Bioresource Technology 161 (2014) 20–28

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

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

Luis Luque [a], Roel Westerhof [a,b], Guus Van Rossum [a,b], Stijn Oudenhoven [b], Sascha Kersten [b], Franco Berruti [a], Lars Rehmann [a,*]

[a] Department of Chemical and Biochemical Engineering, University of Western Ontario, 1151 Richmond Street London, Ontario, Canada
[b] Sustainable Process Technology, Faculty of Science and Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

HIGHLIGHTS

- Ethanol production from pinewood after pyrolysis at 41% theoretical yield.
- Inhibition profiled modeled based on micro-scale fermentation.
- High levoglucosan yield through pre-treatment of wood with pyrolysis product.

ARTICLE INFO

Article history:
Received 18 December 2013
Received in revised form 28 February 2014
Accepted 4 March 2014
Available online 13 March 2014

Keywords:
Ethanol
Biorefinery
Pyrolysis
Modeling
Lignocellulose

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.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Production of lignocellulose-based bioethanol has not yet been established on a commercial scale. The technical and economic challenges associated with pretreatment of biomass, required to hydrolyze and release fermentable sugars, hamper its commercial development (Kazi et al., 2010). An alternative to release these sugars from lignocellulosic material for fermentation, is fast pyrolysis. 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 signification 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

* Corresponding author. Address: Chemical and Biochemical Engineering, University of Western Ontario, 1151 Richmond Street London, Ontario N6A 3K7, Canada. Tel.: +1 519 661 2111x89008.
E-mail address: lrehmann@uwo.ca (L. Rehmann).

http://dx.doi.org/10.1016/j.biortech.2014.03.009
0960-8524/© 2014 Elsevier Ltd. All rights reserved.

(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 depolimerized, 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 2 h 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, monophenols, 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 and Ingram, 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 bentomite 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, 0.35 g 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 Fig. 1.

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 18 wt% on biomass intake) and concentration (up to 37 wt%) 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.

2. Methods

2.1. Pyrolysis oil production and work-up procedure

An overview of the overall experimental scheme is given in Fig. 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 one of the product streams, stream exiting condenser 2, Fig. 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 fluidized bed reactor pilot plant. A detailed description of the pyrolysis and the pine wood 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 2 h (Fig. 1). The pretreated pine wood was then pyrolyzed at 480 °C with a vapor residence time <2 s 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 ± 0.01 bar (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. 90 wt% 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

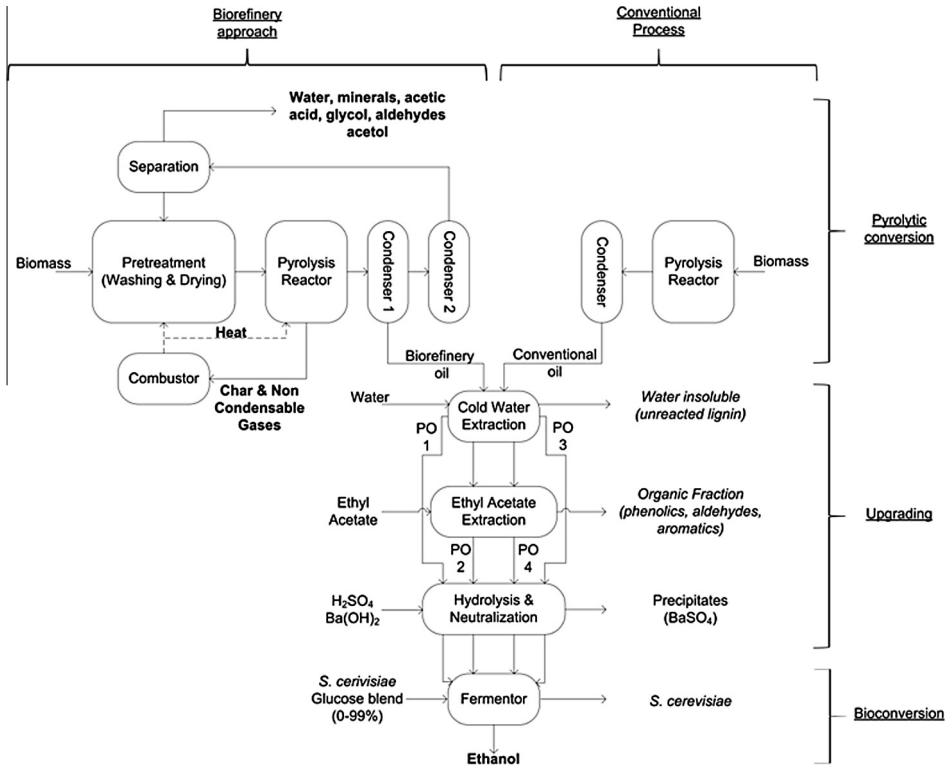

Fig. 1. Process layout comparison for the production of sugars, aromatics and light oxygenates from lignocellulosic biomass via fast pyrolysis (Oudenhoven et al., 2013). Conventional process showed on the right. Streams in bold represent current value-added product streams of the proposed biorefinery approach, while the italicized streams could also be utilized in the future.

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.

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 Milli-Q 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 (0.00–0.20 g/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 300 mm × 7 mm column and a Refractive index detector (Agilent, Santa Clara, USA). 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. 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 ±6 g was prepared as a mixture of 5 wt% pyrolysis oil and 95 wt% acetone. 2 mL 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).

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). 5 g of pyrolysis oil were added drop wise to 50 mL of chilled water (CW) under heavy stirring (900 rpm). 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 0.45 μm cellulose nitrate membrane (Whatman®, UK). Filtrate was centrifuged at 4 °C and 3500 rpm for 20 min (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 1:2 wt% filtrate to EA solution was prepared and mixed for 12 h in an environmental shaker at 150 rpm and 25 °C. After the mixing the sample was left standing for 6 h to secure separation of the phases. The organic layer was separated and remaining EA was removed by evaporation at 50 °C for 24 h in an oven (Isotemp, Fisher Scientific, USA).

Levoglucosan to glucose hydrolysis was realized by transferring extract aliquots of 4 mL to microwave vials (VWR,USA) followed by

the addition of H_2SO_4 (final concentration of 0.5 M) and hydrolysis at 120 °C for 20 min in an autoclave (Bennett et al., 2009) Hydrolyzates were neutralized with solid $Ba(OH)_2$ (Alfa Aesar, USA). After neutralization samples were transferred to 15 mL centrifuge tubes (VWR, Canada) and salt crystals were precipitated by centrifugation at 3500 rpm for 20 min (Sorval ST40R, Thermo Scientific). The supernatant was removed and filtered with a 0.2 μm cellulose acetate syringe membrane (VWR, Canada) and transferred to a new sterile 15 mL 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.

2.4. Bioprocessing

Neutralized and cleaned hydrolyzate was fermented with *S. cerevisiae* DSM 1334 (Braunschweig, Germany) in 96 wells microtiter plates (Costar®, Corning, USA). YPG medium (10 g/L yeast extract (BD, USA), 20 g/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 hydrolyzate (up to 100% hydrolyzate). The final total glucose concentration in the media was kept constant at 40 g/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 hydrolyzate, a range of 5–60% pyrolytic sugar concentration was tested (PO1). As for biorefinery oil EA hydrolyzate, 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 180 μL of the pyrolytic YPG media prepared and inoculated with 20 μL 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 74 rpm using a Tecan M200 micro plate reader (Tecan, Austria). Optical density was measured by the reader in each well at 600 nm every 10 min for 24 h. 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 0.5 mM H_2SO_4 as the mobile phase at a flow of 0.7 mL/min.

2.5. Numerical analysis of yeast growth

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 (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. (1) and (2) respectively, the estimated adaptation time λ for the culture is calculated using Eq. (3).

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

$$\frac{dQ}{dt} = \mu_{max}Q \quad (2)$$

$$\lambda = \frac{\ln\left(1+\frac{1}{Q_0}\right)}{\mu_{max}} \quad (3)$$

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. Results and discussion

3.1. Extraction of pyrolysis oil

From Table 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 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 Fig. 1). After each step samples were drawn to analyze sugar conversion, and TOC loss, as shown in Table 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

Table 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	8.90	7.15	0.00	0.36	0.00
	PO3 hydrolyzed	8.25	1.05	3.91	0.06	0.19
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	17.22	7.90	0.00	0.20	0.00
	PO4 hydrolyzed	14.78	2.75	3.80	0.08	0.10

Table 2
Chemical detection (GC/MS) of known fermentation inhibitors in pyrolysis oils at various stages of the process. All the concentrations are in wt%.

Compound group	Biorefined oil/Conventional oil		
	Original oils	After water extraction	After EA extraction and hydrolysis
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

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 in 14%.

The data in Table 1 also shows that biomass demineralization and fractional condensation play an essential role by increasing the levoglucosan concentration after pyrolysis; concentration increased fivefolds (7.9–44.6 g/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.

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 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 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.

3.2. Fermentation

POs extracts (Fig. 1) were tested as fermentation substrates. Microscale fermentations experiments were performed with standard medium and 40 g/L glucose. To assess the respective fermentability of the 4 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 2), 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% (3.80–3.9 g/L) of the total glucose in the medium, were achievable with the given glucose concentration of the hydrolyzate. In contrast, biorefinery PO extracts (PO1 and PO2) had substantially higher glucose levels (41.8–43.4 g/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 40 g/L without the addition of any other glucose. A common pattern in the growth profile of yeast on all extracts (Fig. 2A–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. 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 Fig. 2B and D (EA extract, PO2 and PO4) with Fig. 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 Fig. 2B and D. In the case of conventional oil PO3 (Fig. 2A), cell growth was only observed when the fraction of pyrolytic sugar was up to a 3% contrasting with a 5% maximum of hydrolyzate added reported by Wang and collaborators (Wang et al., 2012), where the hydrolyzate 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, Fig. 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 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 Figs. 2B and D. Fig. 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 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

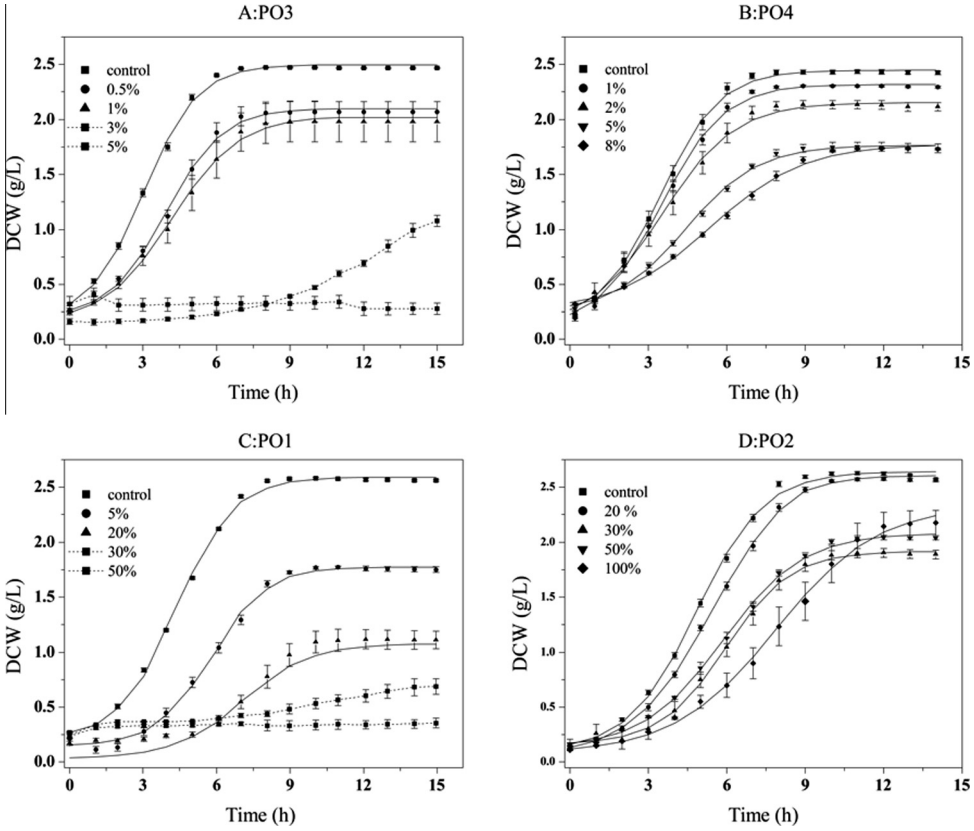

Fig. 2. Pyrolytic substrate fermentation growth profiles on two different types of pyrolysis-oil extract as a function of the pyrolytic sugar fraction. A and B correspond to conventional pyrolysis oil extract. C and D correspond to bio-refined pyrolysis-oil extract. Results on the left graphs correspond 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 (where possible), while dotted lines are used to simply connect data points for visualization purposes when no sufficient regression data could be obtained.

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. 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 Fig. 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 Fig. 3A–D, show an expected inverse relationship between lag time (λ) and the specific growth rate (μ_{max}). The lag time increases, while the specific 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 Fig. 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 pyroltic 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 a ~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 2) when biomass is demineralized (Fig. 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 Fig. 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 fourfolds, from 1.5 h in the control to almost 6 h when the hydrolyzate concentration of demineralized PO1 is only 20%, as shown in Fig. 3C. Interestingly, no significant difference of λ could be seen for an increase in PO concentrations after EA extraction (Fig. 3D). The clear tendency of a decreasing μ_{max} in Fig. 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 Fig. 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.,

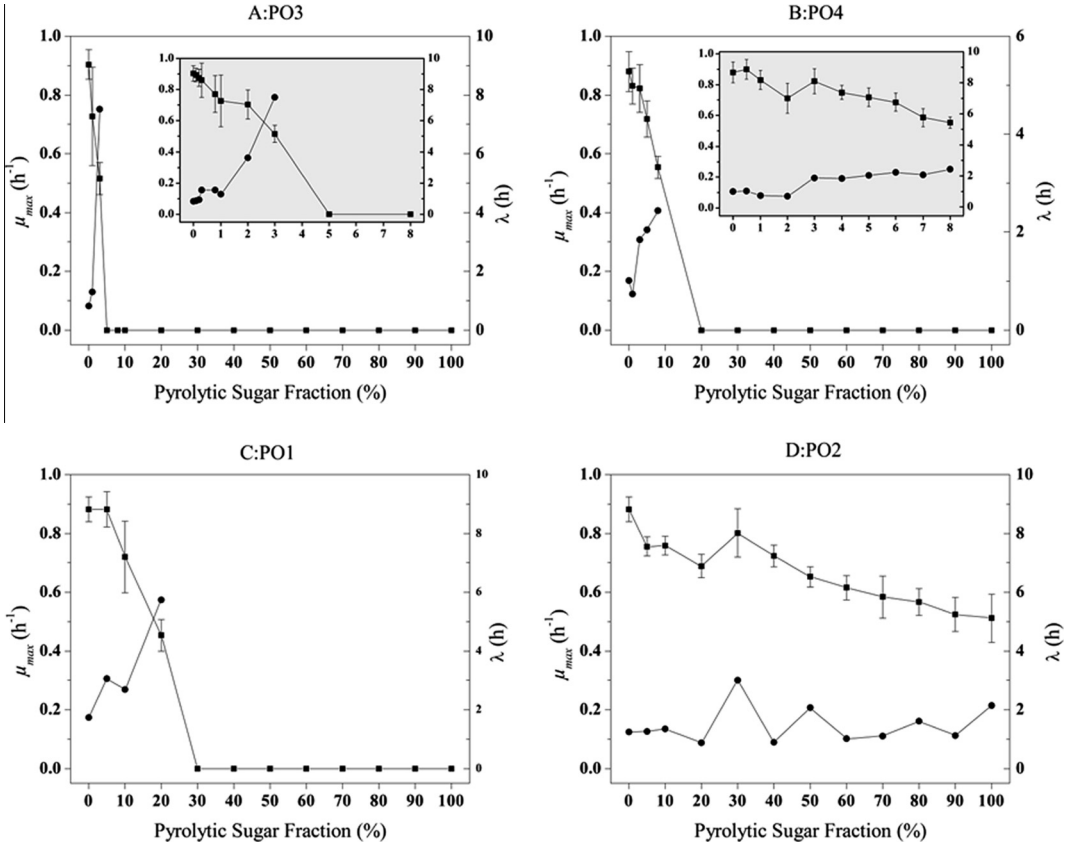

Fig. 3. Estimated model parameters for microfermentations conducted with varying glucose fractions derived from pyrolysis oils. A and B correspond to fermentations of conventional biomass pyrolysis oil. C and D correspond to demineralized biomass pyrolysis oil (biorefinery oil). Results on the left graphs correspond to only cold water extraction, PO1 and PO3, on the right to EA extract fermentation, PO2 and PO4. The specific growth rate estimates, μ_{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.

1999), 5-hydroxymethyl furfural (5-HMF), vanillin, syringaldehyde, coniferyl aldehyde (Delgenes et al., 1996) and 4-hydrozybenzaldehyde (Klinke et al., 2003). 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.

3.4. Ethanol and biomass production

The theoretical yield of ethanol produced from glucose is 0.511 g/g. The maximum yield achieved in this study was 0.49 g 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 15 h and samples for ethanol analysis were drawn at the end-point of each microfermentation. The effect on ethanol yield of increasing pyrolytic sugar fractions is shown in Fig. 4. As expected based on cell growth data (Fig. 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 (Fig. 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 (Fig. 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 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 20 g/L, as shown in Fig. 4D.

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 15 h. Ethanol production on samples containing lower fractions of pyrolytic sugars, will likely have completed faster (see higher values for μ_{max} in Fig. 3, or growth profile in Fig. 2), giving time for ethanol to evaporate

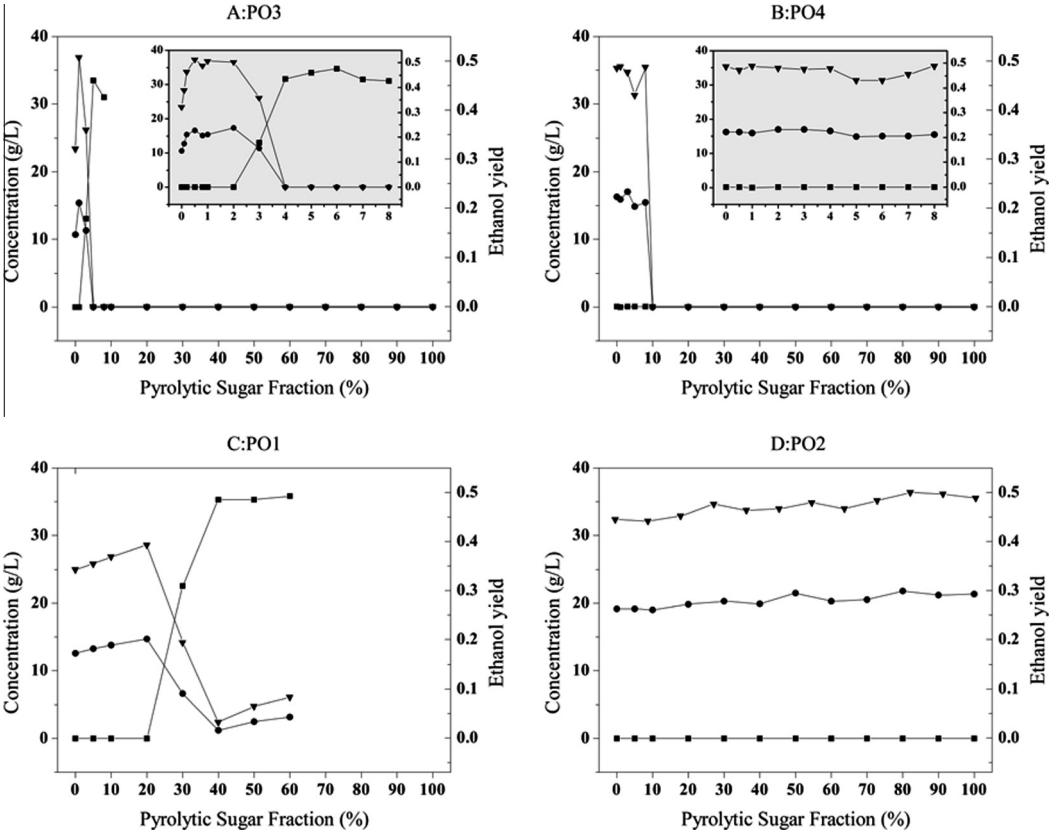

Fig. 4. Calculated glucose consumption and ethanol production. A and B correspond to fermentations of non-demineralized biomass pyrolysis oil. C and D correspond to demineralized biomass pyrolysis oil. Results on the left graphs correspond to only cold water extraction, PO1 and PO3, on the right to EA 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.

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 Fig. 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. Generally, final ethanol concentrations ranged from 18 g/L to 20 g/L corresponding to a range in ethanol yields between 0.45 and 0.5 g ethanol/g glucose (Fig. 4D). Based on the most suitable substrate (PO) a total amount of 8.2 g ethanol could be produced per 100 g pine wood, corresponding 41.3% of the theoretical maximum value (Table 3), based on the assumption that all cellulose in pinewood, approximately 36 wt% (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

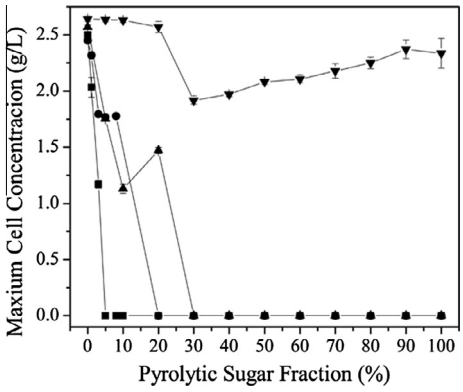

Fig. 5. Maximum cell concentration reached after fermentation process with different pyrolysis oil extracts. (square) PO1 (circle) PO2 (triangle) PO4, (inverted triangle) PO3.

process are biochar and biogas, as well as acidic acid as shown in Fig. 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 shows that the yield of ethanol from the available pyrolysis derived glucose is very high (8.2 g vs. the theoretical maximum of 8.5 g). The efficiency of cellulose to

Table 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%	

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. 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.

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.

Acknowledgements

The authors are grateful to the Natural Sciences and Engineering Research Council of Canada and the Canada Foundation for Innovation for financial support, as well as Dr. Jeff Wood for advice on the numerical analysis.

References

Baranyi, J., Roberts, T.A., 1994. A dynamic approach to predicting bacterial growth in food. Int. J. Food Microbiol. 23, 277–294.
Ben, H., Ragauskas, A.J., 2013. Comparison for the compositions of fast and slow pyrolysis oils by NMR characterization. Bioresour. Technol. 147, 577–584.
Bennett, N.M., Helle, S.S., Duff, S.J.B., 2009. Extraction and hydrolysis of levoglucosan from pyrolysis oil. Bioresour. Technol. 100, 6059–6063.
Bridgwater, A.V., Toft, A.J., Brammer, J.G., 2002. A techno-economic comparison of power production by biomass fast pyrolysis with gasification and combustion. Renew. Sustain. Energy Rev. 6, 181–246.
Chi, Z., Rover, M., Jun, E., Deaton, M., Johnston, P., Brown, R.C., Wen, Z., Jarboe, L.R., 2013. Overliming detoxification of pyrolytic sugar syrup for direct fermentation of levoglucosan to ethanol. Bioresour. Technol. 150, 220–227.
Czernik, S., Bridgwater, A.V., 2004. Overview of applications of biomass fast pyrolysis oil. Energy Fuels 18, 590–598.
Delgenes, J.P., Moletta, R., Navarro, J.M., 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehatae*. Enzyme Microb. Technol. 19, 220–225.
Dobele, G., Dizhbite, T., Rossinskaja, G., Telysheva, G., Meier, D., Radtke, S., Faix, O., 2003. Pre-treatment of biomass with phosphoric acid prior to fast pyrolysis. J. Anal. Appl. Pyrolysis 68–69, 197–211.
Eklund, R., Zacchi, G., 1995. Simultaneous saccharification and fermentation of steam-pretreated willow. Enzyme Microb. Technol. 17, 255–259.
Garcia-perez, M., Wang, S., Shen, J., Rhodes, M., Lee, W.J., 2008. Effects of temperature on the formation of lignin-derived oligomers during the fast pyrolysis of mallee woody. Biomass, 2022–2032.
Kazi, F.K., Fortman, J.A., Anex, R.P., Hsu, D.D., Aden, A., Dutta, A., Kothandaraman, G., 2010. Techno-economic comparison of process technologies for biochemical ethanol production from corn stover. Fuel 89, S20–S28.
Klinke, H.B., Olsson, L., Thomsen, A.B., Ahring, B.K., 2003. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: wet oxidation and fermentation by yeast. Biotechnol. Bioeng. 81, 738–747.
Klinke, H.B., Thomsen, A.B., Ahring, B.K., 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl. Microbiol. Biotechnol. 66, 10–26.
Layton, D.S., Ajjarapu, A., Choi, D.W., Jarboe, L.R., 2011. Engineering ethanologenic *Escherichia coli* for levoglucosan utilization. Bioresour. Technol. 102, 8318–8322.
Lian, J., Chen, S., Zhou, S., Wang, Z., O'Fallon, J., Li, C.-Z., Garcia-Perez, M., 2010. Separation, hydrolysis and fermentation of pyrolytic sugars to produce ethanol and lipids. Bioresour. Technol. 101, 9688–9699.
Lian, J., Garcia-Perez, M., Coates, R., Wu, H., Chen, S., 2012. Yeast fermentation of carboxylic acids obtained from pyrolytic aqueous phases for lipid production. Bioresour. Technol. 118, 177–186.
Lin, S.-H., Juang, R.-S., 2009. Adsorption of phenol and its derivatives from water using synthetic resins and low-cost natural adsorbents: a review. J. Environ. Manage. 90, 1336–1349.
McMillan, J.D., Newman, M.M., Templeton, D.W., Mohagheghi, A., 1999. Simultaneous saccharification and cofermentation of dilute-acid pretreated yellow poplar hardwood to ethanol using xylose-fermenting *Zymomonas mobilis*. Appl. Biochem. Biotechnol. 77–79, 649–665.
Oudenhoven, S.R.G., Westerhof, R.J.M., Aldenkamp, N., Brilman, D.W.F., Kersten, S.R.A., 2013. Demineralization of wood using wood-derived acid: Towards a selective pyrolysis process for fuel and chemicals production. J. Anal. Appl. Pyrolysis 103, 112–118.
Palmqvist, E., Grage, H., Meinander, N.Q., Hahn-Hägerdal, B., 1999. Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnol. Bioeng. 63, 46–55.
Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresour. Technol. 74, 17–24.
Radlein, D.S.T.A.G., Grinshpun, A., Piskorz, J., Scott, D.S., 1987. On the presence of anhydro-oligosaccharides in the sirups from the fast pyrolysis of cellulose. J. Anal. Appl. Pyrolysis 12, 39–49.
Salehi, E., Abedi, J., Harding, T., 2009. Bio-oil from sawdust: pyrolysis of sawdust in a fixed-bed system. Energy Fuels 23, 3767–3772.
Schwab, K., Wood, J.A., Rehmann, L., 2013. Pyrolysis by-products as feedstocks for fermentative bio-fuel production: an evaluation of inhibitory compounds via a synthetic aqueous phaseInd. Eng. Chem. Res. 52 (51), 18234–18240.
Shafizadeh, F., Stevenson, T.T., 1982. Saccharification of douglas-fir wood by a combination of prehydrolysis and pyrolysis. J. Appl. Polym. Sci. 27, 4577–4585.
Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. 83, 1–11.
Vispute, T.P., Huber, G.W., 2009. Production of hydrogen, alkanes and polyols by aqueous phase processing of wood-derived pyrolysis oils. Green Chem. 11, 1433.
Wang, H., Livingston, D., Srinivasan, R., Li, Q., Steele, P., Yu, F., 2012. Detoxification and fermentation of pyrolytic sugar for ethanol production. Appl. Biochem. Biotechnol. 168, 1568–1583.
Weil, J.R., Dien, B., Bothast, R., Hendrickson, R., Mosier, N.S., Ladisch, M.R., 2002. Removal of fermentation inhibitors formed during pretreatment of biomass by polymeric adsorbents. Ind. Eng. Chem. Res. 41, 6132–6138.
Westerhof, R.J.M., Brilman, D.W.F., Garcia-Perez, M., Wang, Z., Oudenhoven, S.R.G., van Swaaij, W.P.M., Kersten, S.R.A., 2011. Fractional condensation of biomass pyrolysis vapors. Energy Fuels 25, 1817–1829.
Westerhof, R.J.M., Kuipers, N.J.M., Kersten, S.R.A., van Swaaij, W.P.M., 2007. Controlling the water content of biomass fast pyrolysis oil. Ind. Eng. Chem. Res. 46, 9238–9247.
Yu, Z., Zhang, H., 2003. Pretreatments of cellulose pyrolysate for ethanol production by *Saccharomyces cerevisiae*, *Pichia* sp. YZ-1 and *Zymomonas mobilis*. Biomass Bioenergy 24, 257–262.
Zaldivar, J., Ingram, L.O., 1999. Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01. Biotechnol. Bioeng. 66, 203–210.
Zaldivar, J., Martinez, A., Ingram, L.O., 2000. Effect of alcohol compounds found in hemicellulose hydrolysate on the growth and fermentation of ethanologenic *Escherichia coli*. Biotechnol. Bioeng. 68, 524–530.
Zhuang, X., Zhang, H., 2002. Identification, characterization of levoglucosan kinase, and cloning and expression of levoglucosan kinase cDNA from *Aspergillus niger* CBX-209 in *Escherichia coli*. Protein Expr. Purif. 26, 71–81.