K^+, Na^+, Ca^{2+} and Mg^{2+}) increased levoglucosan yields as these ions have been found to catalyze levoglucosan-to-inhibitors degradation reactions (Kuzhiyil et al., 2012). Westerhof and collaborators (2011) observed

that by modifying the temperature of a two condensers set in series, vapors produced in the pyrolysis process could be collected in different fractions with varying properties. Temperatures above 70°C resulted in a pyrolytic oil with a lower water and volatile molecules content, hence increasing the fraction of oligomers in the resulting pyrolytic oil (Westerhof et al., 2007). Other carbohydrates like glucose, mannose and galactose have been reported as pyrolysis products found in the aqueous phase but at much lower concentrations (Fabbri and Chiavari, 2001). Different sugars such as sorbitol, cellobiosan, cellobiose and arabinose have also been reported (Lian et al., 2010). It is important to highlight that these carbohydrates can be found along several other compounds known to be fermentation inhibitors due to their toxic nature towards microorganisms are also found.

2.6 Fermentative microorganisms

Fermentation is a process that can be carried out by different microorganisms. It has been used for thousands of years in the making of bread, wine and cheese among other food products. When setting up a fermentation process it is important to know what kind of substrate is available for use, and what are the desired products. The products of a fermentation range from alcohols up to antibiotics including lipids (Lian et al., 2013). These processes can be carried out by eukaryotic and prokaryotic microorganism. The most common microorganisms for ethanol fermentation are *Saccharomyces cerevisiae* and *Zymomonas mobilis* and for butanol fermentation *Clostridium acetobutylicum* and *Clostridium beijerinckii* are the most common species. Each of these species are comprised of different strains which have been improved industrially via adaptative evolution and genetic engineering over the years in order to achieve higher yields of the desired product.

2.6.1 *Saccharomyces cerevisiae*

This yeast is the most commonly used microorganism for ethanol production. It is used in baking, brewing and wine industries. The main metabolic pathway responsible for ethanol production is glycolysis, triggered by the Crabtree effect. In this pathway, glucose is metabolized to pyruvate, under aerobic conditions, and then this pyruvate is reduced to ethanol producing CO_2. Several studies regarding the capacity of *S. cerevisiae* to ferment decomposed cellulose substrate are available, however most are based on cellulose that has been acid hydrolyzed (Yu and Zhang, 2004) or when

the cellulose pyrolysis products have been pretreated (Yu and Zhang, 2003). More recently, the aqueous phase from pyrolysis has been studied as a fermentation substrate due to its high anhydrous sugar content (Bennett, Helle, & Duff, 2009). Even though extensive research exists, it is known that inhibition occurs when decomposed cellulose, hemicelluloses or lignin are used as a substrate.

2.6.2 *Zymomonas mobilis*

Z. mobilis is a gram negative bacterium and facultative anaerobe notable for its ethanol producing capabilities. This bacterium degrades sugars to pyruvate which is then fermented to produce ethanol and carbon dioxide. The pathway used by this bacterium is called the Entner-Doudoroff pathway (Stevnsborg & Lawford, 1986). It presents some advantages over *S. cerevisiae* regarding the ethanol production since it has a higher sugar uptake and a higher ethanol yield, lower biomass production, higher ethanol tolerance, it does not require controlled addition of oxygen during the fermentation and is easier to genetically manipulate. This high tolerance to ethanol comes from the hopanoids content in the plasma membrane, which resemble eukaryotic sterols. Despite these advantages it has a severe limitation as it is restricted to a small range of substrates for fermentation, namely glucose, fructose and sucrose. This limited range has the potential to expand since research has shown that genetic engineering with genes from other species such as *E. coli* have the potential to optimize the bioethanol production (Ranatunga et al., 1997). *Z. mobilis* has been used as a biocatalyst in the fermentation of acid hydrolysis pretreated cellulose pyrolyzate (Yu and Zhang, 2003) but little is known about its performance utilizing pyrolytic derived carbohydrates likely due to the limited flexibility in sugar utilization. Additionally, *Z. mobilis* ethanol fermentation is hindered by inhibitory substances present in this pyrolysis by-product such as organic acids, phenolics and carbohydrate degradation products (Ranatunga et al., 1997)

2.6.3 *Clostridium species*

Clostridium is a diverse genus of gram positive obligate anaerobic microorganism capable of producing endospores, some of the species are pathogenic and some non-pathogenic. The non-pathogenic strains have the ability to produce acetone and butanol. The industrial significance of acetone-butanol production decreased in the early part of the 1960s due to unfavorable economic

conditions brought about by competition with the petrochemical industry (Ezeji et al., 2007). The most common species used in the production of biobutanol are *C. acetobutyliicum, C. saccharobutylicum, C. butylicum and C. beijerinckii*. Extensive literature has covered the Acetone-Butanol-Ethanol fermentation, known as ABE fermentation, on different kinds of substrates but when it comes to the pyrolysis products little research has been done to date. Bacterium belonging to this genus show high sensitivity for compounds produced in the hydrolysis of the cellulose, just as the previous described microorganism. As described by Ezeji and coworkers (2007) *p*-coumaric and ferulic acids decrease the ABE production but compounds such as furfural and hydroxymethyl furfural stimulate the microorganism growth, enhancing the ABE production. As a fuel, butanol has many advantages over ethanol including lower water solubility and higher miscibility with gasoline. This makes the study of butanol production from agricultural wastes a promising avenue for developing renewable fuels and fuel supplements.

2.6.4 Oleaginous yeasts

As butanol, biodiesel is considered a drop-in fuel for established diesel vehicles and boiler engines. It is highly degradable and non-toxic that if combusted emits lower CO, CO_2 a SO and particulate matter levels (Atabani et al., 2012). Biodiesel is synthesized via a transesterification reaction of triacylglycerides into fatty acid methyl esters (FAMEs) (Sitepu et al., 2013). Yeasts capable of accumulating more than 20 wt% oil are considered to be oleaginous. This microbial oil has similar composition and energy values as the plant oils biodiesel is currently being derived from, but with the advantage that production does not compete with food production nor land utilization. Oil production from lignocellulosic hydrolysates has been achieved by a few yeast *Rhodosporidium toruloides* (Wang et al., 2012; Yu et al., 2011) *Cryptococcus curvatus, Rhodotorula glutinis, Lipomyces starkeyi, Yarrowia lipolytica* (Yu et al., 2011) and *Trichosporon fermentans* (Zhan et al., 2013). Other recent studies have shown utilization of carboxylic acids (Lian et al., 2012) or levoglucosan (Lian et al., 2013) derived from pyrolysate fractions for oil production. Moreover as shown by Sitepu and collaborators (Sitepu et al., 2014) some yeast are also capable of producing additionally value products such as carotenoids, but their performance in a pyrolytic derived media is still to be determined. This type of microorganisms show and incredible plasticity to different

carbon sources (Sitepu et al., 2014) and depending on the pyrolytic oil and their tolerance to fermentation inhibitors they are potential candidates to be used in such a process.

2.6.5 Microalgae

Microalgae are single celled photosynthetic microorganism capable of transforming carbon dioxide and water into lipids (Fu et al., 2010) which can be trans-esterified to produce biodiesel (Chisti, 2007). In addition to its photoautotrophic growth capacity, some microalgae species are capable of growing under heterotrophic or mixotrophic conditions (Gélinas et al., 2015). Heterotrophic and mixotrophic culture conditions require the addition of organic carbon, which can enhance lipid accumulation and cell division (Gélinas et al., 2015). During heterotrophic growth, microalgae utilize organic compounds as a carbon and energy source (Wang et al., 2014). As this mode is independent of light, heterotrophic cultivation of microalgae has the potential to avoid photolimitation challenges encountered in photoautotrophic cultivation, therefore achieving higher biomass productivity (Liang et al., 2009; Liu et al., 2011). Lipid accumulation under heterotrophic conditions is comparable or higher than the obtained under photoautotrophic conditions (Miao and Wu, 2004; Xu et al., 2006) translated into higher lipid productivity. Some of the disadvantages with heterotrophic cultivation includes an increased cost due to the bioreactors needed for cultivation (Zhang et al., 2013) and increased risk of contamination by other microorganism related to the organic compounds used (Chen et al., 2011).

As oleaginous yeast, utilizing microalgae for 2nd generation biofuels would not compromise food production (Chisti, 2007) as they have higher oil yields per hectare than current sources and are capable of utilizing different nutrient sources (Mata et al., 2010). Lipids produced from microalgae are different from current oil vegetables and as a result biodiesel quality might not meet the required diesel standard (Chisti, 2007). However, alternating the environmental conditions to algae cultivation can shift the biosynthesis of fatty acids significantly (Los and Murata, 2004). These observations have prompted studies of lipid productivity from different waste water streams (Lu et al., 2015; Sacristán de Alva et al., 2013; Shin et al., 2015) and some from lignocellulosic hydrolysates (Li et al., 2011; Liang, 2013) which have shown that microalgae can accumulate lipid in a variety of environments. To the best of our knowledge, cultivation of algae in pyrolytic fractions has not been

studied, rather residual algal biomass is used as a pyrolytic feedstock (Xie et al., 2015; Zhao et al., 2015).

2.6.6 Inhibition on fermentative microorganism

Some of typical biomass decomposition compounds, showed in Table 2.5, have been extensively studied with regard to their inhibitory characteristics on ethanol fermentative microorganisms such as *Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, Candida shehatae* by Delgenes and collaborators (Delgenes et al., 1996) and *Escherichia coli* by (Zaldivar and Ingram, 1999; Zaldivar et al., 2000, 1999). In addition, these inhibitory effects have also been recorded on butanol fermentative microorganisms, such as *Clostridium beijerinckii* (T. Ezeji et al., 2007). The majority of the reviewed papers focus on compounds formed by the acid hydrolysis of biomass, however since pyrolysis is also a process in which biomass is decomposed, many of the resulting compounds overlap giving a clear idea of the effects of these compounds on microorganisms.

Several of the compounds described in the literature present synergistic effects. Zaldivar et al. (Zaldivar and Ingram, 1999; Zaldivar et al., 2000, 1999) analyzed the effects of different compounds divided into families: alcoholic compounds, aldehydes and organic acids in three different studies. The studies concluded that some components were more toxic than others, methylcathecol for the alcohols studied and furfural for the aldehydes. In the case of the organic acids they were unable to show significant difference between their inhibitions.

Zaldivar and collaborators (Zaldivar et al., 2000) also concluded that the alcohols tested showed a decreased inhibition when compared to the aldehydes and the organic acids. In addition, the three studies reported a synergistic effect of the compounds used in the study, which suggests that if all the compounds are found in the aqueous phase the fermentation microorganisms will be more prone to inhibition, independent of the microorganism. Concentrations required to inhibit the ethanol production by organic acids are depicted on Table 2.6.

Table 2.5 Inhibition on fermentative microorganisms by organic acids derived from lignin decomposition adapted from (Zaldivar and Ingram, 1999)*.

Acid	Concentration (g/L)	Inhibition (%)	Microorganism
Acetic	6	74	S. cerevisiae[1]
Levulinic	40	50	S. cerevisiae[2]
Caproic	0.064	46	Z. mobilis[3]
Gallic	0.173	19	Z. mobilis[3]
4-Hydroxybenzoic	1	30	S. cerevisiae[4]
Syringic	1	-17	S. cerevisiae[4]
Vanillic	3.7	50	S. cerevisiae[2]

[1] (Phowchinda, Deliadupuy, & Strehaiano, 1995) [2] (Clark & Mackie, 1984) [3] (Ranatunga et al., 1997) [4] (Ando, Arai, Kiyoto, & Hanai, 1986)

*adapted with permission from (Zaldivar and Ingram, 1999). Copyright 1999 John Wiley & Sons, Inc.

This interesting behaviour was addressed by Palmqvist and collaborators studying the main interaction effects of acetic acid, furfural and p-Hydroxybenzoic acid on growth and ethanol productivity of yeast (Palmqvist et al., 1999). In this study four variables were measured: cell yield, specific growth rate, ethanol yield and volumetric ethanol productivity. Table 2.6 displays a summary on how the different compounds affected the response variables. *p*-Hydrozybenzoic acid showed no significant effects on any of the variables which contrasts with previous reports (Ando et al., 1986). The difference regarding the two studies was attributed to the difference strains used and the experimental setup. This highlights the difficulty in ensuring reproducible, general results for the effect of inhibitory compounds. It is important to highlight that some compounds have even positive effects on the growth and can account for some inhibition compound removal after a period of adaptation as reported by Chan and coworkers (Chan and Duff, 2010).

Table 2.6 Effects of acetic acid and furfural on four response variables in *S. cerevisiae* using a central composite design. (-) negative effect (+) positive effect. Specific growth rate (μ), cell mass yield ($Y_{x/s}$), ethanol productivity (Q_{EtOH}) and ethanol yield (Y_{EtOH}) adapted from (Palmqvist et al., 1999)*

Compounds	**Responses**			
	μ ***(h^{-1})***	***$Y_{x/s}$*** ***($g\ g^{-1}$)***	***Q_{EtOH}*** ***($g\ L^{-1}\ h^{-1}$)***	***Y_{EtOH}*** ***($g\ g^{-1}$)***
Acetic acid	No effect	No effect	+ (0 - 9 g/L) - (9 - 10g/L)	+(0 - 10 g/L)
Furfural	- (0 - 3 g/L)	+ (0 - 2 g/L) - (2 - 3 g/L)	+ (0 - 3 g/L)	+ (0 - 2 g/L) - (2 - 3 g/L)
Acetic Acid and furfural	-	-	No Interaction	-

*Adapted with permission from (Palmqvist et al., 1999). Copyright 1999 John Wiley & Sons Inc.

As previously mentioned, the aqueous phase of pyrolysis-oil is a complex mixture containing over 300 different compounds (Bridgwater et al., 1999). Recent studies trying to unveil the industrial applications of fast pyrolysis in biofuels and biochemical applications showed that only 40% wt of the pyrolyzate was able to be detected with GC/MS-FID (Butler et al., 2013). Hence evaluating the inhibition exerted by each individual component has limited value due to the demonstrated interaction/synergistic effects which have been demonstrated, as well as extremely time-consuming. Rather, studies have focused in selecting one compound to represent an specific class like in the study performed by Palmqvist et al. (2000) where *p*-hydroxybenzoic was chosen as the representative of the total phenolics, since it makes up a large fraction of phenolics derived from the hydrolysis of lignin (Abnisa et al., 2011).

2.6.7 Upgrading of the pyrolytic oil for fermentation purposes

The complex mixture of compounds found in the aqueous phase shows the potential for many uses, in many different industries. Compounds ranging from low molecular weight organic acids to larger steroid molecules are found dissolved in this phase (Pakdel and Roy, 1996). Nonetheless many of the compounds can be removed when the aqueous phase is fractionated with solvents (Mohan et al.,

2006). Some of the more valuable compounds which remain after this fractionation are the anhydrous sugars and sugars that can be processed using biocatalysts. However they are not ready to be directly used in fermentation. Firstly, because many compounds present in the mixture are toxic and hinder ethanol production through different mechanisms and secondly because the most abundant carbohydrate component is levoglucosan, a sugar which cannot be broken down by the majority of microorganisms but which can be hydrolyzed to yield glucose, an easily fermentable sugar.

Several approaches to ease the problems associated with the toxic compounds range from neutralization of acids using excessive base (hydroxides) to sorption to different matrices and combinations of the two (Yu and Zhang, 2004). Sorption matrices such as activated carbons, diatoms, bentonite and zeolites were studied by Yu and Zhang (2004). More recently Klasson et al. (Klasson et al., 2011) assessed the feasibility of removing furfurals from sugar solutions using activated biochars made from pyrolysis of agricultural wastes, meaning that pyrolysis has the potential of producing inhibitory compounds but at the same time may potentially provide a method removing those same compounds. Polymeric adsorbents like XAD-4 and XAD-7 were used to removed fermentation inhibitors formed during pretreatment of biomass (Weil et al., 2002) and showed promising results adsorbing furfural, which is one of the primary inhibitory compounds identified in pyrolysis-oil. This study also reported that resin hydrophobicity was the main component responsible for the attraction of the inhibitor compounds to the resin.

2.7 Conclusions

Some of the challenges associated with conventional pretreatments of lignocellulosic biomass are also common to biomass pyrolysis, however, pyrolysis utilization offers several advantages as this process is capable of releasing different compounds distributed among three main phases, including fermentable sugars and platform chemicals. Contrasting with the other pretreatments, the liquid fraction produced in pyrolysis can serve not only as a source for fermentable substrates but it can also be regarded as a source of different platform chemicals and fuel additives. In the case petrochemical products, the process would differ from a process aiming for a sugar rich stream. Yet, when isolating the sugars undesired compounds are also isolated and become one of the challenges in the assimilation of these sugars by biocatalysts to produce biofuels. Thus further improvement of the

upgrading strategies and characterizing how these strategies affect biofuel production is necessary to design a robust pyrolysis based biorefinery capable of utilizing different feedstocks to produce a wide range of biofuels and chemicals.

2.8 References

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Chapter 3

3 Pyrolysis based bio-refinery for the production of bioethanol from demineralized lignocellulosic biomass

Luis Luque, Roel Westerhoff, Stijn Oudenhoven, Guus van Rossum, Sascha Kersten, Franco Berruti and Lars Rehmann.

The information in this chapter has been slightly changed to fulfill formatting requirements. This chapter is substantially as it appears in Bioresource Technology, June 2014, Vol 161, pages 20-28.

This chapter describes a novel biorefinery approach for the exploitation of underutilized pyrolytic oils as a source for fermentable sugars for ethanol production. It had been previously observed that sugar yields in pyrolytic oils could be improved by demineralized the biomass with dilute acid solutions prior to pyrolysis (Oudenhoven et al., 2013). In this study, fast pyrolysis of demineralised and non-demineralised lignocellulosic biomass with fractional condensation of the products was used as the thermochemical process to obtain a pyrolysis-oil rich in anhydrous sugars (levoglucosan) and low in fermentation inhibitors. To isolate the sugars from the inhibitors in the oil, two sequential liquid extractions were performed. As a result, an aqueous solution containing levoglucosan was obtained and used to obtain glucose via acid hydrolysis making it the last upgrading step. The obtained pyrolytic glucose was compared to laboratory grade glucose for its fermentability potential in a high throughput fermentation experiment. This fermentation allowed to evaluate in real time the ability of an representative ethanol producing yeaast to produce ethanol from increasing fractions of pyrolytic sugars. Consequently, inhibition increased as compounds were added along with the pyrolytic glucose.

Even though some of these compounds are well known for their potential to hinder growth and ethanol production (Palmqvist et al., 1999), establishing the effects of a pyrolytic fraction had not been previously quantified. The inhibitory effect of thermochemically derived fermentation substrates was quantified numerically to compare the effects of different process configurations and upgrading steps within the biorefinery approach. Ethanol yields comparable to traditional biochemical processing were achieved (41.3% of theoretical yield based on cellulose fraction).

Additional benefits of the proposed biorefinery concept comprise valuable by-products of the thermochemical conversion like bio-char, mono-phenols (production of BTX) and pyrolytic lignin as a source of aromatic rich fuel additive.

The study described in this chapter fulfilled the first four objectives of this thesis. First, development of a high throughput methodology to evaluate the fermentation potential of pyrolytic derived substrates. Achieving this objective allowed to monitor inhibition effects in real time, and enabled the generation of sufficient data, to quantify three growth parameters to measure the degree of inhibition. Second, evaluating the effects of biomass demineralization on growth and ethanol production. Removing minerals from the biomass not only increased the levoglucosan concentration, but facilitated the upgrading of the pyrolytic oil as well as improved ethanol titers. The third objective was to determine to what extent each upgrading process improved the fermentability of the obtained sugars. It was observed that to achieve fermentation of a 100 % pyrolytic substrate the three studied steps were necessary as they were found to complement each other. Lastly, the fourth objective was to characterize the inhibition effects. Determination of three growth parameters, adaptation time, maximum growth rate and maximum cell density, allowed to compare between the different configurations studied, in addition it permitted to correlate the inhibition with the ethanol yields.

Abstract

This paper evaluates a novel biorefinery approach for the conversion of lignocellulosic biomass from pinewood. A combination of thermochemical and biochemical conversion was chosen with the main product being ethanol. Fast pyrolysis of lignocellulosic biomasss with fractional condensation of the products was used as the thermochemical process to obtain a pyrolysis-oil rich in anhydro-sugars (levoglucosan) and low in inhibitors. After hydrolysis of these anhydro-sugars, glucose was obtained which was successfully fermented, after detoxification, to obtain bioethanol. Ethanol yields comparable to traditional biochemical processing were achieved (41.3% of theoretical yield based on cellulose fraction). Additional benefits of the proposed biorefinery concept comprise valuable by-products of the thermochemical conversion like bio-char, mono-phenols (production of BTX) and pyrolytic lignin as a source of aromatic rich fuel additive. The inhibitory effect of thermochemically derived fermentation substrates was quantified numerically to compare the effects of different process

configurations and upgrading steps within the biorefinery approach. The fourth objective was to quantify the inhibition with three growth parameters

3.1 Introduction

Fast pyrolysis is a thermo-chemical process in which biomass is converted, in the absence of oxygen and at temperatures between 400 and 550°C, to char, gas and pyrolysis oil (Bridgwater et al., 2002). Pyrolysis oil is a promising intermediate, suitable for transportation, storage, and further processing through traditional petrochemical processes. However, integrating pyrolysis oil into traditional petrochemical refineries can be challenging and has not been realized at commercial scale, largely due to its complex and variable composition and, especially, its high oxygen and water concentrations. Based on biomass type and operating condition, pyrolysis can yield up to 75 wt% pyrolysis oil containing a significant amount of anhydrosugars (Czernik and Bridgwater, 2004). Recently, substantial efforts have been made at increasing the yield of anhydrosugars with the goal of subsequent fermentative conversion to ethanol (Oudenhoven et al., 2013).

It is well understood that anhydrosugars concentration in pyrolytic oils can be increased if biomass is pretreated via acid washing. Several researchers studied the removal of hemicelluloses and inorganic ash prior to pyrolysis (Shafizadeh and Stevenson, 1982) by pretreating via mild acid hydrolysis (Radlein et al., 1987) and strong acid impregnation of the biomass, where the levoglucosan (main sugar product of pyrolysis) yield increased to up to 15% of the biomass used (Dobele et al., 2003). The acid treatment removes alkali ions known to decrease levoglucosan yields by two connected pathways. Ions hinder cellulose depolymerisation into anhydrous sugars, and, once depolymerized, ions serve as catalysts in anhydrosugar fragmentation reactions (Radlein et al., 1987). Oudenhoven and collaborators studied the effect of demineralizing biomass using diluted acetic acid at 90°C and 800 rpm for 2h and reported an increase of 18 wt% on the levoglucosan yield, demonstrating that mineral acids can be substituted by actual pyrolysis products (e.g. acetic acid) (Oudenhoven et al., 2013). Anhydrosugars can be converted to glucose through hydrolysis, a substrate that can directly be used for ethanol production (Vispute and Huber, 2009). In addition to sugars, pyrolysis oil contains many other compounds, such as acids, aldehydes, phenols, ketones and alcohols. After utilization of the sugars, these other compounds can also be used for chemicals

production (e.g. acetic acid, mono-phenols, etc.) or for the production of transportation fuels (large water insoluble lignin derived oligomers can be converted by hydrotreating processes) (Westerhof et al., 2011).

Previous studies have shown that some of the pyrolysis oil compounds substantially inhibit the ethanol fermentative microorganisms (Oudenhoven et al., 2013; Palmqvist and Hahn-Hägerdal, 2000; Zaldivar et al., 1999). To date, pyrolysis oil has not been fully characterized and, therefore, not all potential inhibitors are known. Characterization is commonly done by only identifying groups of compounds or identifying highly resolved peaks (Ben and Ragauskas, 2013; Salehi et al., 2009). Several compounds such as furfural, p-hydroxybenzoic acid, alcoholic compounds, aldehydes, acetic acid and other organic acids have been investigated separately and combined to determine to which extent the fermentation is hampered or enhanced (Palmqvist and Hahn-Hägerdal, 2000; Schwab et al., 2013; Zaldivar and Ingram, 1999; Zaldivar et al., 2000). These studies provide some insight in how these compounds inhibit growth, some including important synergistic effects. Lian and collaborators, used the whole pyrolysis oil and found that phenols are strong inhibitors in fermentation processes. Thus, removal of these compounds (detoxification) has been proposed as an additional process step prior to fermentation (Lian et al., 2012).

Detoxification approaches encompass different methods, such as adsorption of the resulting hydrolyzate on different polymer matrices such as amberlite XAD-4 or XAD 7, evaporation (Weil et al., 2002), adsorption on activated carbon (Lin and Juang, 2009; Wang et al., 2012) or on bentonite or zeolites (Yu and Zhang, 2003), overliming (Chi et al., 2013), air stripping (Wang et al., 2012), and solvent extraction (Lian et al., 2010; Wang et al., 2012). The main limitations of using adsorption matrices are the high cost associated either with the matrices or with the high costs of regenerating them. These high prices of synthetic resins and activated carbon created recent interest research on low cost alternatives such as natural zeolites (Lin and Juang, 2009). Alternatively, adaptative evolution of ethanol fermentative microorganisms has been proposed (Lian et al., 2010). Some natural occurring organisms are also able to directly metabolize levoglucosan into itaconic and citric acid (without the need to chemically convert it to glucose) (Zhuang and Zhang, 2002) and a genetically engineered strain of *Escherichia coli* has been created for direct ethanol production from pure levoglucosan (Layton et al., 2011). The modified strain could produce, 0.35g ethanol/g (pure)

levoglucosan, nevertheless, direct fermentation of levoglucosan present in pyrolysis oil, and thus in the presence of inhibitors, has yet to be realized. This study presents a proof of concept for producing relevant amounts of ethanol from lignocellulosic biomass via a fast pyrolysis biorefinery approach as illustrated in Figure 3.1

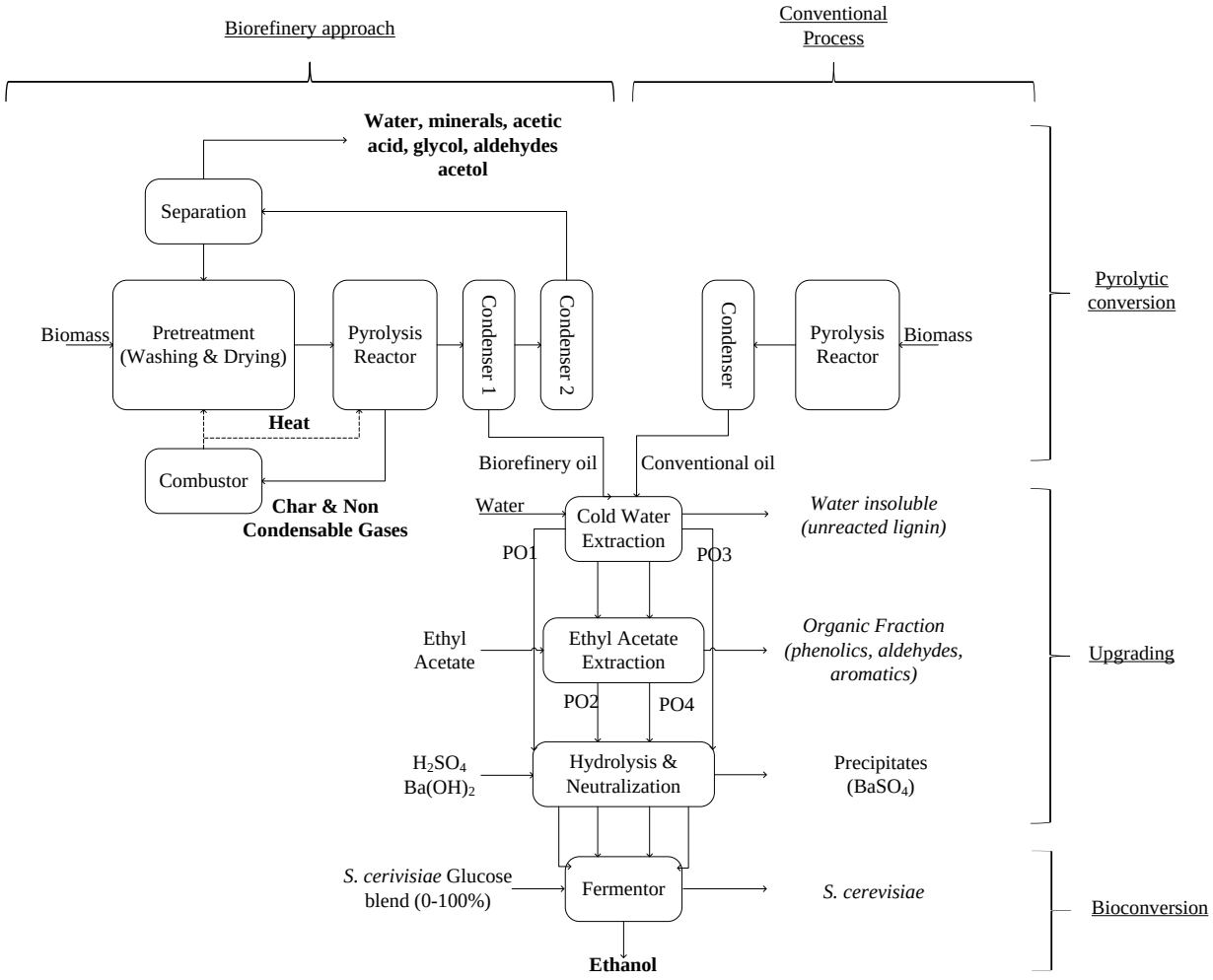

Figure 3.1 Process layout comparison for the production of sugars, aromatics and light oxygenates from lignocellulosic biomass via fast pyrolysis (Oudenhoven et al., 2013). Conventional process showed on the right. Streams in italics represent current value-added.

The proposed process configuration results, amongst other streams, in a concentrated sugar stream, which can subsequently be biologically converted to ethanol without the need for major upgrading

prior to the fermentation. In the proposed process, three distinct chemical classes can be identified in the condensable fraction, a water rich fraction containing light oxygenated compounds (including acids), sugars, and aromatics. High anhydrosugar yield (up to 18wt% on biomass intake) and concentration (up to 37wt%) in the condensates can be obtained via a combination of fractional condensation (separating the water-rich phase and acids from sugars and aromatics) and biomass demineralization (increasing sugar yield) (Oudenhoven et al., 2013). The high acid content stream (mainly acetic acid) can be recycled and used for biomass pretreatment by demineralization prior to pyrolysis. The anhydrosugars can then be separated from the aromatics via the addition of water and further purification via an extraction step. Therefore, a fermentable substrate is obtained bypassing adsorption, absorption, adaptative evolution and overliming steps as previously reported. However an in depth techno-economical study, outside the scope of this study, is necessary in order to draw ultimate conclusions for comparison with otherwise suggested designs

3.2 Materials and Methods

3.2.1 Pyrolysis oil production and work up procedures

An overview of the overall experimental scheme is given in Figure 3.1. Two pyrolysis oils generated from pinewood were tested for their suitability as a substrate for traditional ethanol fermentation. One of the oils was produced through an integrated biorefinery approach including biomass demineralization with the stream exiting condenser 2, Figure 3.1, and fractional condensation, as outlined by (Oudenhoven et al., 2013). The second oil was produced via conventional pyrolysis. Both pyrolysis experiments were performed in the same pilot plant scale fluidized bed reactor. A detailed description of the pyrolysis and the pinewood pretreatment methods can be found elsewhere (Oudenhoven et al., 2013). Briefly, pinewood pretreatment consisted of adding pine wood and condenser two liquid (ratio 1:10) to a stirred batch reactor. The temperature in the reactor was kept at 90°C for 2h (Figure 3.1). The pretreated pine wood was then pyrolyzed at 480°C with a vapor residence time <2s in a fluidized bed reactor. The produced vapors were fractionated according to their boiling point in two condensers. In the first condenser, operated at 80°C, oil rich in sugars and aromatics was obtained. The second condenser, operated at 20°C, yielded oil rich, among others, in acetic acid and water. The second condenser liquid was then used for acid washing (demineralization)

of the pine wood. Both condensers were kept at 1.1±0.01bar (Westerhof et al., 2011). Conventional pyrolysis oil was obtained through the pyrolysis of pinewood in the same set-up where both condensers were operated at 20°C. Almost all of the oil (approx. 90wt% of the total oil) including acids and water were collected in the first condenser. Both oils (produced from acid washed pine wood and condensed at 80°C; and produced from raw pine wood as received and condensed at 20°C) were used for comparison of its performance in the fermentation process.

Both pyrolysis oils were cold water extracted and filtered to remove insoluble lignin. The resulting filtrate was either further extracted with ethyl acetate, or directly acid hydrolyzed, neutralized and supplemented with glucose prior to fermentation (co-fermentation). Phenolics were selectively removed as a result of this additional extraction, leaving an aqueous phase rich in anhydrous carbohydrates (Lian et al., 2010). Glucose was produced as a result of acid hydrolysis. Original acids, e.g. formic and acetic acids, as well as sulfuric acid used in the hydrolysis, were neutralized. Precipitates were removed via centrifugation and a subsequent filtration. The filtrate was supplemented and co-fermented with pure glucose by *Saccharomyces cerevisiae* to produce ethanol.

3.2.2 Pyrolysis oil characterization

Total organic carbon analysis was performed to calculate carbon losses in every process step. A Shimadzu TOC-V series system was used (Shimadzu, Kyoto, Japan). Hundredfold dilutions in dionized water (Milli-Q Integral 5, EMD Millipore, USA) at each process step were prepared and analyzed in triplicates. The TOC calibration curve was linear in the range studied (0.00–0.20g/L).

Sugar content in pyrolysis oil, water extract and ethyl acetate residue were quantified by liquid chromatography using an Agilent LC 1200 infinite system equipped with a Hi-Plex H 300mm×7mm column and a Refractive index detector (Agilent, Santa Clara, USA). 0.5mM H_2SO_4 at a $0.7 mL min^{-1}$ was utilized as the mobile phase. Injection volume of the samples was 20µL. The temperature in the column was held constant at 60°C, while the temperature in the RI detector was held constant at 55°C. The method allowed for the separation of glucose, levoglucosan, cellobiosan, xylose, mannose and arabinose.

Karl Fischer titration was used to determine the water content of the oils. Briefly, samples were diluted with methanol in a 1:2 ratio to reduce viscosity whenever fractional condensation was used. When single condensation was used, conventional oil samples were dissolved in a mixture of methanol and dichloromethane in a 3:1 ratio. Subsequently. a 787 Titrino 703 Ti-Stand (Metrohm, Switzerland) with hydranal composite 5 (Sigma, USA) as the water titrant were used to determine moisture content. Before each sequence and after each 6 measurements a demi-water sample was measured to check the calibration. Each sample was measured in duplicates with a maximum error of 0.5%. Inhibitor compounds (aldehydes, furans and mono-phenols) in the oils were analyzed using GC–MS. A sample of ±6g was prepared as a mixture of 5wt% pyrolysis oil and 95wt% acetone, 2mL of this sample was filtered and analyzed using a GC (Agilent Technologies GC 7890A) equipped with a MS detector Agilent Technologies 5975C. Additional GC analysis was done on an Agilent 6890 series equipped with a 5973 MS detector and a capillary column (HP-INNOwax).

3.2.3 Upgrading

Cold water extraction of the pyrolysis oil was carried out for all samples using chilled water kept at a constant temperature of 4°C (Garcia-perez et al., 2008). 5g of pyrolysis oil were added drop wise to 50mL of chilled water (CW) under heavy stirring (900rpm). Baffles were used to secure proper homogenization of the added pyrolysis oil. Water insolubles were measured gravimetrically and separated by filtration of the emulsion using a previously dried and weighed 0.45μm cellulose nitrate membrane (Whatman®, UK). Filtrate was centrifuged at 4°C and 3500rpm for 20min (Sorval ST40R, Thermo Scientific, USA). The sugar-containing supernatant was separated from the pellet, collected in falcon tubes and stored at 4°C.

Selected samples were further extracted with ethyl acetate (EA) to remove organic compounds, known to be inhibitory for yeasts (e.g. phenolics, furans and aldehydes). A 1:2wt% filtrate to EA solution was prepared and mixed for 12h in an environmental shaker at 150rpm and 25°C. After the mixing the sample was left standing for 6h to secure separation of the phases. The organic layer was separated and remaining EA was removed by evaporation at 50°C for 24h in an oven (Isotemp, Fisher Scientific, USA).

Levoglucosan to glucose hydrolysis was realized by transferring extract aliquots of 4mL to microwave vials (VWR,USA) followed by the addition of H_2SO_4 (final concentration of 0.5M) and hydrolysis at 120°C for 20min in an autoclave (Bennett et al., 2009). Hydrolysates were neutralized with solid $Ba(OH)_2$ (Alfa Aesar, USA). After neutralization samples were transferred to 15mL centrifuge tubes (VWR, Canada) and salt crystals were precipitated by centrifugation at 3500rpm for 20min (Sorval ST40R, Thermo Scientific). The supernatant was removed and filtered with a 0.2µm cellulose acetate syringe membrane (VWR, Canada) and transferred to a new sterile 15mL tube (BD, USA). It is important to notice that the detoxification steps are experimental approaches and are not optimized in terms of process efficiency and amounts of solvents and neutralizing agents used.

3.2.4 Bioprocessing

Neutralized and cleaned hydrolysate was fermented with *S . cerevisiae* DSM 1334 (Braunschweig, Germany) in 96 wells microtiter plates (Costar®, Corning, USA). YPG medium (10g/L yeast extract (BD, USA), 20g/L peptone (BD, USA)) was used for the fermentation. The glucose required for ethanol production (G of YPG medium) was provided as a blend of pure glucose and hydrolysate (up to 100% hydrolysate). The final target glucose concentration in the media was kept constant at 40g/L.

Doing so, a pyrolytic sugars concentration range was created, allowing to evaluate the yeast's performance under an increasing presence of unremoved inhibitors. For the biorefinery oil CW hydrolysate, a range of 5–60% pyrolytic sugar concentration was tested (PO1). As for biorefinery oil EA hydrolysate, a range of 5–100% of pyrolytic sugar was tested (PO2). The same media was used for standard pyrolysis oil. However, due to a low glucose concentration it was only possible to evaluate the samples with a fraction of 0.1–8% pyrolytic sugar (PO3 and PO4).

Microtiter plate wells were filled with 180µL of the pyrolytic YPG media prepared and inoculated with 20µL of active seed culture of *S . cerevisiae* . Inoculated microtiter plates were sterile sparged with nitrogen and sealed with a sterile adhesive PCR film (Thermo Scientific, USA). The film was punctured with a sterile needle to allow gas exchange and the medium was incubated at 30°C and 74rpm using a Tecan M200 micro plate reader (Tecan, Austria). Optical density was measured by the reader in each well at 600nm every 10min for 24h. The reader was equipped with a gas-control unit

(Tecan, Austria) to maintain anaerobic conditions (nitrogen atmosphere). Sugars and ethanol were measured by high pressure liquid chromatography at the end of the fermentation, using a Hiplex H Column kept at 60°C, RI detector at 50°C with 0.5mM H_2SO_4 as the mobile phase at a flow of 0.7mL/min.

3.2.5 Numerical Analysis

To quantify the effects of inhibition, associated kinetic parameters were determined by fitting the measured growth kinetics data to the model of Baranyi and Roberts (Baranyi and Roberts, 1994), which describes biomass density as a function of time with three parameters: μ_{max}, the maximum theoretical growth rate; Q_0, the initial adaptation of the microorganism to its environment; and N_{max}, the maximum biomass density achieved when the cells reach stationary phase. The differential equations describing the biomass density (N) and culture adaptation to environment (Q) are given below in Eqs. (3.1) and (3.2) respectively, the estimated adaptation time λ for the culture is calculated using Eq. (3.3)

$$\frac{dN}{dt} = \mu_{max}\left(\frac{Q}{1+Q}\right)\left(1-\frac{N}{N_{max}}\right)N \quad (3.1)$$

$$\frac{dQ}{dt} = \mu_{max}Q \quad (3.2)$$

$$\lambda = \frac{ln\left(1+\frac{1}{Q_0}\right)}{\mu_{max}} \quad (3.3)$$

Least-squares fits were performed using MATLAB with the differential Eqs. (1 and 2) solved numerically. Fit quality was assessed by confirming the normality of residuals (normal probability plots). This model makes use of an adjusting function (Q) in order to account the adaptation time, λ, to new media. In this case maximum specific growth rate, μ_{max}, differs from that specified by Monod-type kinetics and is described as a maximum potential growth rate vs. a specific measured value (Baranyi and Roberts, 1994).

3.3 Results and discussion

3.3.1 Extraction of Pyrolysis oil

From Table 3.1 it can be seen that the concentration of levoglucosan in the pyrolysis oil is much higher when biomass is demineralized and fractional condensation is applied (PO1 and PO2), as it was expected. The concentration of well-known inhibitors like phenols, aldehydes and furans in the sugar rich pyrolysis oil is also decreased significantly, as illustrated in Table 3.2. The removal of acids from the oil and thus their collection in the second condenser as washing liquid for the next batch is mandatory in this process. Both POs were subjected to cold water extraction to remove water insoluble lignin oligomers. The supernatants were split in equal fractions; one fraction was further extracted with EA. All four resulting extracts were subjected to acid hydrolysis and neutralization under the conditions previously described. As a result of the upgrading processes, four different types of POs were obtained (see Figure 3.1). After each step samples were drawn to analyze sugar conversion, and TOC loss, as shown in Table 3.1.

TOC level decreases by almost 50% when CW extraction was followed by EA extraction for conventional pyrolysis-oil (PO3 vs. PO4), this carbon decrease did not affect the levoglucosan levels in the same way, accounting only in a 9.5% loss of the total levoglucosan present in the original CW extract. The fraction of levoglucosan carbon of the total organic carbon increased from 0.20 to 0.36, showing the selectivity of the method. The decrease of carbon levels in the aqueous phase after EA extraction likely corresponds to a removal of phenols and furans, as shown by Lian et al. when extracting similar compounds from biodiesel (Lian et al., 2010). The same study reports presence of polar compounds, such as levoglucosan, acetol and acetic acid, in the water phase. After acid hydrolysis of the extract and a subsequent neutralization with $Ba(OH)_2$, a slight decrease of TOC was observed, possibly due to a precipitation of some of the soluble organics acids after EA extraction. In addition, EA extraction helps to improve levoglucosan hydrolysis to glucose by 14%.

Table 3.1 Carbohydrate composition of PO streams before and after hydrolysis. The molar yields of the levoglucosan to glucose conversion were 0.49, 0.88, 0.43, and 0.84 for PO1, PO3, PO2 and PO4, respectively. The levoglucosan and glucose carbon fraction is calculated as the mass of carbon present in the respective carbohydrate forms over the total organic carbon measured as TOC.

	PO sample	TOC (g/L)	Levoglucosan (g/L)	Glucose (g/L)	Levoglucosan carbon fraction	Glucose carbon fraction
Water Extracts	PO1	46.90	44.60	0.80	0.42	0.00
	PO1 hydrolyzed	38.50	1.00	41.80	0.01	0.43
	PO3	17.22	7.90	0.00	0.20	0.00
	PO3 hydrolyzed	14.78	2.75	3.80	0.08	0.10
Ethyl Acetate Extracts	PO2	41.30	44.50	0.00	0.48	0.00
	PO2 hydrolyzed	36.70	1.32	43.40	0.02	0.47
	PO4	8.90	7.15	0.00	0.36	0.00
	PO4 hydrolyzed	8.25	1.05	3.91	0.06	0.19

The data in Table 3.1 also shows that biomass demineralization and fractional condensation play an essential role by increasing the levoglucosan concentration after pyrolysis; concentration increased fivefold (7.9–44.6g/L) in the water extract (PO3 vs. PO1). EA extraction decreases the TOC (PO1 vs. PO2) by 12%, contrasting with the almost 50% TOC reduction when the PO comes from a non-demineralized biomass (PO4 vs. PO3). This suggests a significant reduction of water soluble organic compounds found in the demineralized POs, agreeing with previous reports where anhydrosugars degradation is low when inorganic ash is removed (Radlein et al., 1987). The levoglucosan carbon fraction increased from 0.42 to 0.48 after the extraction.

Table 3.2 Chemical detection by GC/MS-FID of known fermentation inhibitors in pyrolysis oils through the process. All the concentrations are in wt %. PO2 and PO4 refer to the streams after the hydrolysis and neutralization step. Water content determined by Karl Fischer titration

	Biorefined oil / Conventional oil		
Compound group	**Original oils**	**PO1 / PO3**	**PO2 / PO4**
Water	1.1 / 1.3	n.d / n.d	n.d / n.d
Water insolubles oligomers	13 / 22	<0.1 / < 0.1	<0.1 / < 0.1
Acetic Acid	<1 / 6.1	<0.1 / 0.36	0.14 / 0.19
Hydroxy-Acetaldehyde	<0.1 / 2.2	<0.01 / 0.32	<0.01 / 0.37
Furans	<0.1 / 1.3	<0.01 / 0.1	<0.01 / 0.13
Mono-phenols	1.6 / 5.4	0.17 / 0.53	<0.01 / 0.1

Ethyl acetate extraction causes a nominal loss of levoglucosan, however it is relatively selective and predominately removed other background organics, as can be seen in the increase levoglucosan fraction of total organic carbon. Other detoxification techniques, such as treatment with activated carbon and adsorption into polymeric matrices, air stripping, and solvent extractions also show some overall sugar reduction, even though they are applied later in the process after the hydrolysis step. Wang and collaborators compared these technologies and achieved their best results with activated carbon, losing only 3.8% of the original sugar (Wang et al., 2012).

The reason for performing detoxification steps prior to acid hydrolysis is due to the well-known generation of additional inhibitory compounds during this high temperature/low pH process (Sun and Cheng, 2002). Additionally, organic acids precipitation suggests that neutralization complements previous detoxification steps.

Ethyl acetate extraction favors the hydrolysis reaction and increases the glucose molar yield. After neutralization, 11–18% of the original total carbon is lost as shown in Table 3.1. As previously explained, this decrease is likely due to a precipitation of organic compounds previously reported to be found in pyrolysis-oil, which account for the low pH and corrosiveness of pyrolytic oil (Sun and

Cheng, 2002). Acid hydrolysis was capable to convert 84–88% of the levoglucosan to glucose (Table 3.1). These high yields agree with previously described results (Lian et al., 2010; Yu and Zhang, 2003). Higher glucose hydrolysis yields, up to 240%, have been reported elsewhere (Bennett et al., 2009). The surplus glucose was likely generated from additional anhydrous carbohydrate oligomers present in the oil used by Bennett et al (2009). Largely due to differences in operating conditions during the pyrolysis, such an effect was not observed in this study. It is however anticipated that hydrolysis yield can be further increased as the process variables have not been optimized in this study.

3.3.2 Fermentation

POs extracts (Figure 3.1) were tested as fermentation substrates. Microscale fermentations experiments were performed with standard medium and 40g/L glucose. To assess the respective fermentability of the four POs, varying fraction of the total glucose were provided through blending the glucose stock solution with the POs. Due to the low glucose concentration of the conventional PO extracts (Table 3.1), only a small fraction of the total glucose could be provided from these POs (PO3 and PO4). Ranges of pyrolytically derived glucose between 0.5% and 8% (3.80–3.9g/L) of the total glucose in the medium, were achievable with the given glucose concentration of the hydrolysate.

In contrast, biorefinery PO extracts (PO1 and PO2) had substantially higher glucose levels (41.8–43.4g/L). Both PO1 and EA extract from PO2 were co-fermented in different proportions creating a pyrolysis sugar range profile from 5% to 60% and 5% to 100%, respectively.

The reason for diluting the extracts was to determine an inhibition profile or the tolerance level of ethanol fermentative microorganism to the expected residual inhibitors (Lian et al., 2012; Sun and Cheng, 2002). Inhibition in one form or the other can be seen for all extracts with an increase of pyrolytic sugars, however, the EA extract of the demineralized PO could be converted at 40g/L without the addition of any other glucose. A common pattern in the growth profile of yeast on all extracts (Figure 3.2 A–D) is a “shifting” of the curves to the right and a lower cell yield as the concentration of pyrolytic sugar in the media increases.

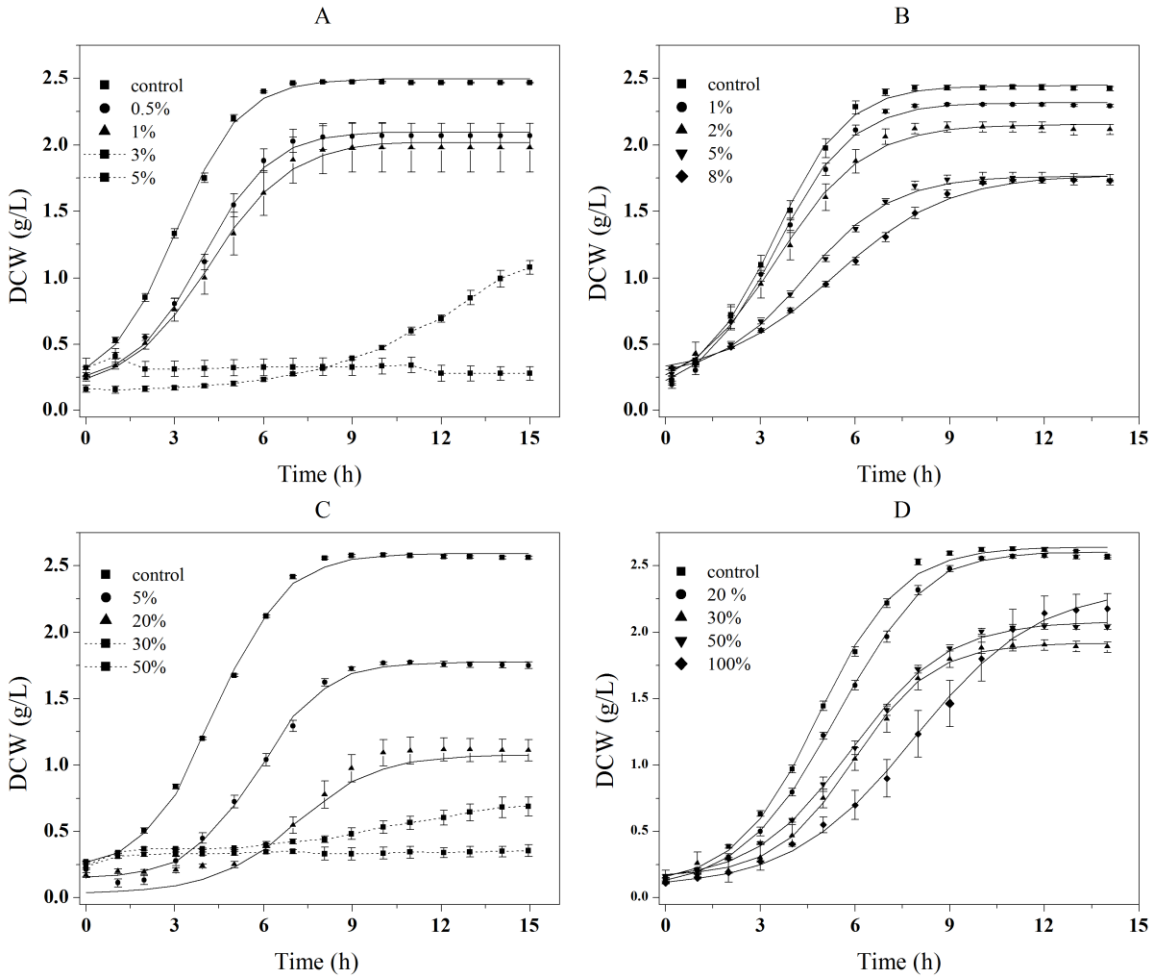

Figure 3.2 Pyrolytic substrate fermentation growth profiles on two different types of pyrolysis-oil extracts as a function of the pyrolytic sugar fraction. A and B correspond to conventional pyrolysis oil extract. C and D correspond to bio-refined pyrolysis-oil extract. Results on the left graphs correspond to only cold water extraction, PO1 and PO3, on the right to EA extract fermentation, PO2 and PO4. The solid lines represent the best fit.

As a result of increasing the pyrolytic sugar, a higher adaptation time to the media is required by the yeast. Once the tolerance level is surpassed, the growth curve becomes flat with no increase in cell concentration. Contrasting Figure 3.2 B and D (EA extract, PO2 and PO4) with Figure 3.2A and C (CW extract, PO1 and PO3) shows the effect of a solvent extraction on the cell growth; as phenolic compounds are removed during EA extraction, the inhibition decreases, and, as a result, the cell concentration increases as the lag phase (adaptation time) decreases, as illustrated in Figure 3.2 B and D. In the case of conventional oil PO3 (Figure 3.2 A), cell growth was only observed when the

fraction of pyrolytic sugar was up to a 3% contrasting with a 5% maximum of hydrolysate added reported by Wang and collaborators (Wang et al., 2012), where the hydrolysate was not yet detoxified and derived from a pyrolysis oil where mild acid washing was applied to biomass. In this study growth was achieved when up to 20% of the glucose was derived pyrolytically without detoxification in the case of demineralized pyrolysis oil (PO3, Figure 3.2C). This represents almost a 7-fold increase in fermentability when demineralized PO is used.

An explanation for this might be the fact that pyrolysis oil contains considerably lower concentrations of inhibitors like aldehydes, furans and mono-phenolics, see Table 3.2, in addition to an already reduced amount of acetic acid due to its consumption in the demineralization step. The same trend applies to the findings illustrated in Figure 3.2 B and D. Figure 3.2D shows growth curves in the presence of EA extracted demineralized PO (PO2), and proves that pyrolytic sugar can be used completely as a substrate.

In addition, Table 3.2 depicts the concentrations of some important inhibitors previously identified in literature (Oudenhoven et al., 2013). A clear reduction of most compounds can be seen after the respective upgrading steps. A slight increase in acetic acid is noticeable after hydrolysis; this might be glucose a degradation product and further highlights the need to optimize the hydrolysis conditions. The pyrolytic oil is a very complex mixture and only selected model compounds were analyzed, it is very likely that additional unknown inhibitory compounds are present in the original oils.

3.3.3 Numerical evaluation

The time course data was fitted to the Baranyi model using MATLAB (The MathWorks, Inc) via least squares regression. The model parameters λ (adaptation time), μ_{max} (maximum growth rate) and N_{max} (maximum biomass density) could only be determined for data sets that showed a characteristic sigmoidal growth. The solid lines shown in Figure 3.2 are the respective best fits and it can be shown that the model is in good agreement with the experimental data. The parameters obtained can, therefore, be used to quantify the effect of inhibitors in the pyrolytic sugars.

The parameters calculated from the experimental data presented in Figure 3.3A–D, show an expected inverse relationship between lag time (λ) and the specific growth rate (μ_{max}). The lag time increases, while the maximum growth rate (μ_{max}) decreases with increasing amount of pyrolytic sugars in the medium. This tendency results from increasing concentration of inhibitors being added to the media with the PO. For water extracts of conventional PO, full inhibition takes places when having only 5% of pyrolytic sugar in the media, as clearly seen in Figure 3.3A by a rapid decrease in μ_{max}.

These findings are in contrast to previous studies where a 5% fraction of pyrolytic sugar resulted in high yields after water extraction only (Bennett et al., 2009), further highlighting potentially different outcomes when different methods are used to generate pyrolytic sugars, and the resulting need in screening technologies as demonstrated in this study. If the conventional PO is further extracted with EA, then up to 8% can be used, however with an approximate 40% decrease in μ_{max}.

It is possible that higher fractions could be fermented; however, 8% of pyrolytic sugar was the maximum that could be added for conventional oil due to low initial levoglucosan concentrations. The inhibitory effect of unremoved compounds mixed with the pyrolytic sugars is clearly decreased, (see Table 3.2) when biomass is demineralized (Figure 3.3C), and particularly when a further EA extraction reduces the total phenolics and furans concentration as previously reported (Lian et al., 2010), as shown in Figure 3.3D. The last quantifiable value of μ_{max} for the water extract (PO1) was at 20% pyrolytic sugar. At this point μ_{max} was reduced to less than 50% of its initial value. The decrease in μ_{max} is far less prevalent after EA extraction. An approximately 30% decrease of μ_{max} was observed for 100% pyrolytic sugar.

The effect of pyrolytic sugars on λ, is correlated to the changes in μ_{max}. The estimated value of the parameter increases fourfold, from 1.5h in the control to almost 6h when the hydrolysate concentration of demineralized PO1 is only 20%, as shown in Figure 3.3C. Interestingly, no significant difference of λ could be seen for an increase in PO concentrations after EA extraction (Figure 3.3D). The clear tendency of a decreasing μ_{max} in Figure 3.3D as pyrolytic sugar increases, might be caused by the presence of furans and phenols which have the particular characteristic of affecting ethanol productivity by inhibiting growth, but not ethanol yields (Klinke et al., 2004). The yields remained constant, as shown in Figure 3.4D.

Inhibition studies on *S. cerevisiae* have been performed by several researches analyzing the effect of individual compounds such as 4-hydrobenzoic acid, furfural, acetic acid (Palmqvist et al., 1999), 5-hydroxymethyl furfural (5-HMF), vanillin, syringaldehyde, coniferyl aldehyde (Delgenes et al., 1996) and 4-hydrozybenzaldehyde (Klinke et al., 2003).

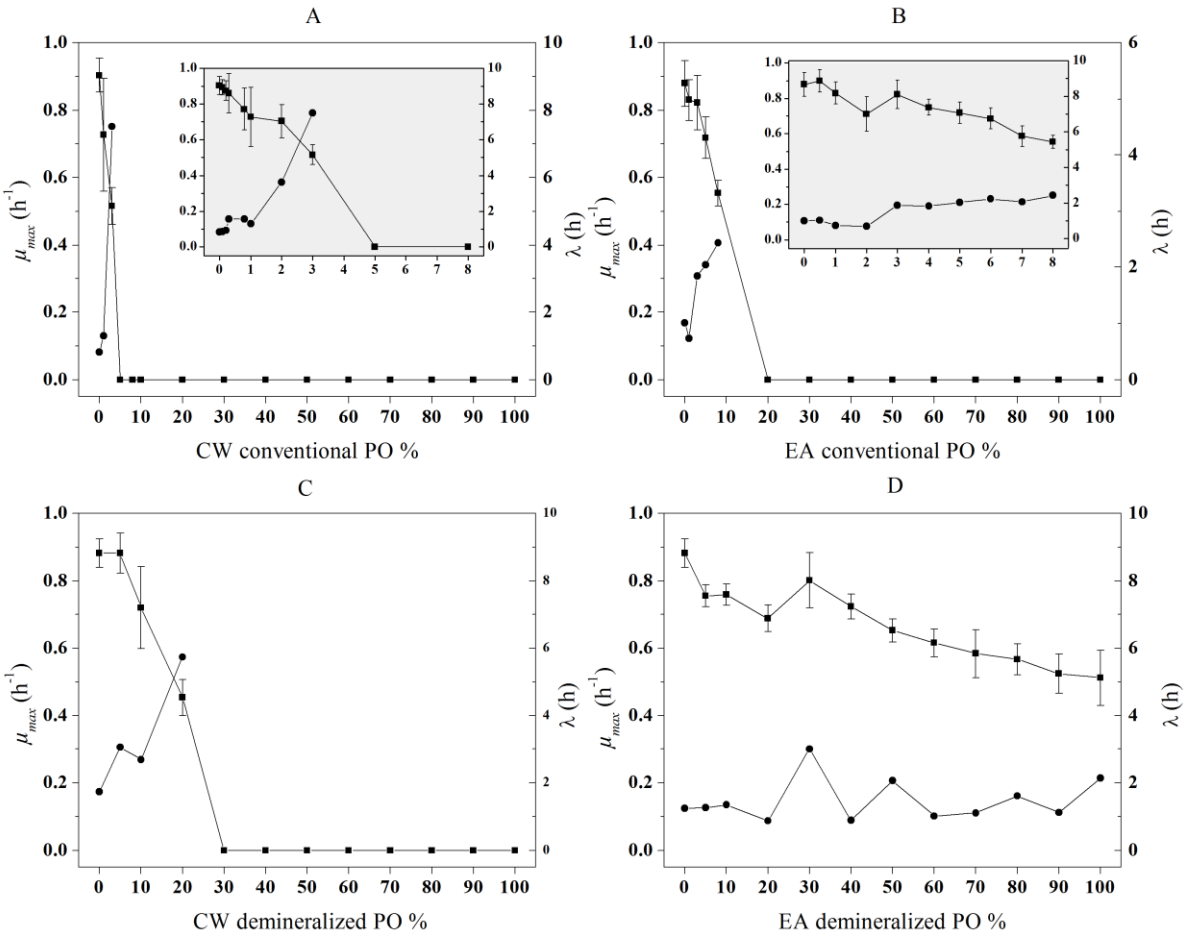

Figure 3.3 Estimated model parameters for microfermentations conducted with varying glucose fractions derived from pyrolysis oils. A and B correspond to fermentations of conventional biomass pyrolysis oil. C and D correspond to demineralised biomass pyrolysis oil (biorefinery oil). Results on the left graphs correspond to only cold water extraction, PO1 and PO3, on the right to EA extract fermentation, PO2 and PO4. The maximum growth rate estimates, $\mu_{max,}$ are represented by the squares, the lag time, λ, by the circles. The subplots on A and B show a detailed trend at low PO concentrations.

The values for μ_{max} in these studies are based on directly measured doubling rates, while the μ_{max} value of the Baranyi model is representing a 'theoretical' maximum growth rate, based on the inflection point of the curve. The numerical values are therefore different (different model used) and direct comparisons between the herein reported values cannot be made, however trends such as relative decrease in growth rates are comparable.

The Baranyi model was chosen, as it is more suitable for complex inhibition kinetics. Modeling of the lag phase is a concept mostly known to food microbiology (Baranyi and Roberts, 1994) and is not a parameter reported in any of the previously mentioned studies. It is however a highly important parameter that will help establish and characterize the pyrolysis oil as a whole inhibitory entity rather than just evaluating singles compounds or simple mixtures of these compounds and their effects on growth.

3.3.4 Ethanol and biomass production

The theoretical yield of ethanol produced from glucose is 0.511g/g. The maximum yield achieved in this study was 0.49g ethanol/g glucose (96% of the theoretical value). Yield calculations were done based on glucose only. Other hexoses such as galactose and mannose, which could be present after pyrolysis and hydrolysis (Lian et al., 2010), were not quantified and hence not taken into account. The fermentation process lasted 15h and samples for ethanol analysis were drawn at the end-point of each micro-fermentation. The effect on ethanol yield of increasing pyrolytic sugar fractions is shown in Figure 3.4. As expected based on cell growth data (Figure 3.2), ethanol production was achieved with a higher fraction of pyrolytic sugars when the POs were also extracted with ethyl acetate. Demineralization was directly responsible for a 10-fold increase in the pyrolytic sugar fraction that could be converted to ethanol seen directly by comparing PO3 and PO1 (Figure 3.4A and C) were the highest fermentable pyrolytic sugar fraction increased from 2% to 20%. As expected, this increase continued for the ethyl-acetate extracted PO4, were ethanol production was realized from 100% pyrolytic sugar (Figure 3.4D).

Ethanol production from hydrolyzate, detoxified via solvent extraction and activated carbon, has been previously reported (Lian et al., 2010). However, a more complex detoxification processing was

employed and full substrate fermentation is shown in this study for the first time using ethyl acetate extraction as the only direct detoxification method prior to acid hydrolysis. This is likely possible due to the initial lower concentration of inhibitors (see

Table 3.2) in this oil, despite the undoubted presence of a partition coefficient of inhibitors between both phases (ethyl acetate and aqueous sugar rich phase). The hydrolyzate was fully fermentable (no need of supplementing with pure glucose) after the solvent extraction, achieving an ethanol concentration of almost 20g/L, as shown in Figure 3.4.

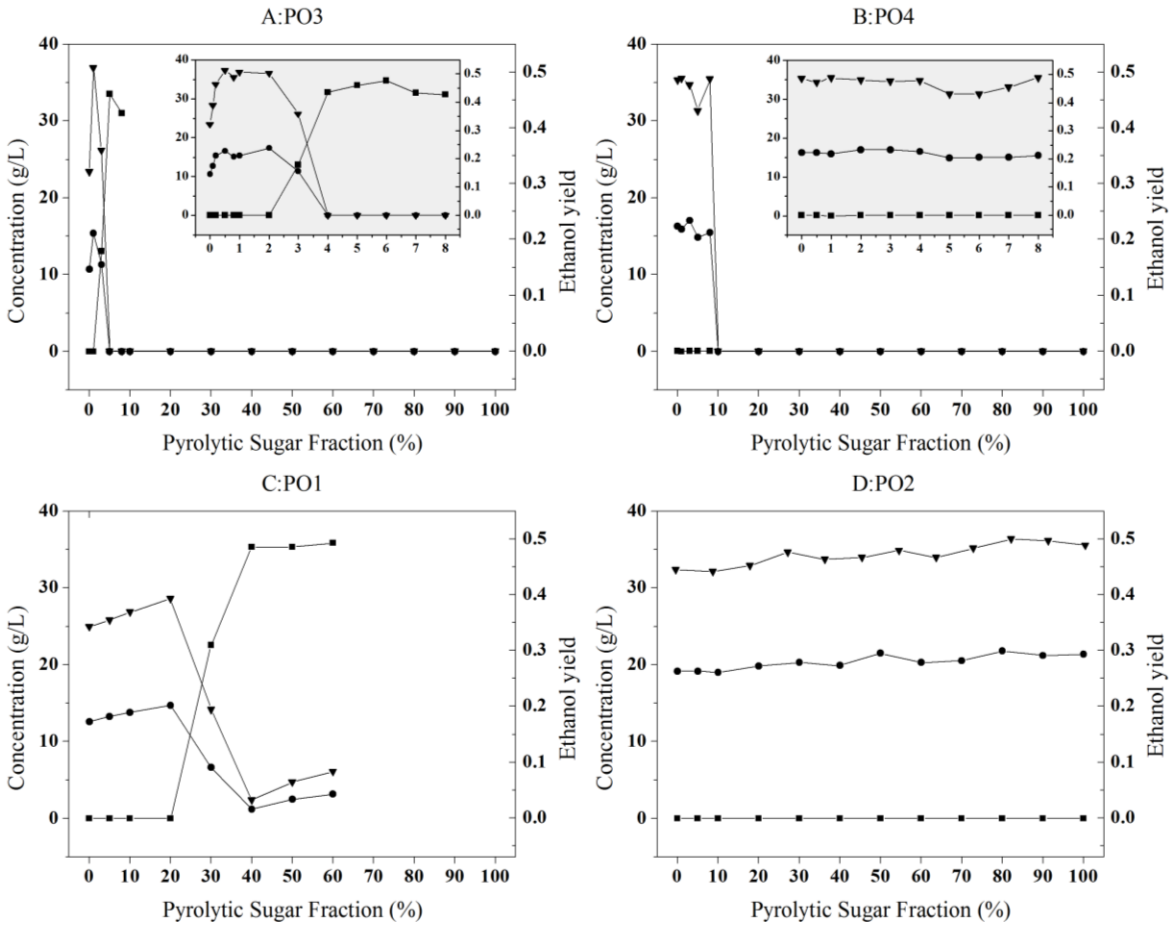

Figure 3.4 Calculated glucose consumption and ethanol production. A and B correspond to fermentations of non-demineralised biomass pyrolysis oil. C and D correspond to demineralised biomass pyrolysis oil. Results on the left graphs correspond to only cold water extraction, PO1 and PO3, on the right to EA extract fermentation, PO2 and PO4. Ethanol yield is read on the left y-axis. Right y-axis corresponds to Concentration. 0 stands for control (fresh YPG media). X-axis shows

amount of pyrolytic sugar (pyrolytic glucose) present in the fermentation media. (triangle) Ethanol yield, (circle) Ethanol g/L, (square) Glucose g/L.

The presented data suggest a slight increase in the ethanol concentration and yields as pyrolytic sugar concentration increases in the media. This might be a result of the experimental design, as samples were only analyzed after 15h. Ethanol production on samples containing lower fractions of pyrolytic sugars, will likely have completed faster (see higher values for μ_{max} in Figure 3.3, or growth profile in Figure 3.2), giving time for ethanol to evaporate amplified by the high surface area to volume ratio resulting from the small scale experiment setup. It is also possible that the other small molecules (e.g. organic acids) (Palmqvist et al., 1999) present in the pyrolytic sugar solution acted as an additional carbon source that was converted to ethanol.

The maximum yeast concentration was also effected by the addition of pyrolytic sugars, as shown in Figure 3.5 for all four investigated substrates. For PO2, the only substrate that could completely replace glucose in the medium, a decrease in N_{max} is observed, as the pyrolytic sugar fraction increases. The previously observed increase in ethanol yield might therefore also be caused by a diversion of carbon flux from biomass (yeast) production to ethanol production. A detailed analysis of these effects however, is beyond the scope of this study.

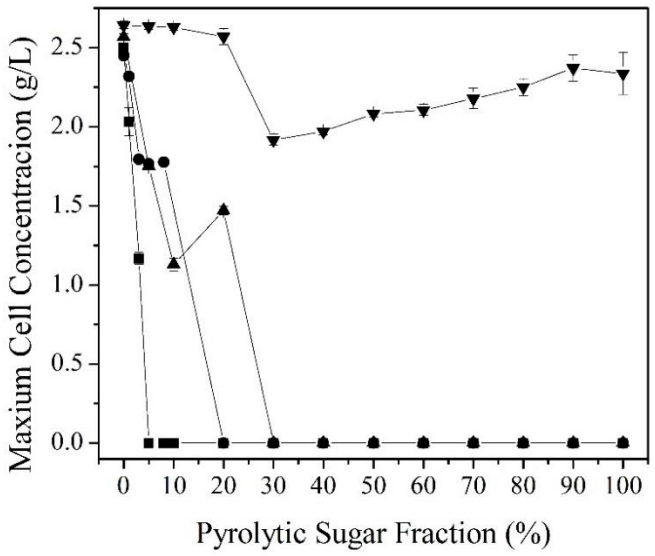

Figure 3.5 Maximum cell concentration reached after fermentation process with different pyrolysis oil extracts. (square) PO1 (circle) PO2 (triangle) PO4, (inverted triangle) PO3.

Generally, final ethanol concentrations ranged from 18g/L to 20g/L corresponding to a range in ethanol yields between 0.45 and 0.5g ethanol/g glucose (Figure 3.4D). Based on the most suitable substrate (PO) a total amount of 8.2g ethanol could be produced per 100g pine wood, corresponding 41.3% of the theoretical maximum value (Table 3.3), based on the assumption that all cellulose in pinewood, approximately 36wt% (Westerhof et al., 2007), can be converted to glucose and subsequently ethanol. Traditional lignocellulosic ethanol processes reported in the literature typically achieve values between 54% and 85% for simultaneous and separate saccharification and fermentation based on the available hexoses (Eklund and Zacchi, 1995; McMillan et al., 1999).

The proposed process approaches this range, despite only being demonstrated at the micro-scale without any optimization attempts to improve yields. The process is further an initial attempt on an integrated biorefinery approach not focusing exclusively on ethanol production. Additional valuable products of this process are biochar and biogas, as well as acidic acid as shown in Figure 3.1. Other streams such as the insoluble lignin fraction, phenolics and other aromatics can easily be separated and could be potentially be used as value added products (Lian et al., 2012). This study is a proof of concept, showing that effective ethanol production can be achieved in combination with pyrolytic biomass conversion. A detailed economic evaluation of the process is beyond the scope of this study but will be attempted in future work.

A detailed look at the data in Table 3.3 shows that the yield of ethanol from the available pyrolysis derived glucose is very high (8.2g vs. the theoretical maximum of 8.5g). The efficiency of cellulose to levoglucosan conversion is at approximately 51%, and substantial improvements trough manipulating operating conditions and process design might be possible. Additional potential of improvement is in the upgrading steps.

Table 3.3 Carbon mass balance for PO2.

Compound	Conversion Step	Theoretical accumulated maximum [g]	Achieved value [g]	Theoretical maximum based on last conversion only [g]
Pinewood	Starting material	100.0	100.0	100.0
Cellulose	Starting material	35.0	35.0	35.0
Levoglucosan	Pyrolysis	35.0	18.0	35.0
Levoglucosan	CW Extraction	35.0	18.0	18.0
Levoglucosan	AE Extraction	35.0	17.1	18.0
Glucose [g]	Hydrolysis	38.9	16.7	19.0
Ethanol [g]	Fermentation	19.8	8.2	8.5
Ethanol % of theoretical max			41.3	%

A substantial fraction of the losses during these steps are due to experimental difficulties associated with the small scale of the experiment (e.g. the material attached to pH probe during pH adjustment becomes significant at the micro-scale) and would not occur at a larger scale. Overall it is expected that it is possible to achieve ethanol yields well within the range of conventional processes, while also producing additional valuable by-products.

3.4 Conclusions

Ethanol yields in the presented study approach values found in traditional pretreatment and fermentation processes. The sugar rich pyrolysis oil with low concentration of inhibitors requires only simple extraction processes to reduce inhibition during fermentative conversion, achieving high ethanol yields (96% of theoretical). The inhibitory effect of compounds in the sugar rich pyrolysis oil can be easily quantified at micro-scale, simplifying the analysis of pyrolysis oils fractions and their suitability for fermentation. The proposed pyrolysis based biorefinery turned is an interesting alternative to traditional lignocellulosic ethanol production in which hydrolysis of biomass is used as

pretreatment step. The main objectives of this chapter were to evaluate how pyrolytic sugars affected ethanol yields. The approach to detoxify the pyrolytic oil and the high through put screening methodologies are strategies which could be applied further when evaluating production of different chemicals such as lipids or butanol, or assessing different pyrolytic oils derived from several feedstocks. Any small increase during the process could translate into higher biofuel productivity.

3.5 References

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Chapter 4

4 The Effect of Individual Pyrolytic-oil Components

Selected data presented in this chapter are part of a journal article authored by Jeffery Wood, Valerie Orr, Luis Luque, Vivek Nagendra, Franco Berruti and Lars Rehmann.

The published work is focused on developing a numerical model to evaluate the effect of pretreatment byproducts (largely focused on acid hydrolysis) on yeast fermentation. This chapter utilizes some of the control data presented in the paper and applies the model to inhibitors found in pyrolytic sugars while evaluating the suitability on the proposed model to three different pyrolytic substrates that were used throughout this thesis and were extensively characterized in this chapter, hence deviates substantially from the published work.

Inhibition by pyrolysis derived compounds has been previously described and has been linked to many factors including biomass and pretreatment types (Czernik and Bridgwater, 2004; Demirbas, 2009; Maity, 2014). In this study different pyrolytic oils produced in the same reactor but from different biomass were upgraded and fermented using the same process described in Chapter 3. After a literature survey, quantification and screening possible candidates to explain the overall inhibition observed from this pyrolytic oils were selected. The obtained concentrations of these compounds were analyze in the respective pyrolytic oils. A response surface polynomial was used to describe the effect of these inhibitors on the growth rate of yeast. The model worked well on pure compounds and was used with the measured concentrations in the pyrolytic sugars and the predictions were with the observed experimental data. The results highlighted the need for a more robust quantification method or an alternative model to describe the observed inhibition exerted by the pyrolytic oils.

4.1 Introduction

Fast pyrolysis is a thermochemical process in which biomass is transformed in the absence of oxygen into a liquid known as pyrolysis oil (PO). POs are complex organic mixture with more than 400 chemical components (Garcia-Perez et al., 2007; Lian et al., 2012) which concentration depend on

feedstock and operation conditions of the pyrolysis process (Maher and Bressler, 2007). Advancements in fast pyrolysis technologies have increased carbohydrates yields in POs (Westerhof et al., 2011). The most abundant carbohydrate found in these pyrolytic oils is levoglucosan (LG), an anhydrous sugar, product of cellulose breakdown, which can be easily hydrolyzed to produce glucose for fermentation into ethanol (Lian et al., 2010; Luque et al., 2014). However, the extraction of LG from pyrolytic oils also carries over some compounds that depending on their concentrations and nature can be detrimental to downstream processing (fermentation). Furfural, hydroxymethylfurfural, vanillin, 4-hydroxybenzaldehyde, *m*-cresol and guaiacol are among some of the compounds associated with biomass decomposition which have been reported to inhibit growth and therefore fermentation in microorganisms (Palmqvist and Hahn-Hägerdal, 2000; Palmqvist et al., 1999; Schwab et al., 2013). Inhibition studies of these toxic compounds have been extensively studied on ethanol producing microbes such as *Z. mobilis S. cerevisiae* (Delgenes et al., 1996) *E. coli* (Zaldivar et al., 2000, 1999) all of them centered on compounds found in biomass hydrolysates.

Due to the large amount of compounds yielded in biomass pyrolysis (Bridgwater et al., 1999), the list of possible inhibitors increases substantially, more over when the compounds profile depends in part to the biomass itself (Maher and Bressler, 2007). Hence, the potential of POs as a source of fermentable substrates depends to a great extent on the ability to identify and assess in a quick and effective manner the effects on growth and ethanol productivity of these compounds and their mixtures (Schwab et al., 2013; Yu and Zhang, 2003). This type of assessment would enable to evaluate the suitability of biomass pretreatments and to measure the performance of different detoxification technologies.

This work attempted to identify common inhibitory compounds found in different pyrolytic oils produced from pretreated and untreated biomasses (switch grass and corn cobs) and to follow the fate of these compounds through the detoxification steps proposed in the biorefinery approach described in Chapter 3. Using a high throughput screening inhibition effect of individual and mixtures of inhibitory compounds allowed to quantify the impact on growth and ethanol productivity on *S. cerevisiae.*

4.2 Materials and Methods

4.2.1 Biomass pretreatment and pyrolytic oils production

Pinewood, switch grass and corn cobs were demineralized utilizing the procedure described in Chapter 3. Section 3.2.1. In brief, demineralization was carried out by adding biomass and an acetic acid solution 10 % v/v (final ratio of 1:10) to a stirred batch reactor at 50°C for 2 hours. Once the demineralization was completed, biomass was rinsed with 1 L batches of deionized water (Milli Q, Millipore, USA) and stirred for 5 minutes at 25°C. Rinsing was performed until output water stream conductivity approached zero and remained unchanged (Pinnacle Series, Nova Analytics, USA). Biomass was then collected and dried (Oudenhoven et al., 2013). Pretreated biomass were pyrolyzed at 480°C with a vapor residence time of <2s in a fluidized bed reactor. A condensation train composed of two condensers were used to collect the produced vapors. The first condenser was operated at 80 °C to obtain an oil rich in sugars with low moisture content. A second condenser was operated at 20°C where water and acetic acid were collected. Both condensers were kept at 1.1±0.01 bar (Westerhof et al., 2011).

4.2.2 Pyrolytic oil upgrading

Upgrading of the pyrolytic oil was achieved following slightly modifying the procedure described on Chapter 3. Section 3.2.3. In brief, insoluble lignin was precipitated via cold water precipitation. Oil were added dropwise to cold water 4°C to a final ratio of 1:10 and mixed at 900 rpm. Water insoluble were measured gravimetrically and removed via vacuum filtration with a predried 0.45 µm cellulose acetate membrane (Whatman, UK).

Glucose hydrolysis was realized by transferring 7 mL aliquots of the obtained filtrates into a microwave vial (VWR, USA) and adding H_2SO_4 to a final concentration of 0.5M. Hydrolysis was performed at 120°C in an autoclave for 20 mins as reported elsewhere (Luque et al., 2014). Hydrolysate was neutralized with $Ba(OH)_2$) (Alfa Aesar, USA). After neutralization, samples were transferred to 15 mL centrifuge tubes (Thermo, USA) and insoluble salts were precipitate by centrifugation at 3500 rpm for 20 mins (Sorval ST40R, Thermo Scientific, USA). The supernatant

was removed and filtered with a 0.2 µm cellulose acetate syringe membrane (VWR, Canada) transferred to a new sterile 15 mL tube (Thermo, USA) and store at -20°C until further use.

Neutralized hydrolysates were further extracted with ethyl acetate (EAc) to compounds known recognized for their inhibitory properties on *S. cerevisiae*. A 1:2 wt% filtrate to EAc solution was prepared and mixed for 12 h at 150 rpm and 25°C. After mixing samples were added to a decantation funnel and left standing for 6 h to secure proper phase separation. The organic layer was separated and remaining EAc in the aqueous fraction was removed in an environmental shaker at 40°C at 150 rpm.

4.2.3 Compound selection and screening

Pyrolytic oil samples were analyzed for possible inhibitor compounds utilizing gas chromatography. Identification and screening of inhibitory compounds in upgraded pyrolytic oil fractions was performed via LC-MS

4.2.3.1 GC/MS

Compounds in selected pyrolytic oils were identified by gas chromatography following the protocol described in Chapter 3. Section 2.2

4.2.3.2 LC/MS

Inhibitory compounds screening was performed via liquid chromatography in a Thermo LTQ XL system (Thermo Scientific, USA) equipped with a mass spectrometer LTQ Orbitrap Discovery (Thermo Scientific, USA). Standards of selected compounds were prepared to a final concentration of 1 mg/mL in liquid chromatography grade acetonitrile (Fischer, USA) and filtered with a 0.2 µm GPH syringe membrane (Pall, USA). Injection volume was set to 10 uL. Compound resolution was achieved utilizing 0.1 M formic acid (Fischer, USA) solution in LC grade acetonitrile (Fischer, USA) as the mobile phase at a 0.4 mL/ min flow rate in a Cortecs C18 column (Waters, USA) set to 25 °C.

4.2.4 Selected compounds quantification

Furfural, Hydroxymethylfurfural (HMF), cresol, guaicol and vanillin have all been previously studied for their inhibition on *S.cerevisiae*. Standards of the samples were prepared and identified by two analytical techniques.

4.2.4.1 GC/FID

Guaicol, *m*-cresol, vanillin and 4 hydroxybenzaldehyde were quantified in upgraded pyrolytic fractions via gas chromatography equipped with a flame ionization detector Agilent 7890A (Agilent Technologies, USA) with a DB Wax Column (Agilent Technologies, USA) and utilizing He as the carrier gas. The injector was set to a split ratio of 5:1 of 2 µL injections. Oven temperature was held constant at 50 °C for 5 min. A temperature ramp to 150 °C at a rate of 3°C/min followed by a second ramp to 230 °C at a ratio of 6 °C/min held for 10 mins was used to resolve the analyzed compounds. Calibration curves of the selected standards were linear in the range studied.

4.2.4.2 Liquid Chromatography

Sugar, furfural and hydroxymethyl furfural content in pyrolysis oil, water extract and were quantified by liquid chromatography utilizing an Agilent LC 1200 infinite system equipped with a Hi-Plex H 300 mm × 7 mm column and a Refractive index detector (Agilent, Santa Clara, USA) and a diode array detector (Agilent, Santa Clara, USA) set to wavelength of 280 nm. 0.5 mM H_2SO_4 at a 0.7 mL min^{-1} was utilized as the mobile phase. Injection volume of the samples was 20 µL. The temperature in the column was held constant at 60 °C, while the temperature in the RI detector was held constant at 55 °C.

4.2.5 Bioprocessing of the pyrolytic oil upgraded extracts.

Growth of *S. cerevisiae* was performed by the HTP methodology discussed in Chapter 3. Section 2.4

4.2.6 Growth kinetics and numerical analysis of yeast growth

Maximum growth rate was used as the main parameter to quantify the effects of inhibition by fitting the measured growth kinetic data to the Baranyi and Roberts model (Baranyi and Roberts, 1994) as previously discussed in Chapter 3. Section 2.5. The use of this model was chosen as it accounts for adaptation times in new and inhibitory media.

4.3 Results

4.3.1 Inhibitor compounds selection

Fast pyrolysis of biomass has been reported to yield close to 300 compounds (Butler et al., 2013). However, the compound distribution profile varies significantly between different types of biomass, hardwoods, softwoods and herbaceous, more over product profile is also a function of fast pyrolysis process conditions, in particular temperature and vapor residence time (Czernik and Bridgwater, 2004). Some of these compounds have also been found in other lignocellulosic biomass pretreatments. In order to fully understand the possible inhibition of these compounds efforts have focused to investigate model compounds at different concentration ranges (Zaldivar and Ingram, 1999; Zaldivar et al., 2000, 1999). However when comparing the results obtained utilizing this models compounds falls short to explain the inhibition exerted by the rest of the compounds.

Based on the expected compounds found in literature Table 4.1 and preliminary GC/MS measurements of three differently obtained pyrolytic oils Table 4.2, an additional LC-MS screening was utilized to identify possible inhibitory compounds carried over in the water extraction process, Table 4.3. The list of compounds was extended by adding several compounds that are known to be important phytochemicals such as the building blocks of lignin and some possible lignin degradation products pinpointing common compounds in different types of bio-oils.

The main purpose of this screening was to identify possible compounds responsible for growth inhibition observed in *S. cerevisiae* when grown in sugars obtained by upgrading the selected pyrolytic oils as discussed on Chapter 3.

Table 4.1 Compound list extracted from literature. The data in this table is based on a review published elsewhere (Islam et al., 2015)*.

Compound	Range wt%	Reference
Levoglucosan	0.1 – 30.5	(Bertero et al., 2012; Demirbas, 2009)
Cellobiosan	0.4 – 3.3	(Demirbas, 2009)
2-5H-Furanone	0.1 – 1.1	(Ioannidou et al., 2009)
Furfuryl Alcohol	0.1 – 5.5	(Demirbas, 2009; Milne et al., 1997)
Furfural	1.5 – 3.0	(Demirbas, 2009)
Syringaldehyde	0.1 – 1.5	(Demirbas, 2009; Ioannidou et al., 2009)
Syringol	0.7 – 4.8	(Milne et al., 1997)
Eugenol	0.1 – 2.3	(Ioannidou et al., 2009)
Acetic Acid	0.2 – 17.0	(Bertero et al., 2012; Demirbas, 2009)
Formic Acid	0.3 – 9.1	(Milne et al., 1997)
Cresol	1.03 – 2.5	(Demirbas, 2009)
Phenol	0.1 – 3.8	(Milne et al., 1997)
Cathecol	0.5 – 5.0	(Demirbas, 2009)
Guaiacol	2.1 – 2.8	(Bertero et al., 2012; Demirbas, 2009)
Formaldehyde	0.1 – 3.3	(Milne et al., 1997)
Eugenol	0.1 – 2.3	(Ioannidou et al., 2009)
Acetol	0.2 – 7.4	(Demirbas, 2009; Milne et al., 1997)

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Table 4.2 Analysis of pyrolytic oils by GC/MS-FID grouped in families

	Concentration in pyrolytic oil wt%		
Compound	**Pinewood Oil****	**Corn cobs oil**	**Switch grass oil**
Water	1.1	1.2	1.5
Water insoluble	13	10.7	11.2
Acetic acid	<1	0.3	0.5
Hydroxyacetaldehyde	<0.1	1.2	1.4
Furans	<0.1	0.1	0.1
Mono phenols	1.6	0.1	1.1

** Values previously shown on Chapter 3 section 3.3.1

Three compounds identified in every step of the upgrading process were hydroxymethylfurfural, furfural and vanillin (IDs # 3 #5 #15 *Table 4.3*), agreeing with the literature survey and some reports focusing on lignocellulosic biomass pretreatment applications (Lian et al., 2013; Palmqvist and Hahn-Hägerdal, 2000). Furfural, HMF and vanillin have been studied as growth inhibitors of different microorganisms (Almeida et al., 2007; Ranatunga et al., 1997; Schwab et al., 2013; Wood et al.,

2014). Common lignin degradation products such as phenol, catechol and guaiacol were not identified in the LC/MS analysis. This absence can be attributed to earlier elution times, or to relative low concentration of the compounds rather than a complete absence in the oils. Absence of lighter molecules such as acetic acid, formic acid, acetaldehyde and formaldehyde can be explained as the majority of these molecules are collected in the second condenser operated at 20°C (Westerhof et al., 2011)

The principal monomers of lignin synapyl alcohol, coniferyl alcohol and coumaric acids (Moldoveanu, 2005) and some of their possible degradation products mainly (hydroxybenzoates) (Kuroda et al., 2001) were included in LC-MS screening, *Table 4.3*. From the six hydroxybenzoates analyzed, 3-hydroxybenzoic acid (ID # 14 on *Table 4.3*) was not identified in the any of the extracts studied. The two most common hydroxybenzoates identified were 3-5 dihydroxybenzoic acid and 4-hydroxybenzaldehyde (ID # 9 and ID# 12 *Table 4.3*). 4-hydroxybenzaldehyde, is an important lignin derivative which has been linked with growth inhibition in fermentative microorganisms (Palmqvist et al., 1999) and which has been identified in lignocellulosic hydrolyzates (Klinke et al., 2003; Zaldivar and Ingram, 1999)

Contrasting the results obtained from the LC-MS screening with the preliminary results obtained by GC-MS and the values gather from literature, six compounds were chosen to be quantified by additional analytical techniques. 5-Hydroxymethylfurfural and furfural were quantified by liquid chromatography, while guaicol, *m*-cresol, vanillin and 4-hydroxybenzaldehyde were quantified via GC-FID. Concentrations of selected compounds were quantified in five different pyrolytic oil water extracts previously upgraded and ready for fermentation, *Table 4.4*.

Table 4.3.LC/MS analysis of pyrolytic oil water extracts throughout the upgrading process.

		Corn Cobs							Switch Grass						
		Water extracts			After hydrolysis		After Solvent Extraction		Water extracts			After hydrolysis		After Solvent Extraction	
ID #	Pretreatment / Compound	Untreated	Acetic Acid	Nitric Acid	Acetic Acid	Nitric Acid	Acetic Acid	Nitric Acid	Untreated	Acetic Acid	Nitric Acid	Acetic Acid	Nitric Acid	Acetic Acid	Nitric Acid
1	2-5H-Furanone	-	+	+	-	-	-	-	-	-	-	-	-	-	-
2	levulinic acid	+	+	+	+	+	-	-	+	-	-	+	+	-	-
3	5 HMF	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	furfuryl alcohol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	Furfural	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	resorcinol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	2-6 dyhydrobenzoic acid	+	-	-	-	-	-	-	-	-	-	-	-	-	-
8	4 Hydroxy benzoic acid	+	-	-	-	-	-	-	+	+	-	-	-	-	-
9	3-5 dihydroxy benzoic acid	+	+	+	+	+	+	+	+	-	-	-	-	-	-
10	4 hydroxy3methoxy benzoic	+	-	+	-	-	-	-	-	+	+	-	-	-	-
11	syringic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	4-hydroxy-benzaldehyde	+	+	+	-	-	-	-	+	+	+	-	-	-	-
13	*p* -coumaryl alcohol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	3-hydroxy-benzaldehyde	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	vanillin	+	+	+	+	+	+	+	+	+	+	-	-	+	+
16	Conyferyl alcohol	-	-	-	-	-	-	-	+	-	-	-	-	-	-
17	*p*- coumaric acid	-	+	+	-	-	-	-	-	-	-	-	-	-	-
18	syringaldehyde	+	+	+	-	-	+	+	+	+	+	-	-	-	-
19	*m* -coumaric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	syringol	-	-	-	-	-	-	-	+	-	-	-	-	-	-
21	*o* -coumaric acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	1-2 dimethoxybenzene	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	Eugenol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	Acetic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	Acetonitrile	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	Formic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	Fumaric Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	glycolic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	m cresol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	phenol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	cathecol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	guaiacol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	levoglucosan	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	acetaldehyde	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36	formaldehyde	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	glycolic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Different pretreatments were applied to the biomass in an attempt to produce cleaner, thus more fermentable substrates. Even though concentrations among the different biomasses for the selected compounds changed slightly for the selected compounds *Table 4.4*, the synergistic effects can be greatly altered.

Table 4.4. Concentration of selected inhibitory compounds in different upgraded water extracts derived from five different pyrolytic oils. Pretreatment type refers to the demineralization process used. AA stands for acetic acid, NA stands for nitric acid.

Biomass	Pretreatment Type	LC Detectables (g/L)		GC Detectables (g/L)			
		5-HMF	Fufural	Guaiacol	m-Cresol	Vanillin	4-Hydroxybenzaldehyde
Pinewood	AA	0.02	0.36	0.02	0.09	0.02	0.00
Switch grass	AA	0.03	0.37	0.03	0.17	0.02	0.00
	NA	0.01	0.36	0.02	0.16	0.04	0.00
Corn Cobs	AA	0.02	0.37	0.02	0.16	0.03	0.00
	NA	0.03	0.37	0.02	0.20	0.05	0.00

4.3.2 Microbial Response to Identified Compounds

Based on the compounds quantified in Table 4.4 a central composite design was used to evaluate the response of *S. cerevisiae* to these inhibitory compounds over a relevant concentration range as shown in Table 4.5. Between the two observed hydroxybenzoates, 4-Hydroxy-benzaldehyde (BLZ) was selected, as it is commonly found in hydrolysates of lignocellulosic biomass (Klinke et al., 2003; Lee et al., 1999; Zaldivar and Ingram, 1999)

Table 4.5. Central composite design coding with corresponding inhibitory range concentrations adapted from (Wood et al., 2014)*.

Compound	Acronym	Level	Coded level and concentration (g/L)				
			-1.414	-1	0	1	1.414
Furfural	FF	x_1	0	0.146	0.500	0.854	1.000
5-Hydroxymethylfurfural	HMF	x_2	0	0.220	0.750	1.280	1.500
Vanillin	VA	x_3	0	0.293	1.000	1.707	2.000
4-Hydroxy-benzaldehyde	BZL	x_4	0	0.044	0.150	0.256	0.300
Guaiacol	GL	x_5	0	0.293	1.000	1.707	2.000
m-Cresol	CL	x_6	0	0.146	0.500	0.854	1.000

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The corresponding growth rates were analyzed as previously described in Chapter 3 and linear regression analysis was used to correlate the specific growth rate to the coded inhibitor levels via a polynomial expression. The obtained coefficients are shown in *Table 4.6*.

Table 4.6 growth rate response surface polynomial coefficients adapted from (Wood et al., 2014)*.

Coefficient Label	Corresponding factors	Coefficient Value	Standard Error	*p*-value
a_0	1	0.32	0.007	<0.001
a_1	FF	-0.024	0.005	<0.001
a_2	HMF	-0.02	0.005	<0.001
a_3	VA	-0.022	0.005	<0.001
a_4	BZL	-0.049	0.005	<0.001
a_6	CL	-0.011	0.005	0.03
a_{16}	FF × CL	-0.018	0.005	<0.001
a_{45}	BZL × GL	-0.014	0.005	0.004
a_{46}	BZL × CL	-0.013	0.005	0.008
a_{56}	GL × CL	-0.024	0.005	<0.001
a_{66}	CL × CL	0.051	0.008	<0.001

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The regression data is in good agreement with the experimentally obtained values as shown in the parity plot presented in *Figure 4.1*. The overall ethanol yield was not affected, and agrees with previous results (Luque et al., 2014). The complexity of pyrolytic oil fractions is a key factor to take into consideration when evaluating their fermentation potential. Small concentration changes of inhibitors, *Table 4.4*, result in small alteration when evaluated with the CCD polynomial, however when compared to the values obtained experimentally the results vary significantly, even for the same biomass type. It is worth noting that all the pyrolytic oils were obtained from the same pyrolysis reactor operated under the same conditions, the only variables changed were pretreatment of the biomass, acid or nitric acid leaching. Biomass composition plays a key role in the product profile from pyrolysis, yielding different inhibitors concentrations (Czernik and Bridgwater, 2004)

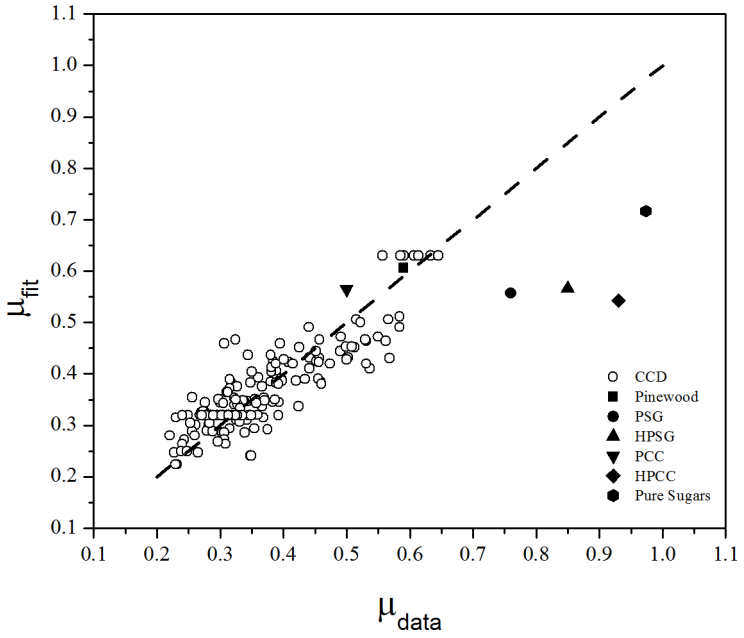

Figure 4.1 Correlation between the observed data both in the CCD and the calculated growth data for concentrations of the six compound selected in different pyrolytic oils. PSG refers to acetic acid treated switchgrass, HPSG corresponds to nitric acid pretreated switchgrass. PCC stands for acetic acid pretreated corn cobs whereas HPCC corresponds to nitric acid pretreated corn cobs.

4.3.3 Application of Regression Model to Pyrolytic sugars

The regression model was used to predict the specific growth rates that could be achieved on selected pyrolytic sugars, based on the measured concentrations of the respective inhibitors. The measured and the predicted values are shown in *Table 4.7* and also represented by the black symbols in *Figure 4.1*. It can clearly be seen that the model, though very effective if used with pure compounds, does not fully capture the effect of pyrolysis by-products on ethanol fermentation. The six compounds selected can therefore not be considered as suitable representatives when evaluating the toxicological effects of pyrolytic sugars on yeast. The model might be improved by extending the list of compounds, however it would further complicate the analysis and the desired outcome might not be achieved. A more generalized indicator for the inhibitory effect would therefore be desirable.

Table 4.7 Obtained parameters utilizing the coded concentrations obtained from HPLC and GC FID analysis. Pretreatment type refers to the demineralization process used. AA stands for acetic acid, NA stands for nitric acid. The maximum growth rate (μ_{max}) is reported as the fraction of un-inhibited growth.

Biomass	Pretreatment Type	Parameters	
		μ_{max} (h^{-1})	
		Model Prediction	Experimental
Pinewood	AA	0.61	0.59
Switch Grass	AA	0.56	0.76
	NA	0.57	0.85
Corn Cobs	AA	0.56	0.50
	NA	0.54	0.93

4.4 Conclusion

The selected compounds that were used for the CCD are not representative for the the inhibition exhibited by the pyrolytic sugars. The inclusion of additional compounds might result in a better model, however it will also result in additional analytical challenges. An empirical model based the concentrations of selected compounds is therefore likely not a feasible approach for pyrolytic sugar samples and a different way to quantify the inhibitory potential of the resulting compound cocktails is needed.

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Chapter 5

5 Comparison of ethanol production from corn cobs and switchgrass following a pyrolysis-based biorefinery approach

Luis Luque, Roel Westerhoff, Guus van Rossum, Stijn Oudenhoven, Sascha Kersten, Franco Berruti and Lars Rehmann.

The information in this chapter has been slightly changed to fulfill formatting requirements. This chapter is substantially as it will be submitted to Biotechnology for Biofuels.

This chapter assesses the robustness of the pyrolysis based biorefinery as proposed in Chapter 3 by evaluating two different Canadian types of biomass for their suitability to produce pyrolytic oils rich in anhydrous sugars. In addition to the established demineralization strategy (acetic acid rinsing), a different strategy was investigated to evaluate the reduction of catalytic centers that would redirect levoglucosan to degradation reactions. The new demineralization strategy showed to enhance the ash level reduction in corn cobs, but not in switchgrass, which translated to higher levoglucosan levels in corn cobs but not in switchgrass. Moreover, as tracing all the possible compounds that could be produced in a pyrolysis reaction is not possible, a new quantification technique was developed based on absorbance spectra of compounds present. This quantification technique allowed to predict which upgrading process would achieve a cleaner fraction thus establishing an improved detoxification route. The results showed that water extraction followed by acid hydrolysis and solvent extraction was the best upgrading strategy. The highest ethanol yields based on the initial cellulose content were 27.8 % for switch grass and 27.0 % for corn cobs and fermentation performance on both feedstock, correlated well with the integral of the UV signal. The study demonstrates that ethanol production from switch grass and corn cobs is possible following a combined thermochemical and fermentative biorefinery approach. However, the ethanol yields achieved were still lower than yields reported for conventional pretreatments and fermentation processes. The feedstock-independent fermentability can easily be assessed with a simple assay.

This study fulfills three proposed objectives (5-7) described on Chapter 1. Firstly, a quantification technique was successfully developed and applied to quantify the overall inhibitor concentration. Secondly, the research showed that high ethanol yields from agricultural residues and energy crops

are possible via fast pyrolysis and as described in chapter 3, several of the streams produced in the biorefinery could serve as platform chemicals or fuel additives. Moreover it showed that demineralization of the biomass is a crucial step as it increases no only yields but it also eases he load on the upgrading processes. This research also suggests that the applications of the realized biorefinery concept can be expanded to other kinds of biomasses and for the production of other kind of biofuels.

5.1 Abstract

One of the main bottlenecks in lignocellulosic ethanol production is the necessity of pretreatment and fractionation of the biomass feedstocks to produce sufficiently pure fermentable carbohydrates. Additionally, the by-products (hemicellulose and lignin fraction) are of low-value, if compared to dried distiller's grains (DDG), the main by-product of corn-ethanol. Fast pyrolysis is an alternative thermal conversion technology for processing biomass. It has recently been optimized to produce a stream rich in levoglucosan, a fermentable glucose precursor for biofuel production. The additional product streams might be of value to the petro-chemical and agricultural industry. However, biomass heterogeneity is known to impact the composition of the pyrolytic product streams, as a complex mixture of aromatic compounds is recovered with the sugars, interfering with subsequent fermentation. The present study investigated the feasibility of fast pyrolysis to produced fermentable pyrolytic glucose from two abundant lignocellulosic biomasses in Ontario, switch grass (potential energy crop) and corn cobs (by-product of corn industry).

Demineralization of biomass removes catalytic centers and increases the levoglucosan yield during pyrolysis. The ash content of biomass was significantly decreased by 82 and 90% in corn cobs when demineralized with acetic or nitric acid respectively. In switch grass only a reduction of 50% for both acids could be achieved. Conversely, levoglucosan production increased 9- and 14-fold in corn cobs when rinsed with acetic or nitric acid respectively, and 11-fold increase in switch grass regardless of the acid used. After pyrolysis, different configurations for upgrading the pyrolytic sugars were assessed and the presence of potentially inhibitory compounds was approximated at each step as the double integral of the UV-spectrum signal of an HPLC assay. The results showed that water extraction followed by acid hydrolysis and solvent extraction was the best upgrading strategy.

Ethanol yields achieved based on initial cellulose fraction were 27.8 % in switchgrass and 27.0 % in corn cobs.

The study demonstrates that ethanol production from switch grass and corn cobs is possible following a combined thermochemical and fermentative biorefinery approach with ethanol yields comparable to results in conventional pretreatments and fermentation processes. The feedstock-independent fermentability can easily be assessed with a simple assay.

5.2 Introduction

Presently, ethanol production in the United States and Canada is predominately derived from corn grains. The additional utilization of plant residues such as corn cobs or stover can potentially increase the ethanol yield per unit area and utilize existing conversion and distribution infrastructure (Christiansen, 2009). Corn cobs were found to yield higher glucose concentrations than other corn residues like the stalks or the leaves if enzymatic hydrolyzed and are removed from the fields during conventional harvest (Crofcheck and Montross, 2004). As an alternative to food crops, perennial grasses have also been proposed feedstocks for liquid fuels production. Switchgrass (*Panicum* virgatum) is a suitable crop to be grown on marginal lands, and requires less water and nutrients compared to other sources of biomass used in fuel production (Sanderson et al., 2006). However, the common challenge for lignocellulosic biomass is the high recalcitrance to biological conversion technologies and thus the requirement of pre-treatment in commercial processes (Kazi et al., 2010). A multitude of technologies is available with different advantages and disadvantages as recently reviewed elsewhere (Banerjee et al., 2009; Jacquet et al., 2015; Liu et al., 2015; Martín et al., 2007; Menon and Rao, 2012; Zhang, 2008). Fast pyrolysis is commonly used as a tool to increase the energy density of bulky biomass through thermal cracking (400 and 550°C in the absence of oxygen); it can alternatively be used as a pretreatment technology combined with biochemical conversion (Bridgwater et al., 2002; Lian et al., 2010; Luque et al., 2014; Rover et al., 2014). Pyrolysis of biomass typically yields condensable (‘bio-oil’) and non-condensable gases (often used as fuel gas to power the process) and char (‘bio-char’, a possible soil amendment)(Czernik and Bridgwater, 2004; Lian et al., 2013; Purakayastha et al., 2016; Wang et al., 2015). The composition of the pyrolysis oil depends heavily on the operating conditions during the pyrolysis process as well as the type of biomass, which

also determine the product distribution with liquid yields of up to 75% wt based on biomass intake (Czernik and Bridgwater, 2004). The most abundant carbohydrate found in pyrolysis oil is levoglucosan, an anhydrosugar which can be easily converted to glucose via acid hydrolysis followed by ethanol production (Vispute and Huber, 2009). Recent studies have focused on ways to increase levoglucosan yields in pyrolytic oils (Oudenhoven et al., 2013) and in its integration to a fermentation process (Lian et al., 2010; Luque et al., 2014).

Anhydrous sugars yields depend on the cellulose content of the biomass but also on the presence of alkali and alkaline earth metals which in turn can vary significantly depending on the growth conditions of the plants as well as harvesting time and conditions (Trendewicz et al., 2015). Studies have shown that decreasing the presence of these metal ions via mild or strong acid rinsing (Radlein et al., 1987; Shafizadeh and Stevenson, 1982) increases levoglucosan. Yields of 30% and 52% g levoglucosan/ g cellulose have been achieved when acid rinsing the biomass (Dobele et al., 2003; Oudenhoven et al., 2013). The most abundant metals present in biomass are magnesium, calcium, sodium and potassium (Trendewicz et al., 2015). Even though the effect of these inorganic elements on pyrolysis has been broadly described in several studies (Antal and Varhegyi, 1995; Pan and Richards, 1989; Scott et al., 1985; Williams and Horne, 1994) a detailed and well established mechanism has not yet been realized. Nevertheless, studies have shown that metals catalyze cellulose depolymerization, and once depolymerized, further catalyze the decomposition of anhydrous sugars. This effect translates into changes in the composition and yield of pyrolytic oils as water and char generation is enhanced (Antal and Varhegyi, 1995) along with several other molecules such as acids, ketones, aldehydes, furans and phenols (Westerhof et al., 2011). Studies involving the fermentation of biomass pyrolyzates have found that these compounds hamper ethanol production by inhibiting the growth of fermentative microorganisms (Palmqvist and Hahn-Hägerdal, 2000; Zaldivar et al., 1999). A complete avoidance of such by-product formation is technically not possible, therefore detoxification approaches to clean the pyrolyzates before fermentation are needed. Possible options are adsorption on activated carbon (Lin and Juang, 2009; Wang et al., 2012) and polymer matrices such as XAD 4 XAD 7 (Weil et al., 2002) overliming (Chi et al., 2013) air stripping (Wang et al., 2012) and solvent extractions (Lian et al., 2012; Luque et al., 2014; Wang et al., 2012). Studies have also shown that possible combinations of these detoxifications routes renders a cleaner extract (Lian et al., 2012).

In a previous study, using a pyrolysis-based biorefinery approach, pyrolytic oil from demineralized pinewood was utilized to prepare fully fermentable pyrolytic sugar. Pyrolytic oils were detoxified via water and solvent extraction followed by acid hydrolysis. The growth and ethanol production kinetics were determined via non-linear regression analysis of online process data, allowing to quantify residual inhibitory effects of by-products in the pyrolytic sugars. Ethanol yields in the fermentation step reached 96% of the theoretical value corresponding to 41.3% of the maximum theoretical value assuming all glucan in the initial biomass to be converted to ethanol (Luque et al., 2014). However, only one source of biomass was tested, and no attempt was made to correlate inhibition to the presence of inhibitors.

The purpose of this study was therefore to evaluate the production of ethanol using a modified pyrolysis based biorefinery approach (Figure 5.1) from two underutilized source of biomasses available in Canada, corn cobs and switch grass. Two demineralization steps were evaluated to determine how alkaline ion removal from the biomass affects ethanol yields. Further a simple HPLC assay was developed to estimate the sugar to inhibitor ratio, which was subsequently used as a substrate independent indicator for fermentability. To facilitate the reader's following of the process, the abbreviation used in this chapter are included in Table 5.1.

Table 5.1 Abbreviation of streams and upgrading steps used in the present study

Acronym	**Definition**
EAc	Ethyl acetate
AACC	Acetic acid pretreated corn cobs
NACC	Nitric acid pretreated corn cobs
AASG	Acetic acid pretreated switch grass
NASG	Nitric acid pretreated switch grass
W-H	Water extraction followed by hydrolysis upgrading route
W-H-EAc	Water extraction followed by hydrolysis proceeded by ethyl acetate extraction
W-EAc-H	Water extraction followed by ethyl acetate extraction followed by hydrolysis

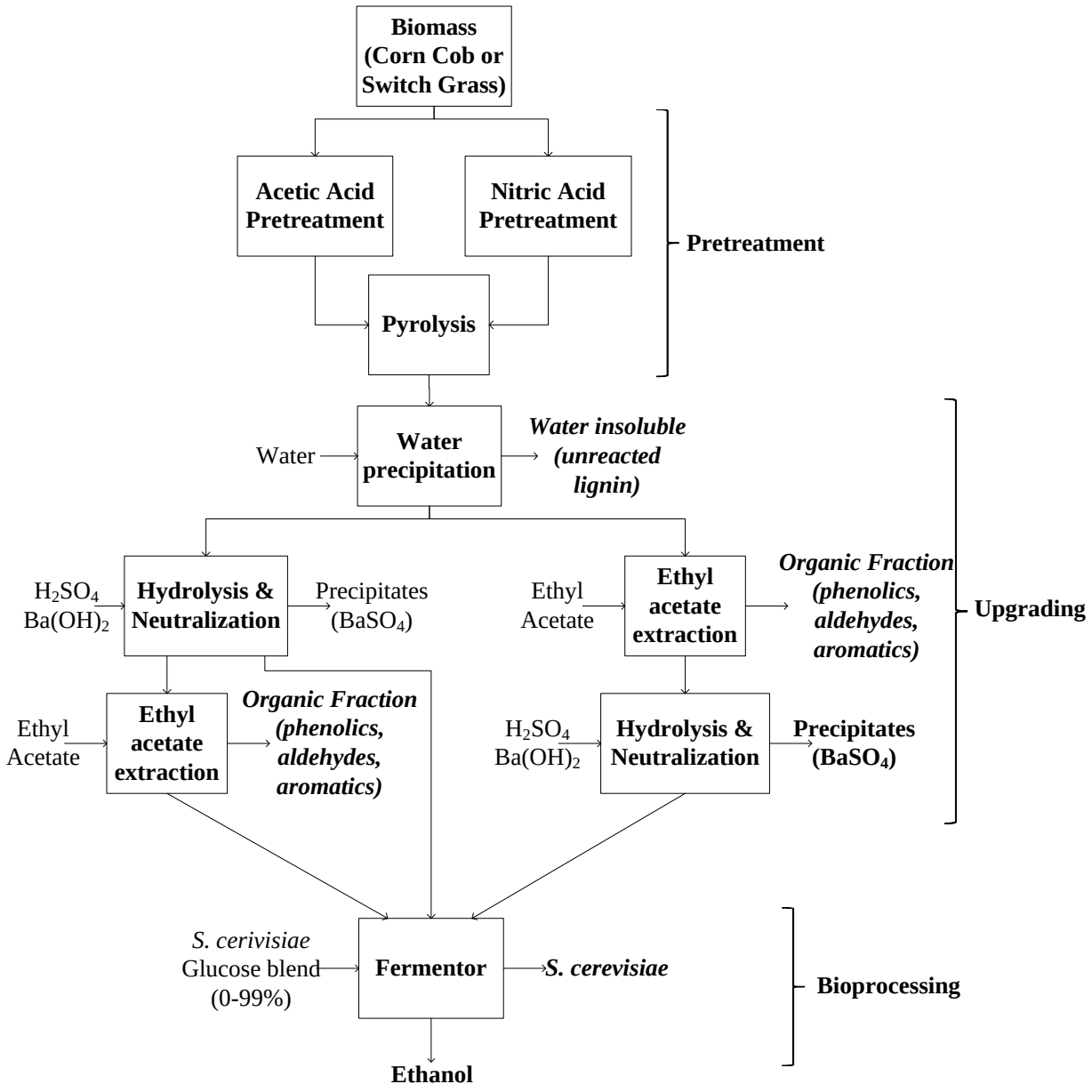

Figure 5.1 Process diagram for the production of sugars via fast pyrolysis using the biorefinery approach. Italized streams represent proposed added value products of the present approach. Underlined are the names given to each of the detoxification routes. Adapted from (Luque et al., 2014).

5.3 Materials and Methods

5.3.1 Biomass pretreatment and characterization

Once reduced to the required particle size, biomass was subjected to demineralization with a weak acid solution (Acetic Acid 10 % V/V) or a strong acid solution (HNO_3 10 % V/V). Biomass was added to the acid solution in a 1:10 ratio. The mixture was stirred for 2 hours at 1200 rpm and 50 °C in a jacketed vessel to secure proper contact of the biomass with the solution (Oudenhoven et al., 2013). Once the stirring was completed, the biomass was rinsed to remove the acid solution by adding dionized water (Milli-Q Integral 5, EMD Millipore, USA) in batches of 1L and stirred for 5 minutes at room temperature. The final point for rinsing was determined by monitoring conductivity (Pinnacle Series, Nova Analytics, USA) of output water stream until the value approached zero and remained constant.

In order to reduce moisture rinsed biomass was dried at a 105 °C for 24 hours in a convection oven (Thermo Scientific, USA). Final moisture was recorded using a moisture analyzer (ADAM, USA).

5.3.2 Anhydrous sugars production

Anhydrous sugars were produced during the fast pyrolysis step in the biorefinery approach detailed in Figure 5.1. Two different oils for each biomass were produced, in order to compare demineralization approaches and their impact on the pyrolytic oil potential as fermentative substrates for ethanol production. Batches of 100 g of dried biomass were thermally decomposed in a fluidized bed pyrolyzer at 480 °C with a vapor residence time <2s. Fractional condensation of vapors was achieved using two condensers in series kept at 1.1±0.01bar. The fraction recovered in the first condenser set at 80°C was an oil rich in aromatics and sugars. The second condenser, set at 20°C, yielded a fraction rich in acetic acid and water. This second condenser liquid can be used in the demineralization of the biomass, due to its high acetic acid fraction as detailed elsewhere (Westerhof et al., 2011)

5.3.3 Upgrading

Insoluble lignin was precipitated from the obtained pyrolytic oil samples via cold water extraction (Garcia-Perez et al., 2008). Pyrolytic oil was added to cold water (4°C) under heavy stirring (900 rpm) in a baffled beaker. Oil was added until the oil to water ratio reached 1:10. Insoluble lignin was measured gravimetrically and removed via filtration using a pre-dried and weighed 0.2 µm membrane. Filtrate was collected and stored at 4°C (Luque et al., 2014). Doing so four different water extracts, each from the four different oils produced, were prepared.

Three different approaches were used to procure the fermentable sugars, Figure 5.1. The first consisted of directly hydrolyzing the water extracts to produce glucose, referred as W-H (water extract to hydrolysis). After hydrolysis, the extract was further treated with ethyl acetate (W-H-EAc).The third approach involved extracting the water extract with ethyl acetate before acid hydrolysis to produce glucose, and referred as W-EAc-H, and previously described on Chapter 3 section 3.3.1.

Solvent extractions aimed to remove organic compounds known to hinder yeast fermentation. A slight modification to the extraction method detailed on Chapter 3 section 3.3.3 of this thesis was implemented. All solvent extractions were performed as follows. Ethyl acetate (EAc) was added to produce a 1:2 wt% filtrate or hydrolyzate (depending on the approach taken) to EAc ratio solution. The solution was then mixed for 12 h at 150 rpm and 25°C in a temperature controlled shaker (Infors, Switzerland). Once mixed, the mixture was transferred to a separating funnel and left to stand for 24 h to ensure proper phase separation. The resulting bottom layer was collected and subjected to evaporation to remove any EAc residue at 50°C using the controlled temperature shaker (Infors, Switzerland). EAc concentration was monitored by analyzing hourly samples using high pressure chromatography until concentration reached a constant value. Sugar concentration was kept constant by adding water.

Glucose was produced via strong acid hydrolysis of levoglucosan. Extract aliquots of 7 mL were transferred to a microwave vial (VWR, USA), proceeded by the addition of H_2SO_4 (Caledon, Canada) to a final concentration of 0.5M. Vials were sealed and hydrolysis was carried out using an

autoclave for 20 mins at 120 °C (Bennett et al., 2009). Hydrolyzate was transferred to a 15 mL centrifuge tube (VWR, Canada) and pH was adjusted to 6.5 by adding $Ba(OH)_2$ (Alfa Aesar, USA). Formed crystals where then precipitated via centrifugation at 3500 rpm for 20 mins (Sorval ST40R, Thermo Scientific). Supernatant was transferred to a new sterile 15 mL centrifuge tube by filtering it with a 0.2 µm cellulose syringe filter (VWR, Canada).

5.3.4 Inhibitors removal quantification

Before and after each detoxification step, *Figure 5.1*, spectra between 190 and 340 nm were measured for 80 minutes with a 2nm step in a diode array detector (DAD) in a high pressure liquid chromatography fitted with a Hiplex H column at 60°C utilizing 5mM H_2SO_4 as the mobile phase at a flow rate of 0.7 ml/min and equipped with a diode array detector (Agilent 1260 series, USA). Raw data was exported and processed in MATLAB (MathWorks Inc, USA). The volume under the recorded spectra was numerically integrated to determine a single value normalized by the sugar concentration of the sample also determined by HPLC. The inhibitor value IV/G was defined as follows (eq. 5.1):

$$IV/G = \int_{t=10min}^{t=80min} \int_{\lambda=190nm}^{\lambda=340nm} S_{DAD}\, dt\, d\lambda\, /C_G \qquad (5.1)$$

Where IV/G is the glucose normalized inhibitor value, t the retention time on the HPLC [min], λ the wavelength of the DAD at time t [nm], S_{DAD} the signal measured at time t and wavelength λ, and C_G the concentration of glucose in the sample [g/L]. Removal performance was measure as changes in the volume under the surface after each complete detoxification step was performed.

5.3.5 Fermentation

After the required detoxification steps, YPG media was prepared using the obtained hydrolysates by adding solid peptone (BD, USA) and yeast extract (BD, USA) to a final concentration of 2 wt% and 1 wt%, respectively. Fresh YPG media with the same peptone, yeast extract and regular glucose concentrations (Alfa Aesar, USA) was prepared and blended with the pyrolytic media in different proportions. The high concentrations of pyrolytic glucose obtained in the extracts allowed to have a pyrolytic sugar fraction between (20 – 100%). Final glucose concentrations in each blend was

2.5wt%. By creating these blends, it was possible to determine the yeast tolerance threshold to unremoved inhibitory compounds dissolved along with the pyrolytic glucose within the media.

Blends were fermented following the protocol realized and explained in Chapter 3 section 3.3.4.

5.3.6 Modelling and determination of yeast growth parameters

In order to calculate inhibition effects on the yeast growth, parameters associated with the growth kinetics were determined by fitting the obtained experimental kinetics data to the model elucidated by Baranyi and Roberts (Baranyi and Roberts, 1994).

The model equations and details are described on Chapter 3 section 3.3.5

5.4 Results and Discussion

5.4.1 Effects of demineralization

Metals such as Ca, K, Mg and Na, occur intrinsically in plant-biomass. However, these metal ions are known to form catalytic centers during pyrolysis and catalyze biomass decomposition beyond desirable intermediates such as levoglucosan, a glucose precursor (Shafizadeh and Stevenson, 1982). Levoglucosan can be subjected to strong acid hydrolysis, producing glucose, which is the preferred carbon source for fermentative microorganisms. In order to maximize levoglucosan yields it is therefore desirable to have low ion concentrations in feedstocks prior to pyrolysis. Acetic and nitric acid (weak and strong acid) solutions were used to reduce the ion content in both, corn cobs and switch grass. The initial switch grass ash content of 40 g/kg and the corn cobs ash content of 27.9 g/kg are within the typical range. Ash content in switch grass can vary between 3.7 (Ewanick and Bura, 2011) and 5.73 g/kg (Greenhalf et al., 2012) and in corn cobs between 2.41 (Zhang et al., 2009) and 8.06 g/kg (Ioannidou et al., 2009). The acid catalyzed biomass demineralization was more pronounced in corn cobs than it was in switch grass (Table 5.2). Post-rinsing ash contents for switch grass decreased to 55.50 % and 54.25% of the original value (40.00 g/kg) after acetic acid and nitric acid washing respectively. Contrasting with the values obtained with corn cobs, 18.2 % and 10.2 %

of the original value (27.90 g/kg). One explanation for the difference post-rinsing ash content is remaining soil traces from the harvesting process. Despite the higher decrease in the ash content for corn cobs, the alkali content in the demineralized biomass, is higher in switch grass 2.03 g/kg and 0.83 g/kg than in corn 0.85 g/kg and 0.47 g/kg with the majority of these percentages corresponding to different ions, Ca^{2+} in switch grass and K^{+} in corn cobs, Table 5.2 .

Table 5.2 Metals ions quantification before and after demineralization. Levoglucosan concentrations obtained after water extraction of the pyrolysis oils. Levoglucosan yields are expressed as mole glucose per mole glucose units that could be released from the cellulose fraction of the respective biomass (38.80 %wt in corn cobs (Zheng et al., 2015) and 37.00 wt% in switchgrass (Gao et al., 2014)).

Ion [g/kg]	Switch Grass			Corn Cobs		
	Untreated	Acetic Acid	Nitric Acid	Untreated	Acetic Acid	Nitric Acid
Ca^{2+}	2.52 ± 0.20	1.94 ± 0..02	0.76 ± 0.03	0.47 ± 0.03	0.17 ± 0.06	0.06± 0.03
K^{+}	11.03 ± 0.20	0.07 ± 0.01	0.05 ± 0.01	15.52 ± 1.47	0.58 ± 0.03	0.34 ± 0.01
Mg^{2+}	0.95 ± 0.06	0.01± 0.00	0.01 ± 0.01	0.71 ± 0.01	0.08 ± 0.06	0.04 ± 0.02
Na^{+}	0.09 ± 0.03	0.01± 0.00	0.02 ± 0.00	0.07 ± 0.01	0.02 ± 0.00	0.04 ± 0.00
Alkali [g/kg biomass]	14.59	2.03	0.83	16.77	0.85	0.47
Ash [g/kg biomass]	40.00	22.20	21.07	27.90	5.09	2.84
% alkali in ash	36.48	9.15	3.96	60.12	16.68	16.50
Levoglucosan [g/L]	1.39	22.42	23.06	2.16	18.06	28.78
Yield [mol/mol]	0.02	0.30	0.31	0.03	0.23	0.37

Alkaline metal ions such as Ca^{2+} and Mg^{2+} have been reported to be catalysts of cellulose dehydration and decompositions reactions where ions such as K+ and Na+ are catalysts of sugar structures derived from cellulose dehydration reactions (Liu et al., 2014). Therefore the presence of K^{+} and Na^{+} can significantly reduce the yield of levoglucosan due to their catalyst activity in decomposition levoglucosan (Kawamoto et al., 2007), and diverting the reaction towards the production of lighter molecules such as hydroxyacetaldehyde, acetol, formic and acetic acid (Zhang and Liu, 2014). In addition to the low levoglucosan yields, formation of these undesirable light products affect

downstream ethanol production, by hindering the growth of fermentative microorganisms (Luque et al., 2014).

The effects of biomass demineralization on anhydrous sugar production are shown on Table 5.2. Levoglucosan production from corn cobs increased nine fold for the acetic acid pretreatment, compared to a 14-fold increase if pretreated with nitric acid. This increase in production is the result of decreasing the ash content from 5.09 g/kg to 2.84 g/kg when nitric acid is used as a rinsing agent in corn cobs, Table 5.2. The importance increasing levoglucosan is not only translates into a higher ethanol production but also an elevated ethanol productivity as less inhibitors are produced, Figure 5.3, enabling a shorter fermentation time as seen on Figure 5.4. In the case of switch grass, levoglucosan production increased almost the same, 16-fold, for both demineralization processes. These increases in levoglucosan concentration after mineral removal are higher than previous results where pinewood demineralization was responsible for increasing levoglucosan by a factor of six (Luque et al., 2014). The benefits of demineralizing the biomass were also observed on the levoglucosan yield based on the initial amount of cellulose available, Table 5.2. The increasing molar yield shows that the levoglucosan is being diverted away from cracking reactions which would create lighter molecules and possible fermentation inhibitors. Nevertheless, molar yields could be further improved by tailoring demineralization to each biomass. These marked contrasts in anhydrous sugar production from different types of biomass, pretreated under the same conditions, can be due to the different biomass compositions and how the pretreatments affects each one directly, for it is known that biomass composition plays a key role in the products profile of pyrolysis (Czernik and Bridgwater, 2004).

5.4.2 Pyrolysis oil upgrading

In order to remove insoluble lignin and hydrophobic inhibitory compounds, all the oils were subjected to a cold water extraction (W) (Garcia-Perez et al., 2008), comprising the first step in the upgrading of the pyrolytic oils, Figure 5.1. Three detoxification approaches were studied. The first comprised of acid hydrolyzing the water extracts to obtained glucose from levoglucosan, followed by a neutralization step (W-H). The second approach was identical but included a solvent extraction using ethyl acetate (W-H-EAc) after the hydrolysis. This step was chosen to remove inhibitory compounds

that remained after the water extraction and also that were generated as a result of the strong acid hydrolysis, as it has been widely documented (Bennett et al., 2009; Palmqvist et al., 1999; Sun and Cheng, 2002). The third approach consisted cold water extraction directly followed by solvent extraction prior to strong acid hydrolysis and neutralization (W-EAc-H). Glucose production from levoglucosan hydrolysis was not affected by the any of the detoxification routes nor by the type of acid used as seen on Table 5.3. Nevertheless, these result contrast with findings on pinewood pyrolysates (Luque et al., 2014) where glucose molar yield was lower, 0.88 but the final glucose concentration was higher 41 g/L. The observed fluctuations are likely a result of residual cellobiose or other oligomers that are also being hydrolyzed to glucose, a know effect that can result in molar yield (glucose per levoglucosan) >1 (Yu and Zhang, 2003). Glucose yields of up to 216% from pyrolysate hydrolysis have been previously reported (Bennett et al., 2009). The difference between the values obtained by Bennett et al. (2009) and the ones obtained in this study could be due to extra anhydrous carbohydrate oligomers not decomposed in the pyrolysis oil used in that study. Bennett et al. (2009) reported increasing glucose levels after levoglucosan depletion (20 mins) in the hydrolysis step.

Table 5.3 Carbohydrates concentrations and molar yields after each detoxification step

		Detoxification Approach								
Biomass	Ion removal type	W-H			W-EAc-H			W-H-EAc		
		Levoglucosan (g/L)	Glucose (g/L)	Molar Yield	Levoglucosan (g/L)	Glucose (g/L)	Molar yield	Levoglucosan (g/L)	Glucose (g/L)	Molar Yield
Corn Cobs	As is	-	-		-	-		-	-	
	Acetic Acid	2.08	18.58	1.05	2.20	17.10	0.97	2.20	19.09	1.08
	Nitric Acid	1.30	28.41	0.93	1.01	29.07	0.94	0.94	28.27	0.91
Switch grass	As is	-	-		-	-		-	-	
	Acetic Acid	1.43	26.62	1.14	1.10	26.54	1.12	1.09	27.54	1.16
	Nitric Acid	1.16	26.15	1.07	1.05	26.83	1.10	1.17	26.55	1.09

Typical by-products of the pyrolysis process that tend to inhibit subsequent fermentation are phenols, furans and aldehydes (Lian et al., 2010; Luque et al., 2014; Palmqvist et al., 1999; Wood et al., 2014).

The cocktail of these compounds is typically very complex and challenging to fully analyze (Bayerbach and Meier, 2009; Garcia-Perez et al., 2007; Oasmaa and Meier, 2005; Schwab et al., 2013; Wood et al., 2014). To the of the author's knowledge a complete characterization (closed carbon balanced) of a pyrolysis product from lignocellulosic biomass has not yet been accomplished. Feedstock variability would also be expected to change to product contribution from biomass to biomass and likely from batch to batch, and is hence not suitable for the purpose of biofuel production. Many of the possible byproducts typically associated with inhibitory effect on the fermentation contain chromophores and can hence be detected in the UV range, where carbohydrates do not show a strong signal. A diode array detector (DAD) was therefore used to record the chromatogram of the pyrolytic sugar samples between 190 and 340 nm during HPLC analysis of the glucose/levoclucosan concentration (quantified via RID). The relative abundance of peaks is an indication for the residual amount of chromophore containing by-products. Selected chromatograms after various detoxification steps can be seen in Figure 5.2.

The peaks shown in Figure 5.2 are not representing the total amount of compounds found in the mixtures, nor was any attempt was made to separate peaks (in the time dimension) by varying the HPLC conditions. The multiple wavelengths give additional resolution; never the less it is very likely that compounds are co-eluding with the given protocol. However, it can be seen clearly that the upgrading steps remove chromophore compounds. Solvent extraction as the last step results in the cleanest samples (Figure 5.2D), likely due to the fact that acid hydrolysis, when performed after solvent extraction (Figure 5.2C) produces its own degradation by-products. The volume under the surface shown in Figure 5.2 was numerically integrated in order to obtain a single numerical value and normalized by the sugar concentration in the sample.

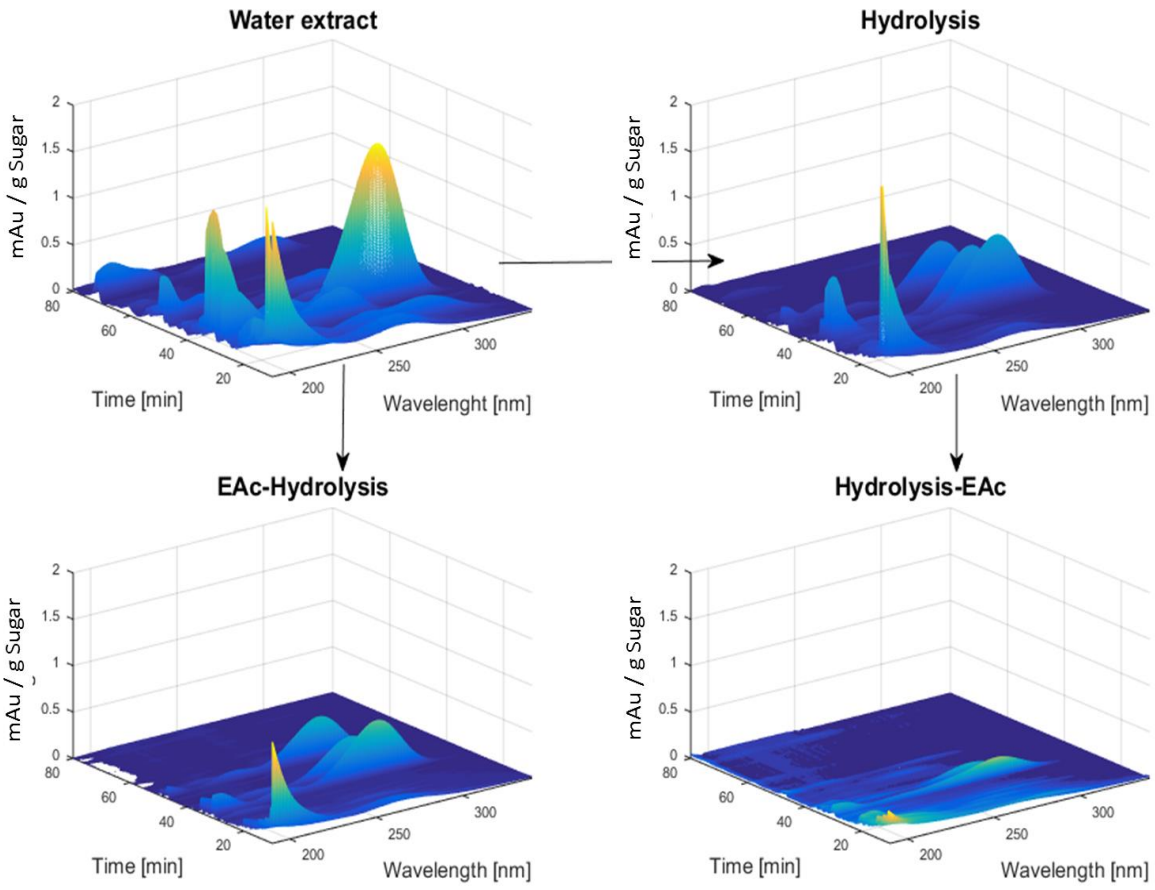

Figure 5.2 Chromatograms as a function of the different detoxification steps. The extract shown correspond to NACC pyrolysis oil upgrading. The arrows indicate the starting point and the order followed in the process.

Figure 5.3 shows IV/G values for the four different pyrolysates at the various upgrading steps. As expected for all the pyrolytic oils, water extracts, the first step in the upgrading train, showed the highest IV/G. Out of the four water extracts, acetic acid pretreated corn cobs (AACC) extracts showed the highest IV/G. NACC water extract levels are double or more if compared to nitric acid pretreated corn cobs (NACC), acetic acid pretreated switch grass (NASG) and nitric acid pretreated switch grass (NASG) after each detoxification approach, Figure 5.3. These high IV/G could be linked to a higher K^+ presence in the biomass before hydrolysis, Table 5.2. For all the samples the steepest decrease was observed after hydrolysis. This reduction can be a result of further decomposition during the hydrolysis step, or through removal during the subsequent $Ba(OH)_2$ treatment (added to increase the

pH). These findings are in accordance with previous reports where a drop in the total carbon levels was observed when water extracts were neutralized after acid hydrolysis (Luque et al., 2014). Conversely, the lowest drop for all the samples was observed when EAc extraction was done to previously hydrolyzed and neutralized samples (W-H-EAc) Figure 5.3.

Having the solvent extraction after the hydrolysis steps helps removing inhibitory compounds that survived the hydrolysis/neutralization step, or that could have been generated while in the process. The numerical IV/G value of a given pyrolytic sugar can be useful when evaluating its fermentability.

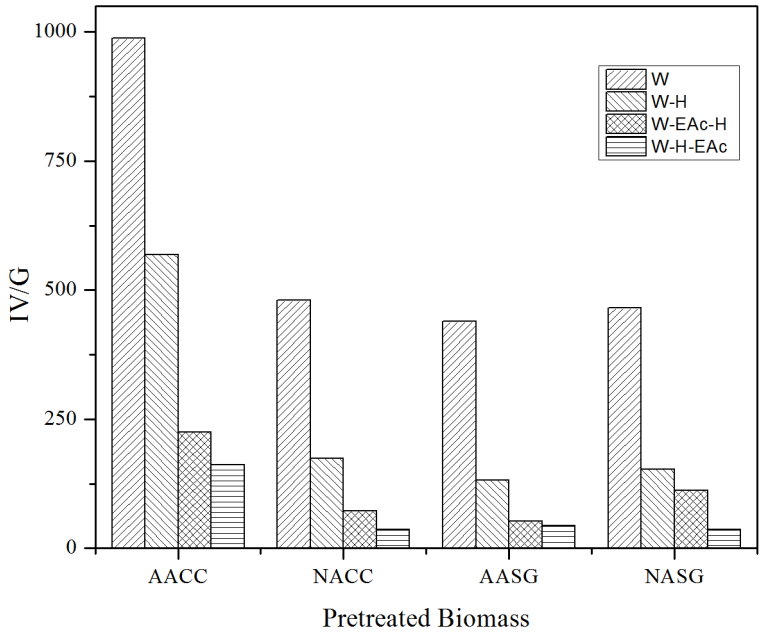

Figure 5.3 IV/G values estimated for each pyrolytic sugar after the respective upgrading step. W stands for the extract of each sample of pyrolytic oil. H stands for hydrolysis and neutralization upgrading step. EAc stands for the ethyl acetate used in the solvent extract upgrading process. In accordance with this nomenclature. The dash in between the letters means the order in which the steps were performed. W-H-EAc is water extract hydrolyzed and neutralized and later treated with ethyl acetate for inhibitors removal.

5.4.3 Pyrolytic sugar bioconversion

Microscale fermentation experiments were conducted to evaluate the pyrolytic oil extracts as fermentation substrates. The total initial glucose concentration was set to 25 g/L and fermentation

broths with various IV/G values were achieved by blending the pyrolytic oil extracts with a glucose stock solution (Luque et al., 2014). In doing so, a range between 20 to 100 % of pyrolytic glucose present in fermentable media was achieved. By having different fractions of pyrolytic sugar, proportional fractions of unremoved inhibitory compounds (represented by the IV/G value) were also present, thus enabling to determine tolerance and threshold levels of *S. cerevisiae* to these compounds (Lian et al., 2012; Sun and Cheng, 2002). Growth curves of *S. cerevisiae* on pure pyrolytic sugars are shown in Figure 5.4. Growth profiles for water extractions only (W-H) showed the strongest inhibition effects as growth in 100% of pyrolytic sugars was not achieved in any of the biomass extracts tested (AACC W-H extracts with 20% of pyrolytic sugars and 40% pyrolytic sugars for NACC, AASG, and NASG). Similarly strong inhibition was also observed with pinewood hydrolyzed water extracts as reported elsewhere (Luque et al., 2014) and confirms that cold water precipitation of the pyrolytic oils fails to extract sufficient quantities of inhibition compounds. Nevertheless, growth on 100% pyrolytic sugars was observed when a solvent extraction (W-EAc-H and W-H-EAc) was performed Figure 5.4, with growth being favored when solvent extraction was the last step in the upgrading train (W-H-EAc).

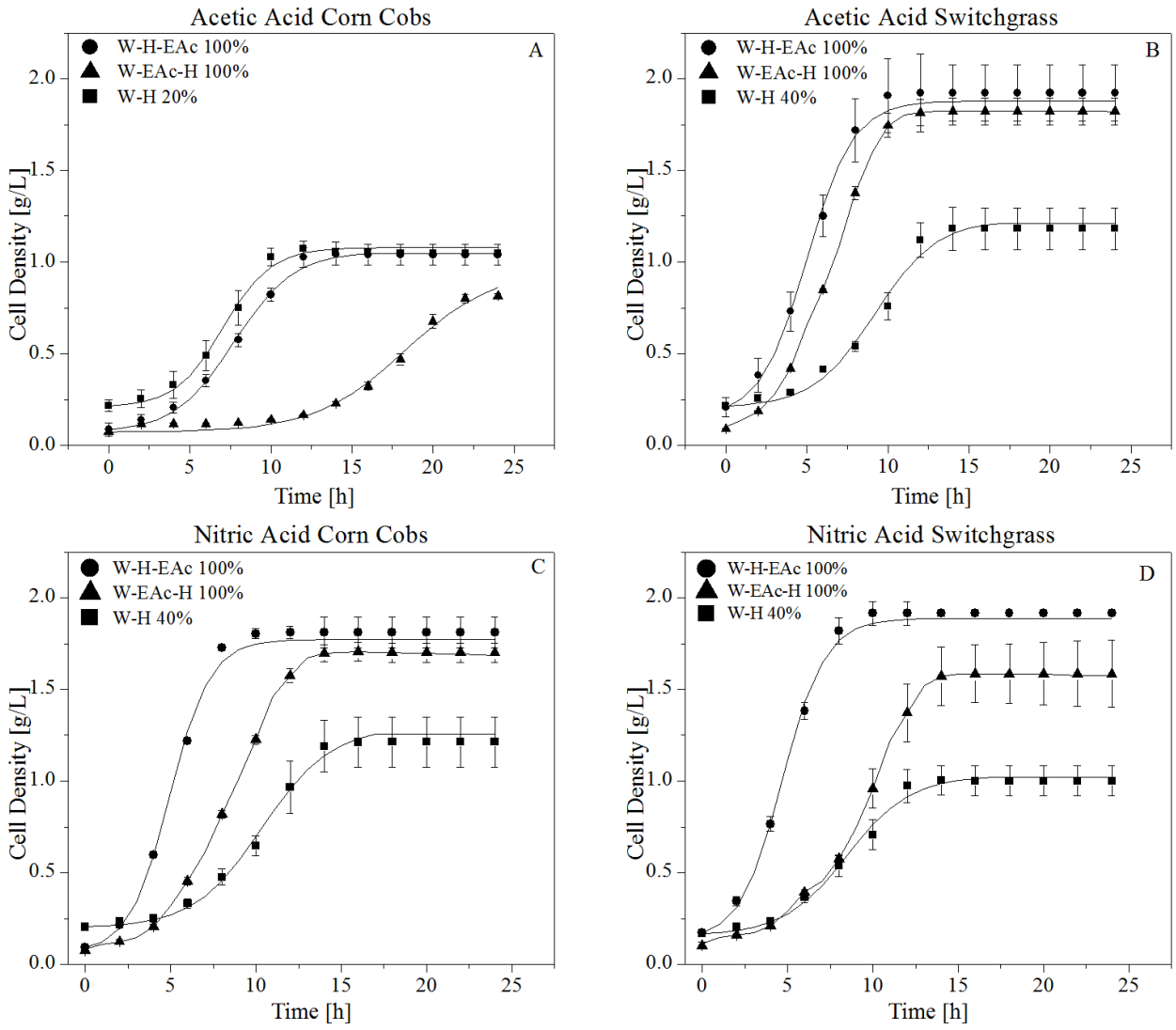

Figure 5.4 Growth profiles corresponding to the highest pyrolytic sugar fractions (highest IV/G values) where growth was achieved for each of the extracts tested. Initial sugar concentration was 25 g/L for all the blends tested. The percentages in the legends represent the fraction of pyrolytic sugar at the beginning of the fermentation.

5.4.4 Kinetic evaluation

Measured growth data was fitted to the Barnayi model using via least squares regression (MATLAB, Mathworks Inc). The model consists of three parameters; μ_{max} (maximum growth rate), λ (adaptation time) and N_{max} (maximum biomass density). The respective best fits are depicted by solid lines for the selected data shown in Figure 5.4. It can been seen that the Baranyi model adequately describes

the data, hence the numerical values of the model parameters can be used to quantify the effect of unremoved inhibitors in the pyrolytic sugar as previously described (Wood et al., 2014).

Figure *5.5* shows the estimated model parameters as well as the measure ethanol yields for the four different biomass samples. The data is shown as a function of the IV/G value of each micro-fermentations, which varied based on the biomass sources as well as the level of upgrading. Additionally, the different blends of each pyrolytic sugar result in further variation of the IV/G value. The distribution of inhibitory compounds in the pyrolytic extracts differs from pyrolytic sugar, and the IV/G value is only an approximation of the total amount of impurities. It can clearly be seen that the model parameters are correlated with the IV/G value.

A lower maximum specific growth rate is a common response of microorganisms subjected to environmental stress. The data is more spread for the estimated lag time and the maximum cell concentration. The lag time quantifies the time microorganisms need to adjust to a changed environment, in this case the presence of an inhibitor cocktail (the pre-cultures were grown on neat glucose). Interestingly, the response with respect to this parameter is more affected by the composition of the cocktails than the maximum specific growth rate. Particularly sugars that have only being upgraded via water extraction and hydrolysis (black symbols) appear to exhibit longer lag phases than samples subjected to solvent extraction (blue and green symbols) with the same IV/G value. Similarly, the maximum cell concentration achieved during fermentation was decreased most in samples subjected to water extraction only. The general decreasing of the final cell concentrations with increasing IV/G values appear to be a logical consequence of inhibition, however the total amount of ethanol produced is not correlated the same way, and does not appear to be effected by the presence of otherwise inhibiting compounds.

The data clearly shows that complex inhibitory cocktails affect microbial growth kinetics in a multitude of ways, with some aspects of the yeast's growth be more sensitive to the composition of the inhibitory mix (lag time and maximum cell concentration) than others such as the maximum specific growth rate. A simply estimate of the inhibitory potential of a pyrolytic sugar can be made based on the proposed parameter IV/G value, particularly for the maximum specific growth rate. The maximum specific growth rate is arguably the most important parameter, as the overall ethanol yield

was not affected over the observed range (for datasets where sigmoidal growth pattern were observed).

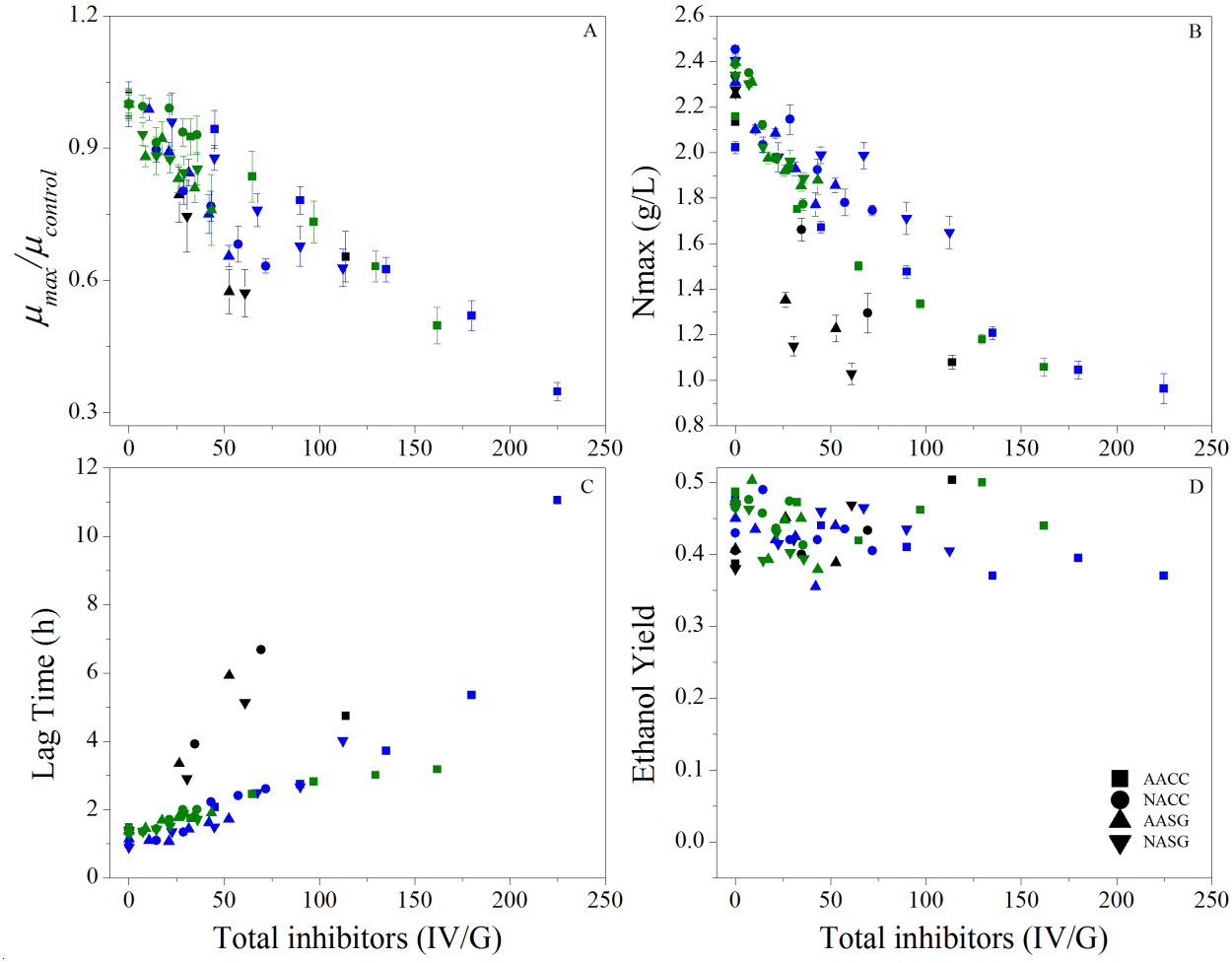

Figure 5.5 Calculated model parameters for fermentation experiments with varying fractions of unremoved inhibitors compounds resulting from the pyrolytic oils, A-C. D Corresponds to the ethanol yields from each of the fermentation experiments. The colors represent a specific detoxification route data shown in black stands for hydrolysis as the only detoxification step (W-H), blue represents the route with a solvent extraction before the hydrolysis (W-EAc-H) while green are the experiments where the solvent extraction came after the hydrolysis. x-Axis shows the relative amount of inhibitory compounds (IV/G) per µL in the total volume of the micro fermentations. AACC stands for acetic acid corn cobs extracts, ANCC nitric acid corn cobs extracts, AASG for acetic acid switch grass and NASG for nitric acid switch grass.

The lag phase can likely be addressed through acclimation of the inoculum, while final yeast concentration is not a typical parameter that would be optimized for in ethanol fermentations. The observed results are in agreement with a previously reported data on pine wood pyrolysate (Luque et al., 2014), as is the fact that the ethanol yield was not affected by the inhibitors, which has been shown before for furans and phenols (Klinke et al., 2004).

The maximum specific growth rate appears to decrease linearly with an increase of the IV/G value, irrespectively of the history of the sample. Linear regression analysis was conducted based on all available data points for the maximum growth rate, *Figure 5.5*A, leading to Equation 5.2:

$$\mu_{max} = 0.9627 - 0.0028 \times IV/G \quad R^2 = 0.85 \tag{5.2}$$

Utilizing eq. 5.2, a comparison between the calculated growth rate with the kinetic fit values, showed a good agreement, Figure 5.6, highlighting the correlation between increased IG/V values and the kinetic parameter. Differing from the results described in Chapter 4, figure 4.1, where the model proposed by Wood and collaborators was used (Wood et al., 2014) R^2 value 0.77 an IV/G value based model is capable of predicting in a more suitable way the synergistic effects of different compounds found in the pyrolytic oil upgraded fractions, R^2 value of 0.85. The better fit could be explained due to a more robust measurement (IV/G) which takes into account the overall fraction rather than six compounds. The applicability of the IV/G value beyond a single type of biomass and a single pre-treatment and upgrading is highly relevant when screening for possible biomass sources and possibly gives this parameter a general meaning beyond this specific study.

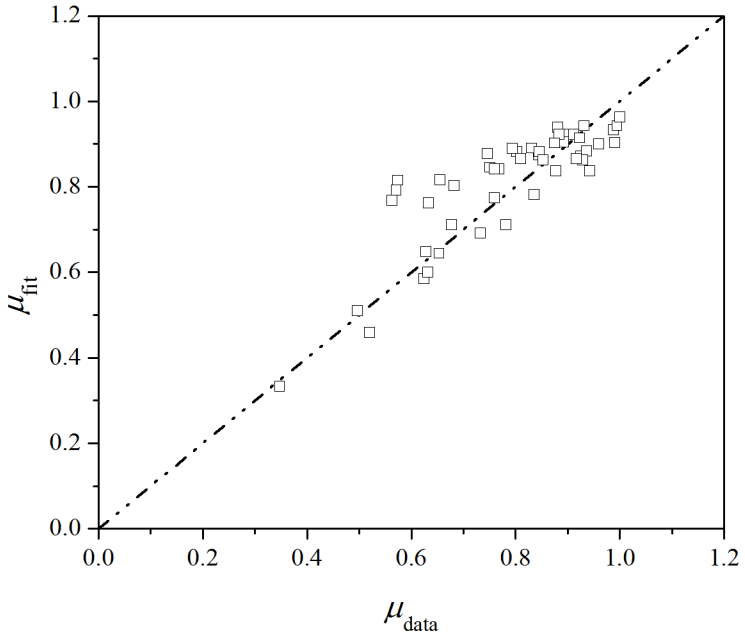

Figure 5.6 Correlation plot of observed experimental growth rate data compared to fitted growth rate

5.4.5 Ethanol Production

The reported ethanol yield was solely based on glucose consumption. Possible ethanol production from other sugars was not considered even though they can be present after pyrolysis and hydrolysis (Lian et al., 2010). The maximum yield achieved was 0.49, corresponding to a 96% of the theoretical maximum. These results agree with previously studies performed on pyrolyzates pinewood (Luque et al., 2014). Samples for ethanol analysis were taken 2 hours after reaching a stationary phase, securing a depletion of glucose and avoiding any possible ethanol loss due to evaporation. Ethanol production was achieved at the highest concentrations of total inhibitors still allowing for cell growth, Figure *5.5*D.

Another important measure of fermentability is the ethanol productivity (rate) (Klinke et al., 2004). The ethanol productivity was defined as the amount of ethanol produced by the cells at the moment they reached stationary phase (relative change in OD_{600nm}<0.025 OD/h) Figure *5.7*, shows the effect on ethanol productivity. EAc extraction after the hydrolysis is responsible for the increases seen in 3 of the 4 biomass extracts used. AACC ethanol productivity increased from 0.16 to 0.5 g/L/h, NACC from 0.63 to 0.88 g/L/h and NASG 0.62 to 0.8 g/L/h, each corresponding to 300, 40 and 30%

increases respectively. These increases in productivity are connected to the total amount of inhibitors, which is reduced if EAc extraction is conducted after the hydrolysis (Figure 5.2). The estimated productivities are largely useful as relative values within this study and cannot be directly compared with typically high values reported in the literature (Klinke et al., 2003), due to the scale and setup of the experimental system (non-optimized seed culture, etc.). Most previous studies only investigated the effects of single inhibitory compounds on ethanol productivity, such as ferulic acid, 4-hydroxycinnamic acid (Larsson et al., 2000) syringic acid (Ando et al., 1986; Klinke et al., 2003; Lee et al., 1999) among others. In this study the hydrolysate is considered as a whole inhibitory unit accounting for overall synergistic effects between the produced compounds.

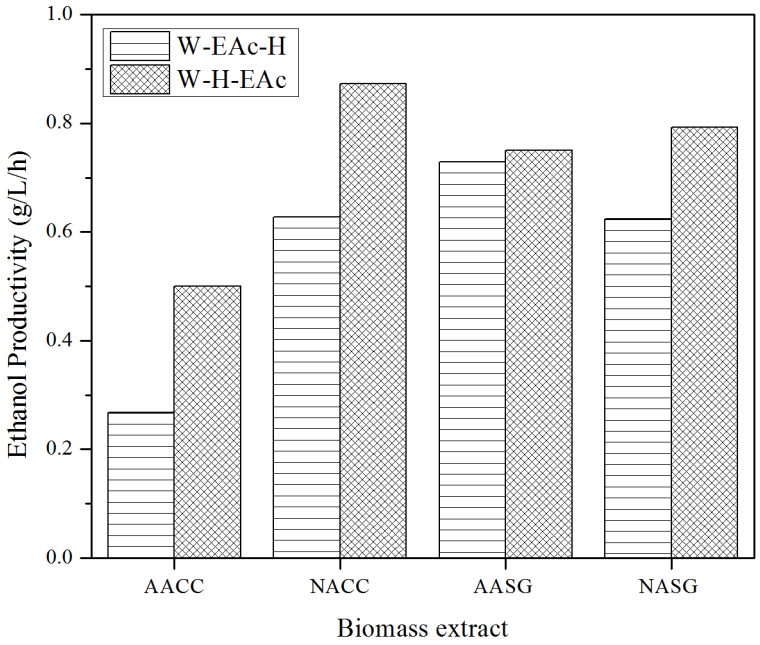

Figure 5.7 Ethanol productivity for fermentation samples with the highest concentration of total inhibitors (blends with 100% of pyrolytic derived sugar.

The total amount of ethanol produced per 100 g biomass was between 3.2 and 6.2 g for corn cobs, between 5.4 and 5.7 g for switchgrass (Table 5.4), corresponding to 14.6 % - 27.8 % and 25.7% - 27% of the theoretical yield (assuming the full conversion of all glucan to ethanol). These values are lower than what has been reported for pinewood (8.2 g ethanol, 41.2 % of the theoretical yield). The difference between the ethanol yields is likely a result of the type of biomass Even though pinewood has a lower cellulose content than corn cobs and switchgrass, 35 wt % vs 38.8 wt% and 37.0 wt% respectively, carbon was loss in the pyrolysis process, the levoglucosan yield after pyrolysis was

higher in pinewood 0.51, as discussed in Chapter 3, contrasted with 0.23 in corn cobs and 0.3 in switchgrass, *Table 5.2*. The strong difference could be due to the ion content as herbaceous biomasses (e.g corn cobs and switch grass) can contained ten times more the amount of alkali and alkaline earth metals than softwood biomasses (pinewood) which translates into a lower levoglucosan yields (Kuzhiyil et al., 2012). Despite the fact of observing lower ethanol values than the ones reported well-established process of lignocellulosic ethanol production (between 54% and 85% based on available hexoses (Eklund and Zacchi, 1995; McMillan et al., 1999)), the entailed process looks at the production of lignocellulosic ethanol as one of many streams generated in a thermochemical biorefinery concept, where valuable products like bio-char and bio-gas are generated in the pyrolysis steps, and where streams branching from the upgrading step, phenols, aldehydes and furans can be used as platform chemicals (Westerhof et al., 2011) or as added value products (Lian et al., 2010).

Table 5.4 Ethanol mass balances based on 100 g of starting biomass material. *Pinewood value was previously reported in Table 3.3 on Chapter 3, and is included for comparison purposes.

		Demineralization type			
		Acetic Acid		Nitric Acid	
Biomass	**Detoxification route**	**Ethanol (g)**	**Ethanol % from theoretical**	**Ethanol (g)**	**Ethanol % from theoretical**
Corn Cobs	1	3.2	14.6	5.9	26.8
	2	3.6	16.5	6.2	27.8
Switch Grass	1	5.7	27.0	5.6	26.8
	2	5.5	26.4	5.4	25.7
Pine wood*	1	8.2	41.3	-	-

This study shows that fermentable substrates for ethanol fermentation can be produced from agro industrial waste biomass, e.g corn cob and switch grass, via fast pyrolysis. Optimization of each steps was beyond the scope of this study but leaves room for further studies in order to increase the feasibility of the process.

5.5 Conclusions

This study demonstrated that switch grass and corncobs showed to be suitable lignocellulosic feedstocks for ethanol production via fast pyrolysis. Biomass demineralization enhanced levoglucosan production and decreased the inhibitors concentration in the resulting pyrolytic oils. The normalized inhibitor value (IV/G) showed to be an efficient tool for quantifying the relative presence of the inhibitors thus rapidly assessing the potential for a pyrolytic oil to be a source of fermentable sugars. A simple extraction reduced the inhibitor fraction enhancing ethanol productivity (0.88g/L/h) while maintaining high ethanol yields (96% of theoretical). Despite the high ethanol yield, it corresponds only to a 28% of the theoretical yield based on the total cellulose available.

5.6 References

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Chapter 6

6 Lipid accumulation from pinewood pyrolysates by *Rhodosporidium diobovatum* and *Chlorella vulgaris* for biodiesel production

Luis Luque, Valerie Orr, Sean Chen, Roel Westerhoff, Stijn Oudenhoven, Guus van Rossum, Sascha Kersten, Franco Berruti and Lars Rehmann.

The information in this chapter has been slightly changed to fulfill formatting requirements. This chapter will be submitted to Bioresource Technology.

The sections included in this chapter describe the application of the devised pyrolysis based biorefinery approach for the production of biodiesel. This study focused on producing a less toxic fraction from pinewood pyrolyzates utilizing the upgrading strategy that procured the least toxic fraction describe on Chapter 5. Two oil producing strains were selected for this study. *Rhodosporidium diobovatum*, has the ability to grow from different carbon sources, including waste glycerol (Munch et al., 2015). In addition, it was reported that the strain was capable of producing lipids in the presence of inhibitory compounds known to be present in pyrolytic oils, hydroxymethylfurfural and vanillin (I. Sitepu et al., 2014). The second strain was *Chlorella vulgaris*, a microalgae capable of accumulating lipids when grown on different waste water streams (Chi et al., 2011; Sacristán de Alva et al., 2013) in addition to lignocellulosic hydrolysates (Li et al., 2011; Miazek et al., 2014). The cleaner pyrolytic glucose solution was utilized to provide the carbon source in two kinds of media used in this study, nitrogen rich and nitrogen limited media. It is known that lipid production in oleaginous yeast and algae can be modified by different culture conditions (Aguirre and Bassi, 2013; Sestric et al., 2014) including inhibitory compounds derived from lignocellulosic biomass pretreatment (Lian et al., 2013; Miazek et al., 2014). Reports on the stress exerted by some of these compounds have demonstrated that if within a certain range, ethanol production in *S. cerevisiae* is enhanced. This study also allowed us to scale up the process to a 24 well plate with 10 times the volume used in previous chapters.

The objectives accomplished with this investigation include an improved upgrading process which rendered a cleaner sugar solution for media preparation. In addition, these experiments demonstrated that stress exerted by the inhibition compounds does not have the same effect than limiting the

nitrogen concentration in the media, as lipid production in pyrolytic media did not exceed 25 % w/w compared to 50 % w/w when nitrogen was limited in *R. diobovatum*. As growth in nitrogen limited media showed to be less as the pyrolytic sugar fraction increased (increased growth inhibition), the possible existing synergy is responsible for the low lipid production in blends > 40%. In contrast, lipid accumulation in *Chlorella vulgaris* was not affected, yet growth in blends >40% was not observed.

6.1 Abstract

This study evaluated the suitability of pinewood pyrolysates as a carbon source for lipid production and cultivation of the oleaginous yeast *Rhodosporidium diobovatum* and the microalgae *Chlorella vulgaris*. Thermal decomposition of pinewood and fractional condensation were used to obtain an oil rich in levoglucosan which was upgraded to glucose by acid hydrolysis. Blending of pyrolytic sugars with pure glucose in both nitrogen rich and nitrogen limited conditions was studied for *R. diobovatum,* and under nitrogen limited conditions for *C. vulgaris*. Glucose consumption rate decreased with increasing proportions of pyrolytic sugars increasing cultivation time. While *R. diobovatum* was capable of growth in 100% (v/v) pyrolytic sugars, *C. vulgaris* growth declined rapidly in blends greater than 20% (v/v) until no growth was detected in blends > 40%. Finally the effects of pyrolysis sugars on lipid composition was evaluated and biodiesel fuel properties were estimated based on the lipid profiles.

6.2 Introduction

Biodiesel is an established alternative to petroleum-derived diesel. It is renewable and matches the fuel properties of diesel (Atabani et al., 2012; I. R. Sitepu et al., 2014). Furthermore, it is easily adopted by consumers as it can be used directly in an unmodified diesel engine or blended with petroleum diesel (Ahmad et al., 2011; Çetinkaya et al., 2005). Currently, biodiesel is largely derived from vegetable oils, wastes fats, and animal fats (Atabani et al., 2012; I. R. Sitepu et al., 2014). Increased demand for edible vegetable oils as a feedstock for the growing biodiesel industry world-wide has resulted in a dramatic increase in the cost of these oils (Atabani et al., 2012). The agricultural production of some of the feedstocks, particularly palm oil are highly controversial (Balat, 2011; Deng et al., 2011). Not only does the increase in demand affect the price available foodstuffs, it also

affect the economics of biodiesel production as feedstock can contribute as much as 75% of the overall cost of a biodiesel process (Atabani et al., 2012). Consequently, the focus of many researchers has shifted towards the development of second generation biodiesel processes which use waste or non-edible oils as their feedstock (Chuah et al., 2015; Karmee and Chadha, 2005; Silitonga et al., 2011).

An alternative to vegetable oil for biodiesel production is the use of oleaginous microorganisms (microorganisms which can amass more than 20% lipid by dry weight) (Meng et al., 2009; I. R. Sitepu et al., 2014). Lipids extracted from single celled organisms can be trans-esterified into fatty acid methyl esters (FAMEs) using the same processes developed for vegetable oils (Chatzifragkou et al., 2011). While microalgae have received the most attention as oleaginous organisms, many others including species of yeast, bacteria, and fungi are also capable of high lipid productivities (Aguirre and Bassi, 2013; Meng et al., 2009; Sitepu et al., 2012). Several oleaginous yeast species have emerged as promising strains for lipid production as they are capable of growing on a variety of different carbon sources including cellobiose, waste from industrial processes such as cheese whey (Chi et al., 2011), olive mill waste water (Gonçalves et al., 2009) or municipal waste water (Chi et al., 2011); and can be grown to higher biomass densities than microalgae in a similar amount of time (Munch et al., 2015; Sestric et al., 2014). While lipid production has been discovered in many yeast species, the amount of lipid is highly dependent on the media composition; requiring either carbon or nitrogen limitation, making it difficult for direct comparison. One of the highest lipid titers reported was achieved using *Lipomyces kockii* grown on nitrogen limit media containing 100g/L glucose. The cells accumulated almost 77.8% wt oil and produced approximately 17 g dry cell weight (DCW)/L (Oguri et al., 2012; Sitepu et al., 2012). However, both lipid accumulation and cell density dropped to 31% wt and 7.1 g DWC/L when the glucose concentration was decreased to 30g/L (Oguri et al., 2012).

Overall lipid yield per glucose molecule has been relatively low (0.12-0.17 g/g compared to the theoretical yield of 0.30 g triacylglyceride/g glucose for *R. toruloides* (Bommareddy et al., 2015; Lian et al., 2010) from heterotrophically grown SOCs, therefore it is necessary to offset the low lipid accumulation (Oguri et al., 2012) by utilizing inexpensive lignocellulosic feedstocks. A similar trend is occurring in the bio-ethanol industry (Cherubini, 2010; Parajuli et al., 2015). However,

lignocellulosic feedstocks need an often energy intensive pretreatment to produce a fermentable substrate. Several studies have been dedicated to the pretreatment of lignocellulosic biomass in order to produce fermentable substrates for the ethanol industry (Cherubini, 2010; Ibrahim et al., 2011; Kazi et al., 2010; Menon and Rao, 2012; Taherzadeh and Karimi, 2008; Zhan et al., 2013). One unconventional pretreatment for the production of sugar from lignocellulose is fast pyrolysis (Jarboe et al., 2011; Lian et al., 2012; Liang, 2013; Luque et al., 2014). Pyrolysis is the thermal decomposition of biomass at temperature typically around 500ºC in the absence of oxygen. Three main products can be obtained (Bridgwater, 1999; Czernik and Bridgwater, 2004; Greenhalf et al., 2012), bio char which can be used as a soil amendment (Purakayastha et al., 2016; Wang et al., 2015), biogas which can be used as combustible process fuel or in the production of liquid fuels via Fischer-Tropsch synthesis (Brown, 2015) and thirdly, condensable gases often referred to as pyrolysis oil which has been successfully hydrotreated to produce transportation fuels (de Miguel Mercader et al., 2010; Isahak et al., 2012) and which has been previous upgraded to produce lipids (Lian et al., 2013, 2012) and ethanol (Luque et al., 2014). A carbohydrate-rich liquid stream can be recovered through factional condensation of the condensable gases, which can be used as the feedstock for several bioconversion processes generating additional value for the pyrolysis process (Lian et al., 2013, 2012; Luque et al., 2014)

Pyrolysis oils can reportedly contain over one hundred other compounds such as acids, aldehydes, phenols, ketones, alcohols, and furans many of which can act as growth inhibitors during the subsequent fermentation if they are not removed (Garcia-perez et al., 2008; Luque et al., 2014; Palmqvist and Hahn-Hägerdal, 2000; Wood et al., 2014). It was previously shown that an upgrading process as shown in Figure 6.1 can decrease inhibitory compounds which can translated into full conversion of pyrolytic sugars to ethanol (Luque et al., 2014).

In this study, a sugar rich fraction low in inhibitory compounds was obtained through a two-step upgrading process. Glucose obtained from this process was used as the main carbon source for lipid accumulation in *R. diobovatum* and *C. vulgaris*. The effect of increasing amounts of pyrolysis inhibitors on growth, lipid accumulation, and lipid composition in these species was evaluated by substituting increasing proportions of pure glucose for pyrolytic sugars. Furthermore, the effects of

pyrolytic sugars on the estimated fuel properties (Cetane number and cold flow plugging point) were calculated from the lipid composition using a previously described model.

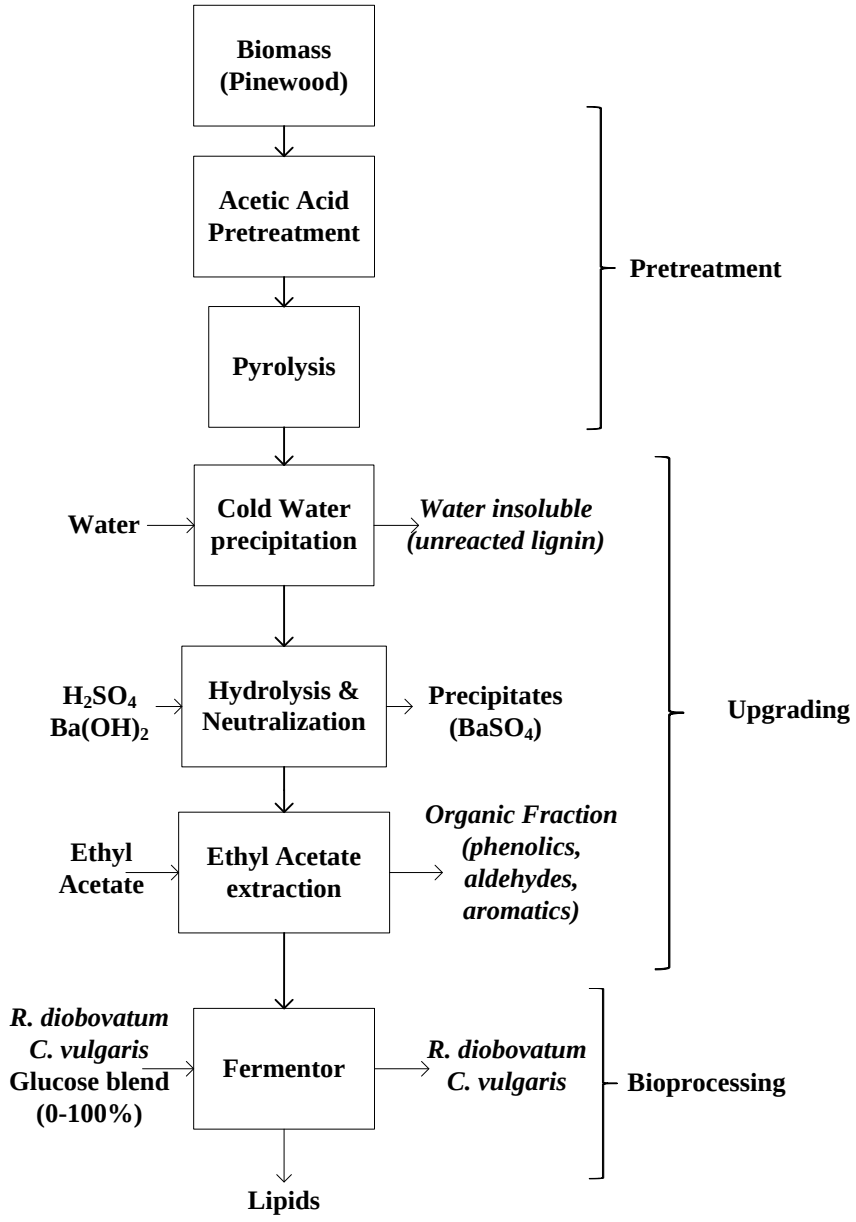

Figure 6.1 Biorefinery approach for lipid production with *Rhodosporidium diobovatum*.

6.3 Materials and Methods

6.3.1 Biomass demineralization

Leaching of pinewood biomass was achieved by mixing the biomass with an acetic acid solution 10% V/V in a jacketed stirred batch reactor, to a final biomass to leaching agent ratio of 1:10 for 2 h at 90°C. (Oudenhoven et al., 2013). Once the leaching was completed, leachate was removed through a perforated plate in the bottom of the reactor. Biomass was then rinsed with Milli Q (Milli-Q Integral 5, EMD Millipore, USA) water in batches of 1L for 5 minutes at room temperature. The final rinse batch was determined by monitoring the conductivity (Pinnacle Series, Nova Analytics, USA) of the output leachate stream until the value approached zero and remained constant. Excess water was removed via evaporation at 105°C for 24 h in a convection oven (Thermo Scientific, USA). Final moisture was determined using a moisture analyzer (ADAM, USA)

6.3.2 Pyrolysis oil production

Demineralized pinewood was pyrolyzed in a fluidized bed reactor at 480°C with a vapor residence time <2s. Two condenser in series were used to fractionate the pyrolytic vapors according to their boiling point. In the first condenser operated at 80°C, an oil rich in anhydrous sugars and lignin-derived aromatics was obtained. The second condenser operated at 20°C, procured an oil fraction rich in acetic acid and water. The pressure in both condensers was held constant at 1.1±0.01 bar (Westerhof et al., 2011). The oil collected in the first condenser was used as the source of sugars for the lipid production experiments.

6.3.3 Upgrading of pyrolysis sugars

Pyrolysis oil rich in anhydrosugars was subjected to cold water precipitation as reported elsewhere (Garcia-perez et al., 2008). Water temperature was kept constant, 4°C, in an ice bath while oil was added dropwise, under heavy stirring (900 rpm) to 1:5 oil to cold water ratio. Insoluble lignin was recovered via vacuum filtration with a previously weighed and dried 0.45 µm cellulose nitrate membrane (Whatman®, UK) and measure gravimetrically (Luque et al., 2014). Resulting filtrate was

centrifuged at 4°C and 3500 rpm for 20 mins (Sorval ST40R, Thermo Scientific, USA). The sugar containing supernatant was recovered from the pellet, collected in falcon tubes and store at -20°C until further use.

After the precipitation, levoglucosan present in the filtrate was acid hydrolyzed to glucose. Briefly, aliquots of filtrate were added to pressure vials. H_2SO_4 was then added to a final concentration of 0.5M. Hydrolysis was performed at 120°C for 20 mins in an autoclave (Bennett et al., 2009; Luque et al., 2014). The hydrolysate pH was adjusted to 6.5 solid $Ba(OH)_2$ (Alfa Aesar, USA). Formed salts and solids were precipitated by centrifugation at a temperature of 4°C, 3500 rpm for 20 mins (Sorval ST40R, Thermo Scientific). Supernatant was recovered and transferred to a sterile 50 mL falcon tube by filtering it with a 0.2 µm cellulose syringe filter (VWR, Canada).

To remove possible growth inhibitors (e.g phenolics, furans, and aldehydes), hydrolyzate was further extracted with ethyl acetate (EAc). A solution containing filtrate and EA in a 1:2 wt% ratio was prepared in a 250 mL Erlenmeyer and sealed with a rubber stopper to prevent loss of EAc to the environment due to evaporation. Solution was homogenized for 12 h at 150 rpm and 25°C. After mixing, the sample was transferred to a 125 mL separation funnel and left standing for 24 h to secure proper phase separation. (Luque et al., 2014). The organic layer (Top) was collected and any remaining EAc in the rich sugar aqueous layer (bottom) was removed by evaporation at 150 rpm and 40°C. Evaporated ethyl acetate was measured gravimetrically and confirmed by samples taken every hour and measured by high pressure liquid chromatography until EA reached a constant value. Sugar concentration was kept constant by adding water.

Sugar content of the pyrolysis oil, water extract, and ethyl acetate residue were quantified by a previously described protocol using high pressure liquid chromatography using an Agilent LC 1200 infinite system equipped with a Hi-Plex H 300 × 7mm column and a RI detector (Agilent, USA) (Luque et al., 2014).

6.3.4 Inhibitory value quantification

Before hydrolysis and after EAc extraction, Figure 6.1, spectra between 190 and 340 nm were measured for 80 minutes by high pressure liquid chromatography fitted with a Hiplex H column at

60°C, and equipped with a diode array detector (Agilent 1260 series, USA). Raw data was exported and processed in MATLAB (MathWorks Inc, USA). Removal performance was measure as changes in the volume under the surface after the detoxification process was performed. The inhibitor value normalized for glucose (or levoglucosan) concentration (IV/G) was previously defined according to equation (6.1):

$$IV/G = \int_{t=10min}^{t=80min} \int_{\lambda=190nm}^{\lambda=340nm} S_{DAD}\, dt\, d\lambda / C_G \quad (6.1)$$

6.3.5 Strain and culture conditions

6.3.5.1 *Rhodosporidium diobovatum*

*R. diobovatum (*08-225) obtained from Munch et al. (2015) were maintained using a slight modification of their reported method. Briefly, *R. diobovatum* was streaked onto YPD agar plates (10 g/L yeast extract, 20 g/L peptone, 30 g/L glucose, 15 g/L agar) and grown at 30 °C for 2 days and stored at 4°C until further use. A seed culture grown overnight at 30 °C from a single colony was used to inoculate either YPD media (N^+; 30 g/L glucose or pyrolysis derived glucose, 20 g/L peptone, and 10 g/L yeast extract) or nitrogen limited media (N^-; 30 g/L glucose or pyrolysis derived glucose, 3 g/L yeast extract, 8 g/L KH_2PO_4, 0.5 g/L MgSO4-7H_2O) at 10% (v/v). Media was adjusted to a pH of 5.5 and sterilized by filtration.

6.3.5.2 *Chlorella vulgaris* cultivation conditions

Chlorella vulgaris strain UTEX 2714 was purchased from The Culture Collection of Algae at the University of Texas Austin. The culture was maintained as an actively growing cultures in liquid media using aseptic technique in 150 mL Tris-acetate-phosphate (TAP) media (20 mM Tris base, 1.58 mM K_2HPO_4, 2.4 mM KH_2PO_4, 7.0 mM NH_4Cl, 0.83 mM $MgSO_4$, 0.34 mM $CaCl_2$, 1 mL/L glacial acetic acid, and 1 mL/L of Hutner's trace elements solution) at pH 7.0 in 500 mL shaker flasks. Cultures were grown and maintained at 25 °C at 150 rpm under cyclic illumination consisting of 16 h on: 8 h off (100 μmol m^{-2} s^{-1}). After 48 h, an exponentially growing seed culture was inoculated into Tris-nitrate-glucose (TNG) media (20 g/L glucose or pyrolysis derived glucose, 20

mM Tris base, 1.58 mM K_2HPO_4, 2.4 mM KH_2PO_4, mM, 2.4 mM $NaNO_3$, 0.83 mM $MgSO_4$, 0.34 mM $CaCl_2$, 1 mL/L Hutner's trace elements solution) at 10% (v/v) and grown under the same conditions as above. The TNG media was adjusted to a pH of 6.8 and filter sterilized.

6.3.5.3 Lipid production using pyrolytic sugars

Media prepared with pure glucose was blended with the same media prepared with pyrolysis sugars to the indicated amounts (% v/v). Final glucose concentration was kept at 30 g/L for the yeast and 20 g/L for the microalgae. Yeast cultures were grown in a 24 well plate in triplicate in a final volume of 2 mL. Plates were sealed with a sterile PCR film (VWR, Canada) and a hole was puncture to allow aeriation using a sterile 18 gauge needle (BD, USA). Plates were incubated at 30 °C and 74 rpm using a Tecan 200m Microtiter plate reader (Tecan, Austria) until glucose levels were depleted as detected by HPLC. Growth was monitored by optical density, OD_{600nm}, at 15 mins intervals. Algae cultures were grown in triplicate in a final volume of 5.5 mL in a shaker incubator at 25 °C and150 rpm with cyclic illumination of 16 h on: 8 h off (100 μmol m^{-2} s^{-1}). Small samples (20 μL) were taken every 24 h to monitor growth by optical density at 680 nm and glucose concentration was detected by HPLC.

6.4 Lipid Analysis

6.4.1 Harvesting and freeze drying

Once glucose was depleted or growth had ceased, approximately 1.5 mL of each culture was transferred to preweighed 2.0 mL centrifuge tubes. Cultures were harvested by centrifugation at 10000 × g in a Spectrafuge 24D microcentrifuge (Labnet International, USA) for 5 min. Cell pellets were resuspended with deionised water and washed three times *via* centrifugation and resuspension to remove residual salts and sugars. The washed cells were frozen at -20 °C for a minimum of 8 h and lyophilized using a 4.5 L freeze-drier (Labconco) for 24 h or until the weight no longer fluctuated.

6.4.2 Analytical Determination of Total FAME Content

The FAME content by weight was determined for triplicate cultures using a slightly modified standard FAME laboratory analytical procedure (NREL/TP-5100-60958). Briefly, approximately 10 mg of dried cells were mixed with 20 μL of the recovery standard pentadecanoic acid methyl ester (C15:0Me at 10 mg/mL), 300 μL of 0.6M HCl, and 200 μL of a trichloromethane methanol mixture (2:1 v/v) and subsequently incubated for 1h at 85°C in a water bath with stirring on a magnetic hot plate at 1000 rpm. After cooling, 1 mL of hexane was added to each sample and mixed at ambient temperature at 1000 rpm. Samples were centrifuged and 450 μL of the clear top hexane phase was spiked with 50 μL of the internal standard undecanoic acid methyl ester (C11:0Me) to have a final concentration of 100 μg/mL. FAME was separated and analysed using an FID equipped Agilent 7890 Series GC and an Agilent DB-Wax capillary column (30m, 0.25 mmm, 0.25 μm). Helium was used as the carrier gas at a constant pressure of 119 kPa, and the FID was operated at 280°C. Samples were injected in split mode with a 1:10 split ratio and eluted using the following oven ramp: 50°C, 1 min, 10°C min-1 to 200°C, 3°C min-1 220°C, 10 min. Individual FAMEs were quantified using analytical standard mixture (Supelco 37, Sigma Aldrich) and the internal standard. Unidentified FAME were quantified by applying the RF factor of the closest known peak. Total FAME content by weight was calculated according to the NREL LAP by adjusting the cumulative FAME mass using the recovery standard C15:0Me and dividing the total by the weight of cells used in the assay.

6.4.3 Estimation of Biodiesel properties based on FAME content

The analytical data obtained from the GC analysis of the FAMEs provided the molecular structures required to estimate important properties of biodiesel produced from the accumulated oils in the yeast. Saponification value (SV), iodine value (IO), cetane number (CN) and the cold filter plugging point (CFPP) were calculated using the equations reported elsewhere (Nascimento et al., 2013a). Briefly, SA and IO were calculated using eqs (6.2) and (6.3), where *M* is the molecular mass of the FAME, *P* the percentage of each individual FAME component by weight and *D* is the number of double bonds present in the FAME:

$$SA = \sum(560 \times P)/M \qquad (6.2)$$

$$IO = \sum 254 \times DP)/M \quad (6.3)$$

Once the values were determined, the cetane number was estimated using eq (6.4)

$$CN = 46.3 + \left(\frac{5458}{SA}\right) - (0.255 \times IO) \quad (6.4)$$

The CFPP was calculated by estimating the long-chain saturation factor (LCS) using eq (6.5)

$$LCS = (0.1 \times C16{:}\,0) + (0.5 \times C18{:}\,0) + (1 \times C20{:}\,0) + (1.5 \times C22{:}\,0) + (2 \times C24{:}\,0) \quad (6.5)$$

and substituting the LCS value on equation (6.6):

$$CFPP = (3.1417 \times LCS) - 16.477 \quad (6.6)$$

6.5 Results and discussion

Pyrolytic sugars were produced through fast pyrolysis of demineralized pine wood followed by fractional condensation as reported elsewhere (Luque et al., 2014). A fraction rich in levoglucosan was then upgraded to glucose through two extraction steps hydrolysis to glucose.

6.5.1 Upgrading of pyrolytic sugars

The upgrading process is shown in Figure 6.1. The first upgrading step consisted of removing insoluble lignin and hydrophobic compounds from pyrolytic oil via cold water extraction (W) (Garcia-perez et al., 2008). Detoxification continued with an acid hydrolysis of the levoglucosan to obtained glucose followed by neutralization and finalized by a solvent extraction with ethyl acetate (W-H-EAc). Ethyl acetate was chosen to be the last step of the detoxification train to remove inhibitory compounds carried over from the water extraction and any that could have been generated as a result of the strong acid hydrolysis as previously described (Bennett et al., 2009; Lian et al., 2012; Palmqvist et al., 1999). The final glucose concentration achieved was 35g/L corresponding to

a molar yield of 0.89 (mol glucose / mol levoglucosan) and a yield based of 0.48 on the total initial cellulose available in the biomass. The values are in agreement with previously reported data (Luque et al., 2014).

Pyrolysis byproducts from lignin decomposition and further sugar degradation such as aldehydes, furans, phenols and organic acids are known to interfere with later bioconversion processes (Luque et al., 2014; Palmqvist, 2000; Palmqvist et al., 1999; Wood et al., 2014; Zaldivar and Ingram, 1999; Zaldivar et al., 2000). Prior characterization studies have identified up to 100 compounds accounting for almost 40% of carbon based on biomass intake, making the task of fully identifying these compounds challenging and time consuming (Butler et al., 2013; Garcia-Perez et al., 2007). The reduction of the inhibitory compounds after upgrading was quantified using an HPLC equipped with a diode array detector (DAD). Many of the potential inhibitory compounds which may be present in pyrolytic sugars contain chromophores which can be detected using UV spectroscopy. The relative abundance of these inhibitors was quantified by numerical integration and normalized to the sugar concentration as the inhibitor value (IV/G) as described in section 6.3.4. A reduction in the number and height of peaks shown in Figure 6.2 demonstrate the removal of absorbing compounds.

In Figure 6.3, the IV/G values for pinewood hydrolysates used in this study were compared to values obtained for corn cobs and switchgrass hydrolysates upgraded using a similar process (Chapter 5). The increased abundance of inhibitors in pinewood hydrolysates may be attributable to the increased proportion of lignin in pine wood; 35 % wt, compared to between 20 to 30 %wt for switchgrass and corn cobs (Mosier et al., 2005).

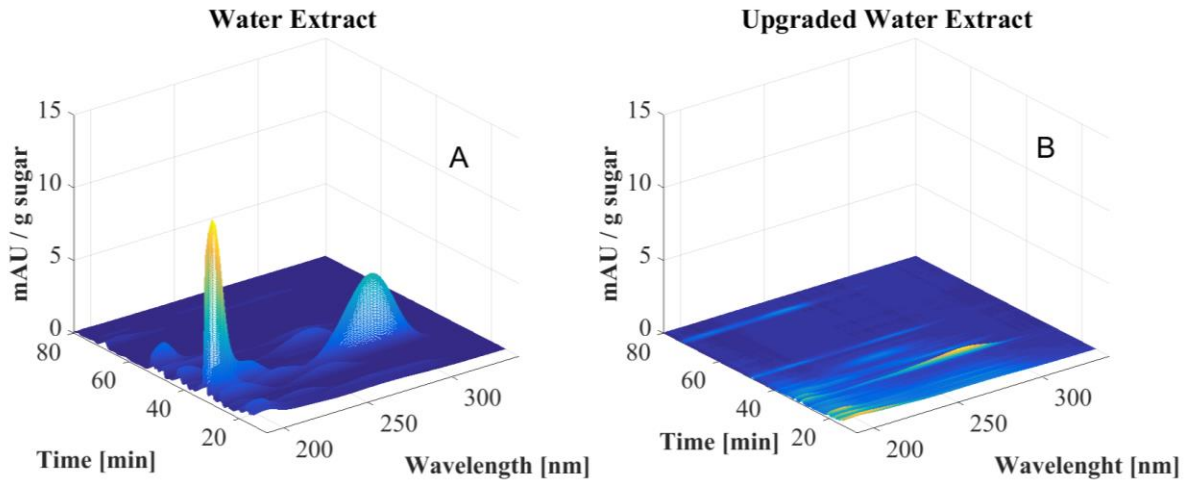

Figure 6.2 Surface change as a function of upgrading train. The figure shows how the spectra of the sample changes as the detoxification process is performed.

The IV/G values show that the detoxification approach evaluated in this study reduces substantially the inhibitor presence in the extracts agreeing with previous reports (Luque et al., 2014) where acid hydrolysis and ethyl acetate showed to reduce the toxicity of the extract.

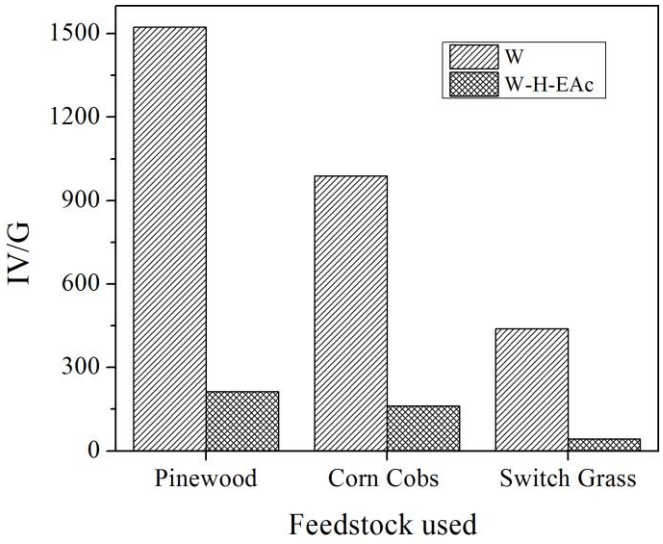

Figure 6.3 IV/G values for different pyrolytic extracts determined with the methodology described above. Corn cobs and switchgrass values were taken from Chapter 4 and included for comparison. W corresponds to values for the water extracts of each water extract. Whereas W-H-EAc indicated

the detoxification route utilized and explained in the materials and methods section, water extract followed by hydrolysis and neutralization and finalized by ethyl acetate extraction.

6.5.2 Bioconversion of pyrolytic sugars

R. diobovatum was cultivated in either YPD media (Figure 6.4A) or nitrogen limited media (Figure 6.4B) with pyrolytic sugars as the sole carbon source. Nitrogen limited media was evaluated in order to stimulate lipid production in this species (Munch et al., 2015). Glucose and xylose consumption is shown on Table 6.1. Media containing pure glucose was blended with media containing pyrolytic sugars in order to evaluate whether the inhibitors present in the pyrolysate affected growth, lipid accumulation, and lipid composition. Growth was marginally affected by increasing blends of pyrolytic sugars in YPD media, however final cell titers as measured by volumetric end point dry cell weight (Table 6.2) indicates that pyrolytic sugars supported significantly higher biomass densities (p <0.05) in all blends compared to the control. This is likely due to the increasing proportion of xylose present in the pyrolysis sugar media which was not added to the control media. Although *R. diobovatum* has not been extensively studied it has been shown that it can grow on a variety of carbon sources (I. Sitepu et al., 2014). Indeed, complete xylose consumption, as reported in Table 6.1, occurred in blends up to 60% which also corresponds to the highest observed biomass density.

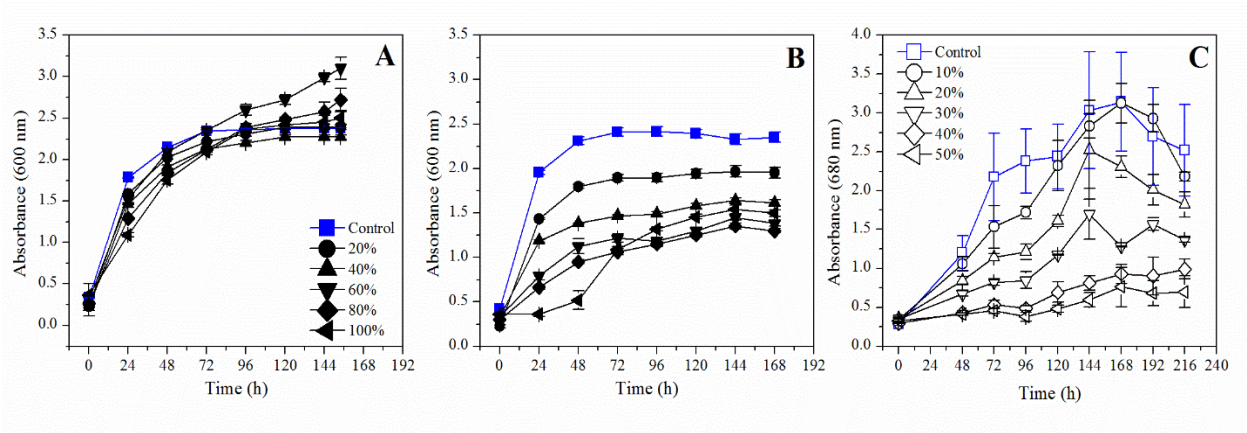

Figure 6.4 Growth profiles of *R. diobovatum* (A & B) and *C. vulgaris* (C) using and increasing proportion of pyrolytic sugars (0-100%). **A.** Nitrogen rich YPD media **B.** Nitrogen limited media **C.** TNG media

Conversely, growth was significantly impaired with increasing blends of pyrolytic sugars under nitrogen limited conditions, *Figure 6.4*B (p <0.05). However, final biomass density was identical in YPD and nitrogen limited controls (11.66 ± 0.05 g/L and 11.59 ± 0.20 g/L, respectively) indicating that on its own nitrogen limitation was not sufficient to affect cell titers. Previous results have shown *R. glutinis*, *R. toruloides*, and *C. curvatus* are capable of growth on 100% pyrolytic sugars (Lian et al., 2013, 2010).

C. vulgaris was cultivated in TNG media with glucose or pyrolytic sugars under mixotrophic conditions (*Figure 6.4*C). Growth was only sustained with up to 30% (v/v) pyrolytic sugar blend after which growth was severely affected. It should be noted that absorbance at 680 nm is highly dependent on the chlorophyll content of the cells which may change in response to the coloration of the media and evaporation of the media over the lengthy trials was greater than 20% (v/v) (Orr and Rehmann, 2014). However, dry cell weights collected at the end point indicate the same trend (Table 6.2). In comparison, growth of the microalgae *Chlamydomonas reinhardtii* on acetic acid rich pyrolytic bio-oil was only possible in blends up to 5.5% (w/w) (Liang et al., 2013; Zhao et al., 2015). The algae could be adapted to grow on up to 50% (w/w) blend of pyrolysis derived acetate, however adaptation took over 170 days and growth on pyrolytic acetate was still delayed compared to the control (Liang et al., 2013). Overall, growth rate and maximum cell density of the microalgae *Chlamydomonas reinhardtii* was found to be more highly correlated with the concentration of inhibitors; particularly phenolic inhibitors, when grown on acetic acid rich bio-oils (Zhao et al., 2015).

Decreasing the IV/G value of upgraded pyrolytic fractions showed to strongly correlate with improved growth kinetics on *S. cerevisiae* as discussed in the previous chapter. Even though IV/G value of upgraded pinewood fraction was higher, 213, than corn cobs, 161, and switch grass, 43.20, full utilization growth in in full pyrolytic sugars was observed on *R. diobovatum* Figure 6.4A. Moreover glucose and xylose utilization was observed, *Table 6.1*. However, the results observed in nitrogen rich media were not the same when nitrogen was limited, evidence that the IV/G value stills needs to be decreased if nitrogen limited media is to be used. Interestingly, overall lipid production by *R. diobovatum* was not affected when grown on nitrogen rich media, *Table 6.2*, as was the case in ethanol production from *S. cerevisiae* (Chapter 5), which suggests operating conditions below the IV/G threshold. Increasing IV/G values in nitrogen limited media for *R. diobovatum*, *Figure 6.4*B, and in *C. vulgaris* growth, *Figure 6.4*C, affected lipid production by inhibiting growth as less cells

results in less lipid collected, *Table 6.2*. This follows the same trend observed in ethanol production by *S. cerevisiae*, where high IV/G value of less detoxified pyrolytic fractions inhibited cell growth on blends >30.

6.5.3 Sugar assimilation

Glucose consumption was monitored to determine fermentation end points in order to avoid any lipid loss due to cell death or re-assimilation, Figure 6.5. Evidently, the presence of inhibitors delays the glucose consumption for both species. Sugar depletion was reached in all experiments when using rich nitrogen media and *R. diobovatum* after 152 hours. This was not the case for nitrogen limited media, where only the control and 20% blend was depleted at 120h. *C. vulgaris* did not deplete the glucose even in the control cultivation possibly indicating nitrogen limitation was too severe.

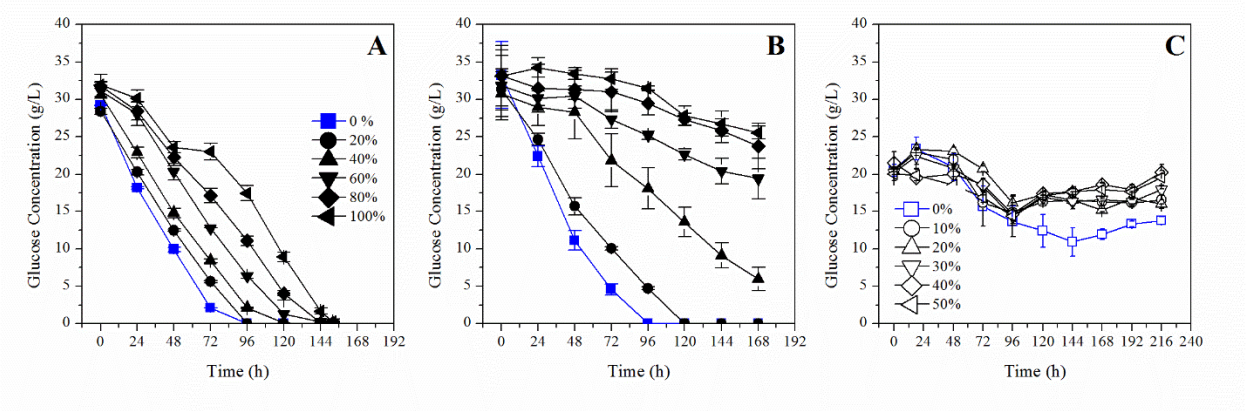

Figure 6.5 Glucose consumption profile in pyrolytic media at different fractions for *R. diobovatum* (A & B) and *C. vulgaris* (C) using and increasing proportion of pyrolytic sugars (0-100%). A. Nitrogen rich YPD media B. Nitrogen limited media C. TNG media.

The effect of pyrolysis sugars on initial glucose consumption rate (Ω) was calculated in the linear region with a linear regression (Matlab, MathWork Inc) and summarized in Table 6.1. As expected, glucose consumption rates for *R. diobovatum* in nitrogen rich media (YPD) were higher than nitrogen limited (N^-). The glucose consumption rate calculated for the 40% blend in nitrogen limited media is approximately half the value of the nitrogen rich YPD media. Glucose consumption in *C. vulgaris* showed a similar trend. Experiments were terminated before glucose was depleted if the glucose consumption rate was excessively small or in the case of *C. vulgaris*, glucose consumption ceased. Xylose consumption was only observed in the nitrogen rich media with *R. diobovatum*. Increases in

xylose concentration indicated in Table 6.1 are likely due to evaporation of liquid during the extensive cultivation times required for these organisms.

Table 6.1 Glucose and xylose consumption (Ω) by *R. diobovatum* (YPD, Nitrogen Limited) and *C. vulgaris* (TNG).

Media Blend	Glucose (g/L)		Ω_{Glc} (g/L/h)	Xylose (g/L)		Ω_{Xyl} (g/L/h)
	Initial	Final		Initial	Final	
***R. diobovatum* – YPD**						
Control	29.22 ± 0.05	n.d	0.37	--	--	--
20%	28.38 ± 0.38	n.d	0.32	4.08 ± 3.30	n.d	0.036
40%	30.58 ± 1.82	n.d	0.31	4.60 ± 0.57	n.d	0.043
60%	31.11 ± 0.76	n.d	0.30	7.01 ± 1.01	n.d	0.052
80%	31.73 ± 1.60	n.d	0.23	11.56 ± 1.24	1.34 ± 0.03	0.062
100%	31.91 ± 0.43	n.d	0.21	13.59 ± 0.86	3.18 ± 0.45	0.058
***R. diobovatum* – Nitrogen Limited**						
Control	33.25 ± 4.47	n.d	0.40	--	--	--
20%	31.34 ± 2.74	n.d	0.28	4.07 ± 0.56	n.d	0.03
40%	30.72 ± 2.99	5.94 ± 1.56	0.16	6.31 ± 0.42	3.18 ± 0.54	0.02
60%	31.91 ± 4.68	19.42 ± 2.74	0.08	7.01 ± 1.01	7.13 ± 0.80	0.00
80%	33.15 ± 4.06	23.76 ± 3.04	0.05	11.56 ± 1.25	8.90 ± 1.04	0.02
100%	33.12 ± 2.75	25.55 ± 0.86	0.05	13.60 ± 0.84	11.40 ± 0.5	0.02
***C. vulgaris* – TNG**						
Control	20.49 ± 0.18[a]	13.76 ± 0.49	0.12	--	--	--
10%	20.49 ± 0.18 [a]	16.59 ± 0.59	0.11	0.76 ± 0.09	1.12 ± 0.04	--
20%	20.49 ± 0.18 [a]	15.99 ± 0.59	0.06	1.16 ± 0.07	1.46 ± 0.13	--
30%	20.25 ± 2.75	17.89 ± 0.65	0.05	1.54 ± 0.11	2.05 ± 0.12	--
40%	21.51 ± 0.60	20.25 ± 0.21	n.a	2.07 ± 0.08	2.95 ± 0.24	--
50%	20.01 ± 0.88	19.62 ± 1.66	n.a	2.44 ± 0.17	3.13 ± 0.18	--

[a] Glucose concentration was calculated based on blend ratio of glucose detected in sterile media

n.a not applicable, n.d. not detected

Using an orthogonal design, varying degrees of glucose consumption inhibition were observed during the cultivation of *R. toruloides* in the presence of multiple inhibitors and synergistic effects were detected between acetic acid, furfural, and vanillin (Zhao et al., 2012). Furthermore, cultures of *R. diobovatum* grown in the presence 5-HMF, acetic acid, and furfural under nitrogen limited conditions experienced growth delay or complete inhibition (I. Sitepu et al., 2014). However, the concentrations of 5-HMF (0.04 g/L) and furfural (0.4g/L) in the 100% blend of pyrolysis sugars used in this study

were below the values previously tested. However, synergies among different inhibitors derived from biomass decomposition have been previously reported in *S. cerevisiae* and *R. toruloides* (Wood et al., 2014; Zhao et al., 2012). Furthermore, HMF and furfural which are known to directly inhibit alcohol, pyruvate and aldehyde dehydrogenases; enzymes involved in the catabolism of glucose by glycolysis (Banerjee et al., 1981) making them likely inhibitors of glucose consumption.

6.5.4 Effects of pyrolysis sugars on lipid accumulation

Lipid accumulation in yeasts and microalgae is significantly affected by cultivation conditions including pH, temperature, nutrient limitation, and trace metals (Beopoulos et al., 2011). Generally, lipids are accumulated when cell growth becomes limited while the carbon source is still in excess. Nitrogen limitation is most commonly used as it is simple to control and is one of the most effective means of limiting biomass growth, this is often referred to as having a high carbon to nitrogen ratio (Beopoulos et al., 2011). As expected, lipid accumulation by *R. diobovatum* in the nitrogen limited media, *Figure 6.6*A, was much greater than the nitrogen rich media in the control cultivations (56.1% (w/w) and 12.3% (w/w) respectively. Additional stress placed on the cells by increasing the amount of blended pyrolysis sugars increased the lipid production in the nitrogen rich media, however, in the nitrogen limited media, the addition of pyrolytic sugars had a negative effect on lipid accumulation. This corresponded to the low levels of glucose consumption in these cultures and lack of glucose depletion in blends > 20% (v/v). Lipid accumulation was not affected by increasing blends of pyrolytic sugars in *C. vulgaris* cultures, *Figure 6.6*B.

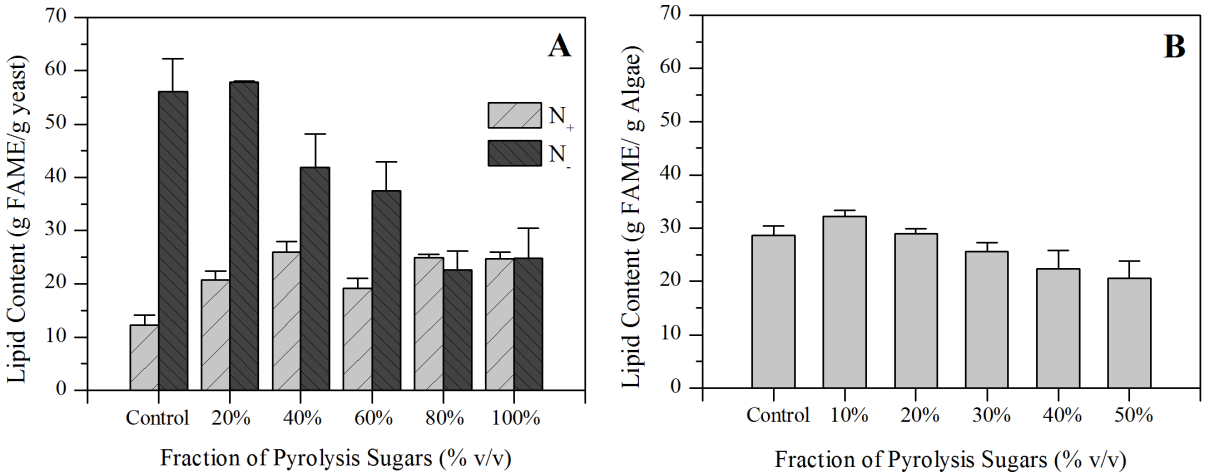

Figure 6.6 Lipid accumulation of **A.** *R. diobovatum* and **B.** *C. vulgaris* using and increasing proportion of pyrolytic sugars (0-100%).

Several important indicators of culture performance (Table 6.2) were calculated for each condition including lipid productivity (g lipid/L/h) and lipid conversion (g lipid/g glucose) (I. R. Sitepu et al., 2014). *R. diobovatum* cultures in nitrogen rich media have a higher lipid productivity at high blend ratio with pyrolytic sugars compared to nitrogen limited media. However, as these cultures consumed more glucose and produced less lipids they had a lower conversion ratio. These differences demonstrate the need for further optimization of growth of oleaginous yeasts on pyrolytic sugars as severe nitrogen limitation may be detrimental to lipid productivity when inhibitors are present. *C. vulgaris* had the highest lipid conversion of 0.25 g/g glucose in the 20% (v/v) blend however, this may be due to simultaneous carbon fixation as they were grown under mixotrophic conditions.

In previous reports, pyrolytic sugars upgraded through an extensive process (ethyl acetate extraction, acid hydrolysis, activated carbon detoxification and rotary evaporation) were converted to lipids using *R. toruloides*, *R. glutinis* and *C. curvatus* (Lian et al., 2013, 2010). The most promising species, *C. curvartus* accumulated up to 68% wt in lipids and produced over 16 g/L of biomass (~0.16 g lipid/g glucose) while *R. glutinis* produced 12 g/L of biomass and accumulated only 46% in lipids (~0.08 g lipid/g glucose) when cultivated on approximately 70 g/L glucose. *R. toruloides* and *R. glutinis* have also been shown to grow directly on levoglucosan, however lipid yields were significantly lower; 3.3 g/L biomass and 23.6%, than when using glucose (Lian et al., 2013). The effect of increasing pyrolytic sugar substitution on lipid accumulation has not previously been

studied. However, independently, furfural has been show to decrease lipid accumulation by up to 60% in *C. curvatus* at concentrations above 0.5 g/L while HMF concentrations up to 3 g/L had no effect (Yu et al., 2011a). Further study of the upgrading process indicated that growth and lipid accumulation was severely affected by the removal of activated carbon detoxification, rotary evaporation, or ethyl acetate extraction during pyrolytic sugar upgrading (Lian et al., 2013). However, a larger study of the effects of inhibitor interactions and inhibitor concentration for oleaginous yeast is need to provide more insight into the inhibition process.

Table 6.2 Culture performance in terms of biomass generation and lipid production by *R. diobovatum* (YPD, Nitrogen Limited) and *C. vulgaris* (TNG).

Media Blend	Dry Cell Weight (g/L)	Volumetric Lipid Production (g/L)	Lipid Productivity (mg/L/h)	Lipid Conversion (g lipid/g glucose)
***R. diobovatum* – YPD**				
Control	11.66 ± 0.05	1.43 ± 0.22	19.85 ± 3.06	0.05 ± 0.01
20%	12.15 ± 0.10	2.52 ± 0.21	35.00 ± 2.95	0.09 ± 0.01
40%	12.52 ± 0.21	3.24 ± 0.18	27.04 ± 1.54	0.11 ± 0.01
60%	19.21 ± 1.90	3.98 ± 0.27	27.66 ± 1.87	0.13 ± 0.01
80%	17.32 ± 0.39	3.98 ±0.47	26.16 ± 3.10	0.13 ± 0.01
100%	17.53 ± 0.42	4.08 ± 0.36	26.85 ± 2.38	0.13 ± 0.02
***R. diobovatum* – Nitrogen Limited**				
Control	11.59 ± 0.19	6.49 ± 0.77	67.65 ± 8.07	0.20 ± 0.02
20%	10.50 ± 0.08	6.05 ± 0.28	50.39 ± 2.33	0.19 ± 0.01
40%	9.43 ± 0.31	3.99 ± 1.23	22.07 ± 5.31	0.15 ± 0.04
60%	6.48 ± 0.20	2.45 ± 0.59	14.58 ± 3.54	0.20 ± 0.05
80%	4.83 ± 0.20	1.08 ± 0.22	6.42 ± 1.31	0.11 ± 0.02
100%	4.93 ± 0.36	1.23 ± 0.36	7.31 ± 2.16	0.16 ± 0.05
C. vulgaris – TNG				
Control	5.99 ± 0.51	1.71 ± 0.13	14.27 ± 1.38	0.18 ± 0.02
10%	4.94 ± 0.08	1.59 ± 0.05	13.27 ± 0.61	0.25 ± 0.01
20%	4.23 ± 0.31	1.22 ± 0.06	10.17 ± 0.60	0.17 ± 0.01
30%	3.00 ± 0.20	1.77 ± 0.09	6.44 ± 0.91	0.17 ± 0.02
40%	1.63 ± 0.20	0.36 ± 0.03	3.01 ± 0.34	n.d
50%	0.69 ± 0.36	0.15 ± 0.08	n.d	n.d

n.d due to lack of glucose consumption or growth

6.5.5 Effect on biodiesel composition and properties

Compositional analysis of lipids based on the FAME profile obtained for each culture condition showed differences in the distributions depending on both the nitrogen content in the media and the fraction of pyrolytic glucose. Fatty acid profiles are available in Tables 6.3-6.5. Cetane number (CN) and cold flow plugging point (CFPP) were calculated from the lipid profiles of each culture in triplicate using the model proposed by Ramos et al. (2009) and are summarized in *Table 6.6*. While the relative composition of lipids isolated from *R. diobovatum* significantly differed between the nitrogen rich and limited media similar changes due to the presence of pyrolytic sugars were detected. The differences between 0% and 100% blends were significant (two tailed heteroscedastic student T test) for palmitic acid (C16:0), stearic acid (18:0), oleic acid (C18:1), linoleic acids (C18:2), and lignoceric acid (C24:0) with $p \leq 0.01$, however many individual step sizes did not significantly alter the fatty acid profile.

In both nitrogen rich (Table 6.3) and limited media (Table 6.4), addition of pyrolytic sugars increased the proportion of C18:0, C18:1, and C24:0, but decreased the content of C18:2 with increasing amounts of pyrolytic sugars. However, C16:0 content decreased in nitrogen limited media and increased in nitrogen rich media. Pyrolytic sugar content had no significant effect on the lipid composition of *C. vulgaris* (Table 6.5) however, *C. vulgaris* produces shorter and more highly unsaturated fatty acids than *R. diobovatum*.

A modest decrease of palmitic acid content from 26.5 to 24.4% was previously reported when *C. curvatus* was cultured in the presence of 1 g/L of the inhibitor 5-HMF (Yu et al., 2011b). Furfural had a greater effect at the same concentration and decreased C16:0 content to 21.4%. Both 5-HMF and furfural are present in the pyrolytic sugars used in this at concentrations of 0.04 g/L and 0.4 g/L respectively. While this is significantly lower than those used by Yu et al. (2011), the presences of other phenolics or synergistic effects may account for the significant effects seen in this study.
Fatty acid composition is known to significantly affect the fuel properties of the synthesized biodiesel (Knothe, 2005; Meher et al., 2006).

Table 6.3 Average relative lipid composition (%) of major fatty acids in triplicate cultures of *R. diobovatum* cultured in YPD media. Fatty acids representing less than 1% of the total are omitted.

	Pyrolytic sugar fraction % (v/v)					
Fatty Acid	**0%**	**20%**	**40%**	**60%**	**80%**	**100%**
C16:0	9.5 ± 0.0	11.4 ± 0.1	11.9 ± 0.1	13.5 ± 0.8	14.0 ± 0.7	14.3 ± 1.3
C16:1	3.1 ±0.1	3.1±0.1	2.3 ± 0.0	1.8 ± 0.1	1.7 ± 0.0	1.6 ± 0.1
C17:1	0.8 ± 0.0	1.1 ± 0.0	0.9 ± 0.0	1.4 ± 0.1	1.2 ± 0.1	1.0 0.1
C18:0	0.6 ± 0.0	1.6 ± 0.1	2.8 ± 0.0	3.7 ± 0.2	4.1± 0.3	4.6 ± 0.6
C18:1	61.3 ± 0.4	71.6 ± 0.4	70.5 ± 0.1	68.8 ± 1.0	68.1 ± 1.5	66.6 ± 2.0
C18:2	15.3 ± 0.3	2.5 ± 0.1	3.3 ± 0.1	1.7 ± 0.1	2.0 ± 0.7	2.7 ± 0.6
C24:0	2.4 ± 0.1	3.0 ± 0.1	3.2 ± 0.1	3.6 ± 0.1	3.4 ± 0.1	3.5 ± 0.2
NI	4.4 ± 0.0	3.4 ± 0.1	2.3 ± 0.0	2.1 ± 0.2	2.1 ± 0.1	1.9 ± 0.2
Total	92.9%	94.3%	94.7%	94.5%	94.6%	94.4%

NI, non-identified

Table 6.4 Average relative lipid composition (%) of major fatty acids in triplicate cultures of *R. diobovatum* cultured in Nitrogen limited media. Fatty acids representing less than 1% of the total are omitted.

	Pyrolytic sugar fraction % (v/v)					
Fatty Acid	**0%**	**20%**	**40%**	**60%**	**80%**	**100%**
C14:0	1.1 ± 0.1	1.2 ± 0.0	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	0.8 ± 0.0
C16:0	24.0 ± 0.9	23.6 ± 0.4	19.8 ± 0.8	17.0 ± 0.7	15.5 ± 0.5	15.7 ± 0.4
C16:1	1.9 ± 0.1	1.9 ± 0.0	1.2 ± 0.0	1.0 ± 0.1	0.9 ± 0.0	0.9 ± 0.0
C17:0	0.2 ± 0.0	0.5 ± 0.0	1.0 ± 0.1	1.6 ± 0.2	2.2 ± 0.1	3.2 ± 0.1
C17:1	0.3 ± 0.0	0.9 ± 0.0	1.6 ± 0.1	2.3 ± 0.3	3.1 ± 0.2	3.7 ± 0.3
C18:0	2.9 ± 0.2	3.3 ± 0.3	4.1 ± 0.3	5.2 ± 0.6	6.3 ± 1.0	5.9 ± 0.6
C18:1	43.7 ± 0.8	46.4 ± 0.4	47.0 ± 0.7	44.7 ± 1.4	45.3 ± 1.2	49.2 ± 1.5
C18:2	19.2 ± 0.7	16.3 ± 0.2	16.7 ± 0.6	16.2 ± 1.1	17.3 ± 0.4	12.4 ± 0.9
C18:3	1.9 ± 0.1	1.4 ± 0.1	1.2 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	0.8 ± 0.1
C24:0	2.1 ± 0.1	1.7 ± 0.5	2.4 ± 0.1	2.8 ± 0.1	3.4 ± 0.2	2.9 ± 0.4
NI	1.3 ± 0.3	1.1 ± 0.2	1.8 ± 0.3	4.8 ± 4.0	2.1 ± 0.2	2.4 ± 0.1
Total	97.3%	97.3%	96.1%	93.0%	96.2%	95.6%

NI non-identified

Table 6.5 Average relative lipid composition (%) of major fatty acids in triplicate cultures of *C. vulgaris* cultured in TNG media. Fatty acids representing less than 1% of the total are omitted.

	Pyrolytic sugar fraction % (v/v)					
Fatty Acid	**0%**	**10%**	**20%**	**30%**	**40%**	**50%**
C12:0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.7 ± 0.2	1.6 ± 0.6
C14:0	0.4 ± 0.1	0.4 ± 01	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.4	1.1 ± 0.3
C16:0	19.2 ± 0.8	19.4 ± 0.6	18.9 ± 0.3	20.0 ± 0.4	22.8 ± 4.9	26.0 ± 1.4
C16:1	1.8 ± 0.3	1.4 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	2.7 ± 2.5	0.4 ± 0.8
C17:0	0.3 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.8 ± 0.1	1.1 ± 1.0	2.5 ± 0.5
C17:1	4.6 ± 0.5	4.3 ± 0.2	4.4 ± 0.1	4.8 ± 0.5	4.6 ± 0.3	4.7 ± 0.8
C18:0	2.8 ± 0.3	3.0 ± 0.9	2.5 ± 0.1	3.0 ± 0.3	3.9 ± 2.3	10.3 ± 32
C18:1	33.3 ± 4.0	38.4 ± 0.6	37.0 ± 0.2	31.5 ± 3.4	27.1 ± 2.3	17.9 ± 3.7
C18:2	20.8 ± 2.2	19.8 ± 0.7	22.3 ± 0.3	22.7 ± 1.6	20.7 ± 1.3	15.4 ± 1.4
C18:3	8.0 ± 0.7	7.8 ± 0.3	7.9 ± 0.2	9.2 ± 1.0	9.8 ± 2.0	12.5 ± 23
NI	7.7 ± 3.4	4.1 ± 0.3	3.7 ± 0.2	4.9 ± 0.6	5.6 ± 3.0	7.5 ± 2.2
Total	99.2%	99.4%	99.3%	99.2%	99.4%	100.0%

NI non-identified

Cetane number, Table 6.6, is one of several performance indicators regulated for biodiesel (Knothe, 2005) and is an indicator of ignition quality. Higher cetane numbers are correlated with lower emissions (Meher et al., 2006). Cetane number increased with increasing pyrolytic sugar substitution in the nitrogen rich media while it had the opposite effect in nitrogen limited media. In pyrolytic sugars blends > 60 % (v/v) in nitrogen limited media, the estimated cetane value decreased below both the American Society for Testing and Materials (ASTM) and European Committee for Standardization (EN) values of 47 and 51 respectively (Knothe, 2005).

The cold filter plugging point (CFPP) is commonly used as an indicator of biodiesel performance at low temperatures and indicates the need for additives for winterization to prevent the precipitation of FAME in cold climates (Knothe, 2005). CFPP is primarily dependent on the proportion of unsaturated fatty acids and longer chain length fatty acids of which *R. diobovatum* produced a larger proportion. Thus, the yeast produced significantly higher CFPP values than the microalgae lipid profiles indicating that microalgae derived biodiesel is more versatile. Many species of microalgae produce much higher proportions of shorter chain length and unsaturated fatty acids resulting in biodiesel with CFPP often below 0°C(Nascimento et al., 2013b). CFPP increased with increasing proportion of pyrolytic sugars for *R. diobovatum* to almost 20°C, indicating the need to further study the effects of

pyrolytic inhibitors on fatty acid composition. Cetane number and CFPP were calculated for fatty acid profiles reported in the literature for oleaginous yeast and algae grown on pyrolytic sugars or acetate. *C. curvatus* produced estimate fuel properties within the same range as *R. diobovatum* (CN 62.8 and CFPP 20.8°C) as did *R. glutinis* (CN 58.4 and CFPP 9.1°C) (Lian et al., 2010). *C. reinhardtii* grown on acetate rich pyrolytic oils had much poorer estimate biodiesel properties (CN 45.3 and CFPP 7.8°) however, this species is more typically used to study photosynthesis mechanisms than lipid production.

Table 6.6 Estimated Cetane number (CN) and Cold Flow Plugging Point (CFPP) obtained from oils accumulated by *R. diobovatum* in nitrogen rich (YPD) and limited media (NL) and *C. vulgaris* in TNG media.

Media Blend	CN	CFPP (°C)
***R. diobovatum* – YPD**		
Control	49.5 ± 0.1	2.5 ± 0.3
20%	52.1 ± 0.1	8.4 ± 0.5
40%	52.9 ± 0.1	12.0 ± 0.3
60%	52.2 ± 0.1	14.9 ± 1.7
80%	53.7 ± 0.1	17.4 ± 0.4
100%	53.7 ± 0.7	18.7 ± 0.5
***R. diobovatum* – Nitrogen Limited**		
Control	53.0 ± 0.2	9.1 ± 0.6
20%	51.2 ± 0.1	7.1 ± 2.5
40%	48.1 ± 0.3	12.3 ± 0.9
60%	46.0 ± 1.5	14.6 ± 1.6
80%	42.5 ± 0.7	19.6 ± 0.7
100%	41.8 ± 0.9	16.0 ± 2.2
***C. vulgaris* – TNG**		
Control	51.3 ± 1.0	-5.6 ± 0.64
10%	51.1 ± 0.6	-5.3 ± 1.3
20%	50.2 ± 0.2	-6.0 ± 0.1
30%	50.2 ± 0.8	-4.8 ± 0.3
40%	51.2 ± 0.5	-2.3 ± 2.1
50%	53.9 ± 0.8	7.9 ± 4.4

6.6 Conclusion

R. diobovatum was found to tolerate up to 100% upgraded pyrolytic sugars under nitrogen rich conditions, however, significant inhibition of growth and lipid accumulation was observed under nitrogen limited growth conditions. *C. vulgaris* was grown on pyrolytic glucose and demonstrated the highest lipid conversion ratio however it also demonstrated the highest sensitivity to pyrolysates. Inhibitors carried over from pyrolysis were found to affect glucose consumption rates, lipid accumulation and composition. Blending of pyrolysis sugars demonstrated that these effects were likely due to increasing concentrations of inhibitors and indicates a need for a more in depth study of the effects of inhibitory compounds on both oleaginous yeasts and microalgae.

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Chapter 7

7 Conclusions and Recommendations

This chapter outlines the main conclusion of the study. In addition, some recommendations for future work are proposed.

7.1 Conclusions

The experimental results outlined in this work demonstrate how fast pyrolysis can be utilized as a pretreatment method for lignocellulosic biomass to produce fermentable substrates that can be converted to ethanol or lipids.

Adaptation of fermentation experiments to 96- and 24- microtiter plates allowed to monitor in a high throughput manner the fermentation and increased the parallelization of the experiments. Data collected utilizing this process permitted to evaluate the effects of increasing pyrolytic fractions on growth kinetics of *S. cerevisiae* fermentations. In addition, the methodology proved beneficial as various conditions could be monitored utilizing smaller sample quantities of raw material (pyrolytic oil). This method was utilizing throughout the entire research Chapters 3 – 6 for every fermentation.

Removing alkaline and alkaline earth metals, from the biomass prior to fermentation showed to increase 5-fold levoglucosan (LG) yield on pyrolytic oils. A higher LG fraction was translated into higher glucose thus higher ethanol titers. In addition, less inhibitor compounds derived from LG degradation reactions were generated thus increasing the fermentability of the upgraded sugars.
Acid demineralization was responsible for incrementing the pyrolytic sugar fraction present in the fermentable substrates from 3 to 20%.

Inhibition properties of extracted pyrolytic oils were mitigated by design an upgrading train which was able to decrease the pyrolytic oil recalcitrance by removing enough inhibitors to enable complete conversion of pyrolysis derived sugars. Acid hydrolysis and neutralization showed to reduce the overall carbon fraction attributed to inhibitor compounds while producing higher amounts of glucose. This contribution to the upgrading process translated into higher ethanol yields at increased pyrolytic

sugars. Coupling of solvent extraction with acid hydrolysis and neutralization, was necessary to achieve ethanol production from 100% of pyrolytic sugars.

The inhibition observed from by different upgraded pyrolytic fractions could not be explained with the six selected compounds. Poor correlation to a previously developed model prompted to develop a more robust technique that incorporated the entire fraction. Accounting for the presence of the overall inhibitors showed a better correlation to the inhibition observed.

Inhibition properties of the pyrolytic oils were quantified by correlating three microbial growth parameters (maximum cell density, N_{max}, maximum growth rate μ_{max}, and lag time, λ) with increasing pyrolytic fraction. The model utilized in Chapter 3 (Baranyi model) proved to be very accurate determining the values of the different parameters. As expected, increasing the pyrolytic sugar fraction in the fermentation media decreased both, N_{max} and $\mu_{max,}$ since higher pyrolytic fraction would add more inhibitory compounds in the media. Increasing relative presence of inhibitors exerted some stress on the fermentative microorganisms, and as a result, higher adaptation times were observed. The increase in lag time also meant that reaching N_{max} took prolonged periods of time. Even though growth was inhibited, final ethanol yield remained constant, 0.49, suggesting that ethanol production is not affected by these harsh conditions. Nevertheless, since the time to reach the same ethanol yield was longer, the ethanol productivity is reduced.

Pyrolytic oils from corn cobs and switch grass were upgraded and converted to ethanol under the conditions found on chapter 3. This explored the robustness of the biorefinery concept explained on chapter 3. A reconfiguration of the upgrading steps improved the fermentability of complete fractions of pyrolytic derived sugars, evidenced by shorter fermentation times and increased ethanol productivity.

The model developed to measure the relative presence of inhibitors demonstrated to strongly correlate with the obtained results and proofed to be rapid and efficient way to predict certain trends. Therefore, this model could be potentially used in the evaluation of different pyrolytic oils for their fermentability potential.

The pyrolysis based biorefiney approach showed to be successful in producing lipids from pinewood pyrolysates, showing the capabilities of the process and the potential to extent further. Xylose fractions were also used showing a benefit which *S. cerevisiae* did not exhibit, thus adding potential to the proposed process.

7.2 Recommendations

From the experience gathered during the completion of this research thesis some suggestions are stated for future work in pyrolytic sugars.

- In chapter 3 the realization of the upgrading process did not involve an optimization process. Rather it was devised by analyzing different information gathered in the literature and integrated as a proof of concept. Therefore, optimization studies in the solvent extraction could yield less solvent usage, and less time removing unwanted solvent.

- Further studies to optimize water:oil and water extract:ethyl acetate ratios can augment biofuel fuel production by increasing the sugar extraction efficiency thus increasing the overall sugar fraction. These studies should also take into account the effects on fermentation as changing quantities could extract more inhibitory compounds.

- Even though ethyl acetate was selected because of its low toxicity to yeast, low affinity for levoglucosan and glucose and high affinity for lignin derived aromatics, different solvents could also be explored to evaluate options tailored to extracting most toxic compounds.

- As mentioned on chapter 3, the advantage of fast pyrolysis over other pretreatments is the vast number of compounds that it yields giving it increased flexibility. However, research on the utilization of these compounds is still in its infancy. The overall process could beneficiate from future studies on the streams leaving the process, especially on the insoluble lignin and ethyl acetate fractions.

- Even though it was beyond the scope of this research identification of the main inhibitory compounds prior to upgrading steps would help tailor the process since solvents and process

conditions can be optimize aiming for their removal. However, this task can prove to be rather time consuming since only approximately 40% of the compounds found in these oils have been identified.

- Chapter 5 briefly showed the robustness of this process by utilizing different biomasses, but to expand the process range studies with new biomasses could prove useful.

- Chapter 6 briefly discussed how *R. diobovatum* is tolerant to pyrolysate fractions in rich nitrogen media but that tolerance drops once nitrogen concentration is decreased. Studies on the Pyrolytic Carbon:Nitrogen ratio can help elucidate the optimal values to enhance the productivity from pyrolytic sugar fractions with a higher glucose consumption rate.

- During the research butanol production was briefly studied. Future studies on butanol production would increase the process product portfolio.

- Techno-economic studies would be valuable to assess which combination of fuels could render the most value from the process.

- In Chapter 6, it was found that pyrolytic xylose was also assimilated by *R.diobovtum* a capability which *S. cerevisiae* did not show. Studies on the utilization of the pyrolysis fractions by both microorganisms simultaneously would be beneficial to increase the process value.

- As stated, these experiments were performed in microtiter plates for several reasons one being the limiting quantities of available pyrolytic oil. However, fermentations in shake flasks would prove beneficial to evaluate the effects of scaling up.

Appendix

A.1 *S. cerevisiae* dry cell weight calibration

A calibration curve for *S. cerevisiae* was determined in YPG (yeast extract, peptone and glucose) media with final concentrations of 1% wt, 2% wt and 3%w for yeast extract peptone and glucose respectively. *S. cerevisiae* was grown in a shake flask for 24 hours in a shake flask at 30°C and 150 rpm in an environmental shaker. Samples were taken and diluted with fresh YPG media to a final volume of 10 mL in different proportions % v/v. Dry cell weight was determined gravimetrically by vacuum filtration of pre dried and weighed 0.2 µm membranes. The optical density (OD_{600nm}) of 200 µL aliquots for each cell dilution was determined on a 96-microtiter plate (Corning, USA) in a Tecan M200 microtiter plate reader (Tecan, Austria).

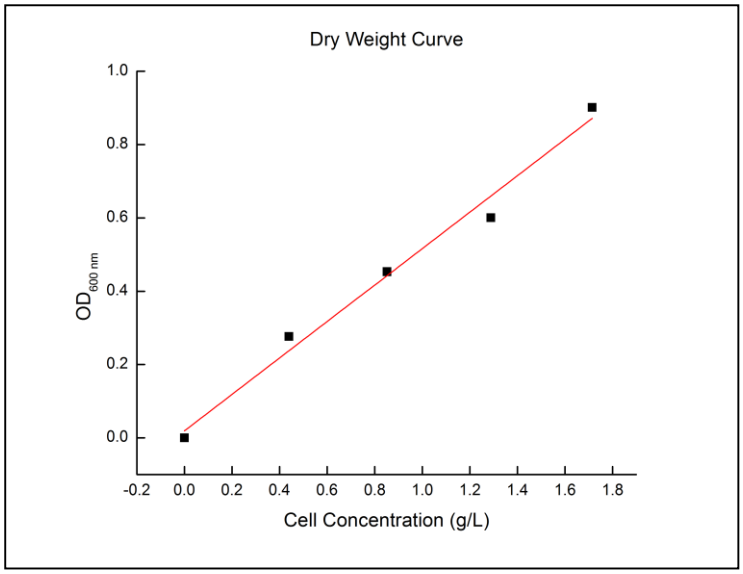

Figure A. 1 Calibration curve of for *S. cerevisiae*.

Table A. 1 Linear regression equation and statistics for the Saccharomyces cerevisiae standard curve

Equation	y = a + b*x		
Adj. R-Square	0.98143		
		Value	**Standard Error**
OD	Intercept	0.01914	0.03584
OD	Slope	0.49729	0.03412

B.1 Ethyl acetate inhibition

As ethyl acetate was chosen for the organic solvent in the upgrading process, it was necessary to assess possible detrimental effects on *S. cerevisiae* growth by the presence of the solvent. Therefore growth of *S. cerevisiae* in the presence of different ethyl acetate fractions. Ethyl acetate was added by weight to complete the volume when the YPG media was prepared. Fractions of ethyl acetate in the media varied from 10% to 100% were 100% is the maximum solubility of ethyl acetate in water (8.8 g ethyl acetate / 100 mL water). YPG media with final concentrations of 1 wt%, 2%wt and 3% wt yeast extract, peptone and glucose were obtained. The results shown on Figure B. 1 suggest that ethyl acetate has no apparent effects on the final cell density obtained. As part of the upgrading process performed, ethyl acetate is evaporated before the fraction is used, hence concentration of 8.8 g ethyl acete/100 ml of water were never achieved.

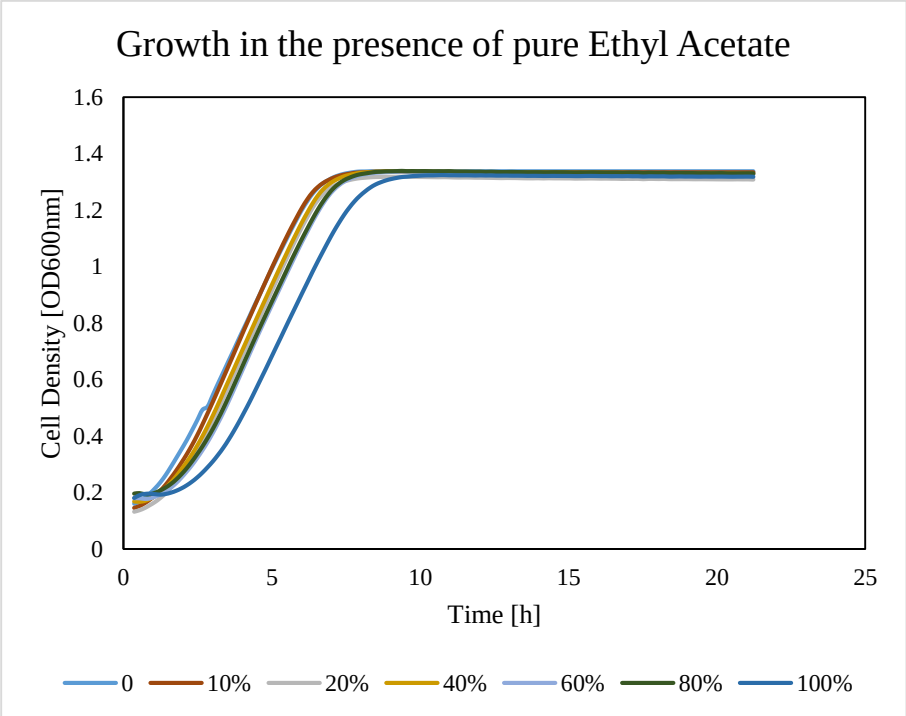

Figure B. 1 *Saccharomyces cerevisiae* grown with different ethyl acetate concentrations. Fractions are based on the maximum solubility of ethyl acetate in water 8.8 g Ethyl Acetate / 100 mL of water.

C.1 HPLC calibration curves

HPLC was used to monitor glucose consumption and ethanol production. At the same time two main inhibitors (furfural and 5-hydroxymethylfurfural) were monitored both with the refractive index detector and with a diode array detector set to a wavelength of 280 nm. In addition to this four compounds levoglucosan was also monitored during the course of this study as levoglucosan is a glucose precursor. The following figures show the calibration curves for each of the compounds. All the calibration curves were linear in the range of concentrations studied.

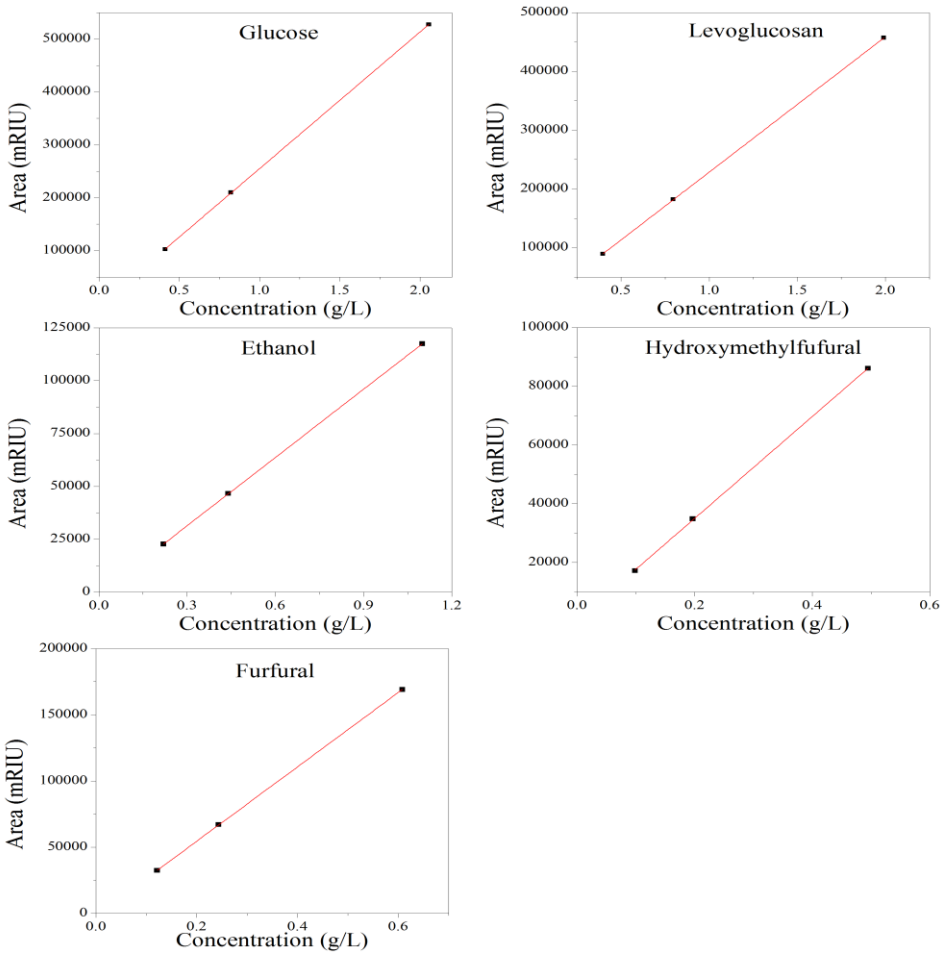

Figure C 1. Refractive index calibration curves for glucose, levoglucosan ethanol 5HMF and furfural. mRIU stands for micro refractive index units, standard units rendered by Agilent software

Table C 1. Retention time, slope, Y-intercept and R^2 values for the calibration curves of five different compounds analyzed by refractive index.

Compound	Retention time [min]	Slope	Y-intercept	R-squared
Glucose	9.604	230586.0411	-2141.91538	0.99998
Levoglucosan	12.402	258343.44	-3190.07692	0.99998
Ethanol	21.948	107617.396	-822.20769	0.99998
Hydroxymethylfufural	30.933	173199.5741	-421.81221	0.99986
Furfural	46.870	280862.2217	-1600.12308	0.99997

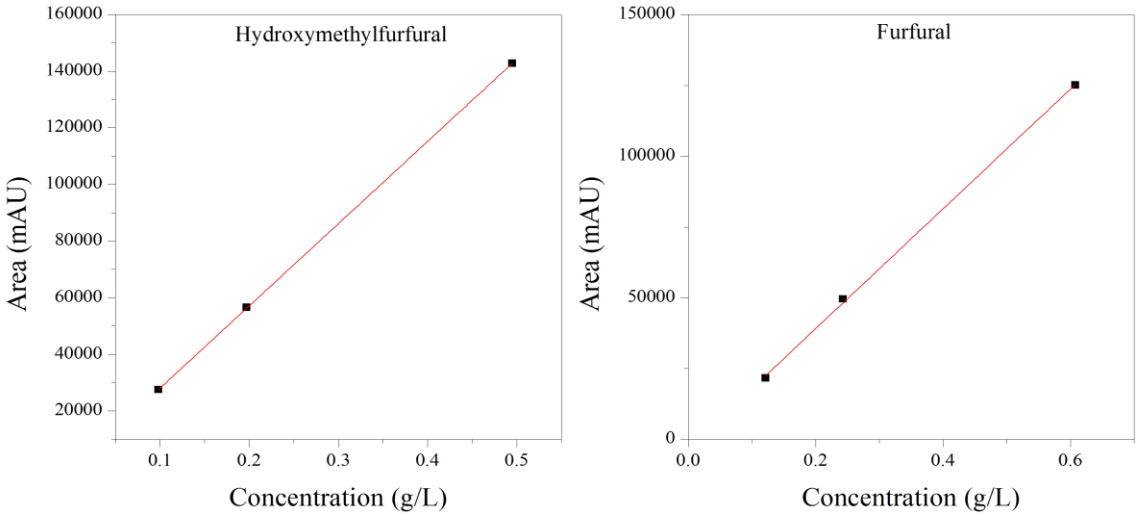

Figure C 2. Diode array detector calibration curves for 5-HMF and furfural. mAU stands for array units, standard units rendered by Agilent software

Table C 2. Retention time, slope, Y-intercept and R^2 values for the calibration curves of two different compounds analyzed by absorbance at 280 nm on the diode array detector.

Compound	Retention time [min]	Slope	Y-intercept	R-squared
Hydroxymethylfufural	31.001	290304.0081	-904.87281	0.99997
Furfural	46.982	211480.5162	-3176.01538	0.99904

D.1 Growth on different pyrolytic oils asd

Experiments on phragmites to analyze the fermentability of some pyrolytic oils produced with a mechanical fluidized bed reactor.

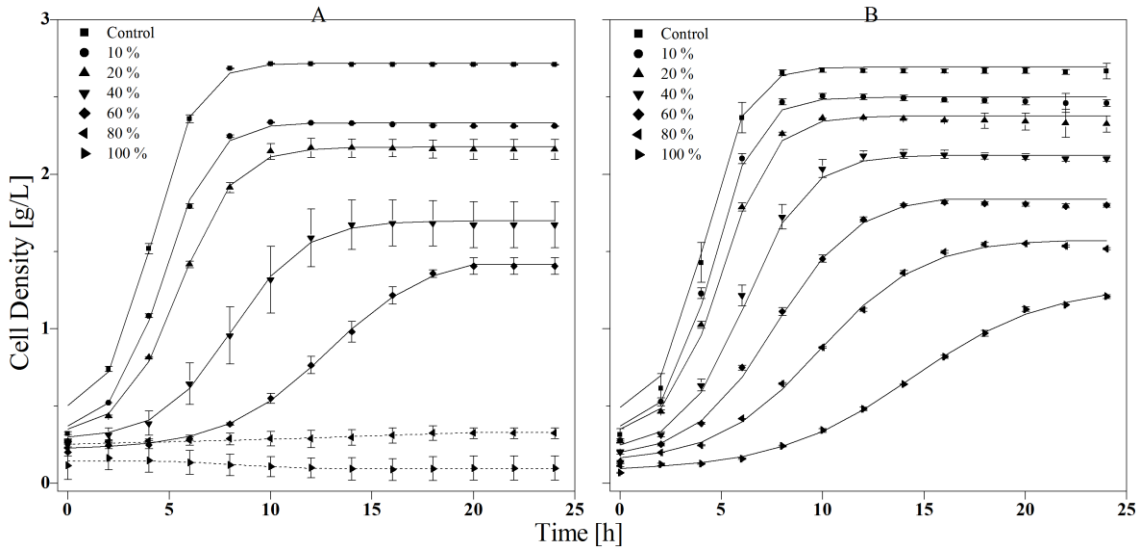

Figure D 1 Growth on pyrolytic sugars obtained from phramites pyrolytic oil. The left graph shows growth after two rounds of ethyl acetate. The graph on the left depicts growth after a third round of ethyl acetate extraction.

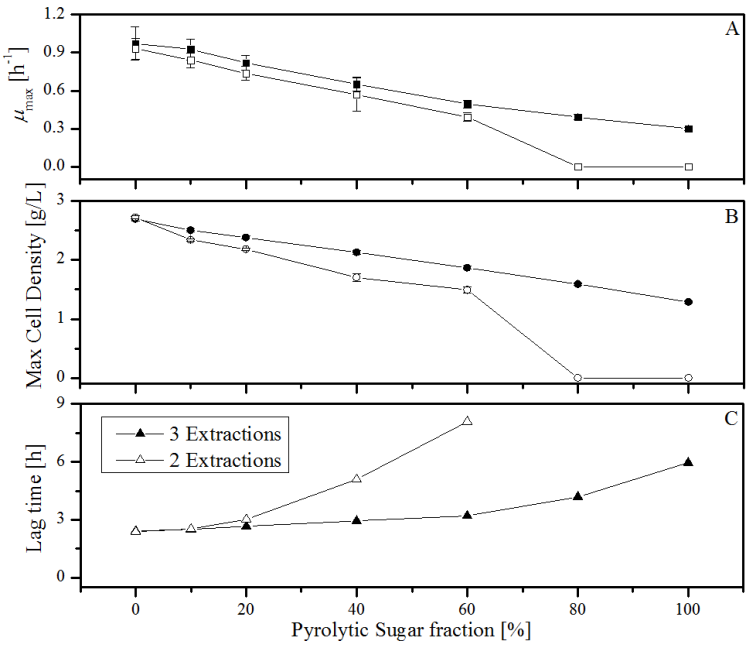

Figure D 2. Calculated growth parameters for experiments on phragmites oil.

E.1 Confirmation of upgrading steps for lipid accumulation.

To confirm the detoxification of the upgrading steps utilized for the pyrolysate on Chapter 5, some of the extract was used to grow *S. cerevisiae* which had grown on a similar pinewood pyrolysate extract.

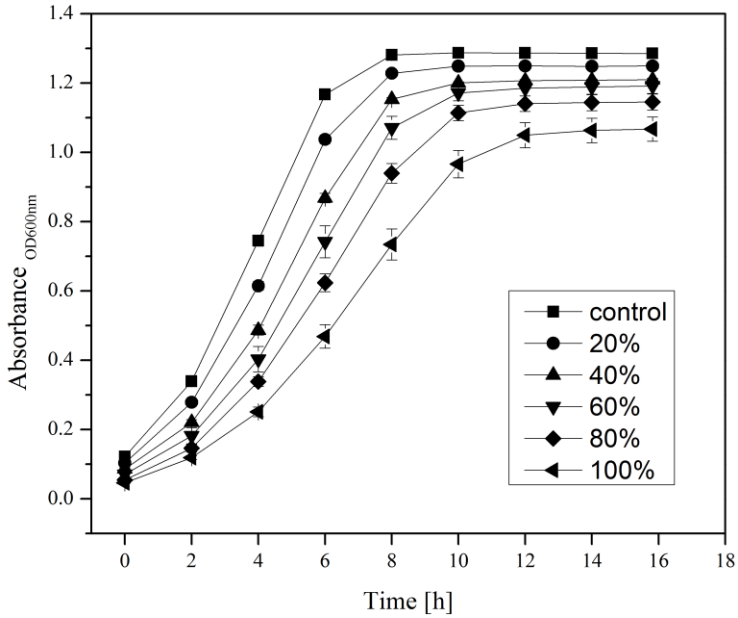

Figure E 1 Growth profiles of S. cerevisiae in the upgraded media used in chapter 5.

Growth of S. cerevisiae served as a validation of the media as all the previous upgrading steps were evaluated with the same strain. Known that this yeast grew confirmed that the detoxification strategies used did not inhibit growth. Ethanol production was not measured for this validation.

F.1 Matlab Routines

The following code solves equations (1) (2) and (3) explained on Chapter 3 and 4. This code was used to determine the growth parameters of when *Saccharomyces cerevisiae* was the fermentative microorganism.

```
function [std,varresid,r2,cor,vcv,varinf]=regdata(param,yfit,ydata,jac)
%[std,varresid,r2,cor,vcv,varinf]=regdata(param,yfit,ydata,jac)
% Calculate and Plot regression statistics from lsqcurvefit.m
% OUT
% std -standard error of each parameter
% varresid- Variance of residuals
% r2    - R^2 Correlation coefficient
% cor   - Correlation matrix for Parameters
% vcv   - Variance Covariance Matrix for Parameters
% varinf- Variance inflation factors >10 implies Multicollinearity in x's
% IN
% param -Least squares parameter values
% yfit  -Response fit using param to get yfit from lsqcurvefit use
yfit=residual+ydata
%                                  where residual is the error matrix from
lsqcurvefit
% ydata -Response data
% jac   -Jacobian value at Least squares parameter values

% Arthur Jutan Univ of Western Ontario Dept of Chemical Engineering
% ajutan@julian.uwo.ca
% Revised 11-20-98,5-19-99

e=yfit(:)-ydata(:); %error vectorize the Y matrix for multiple ouputs
ss=e'*e; % best sum of squares
m=length(yfit);n=length(param);
if (m~=n),varresid=ss./(m-n);else, var=NaN;end % variance of Residuals

% CALC VARIANCE COV MATRIX AND CORRELATION MATRIX OF PARAMETERS
%convert jac to full matrix for ver 5.3
    jac=full(jac);%aj 99
    xtx=jac'*jac;
      xtxinv=inv(xtx);

      %calc correlation matrix cor and variance inflation varinf
    varinf = diag(xtxinv);
    cor = xtxinv./sqrt(varinf*varinf');
```

```
% Plot the fit vs data


      disp(' Least Squares Estimates of Parameters')
      disp(param')
      disp(' correlation matrix for parameters ')
      disp(cor)
      vcv=xtxinv.*varresid; % mult by var of residuals~=pure error
      disp('Variance inflation Factors >10 ==> Multicollinearity in x"s')
      disp(varinf')

%Formulae for vcv=(x'.vo.x)^-1 *sigma^2 where meas error Var, v=[vo]*sigma^2
      std=sqrt(diag(vcv)); % calc std error for each param
      disp('2*standard deviation (95%CL) for each parameter')
      disp(2*std')
%Calculate R^2 (Ref Draper & Smith p.46)
      r=corrcoef(ydata(:),yfit(:));
      r2=r(1,2).^2;
      disp('Variance of Residuals  ' )
      disp(  varresid )
      disp( 'Correlation Coefficient R^2')
      disp(r2)


function [beta tdata Ndata Ncalc lambda jac
residual]=luisKineticFit(tdata,Ndata)
close all;
clc;

[tdata I] = sort(tdata);
Ndata = Ndata(I);

options = optimset('TolFun',1e-8,'TolX',1e-9,'MaxIter',10000,'display','iter');
N0 = mean(Ndata(1:3));
EndPoint = length(Ndata);
newEnd = length(Ndata);

[beta resNorm residual exitflag output LagrangeMul jac] =
lsqcurvefit(@kineticFit,[0.06 0.1 2.6],tdata,Ndata,[0 0 0],[],options,N0);
Q0 = beta(2);
mu_max = (beta(1));

lambda = log((1+1/Q0))/mu_max;
Ncalc = residual + Ndata;

plot(tdata,Ndata,'o',tdata,Ncalc,'-k')
figure(2)
normplot(residual)
[std,varresid,r2,cor,vcv,varinf]=regdata(beta,Ncalc,Ndata,jac);
function G = kineticFit(beta,tdata,N0)

mu_max = beta(1);
Q0 = beta(2);
Nmax = beta(3);
initialCond = [N0 Q0];
```

```
tSpan = [0 110];
sol = ode23s(@growthKinetics,tSpan,initialCond,[],mu_max,Nmax);

G = (deval(sol,tdata,1)');

function dF = growthKinetics(t,F,mu_max,Nmax)

N = F(1);
Q = F(2);

dF(1) = mu_max*Q./(1+Q).*(1-N/Nmax).*N;
dF(2) = mu_max*Q;
dF = dF';
```

This code was used to solve the integral under the surface explained on equation (1) on Chapter 4. The code reads directly from the excel sheets imported directly from Open Lab (Agilent data acquisition software)

```
%% Reading and filtering the data for the water extract
filename = 'HPCC W.xlsx'; % change name to the appropiate name on folder
sheet=1;
intensity_range='B2:BY12001'; % copies signal data values into a matlab matrix
W_Data=xlsread(filename, sheet, intensity_range);
wavelength_range='B1:BY1'; %copies the wavelenght values same for all
intensities
wavelength_vector=xlsread(filename, sheet, wavelength_range);
time_range='A1:A12001'; %copies the time values same for all intensities
time_vector=xlsread(filename, sheet, time_range);

%%% Initiating filtering of the read data from the spreadsheet
[W_Data_peaks,W_Data_FWHH,W_Data_LR]=mspeaks(time_vector,W_Data, 'HeightFilter',
2);
[n_data, m_data]=size(W_Data);
new_W_Data=zeros(n_data,m_data);

for wavelength=1:size(W_Data_LR);
    [n,m]=size(W_Data_LR{wavelength});
    pos=W_Data_LR{wavelength};
    for peak_position=1:n
        tleft=pos(peak_position,1);
        tright=pos(peak_position,2);

        for current_time=1:length(time_vector)
            if time_vector(current_time) > tleft
                if time_vector(current_time) < tright

new_W_Data(current_time,wavelength)=W_Data(current_time,wavelength);
                end
            end
        end
    end
```

```
end

%%% Integration of the filtered data
figure (1)
[wavelenght_matrix,time_matrix]=meshgrid(wavelength_vector,time_vector);
subplot(2,2,1)
mesh(wavelenght_matrix,time_matrix,new_W_Data);
axis([190,340,0,80,0,100])
IntW=trapz(trapz(new_W_Data)*(time_vector(3)-
time_vector(2)))*(wavelength_vector(3)-wavelength_vector(2));
disp(IntW)


%% Copying values for the second set of numbers
filename = 'HPCC WH.xlsx'; % change name to the appropiate name on folder
sheet=1;
WH_Data=xlsread(filename, sheet, intensity_range);

%%% Initiating filtering of the read data from the spreadsheet
[WH_Data_peaks,WH_Data_FWHH,WH_Data_LR]=mspeaks(time_vector,WH_Data,
'HeightFilter', 2);
[WH_n_data, WH_m_data]=size(WH_Data);
new_WH_Data=zeros(WH_n_data,WH_m_data);

for wavelength=1:size(WH_Data_LR);
    [n,m]=size(WH_Data_LR{wavelength});
    pos=WH_Data_LR{wavelength};
    for peak_position=1:n
        tleft=pos(peak_position,1);
        tright=pos(peak_position,2);

        for current_time=1:length(time_vector)
            if time_vector(current_time) > tleft
                if time_vector(current_time) < tright

new_WH_Data(current_time,wavelength)=WH_Data(current_time,wavelength);
                end
            end
        end
    end

end

%%%
figure (1);
subplot(2,2,2)
mesh(wavelenght_matrix,time_matrix,new_WH_Data);
axis([190,340,0,80,0,100])
IntWH=trapz(trapz(new_WH_Data)*(time_vector(3)-
time_vector(2)))*(wavelength_vector(3)-wavelength_vector(2));
disp(IntWH)

%% Copying values for the third set of numbers
filename = 'HPCC WEAH.xlsx'; % change name to the appropiate name on folder
```

```
sheet=1;
WEAH_Data=xlsread(filename, sheet, intensity_range);

%%% Initiating filtering of the read data from the spreadsheet
[WEAH_Data_peaks,WEAH_Data_FWHH,WEAH_Data_LR]=mspeaks(time_vector,WEAH_Data,
'HeightFilter', 2);
[WEAH_n_data, WEAH_m_data]=size(WEAH_Data);
new_WEAH_Data=zeros(WEAH_n_data,WEAH_m_data);

for wavelength=1:size(WEAH_Data_LR);
    [n,m]=size(WEAH_Data_LR{wavelength});
    pos=WEAH_Data_LR{wavelength};
    for peak_position=1:n
        tleft=pos(peak_position,1);
        tright=pos(peak_position,2);

        for current_time=1:length(time_vector)
            if time_vector(current_time) > tleft
                if time_vector(current_time) < tright

new_WEAH_Data(current_time,wavelength)=WEAH_Data(current_time,wavelength);
                end
            end
        end
    end

end

%%%
figure (1);
subplot(2,2,3)
mesh(wavelenght_matrix,time_matrix,new_WEAH_Data);
axis([190,340,0,80,0,100])
IntWEAH=trapz(trapz(new_WEAH_Data)*(time_vector(3)-
time_vector(2)))*(wavelength_vector(3)-wavelength_vector(2));
disp(IntWEAH)

%% Copying the fourth set of data
filename = 'HPCC WHEA.xlsx'; % change name to the appropiate name on folder
sheet=1;
WHEA_Data=xlsread(filename, sheet, intensity_range);

%%% Initiating filtering of the read data from the spreadsheet
[WHEA_Data_peaks,WHEA_Data_FWHH,WHEA_Data_LR]=mspeaks(time_vector,WHEA_Data,
'HeightFilter', 2);
[WHEA_n_data, WHEA_m_data]=size(WHEA_Data);
new_WHEA_Data=zeros(WHEA_n_data,WHEA_m_data);

for wavelength=1:size(WHEA_Data_LR);
    [n,m]=size(WHEA_Data_LR{wavelength});
    pos=WHEA_Data_LR{wavelength};
    for peak_position=1:n
        tleft=pos(peak_position,1);
        tright=pos(peak_position,2);
```

```
        for current_time=1:length(time_vector)
            if time_vector(current_time) > tleft
                if time_vector(current_time) < tright

new_WHEA_Data(current_time,wavelength)=WHEA_Data(current_time,wavelength);
                end
            end
        end
    end

end

%%%
figure (1);
subplot(2,2,4)
mesh(wavelenght_matrix,time_matrix,new_WHEA_Data);
axis([190,340,0,80,0,100])
IntWHEA=trapz(trapz(new_WHEA_Data)*(time_vector(3)-
time_vector(2)))*(wavelength_vector(3)-wavelength_vector(2));
disp(IntWHEA)

int_vec=[IntW,IntWH,IntWEAH,IntWHEA];
figure (3);
subplot(2,2,1)
bar (int_vec);
```

8 Curriculum Vitae

PROFESSIONAL EXPERIENCE

Chemical & Biochemical Engineering PhD Candidate and teaching assistant, Western University, London, Canada. January 2011 – December 2015

Coordinated and managed research focusing on a pyrolysis based biorefinery for biofuels production.

- Developed a high throughput methodology to assess pyrolysis products potential for fermentation.
- Transformed agro-industrial waste into value added products, e.g., ethanol and butanol.
- Published findings in peer-reviewed journals and presented data to global academics and industry leaders in four different Canadian conferences and one international conference.
- Operated and developed Standard Operating Procedures for analytical equipment and bench scale fermenters.
- Led first year engineering student teams to win four design competitions in four consecutive years.
- Proved that ethanol production from three thermal-decomposed (pyrolyzed) waste biomasses is possible, achieving biofuel yields up to 96% of theoretical value.

PhD Visiting Student, University of Twente. Enschede, The Netherlands. February – April 2014

Developed and executed plans to increase carbohydrate content in pyrolytic oils

- Worked at the Sustainable Process Technology Group led by Prof. Dr. Sascha Kersten.
- Characterized biomass following the National Renewable Energy Laboratory (NREL) protocols.
- Reduced alkaline content of two biomasses by 95% to increase fermentable carbohydrates fraction in pyrolysis oil from 2 to 30 wt%.

Research assistant, Co-op Program, National Coffee Research Center. Manizales, Colombia. January to August 2011

Assisted the plant pathology research staff by executing different molecular biology techniques and writing reports.

- Ensured that experiments followed the established protocols.
- Analyzed genetic libraries for coffee blight resistance genes to enhance coffee plants survival rate

EDUCATION

Doctor of Philosophy Candidate, University of Western Ontario, Canada, January 2011 – present

- PhD Thesis title: Thermal decomposition of biomass via fast pyrolysis for fermentable substrate production. Expected graduation October 2015.
- Cumulative average: 86/100 (3.9 GPA)
- Relevant courses: Risk management, Green Fuels and Chemicals, Advanced Statistical Process Design.

Bachelor, Chemical Engineer and Microbiologist, Universidad de Los Andes, Colombia, 2004 – 2009

- Concurrent degrees in Chemical Engineering and Microbiology.
- Emphasis in Industrial processes, Environmental and Industrial microbiology.
- Relevant Courses: Plant Design, Reactor Engineering, Chemical Process modelling. Industrial and Environmental Microbiology.
- Cumulative average: 80/100 (3.7 GPA)

PUBLICATIONS

- Luque, L., Westerhof, R., Van Rossum, G., Oudenhoven, S., Kersten, S., Berruti, F., Rehmann (2014). Pyrolysis based bio-refinery for the production of ethanol from demineralised cellulosic biomass. Bioresource Technology. Vol 161, Pages 20-28.

- Wood, J.A., Orr, V.C.A., Luque, L., Nagendra, V., Berruti, F., Rehmann, L., 2014. High-Throughput Screening of Inhibitory Compounds on Growth and Ethanol Production of *Saccharomyces cerevisiae*. BioEnergy Res. Vol 8, Pages 423–430

- Luque, L., Berruti, F., Kersten, S., Van Rossum., G. Westerhof, R., Rehmann, L.(2013) Alcohol production from pyrolytic sugars obtained from selective fast pyrolysis of pretreated wood in BioEnergy IV. http://dc.engconfintl.org/bioenergy_iv/40/.

CONFERENCES PRESENTATIONS

- Luque, L., Westerhof, R., Van Rossum, G., Kersten, S., Berruti, F., Rehmann, L. Alcohol Production from Pyrolytic Sugars Obtained from Selective Fast Pyrolysis of Pretreated Wood. 16th Ontario-Quebec Biotechnology Conference, May 15th – May 16th, 2014, Toronto, ON, Canada.

- Luque, L., Westerhof, R., Van Rossum, G., Kersten, S., Berruti, F., Rehmann, L. Alcohol Production from Pyrolytic Sugars Obtained from Selective Fast Pyrolysis of Pretreated Wood. BioEnergy IV, June 10th – 14th, 2013, Otranto, Italy.

- Luque, L., Rehmann, L., Berruti, F. Fermentable Substrates Production from Agricultural Waste via Fast Pyrolysis. Research Bridges, May 3rd, 2012, Sarnia, ON, Canada.

- Luque, L., Rehmann, L., Berruti, F. Upgrading of pyrolytic sugars for fermentation. The 61st Canadian Chemical Engineering Conference, October 23rd – 26th, 2011. London, ON, Canada.

AWARDS AND ACCOMPLISHMENTS

- Second place for oral presentation among 31 presenters at Ontario-Quebec Biotechnology Meeting. Toronto. May 15th – 16th, 2014.

- Competed against all the graduate students in biotechnology and won first place for oral presentation at Research Bridges in Biotechnology and Environment category. Sarnia. Ontario. May 3rd, 2012.

EXTRACURRICULAR AND VOLUNTEERING

- Volunteer Spanish translator, The University of Western Ontario (2011- present); Equinox Resort, Manchester, VT, USA (Summer 2007); Yellowstone National Park, WY, USA (Summer 2006).

- Mathematics, English and Spanish Tutor, Alianza Educativa, Bogota, Colombia 2004 - 2006

SKILLS AND INTERESTS

Languages: Spanish: Native proficiency. English: Native proficiency. French: Beginner proficiency.
Software: ASPEN HYSYS, Microsoft Office macros, MATLAB.
Personal Interests: Photography, squash, cooking Colombian food, winter camping, and hiking.