Fermentation of cellulose pyrolysis oil by a *Clostridial* bacterium

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Abstract

The coupling of thermochemical and biological conversion of biomass is a promising strategy to produce chemicals in future integrated biorefineries. Indeed, thermochemical conversion such as pyrolysis is a fast process without any solvent or enzyme for the depolymerization of biomass. In this work, cellulose was pyrolyzed to produce sugars which have been then fermented by bacteria (*Clostridium acetobutylicum*) to produce acetone and butanol. This type of bacteria presents an interesting biological platform: it is resilient, easily up-scalable and *Clostridium* can be genetically engineered to target various other chemicals. Pyrolysis of cellulose was performed in a continuous fluidized bed reactor equipped with a staged condensation system, including a warm electrostatic precipitator. Different bio-oil fractions rich in levoglucosan (LVG) and with different concentrations in inhibitors for the fermentation stage were produced. LVG was found to be non-fermentable by *C. acetobutylicum*. Therefore, the bio-oil fractions were hydrolysed to obtain fermentable glucose. The mechanisms of acid hydrolysis (with diluted H₂SO₄) of LVG and cellobiosan have been revealed by high resolution mass spectrometry. The microorganisms were not able to grow with all hydrolysed bio-oil fractions depending on the concentration in inhibitors (aldehydes and organic acids). The fractions rich in LVG (and then glucose) lead to normal bacterial growth and normal fermentation products pattern without the need of detoxification. These results show the importance of a pyrolysis process with a staged condensation as a preliminary step for fermentation. It opens the road to production of various cellulose-derived chemicals by bacteria.

Keywords: cellulose, pyrolysis, hydrolysis, ABE fermentation

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1 Introduction

Valorisation of lignocellulosic materials in biorefineries offers a renewable and sustainable alternative to crude oil refineries [1]. Cellulose can be extracted from the lignocellulosic matrix through different fractionation processes [2–5]. Different fractionation methods of lignocellulosic biomass have been compared in order to produce a cellulose-rich pulp [ref]. Besides its use in the paper industry [6], cellulose can be converted by catalytic or biological processes into fuels and chemicals [7,8]. Its direct fermentation undergoes long reaction times taking several days (due to its recalcitrance), ending in a relatively low yield of the final product [9,10]. For this reason, the depolymerisation of cellulose is required prior to its fermentation. Cellulose depolymerisation through direct acid hydrolysis produces a maximum glucose yield of about 70 wt.% with reaction times of about 2 hours, but this process requires expensive corrosion-resistant reactor operated at high pressures and temperatures and it leads to high operating cost related with acid recovery and high sugars dilution in water [11,12]. Enzymatic hydrolysis is commonly used for the depolymerisation of cellulose [13]. However, in the bioethanol industry, enzyme cost represents more than 45 % of the minimum ethanol selling price, making this stage the most expensive one of the global process [14].

Fast pyrolysis allows a direct thermochemical depolymerisation of cellulose without solvents or enzymes. This process produces bio-oil (as the main fraction), char and permanent gas [15]. In comparison with enzymatic hydrolysis, fast pyrolysis is not sensitive to inhibitors and can reach higher sugar yields in only seconds, compared to days in the case of biochemical conversions using enzymes [13]. In addition, the depolymerisation process must reduce the production of inhibiting compounds that hinder microbial growth during the fermentation process [16]. To overcome this problem, pyrolysis plants can be equipped with fractional condensation systems that separate pyrolytic vapours according to their condensation point [17]. Therefore, the condensation system allows recovering a heavy oil rich in sugars (for further conversion with microorganisms) separated

from the light oil with the microorganism inhibitory compounds (light oxygenated: aldehydes, alcohols, carboxylic acids) [17,18]. The light fractions can be valorised for other purposes, such as the pre-treatment of biomass for its demineralization in order to increase the sugars yield after fast pyrolysis [19].

Some molecules (glutamic acid, 3-hydroxypropionic acid, isoprene, farnesene, itaconic acid, lactic acid, etc.) [20] can only be synthesized through biological conversion involving enzymes or microorganisms. For instance, fermentation can convert the saccharides into various chemicals of industrial interest such as: butanol, ethanol, xylitol, sorbitol, 1,3-propanediol, biodiesel, lactic acid, succinic acid, isoprene, hydrogen, among others [21,22].

Therefore, it is of tremendous interest to combine pyrolysis of cellulose for a fast depolymerisation without solvents or enzymes and further biological conversion of the as-produced depolymerized products recovered in the bio-oil. Table 1 presents an overview of work dealing with the biological conversion of cellulose pyrolysis oils.

Insert Table 1 here.

While fermentation of biomass-derived saccharides into biofuels and chemicals has been widely investigated [23,24], published studies on the biological conversion of the anhydro-saccharides found in pyrolytic bio-oil are rather rare (see Table 1) [25,26]. Indeed, levoglucosan (LVG) is not a common molecule in nature (unless as a stable marker of wood fires), therefore the microorganisms capable of its direct metabolism are very rare [27]. Some strains have been genetically engineered for this purpose [28]. Otherwise, using mild acidic media, levoglucosan can be easily hydrolysed into glucose that is one of the most common fermentable sugars [29].

Several microorganisms allow the conversion of sugars into fuels and chemicals but not all of them are suitable for industrial production for three main reasons: 1) its adaptability to different environments (pH, temperature, etc.) without adjusting carbon and nitrogen inputs (flow rate and

hydrodynamic), 2) its simplicity of homogenization in the multiphase bioreactor, and 3) the capability of monitoring cell density in a relatively short time [30].

The acetone-butanol-ethanol (ABE) fermentation using *Clostridial* species is one of the most promising biological conversion processes because it has already been developed at industrial scale since World War I [31]. Second, metabolic engineered strains of *C. acetobutylicum* are able to produce a wide variety of interesting platform molecules [32–34]. Third, *Clostridium* bacteria present an important microbial adaptation and a good resistance to various inhibitors [35,36].

A gas (H_2 , CO_2) is also produced by *Clostridium acetobutylicum*. This gas can be further valorised for heat production or for a further chemical valorisation, for instance for the production of butanol by fermentation. Ref.

Another promising way is the fermentation of syngas produced by biomass gasification using specific *Clostridial* strains that are able to convert carbon monoxide and carbon dioxide into alcohols. This approach allows the valorisation of biomass wastes [37,38]. However, the fermentation using carbohydrates presents faster cell growth, more efficient mass transfer (carbohydrates are already present as a soluble molecule in the aqueous phase) and several microorganisms are able to ferment carbohydrates to produce a wide variety of chemicals and biofuels [39]. Therefore, syngas fermentation is of potential interest for valorising wastes syngas but it seems more favourable to convert cellulosic biomass into soluble sugars (than into a syngas) for a further fermentation process.

For all these reasons, *Clostridium* bacteria were chosen in the present work to ferment cellulose pyrolysis oils. The process developed in this work is presented in Figure 1.

Insert Figure 1. here.

Cellulose was pyrolyzed in a fluidized bed reactor using a fractional condensation train to produce a bio-oil with a high sugar content. This oil was hydrolysed to obtain fermentable sugars. The

hydrolysed oil was, then, fermented using *C. acetobutylicum*. To the best of our knowledge, this work reports for the first time the combination of fast pyrolysis with staged-condensation, hydrolysis and ABE fermentation for the valorisation of cellulosic materials into building blocks (Figure 1). This process arrangement could be further used to produce various other targeted chemicals from cellulose by means of metabolic engineering of diverse *Clostridium* strains.

2 Materials and methods

2.1 Reactants

Microcrystalline cellulose Avicel PH-101 (particle size < 50 µm), glucose (99.5 %), sulphuric acid (99.99 %), acetone (99.9 %), ethanol (99.8 %), butanol (>99 %), acetic acid (>99.5 %), lactic acid (>98 %), butyric acid (99 %) were purchased from Sigma Aldrich, San Luis, USA. Levoglucosan (98 %), cellobiose (98 %) and cellobiosan (95 %) standards were purchased from Carbosynth (Compton, United Kingdom).

2.2 Pyrolysis

Fast pyrolysis of cellulose was performed at ICFAR (Canada) in a gas fluidized bed pilot plant (590 mm height and 91 mm internal diameter) equipped with a feeding system allowing an efficient distribution of cellulose in the fluidized zone of the reactor (Figure 2).

Insert Figure 2. here.

The fluidized bed was composed of 1033 g of quartz sand with a particle size of 0.2–0.4 mm. The fluidization gas was nitrogen at 25 standard litres per minute (SLPM). Compressed air was used to activate the valve of the pulsating feeding system [40]. The cellulose feed rate was 16.7 g/min. The average temperature inside the reactor was 475 °C, with a vapour residence time of 2.2 s.

Temperature was monitored at five different locations along the reactor, as shown in Figure S1. A staged condensation system of successive condensers with bath temperatures of 70, 45, and 5 °C, recovered five different bio-oil fractions, as shown in Figure 2. The second condenser was a temperature controlled electrostatic precipitator (ESP) [41]. The permanent gas was vented and not analysed. All the bio-oil fractions were recovered and stored at -20°C for further experiments and analysis.

2.3 Hydrolysis of the pyrolytic oil

An autoclave stirred reactor (stainless steel 316, Parr Instrument Company) of 300 mL was used for the hydrolysis of pyrolytic oil (presented in supplementary material, Figure S2). 10 g of oil were hydrolysed in 100 mL of an acidic water solution (15 g/L of H₂SO₄) at 120 °C for 44 min under 10 bar of nitrogen atmosphere (used to maintain the water in liquid phase) following the method proposed by Bennett et al. [29]. Samples were taken (by a sampling system, see Figure S2b) before heating the reactor, when the target temperature was reached (t=0min, beginning of isothermal conversion at 120°C), after 20 min of reaction at 120°C and at the end, after 44 min of reaction at 120°C. The final solution was then recovered and stored for further analysis and fermentation.

2.4 Microorganism and media

Clostridium acetobutylicum ATCC 824 was selected as an interesting strain for the conversion of cellulose-derived products based on the previous work of our laboratory and of literature. Indeed its capability to use a wide variety of saccharides and to produce platform molecules such as butanol has been demonstrated. Its efficiency under mixtures of glucose/cellobiose (representing cellulose derived sugars) was studied by the authors in a previous work. These previous experiments serve as a control with a common carbon source (glucose) in order to characterize the activity of this strain. ATCC 824 spores were maintained in Difco™ Reinforced *Clostridial* Medium (RCM) at

182 ambient temperature [42]. All precultures were rigorously conducted in a similar way. Each
experiment was started with the spores of *C. acetobutylicum*. The spore culture was diluted to a
184 concentration of 10 % in 10 mL of RCM fresh media (Hungate tubes) and then heat shocked at
80 °C for 20 min to induce germination. Reactivated cultures were incubated in fresh RCM medium
186 at 37 °C for 12 h and then used to inoculate the corresponding cultures containing the bio-oil
within the synthetic medium. The synthetic medium was composed of 0.5 g/L KH₂PO₄, 1.5 g/L
188 (NH₄)₂SO₄, 1 g/L MgCl₂, 0.15 g/L CaCl₂, 1.5 g/L yeast extract, 0.01 g/L FeSO₄·7H₂O, 0.01 g/L
MnSO₄·H₂O, 4x10⁻⁵ g/L biotin. All the chemicals, yeast extract and biotin were provided by Sigma
190 Aldrich.

192 2.5 Fermentation

Batch fermentations were carried out in 50 mL fermentation vials. 40 mL of the synthetic media
194 and 5 mL of each hydrolysed pyrolytic oil were transferred to the fermentation vials and then
sterilized in an autoclave. The vials were initially purged with nitrogen to ensure an anaerobic
196 atmosphere and then inoculated with 5 mL of pre-culture. The medium pH was set to 5 before
inoculation.

198 Throughout all fermentation experiments, temperature was maintained at 37 °C. The starting sugar
concentrations resulted from the pyrolysis-hydrolysis are provided in the results section. Cell
200 growth was impossible to monitor by spectrophotometry due to the dark colour of the mixture
after mixing with the bio-oil sample, but the formation of products is a clear indicator of cell
202 growth. Samples were taken every 12 h. Each experiment was carried out until there was no more
substrate consumption for a maximum of 72 h. All the experiments were performed at least in
204 duplicate. Other fermentability tests with pure glucose and levoglucosan were performed in the
same conditions.

2.6 Analysis of liquids

2.6.1 Total sugars quantification by the phenol/sulphuric acid method

Total sugars present in pyrolysis oil, hydrolysed oil and after fermentation were quantified using the phenol/sulphuric acid assay [43]. The absorbance was measured in a spectrophotometer Multiskan™ GO (Thermo Scientific™) at 490 nm. Glucose was used to determine the calibration curves and the total carbohydrates were calculated as glucose-equivalent. Total non-sugar compounds were calculated as the difference between the total water-soluble compounds (weighted after lyophilisation) and the total carbohydrates.

2.6.2 Analysis of pyrolysis products by GC-FID/MS

Analysis of the bio-oil fractions was performed using gas chromatography coupled with a flame ionization detector and mass spectrometry (GC-FID/MS). 2 g of each bio-oil fractions from the staged condensation system were dissolved in 20 mL methanol. 1 µL of tetradecene was added to the 20 mL as an internal standard. Then, 1 µL of the solution was injected in an Agilent 7890 gas chromatograph with an Agilent HP-5MS column (diphenyl -5% - dimethylpolysiloxane -95%), FID and MS [44]. A split ratio of 10 was used. The temperature program was: 40°C (hold 10 min), then increased 5°C/min until 200°C (hold 21 min). The relative response factor were calculated according to the predictive method of Saint-Laumer et al. [45].

2.6.3 Analysis of levoglucosan and cellobiosan by HILIC-LTQ-Orbitrap MS

Levoglucosan and cellobiosan present in the real pyrolysis oil was monitored during the hydrolysis step by using hydrophilic interaction liquid chromatography coupled to a linear trap quadrupole Orbitrap mass spectrometer analyser (HILIC-LTQ-Orbitrap MS) using a YMC-Pack Polyamine II (250 mm x 4.6 mm) column guarded by a 7.5 mm x 4.6 mm pre-column cartridge, both from YMC Europe GmbH (Dinslaken, DE). The method was presented in a previous article [46].

232

2.6.4 Analysis of sugars by HPAEC-PAD

234 Glucose and cellobiose were analysed by high performance anion exchange chromatography with
pulsed amperometric detection (HPAEC-PAD) [46]. The column used was a CarboPac PA100.
236 The HPLC system was equipped with a quaternary solvent delivery pump. Two solvents were used
in this analysis: de-ionized water (A) and a 100 mM sodium hydroxide solution (B). The method
238 uses a gradient program as follow: 5 min of stabilization at 25 % B, then from 0 to 5 min 25 % B,
from 5 to 10 min a gradient from 25 to 100 % of B and from 10 to 25 min 100 % B.

240

2.6.5 Analysis of the fermentation products by HPLC-UV

242 Acetone, ethanol, butanol, acetic acid, lactic acid and butyric acid concentrations were measured
with a HPLC equipped with a refractive index detector and an ultraviolet-visible spectroscopy
244 (HPLC-RID-UV), using an Aminex HPX 87h column. The samples were filtered with a 0.2 μ m
filter and the injection volume was 10 μ L. The oven of the column was kept at 45 °C. The mobile
246 phase was a 25 mM sulphuric acid (H₂SO₄) solution. The analysis time was 35 min in isocratic
mode.

248

3 Results

250 First, the results on the fast pyrolysis of cellulose are presented in section 3.1. Then the hydrolysis
of bio-oil (section 3.2.) and their fermentation (section 3.3.) are displayed. Finally, the integration
252 of the 3 process steps is discussed in section 3.4.

3.1 Pyrolysis of cellulose

Cellulose pyrolysis in the fluidized bed reactor has produced 73, 22 and 5 wt.% of bio-oil, permanent gas and char respectively, as shown in Figure 3.

Insert Figure 3 here.

The objective of this work was to produce the highest possible yield of bio-oil that can be converted into value-added chemicals by fermentation. The concentration in inhibitory compounds (aldehydes and carboxylic acids) in the bio-oil should be low for the microorganisms used in the fermentation process. Three important features of this pyrolysis set-up enable achieving this goal. The first one is the pulsed intermittent feeder which promotes a good contact between cellulose particles and the hot sand bed leading to a high heating rate and a high yield in bio-oil [47]. The second feature is the staged condensation system for product recovery. Moreover, the vapour residence time in the hot zone of the fluidized bed reactor is short (2.2 s) in order to reduce secondary conversion of levoglucosan [48,49]. The bio-oil was collected with the 5-stage condensation system allowing the separation of the anhydro-saccharides from the lighter compounds (e.g. acetic acid, furans, ketones, etc.). The bio-oils collected from condensers 1, 2 and 3 are called fractions C1, C2 and C3, respectively. The bio-oil collected from condensers 4 and 5 was mixed in one single fraction called C4, since these two condensers are in parallel and at the same temperature. These staged condensers resulted in bio-oil fractional yields (calculated relative to the initial mass of cellulose) of 45, 19, 5 and 4 wt.% for the fractions C1, C2, C3 and C4 respectively. Fractions C1 and C2 consisted of a viscous sugar-rich oil that solidified at ambient temperature. These two condensers represented 87.8 wt.% of the whole bio-oil and they were submitted to subsequent fermentation.

Fractions C3 and C4 were significantly clearer and less viscous than fractions C1 and C2. These two fractions represented, when combined, only 12.2 wt.% of the whole bio-oil and their main purpose was to collect compounds that would inhibit further biological conversion.

The temperature of the gas leaving the reactor was 333 °C. The temperature of the first condenser was set to 70 °C. In this way, most of the levoglucosan and other anhydro-saccharides with higher condensation point was trapped in this first condenser. The temperature of the second condenser was set to 45 °C, in order to recover the remaining anhydro-saccharides. The second condenser was a warm electrostatic precipitator (ESP) in order to recover aerosols formed after the first condenser [41]. This warm ESP presents an interesting feature in order to control the recovery of anhydro-sugars while limiting the condensation of lighter compounds rich in inhibitors for the fermentation. More details about the efficiency of the ESP system can be found in Bedmutha et al. [ref]. The bath temperatures of the downstream condensers were set at 5°C so that the lighter compounds were collected in the last condensers.

The composition and chemical nature of the compounds in these fractions is of crucial importance for a successful fermentation. For this reason, GC/MS-FID characterization was conducted on each fraction. The yield in total sugars was analysed by the phenol/sulphuric acid method. The mass yields of the individual and total saccharides quantified in the bio-oil fractions are presented in Table 2. The sum of individual sugars' yields (as quantified by GC/FID) is lower than the total sugars yield (analysed by the phenol/sulphuric acid method) because some heavy sugars (like cellobiosan) cannot be analysed by gas chromatography.

Insert Table 2 here.

Figures S3a, S3b and S3c, in the supplementary material, show the chromatograms corresponding to the bio-oil fractions C1, C2 and C3 respectively. Fraction C4 did not contained any sugar

(according to the total sugars analysis) and was not suitable for this GC analysis due to its high content of water.

Fractions C1 and C2, presented a successful separation of levoglucosan with almost no light compounds (e.g. carboxylic acids, aldehydes, ketones), as presented in Figure S3a and b. These 2 fractions are mainly composed of anhydrosugars. We were not able to identify by GC/MS the very minor peaks which may be other compounds than anhydrosugars.

Levoglucosan was mainly recovered in the first condenser (19.8wt% based on cellulose, see Table 2) and in the second one (C2: 6.8 wt.%). It is selectively recovered in the first 2 condensers representing 99% of produced LVG. This LVG yield is similar to that reported by Piskorz et al. [48] on a continuous fluidized bed reactor. However, it is lower than that reported by Patwardhan et al. [49]. This is probably because their vapour residence time is lower (~1.3 s), so the secondary reactions are less important in their reactor. Westerhof et al. also showed a yield of 40 wt.% with a vapour residence time of 1.6 s [50]. Small quantities of levoglucosan were also condensed in fraction C3 but no other saccharide was found in this fraction with the analysis performed in this work. The presence of levoglucosan in fraction C3 may be explained by interactions with lighter compounds which may reduce its condensation point. Levoglucosan may also come from fine mist droplets escaped from the electrostatic precipitator C2. C3 is mainly composed of glycolaldehyde, 1-hydroxy-2-propanone, 3-hydroxy-2-butanone, furfural (etc.) which are known products from cellulose pyrolysis [17,51].

Other products of cellulose dehydration reactions identified and quantified by GC-FID-MS were

1,6-anhydro- β -D-glucofuranose, levoglucosenone and 1,4:3,6-dianhydro- α -D-glucopyranose.

1,6-anhydro- β -D-glucofuranose was the second major product found in fractions C1 and C2. This compound has been previously reported as an important pyrolysis product in the literature [52–54]. This compound is an isomer of levoglucosan, with a subsequent rearrangement of the glucopyranose unit at the non-reducing end. Any anhydrous end among the oligomeric compounds are potential precursors of furanolic ends [55].

1,4:3,6-Dianhydro- α -d-glucopyranose and levoglucosenone were only detected in fraction C2 (Table 2). These results show that the conditions of C2 (ESP) are well targeted to collect efficiently these compounds, as it was previously shown in the work done by Pollard et al. [17].

Overall, total sugars represented 80.4 and 59.7 wt.% of the bio-oil fractions C1 and C2 respectively, making them potential substrates for subsequent fermentation.

In all the cases, the sum of the saccharides quantified by GC is less than the total sugars' yields. Other saccharides are not identified by GC analysis. Indeed, the main anhydro-saccharides detected by GC-FID-MS are only monomeric due to the limitation of this analytical method to quantify only relatively volatile compounds. Besides, it has been proven that around 35 % of cellobiosan is converted into levoglucosan inside the GC injector, generating erroneous yields and misunderstandings of the pyrolysis phenomena [56].

For this reason, the two first fractions, C1 and C2, were analysed by HILIC-LTQ-OrbitrapMS looking for any heavy compounds. Nonetheless, cellobiosan was identified only in the C1 fraction. Cellotriosan in the bio-oils was also looked for by HILIC-Orbitrap MS. However, none of bio-oil fractions contained any trimers around this m/z . This compound has higher condensation points than the reaction temperature. It is most probably not devolatilized and further converted to char or volatiles [57,58]. If it was ejected and transferred in aerosol, it may undergo secondary reactions.

These data show that pyrolysis with staged condensation provided a bio-oil fraction with a high concentration of sugars. The sugar-rich fractions (C1 and C2) and the combination of all bio-oil fractions (F) have been used as substrate in a fermentation process using *C. acetobutylicum* in order to produce building blocks. This will be discussed in section 3.3. Fractions C3 and C4 contained too high concentrations of acids and too low concentration in sugars for fermentation. These fractions can be used for other purposes in an integrated biorefinery for instance for biomass leaching prior to pyrolysis [59].

Fermentation tests were performed with these bio-oil fractions and with pure levoglucosan showing no bacterial growth. Cellobiosan was also present in fraction C1. The fermentation test of C1 fraction was also negative. To the best of our knowledge, this work reports for the first time the non-fermentable nature of cellobiosan when used as substrate to cultivate *C. acetobutylicum*. These results show that bio-oils fraction, levoglucosan and cellobiosan are not directly fermentable by *C. acetobutylicum*. These will be discussed further in section 3.3. For this reason, a hydrolysis stage was implemented in order to obtain fermentable saccharides from anhydro-saccharides.

3.2 Hydrolysis of cellulose pyrolysis oils

The anhydro-saccharides produced by pyrolysis, collected in fractions C1 and C2, were hydrolysed in diluted acidic media in order to produce sugars fermentable by *C. acetobutylicum*. Mass yields before and after hydrolysis of the fractionated and complete bio-oil (all fractions) are presented in Figure 4.

Insert Figure 4 here.

This mild hydrolysis enables the fast hydration of levoglucosan and other anhydro-saccharides producing (mostly) glucose.

Total sugars yields (based on cellulose mass) were 49.2, 36.4, 11.4 and 1.3 wt.% for the bio-oils total fractions (F), C1, C2 and C3 respectively. Levoglucosan yields were 27 wt.% for the total fraction F, 19.8 and 6.8 wt.% for the fractions C1 and C2. After hydrolysis, the total sugars yield slightly decreased in all fractions obtaining values of 42.9, 33.6, 8.9 and 0.5 wt.% for the bio-oils F, C1, C2 and C3 respectively. In all the cases, there was no levoglucosan present in the samples after the hydrolysis stage. The loss of total sugars during hydrolysis is probably due to the overreaction (degradation) of sugars into aldehydes and carboxylic acids by dehydration and decarboxylation reactions [60]. One advantage of performing this hydrolysis stage is the conversion of anhydro-

sugars with DP>2 (Degree of polymerization) into sugars (such as cellobiose). The fermentable
behaviour of these sugars has been previously studied [61,62]. HILIC-LTQ-Orbitrap MS has been
used to study more in depth the conversion of levoglucosan and cellobiosan present in the C1
fraction during their hydrolysis.

Figure S4 displays the SIM mode spectra of levoglucosan, glucose, cellobiosan and cellobiose
during the hydrolysis of fraction C1. An isomer of cellobiosan was identified in the initial pyrolysis
C1 fraction. Small quantities of levoglucosan were hydrolysed into glucose when the sample was
dissolved for analysis, whereas no significant quantities of cellobiose were detected at this time
(Figure S4a). Formation of glucose and cellobiose was important when the acid was added and the
reactor heated from ambient temperature to 120°C, as displayed in Figure S4b. Then, during the
44 min of reaction, cellobiose decreases probably hydrolysed into glucose. A simplified scheme
presenting the hydrolysis of LVG and cellobiosan in real bio-oils based on this HILIC-Orbitrap
MS analysis is presented in Figure 5.

Insert Figure 5 here.

Mild acid hydrolysis enables the successful recovery of 92.3 and 78.1 % of the sugars in fractions
C1 and C2. Glucose yields of the hydrolysed samples are higher than the theoretical yield for the
conversion of only levoglucosan. Therefore, these data show that this hydrolysis stage allowed not
only to convert practically all the levoglucosan, but also other pyrolysis-derived saccharides as
glucose (most probably cellobiosan). Table 3 presents a comparison between the main processes
for cellulose depolymerisation.

Insert Table 3 here.

It is known that enzymatic hydrolysis allows cellulose depolymerisation with high selectivity. However, this process presents relatively high reaction times and operational cost due to the price of enzymes and their complex recycling [13]. The hydrothermal depolymerisation in both batch and continuous reactor presented similar and higher yield to pyrolysis respectively. However, these processes dilute the sugars of interest and entrained various by-products in the water flow [63,64]. Fast pyrolysis offers an interesting alternative to produce sugars from cellulose with high selectivity for dimers and monomers (thanks to the staged condensation), lower operational cost and faster reaction times. The subsequent mild acid hydrolysis stage of the bio-oil is easily operated with a high selectivity as demonstrated in this present work and in agreement with Bennett et al. [29]. Our HILIC high resolution mass spectrometry analysis reveals that glucose and cellobiose are the major sugar compounds after hydrolysis of the bio-oils and that no other sugars were present in high yields. Therefore, fast pyrolysis combined with mild hydrolysis leads to a good selectivity into glucose and cellobiose and lower dilution (between 35 and 70 g/L) in water than direct hydrothermal conversion of cellulose without the pyrolysis stage) (around 20 g/L) [65–67].

3.3 Fermentation of glucose, levoglucosan and bio-oil fractions

At this point, fermentable bio-oils with few inhibiting compounds have been produced. In order to study any cross effects of potential inhibitory compounds present in the bio-oil, fermentation by *C. acetobutylicum* ATCC 824 of pure levoglucosan, glucose, alone or in mixture, were first evaluated on both synthetic and reinforced clostridial medium (RCM). Results are reported in Table 4. These two culture medias offer the best conditions for the growth of *C. acetobutylicum*.

Insert Table 4 here.

In all cases, glucose was almost completely converted with a conversion degree of around 90 wt.%. However, levoglucosan utilisation by *C. acetobutylicum* is lower than ~ 5 wt.% (table 4), whatever

the culture medium used. In the synthetic medium, pH values do not decrease with levoglucosan (LVG). This point highlights that a low metabolism activity occurs with LVG. Indeed, the drop in pH results usually from organic acids accumulation such as acetic or butyric acids, which are one of the main products produced during the growth phase. In this study, such products are produced in very low amount when levoglucosan was the only source of carbon. The low conversion of levoglucosan could be explained by levoglucosan hydrolysis at very low rate due to the relatively weak acidic environment of the fermentation media.

Besides, fermentation of levoglucosan by *C. acetobutylicum* was never reported in the literature as well as with other clostridium species [68].

This work demonstrates that *C. acetobutylicum* is unable to use levoglucosan probably because this molecule is not present naturally in raw biomasses. Microorganisms are not commonly exposed to levoglucosan and they have not developed tools for its metabolism. Indeed, levoglucosan is a stable marker of forest fires in the environment.

Consequently, the hydrolysis stage to convert levoglucosan into a fermentable substrate is required. The fermentation of hydrolysed bio-oils by *C. acetobutylicum*, using glucose pregrown inocula, were carried out in the same experimental conditions as the ones studied for pure levoglucosan, glucose and levoglucosan/glucose mixtures. When the hydrolysed whole bio-oil (from fraction “F”) is used in a fermentation process, *C. acetobutylicum* did not exhibit any cell growth, suggesting that inhibitors present in important concentrations in fraction F may have a detrimental effect on the cellular activity [68]. On the contrary, when hydrolysed fractions C1 and C2 are used as the carbon source, a normal cell growth with typical fermentation product pattern is observed. These results confirm that staged condensation efficiently ensure the fermentability of ~88 wt.% of the bio-oil (fractions C1 and C2, see table 2) produced by cellulose pyrolysis.

However, the fermentation of hydrolysed bio-oil fractions by *C. acetobutylicum* required a specific care in the initial pH. Indeed, if the pH value is not adjusted upon addition of bio-oil (initial pH between 2 and 3), *C. acetobutylicum* was unable to grow. Therefore, setting the pH value of the culture

media to a value at least superior than the pKa of the main fermentation acidic products (4.7 – 4.8) is of crucial importance. In this work, setting a pH value of 5 before inoculation was shown to be relevant for the cellular activity. In these conditions, fermentation processes of bio-oil fractions C1 and C2 by *C. acetobutylicum* were carried out during 72 h. Kinetics of sugar utilization and metabolites production are shown on Figure 6. Fermentation of the bio-oil fractions was done separately to study the effects of each composition on the fermentation products.

At the beginning of the fermentation process, the total sugar concentrations were 44.5 and 27.8 g/L for the cultures containing fractions C1 and C2 respectively. Fermentation of fraction C1 exhibited a continuous sugar utilization all along the 72 h of culture, with a final sugar residue of 2.8 g/L. In the case of fraction C2, total sugar consumption was consumed during the first 48 h of fermentation, then stopped and its concentration remained stable at a concentration of 4 g/L until the end of the fermentation at 72 h. Both cultures exhibit a normal ABE fermentation pattern, so they could be combined into one sole fermentable fraction during the process. Final pH was analysed at the end of the fermentation (pH 3.5 with hydrolysed C1 and 3.9 with C2).

Under our experimental conditions, the fermentation of the fraction C1 leads to a butyric acid maximum concentration of 3.1 g/L after 24 h of culture, whereas the maximum butyric acid concentration (2.3 g/L) was obtained only after 36 h of fermentation with the fraction C2, probably because of the differences in composition and in concentration of fermentable sugars. As a result, the production of the solvents (ABE) triggered by acids occurs much later with C2 than with C1, leading to a decrease of the overall fermentation productivity for the C2 fraction (figure S6).

Contrary to butyric acid, acetic acid production was similar for both fractions. Indeed, final concentrations were 1 and 1.1 g/L for the fractions C1 and C2 respectively and the slightly lower acetic acid production with C1 may be explained by its conversion into acetone [69]. Maximum concentration of acetic acid was found after 48 and 36 h of fermentation for fractions C1 and C2 respectively. In agreement with fermentation pattern of *C. acetobutylicum* usually observed with glucose and related substrates [70], lactic acid production was relatively low in both fractions

compared to other fermentation products. Total solvent concentration obtained after 72 h of fermentation were 11 and 5 g/L for fractions C1 and C2 respectively. More specifically, after 72 h of fermentation with fraction C1, *C. acetobutylicum* was able to produce up to 8 and 2.9 g/L of butanol and acetone respectively, whereas only 3.3 g/L of butanol and 1.6 g/L were measured with fraction C2 after the same fermentation time. Acetone production started after 24 h of fermentation for fraction C1 and after 12 h for fraction C2. Ethanol was produced in relatively low concentration after the fermentation process of both fractions (see supplementary material, Figure S5). As a general rule, the ratios between the produced butanol, acetone and ethanol agreed with those of a typical fermentation by *C. acetobutylicum* when glucose is the carbon and energy source [42]. Accordingly, the bio-oil fractions C1 and C2 did not contain any strong inhibitors that altered the fermentation product pattern of *C. acetobutylicum*. Concerning the productivities (mg/L/h) of the solvents produced during the fermentation (presented in figure S6), the highest productivities were mainly achieved after 48 h of fermentation, whereas the highest yields (wt.) were obtained after 72 h fermentation (see Figure S6). During the last 24 h of the process, i.e. from 48 to 72 h of fermentation, the productivities of butanol, acetone and ethanol decreased significantly for the 2 hydrolysed fractions C1 and C2.

3.4 Integration of thermochemical and biological conversion

Figure 7 shows the yields in products obtained by pyrolysis of cellulose, then the hydrolysis of the bio-oil and finally the fermentation of the sugars obtained by *C. acetobutylicum*.

Insert Figure 7 here.

First, 73 wt.% of the initial cellulose was converted into bio-oil by fast pyrolysis. The rest of the cellulose becomes gas and char that can be used to recover energy. The overall cellulose to sugars

yield was 49.2 wt.% (see table 2, fraction F). The sugar-rich bio-oil fraction (trapped in the first two condensers) represented 64.4 wt.% of the initial cellulose (88 wt.% of the whole bio-oil) and accounted for 97 wt.% of total sugars produced. LVG yield is 27wt.% (based on initial cellulose weight). Even if the light oil fraction yield of 9 wt.% was significantly lower than that of the sugar-rich fraction, the fermentation test with the total bio-oil (fraction F) proved that the inhibiting nature of these compounds will stop any cell growth. The hydrolysis stage under mild acidic conditions allowed recovering 92 and 78 wt.% of the sugars contained in fractions C1 and C2 respectively, so 33.6 wt.% and 8.9 wt.% on cellulose basis (figure 4). Glucose represents 89 and 70 wt.% of the sugars recovered after hydrolysis of the bio-oils C1 and C2 respectively. The high content of glucose, that is a known fermentable sugar and frequently the preferred substrate of several microorganisms, increases substantially the cellular performance during the fermentation process. The fermentation of the fractionated-hydrolysed bio-oil converts 33 wt.% of the sugars (43%wt. cellulose base) into building blocks, corresponding to a yield of 14 wt.% relative to the initial cellulose. Table 5 presents a comparison between the main process for the production of butanol from different resources.

Insert Table 5 here.

Butanol mass yield from propylene is considerably higher than those of the processes that use biomass as feedstock. But propylene is not a primary sustainable resource and the global efficiency of butanol production from crude oil is not easily accounted. Several research work attempts developing thermochemical and biochemical processes to produce butanol and other chemicals from renewable resources. In the case of butanol chemical synthesis from ethanol, this pathway still requires the production of the ethanol, since it is not a primary resource. The production of butanol from first generation biomass is in competition with alimentary feedstock, which might

provoke socio-political issues. Second generation biomass is commonly depolymerized using
536 enzymes that add compelling production costs to the global process [13].

Therefore, our approach presents potential benefits compared to other production routes of
538 butanol. Indeed, cellulose is the most available bio-macromolecule on Earth. Its depolymerisation
by pyrolysis does not consume costly enzymes or toxic solvents. Moreover, the hydrolysed sugar-
540 rich bio-oil does not require any detoxification to ensure its fermentability. Nevertheless, our
butanol yield could be further improved. The batch fermentation process using *C. acetobutylicum*
542 converts approximately one third of the carbon source (sugars) in gas and another third in other
cells. The yields and selectivity to ABE products could be improved by a continuous fermentation
544 process, avoiding the consumption of sugars for cell growth and a better control of fermentation
conditions [71,72]. Gas produced by *C. acetobutylicum* is a mixture of hydrogen (H₂) and carbon
546 dioxide (CO₂) that can be further valorised. The hydrolysed bio-oils fractions rich in glucose could
be converted following this same approach by other bacteria or yeast such as glutamic acid, 3-
548 hydroxypropionic acid, 1,3-propanediol, succinic acid, isoprene, farnesene, itaconic acid and lactic
acid [73–75].

4 Conclusion

552 This work reports for the first time the combination of pyrolysis and fermentation using *Clostridium*
acetobutylicum. Cellulose pyrolysis with a staged condensation system recovers 88 % of the bio-oil as
554 a sugar-rich fraction separated from the undesired inhibitory compounds.

Anhydro-saccharides produced by fast pyrolysis were not fermentable by *C. acetobutylicum*.
556 However, a mild and fast acid hydrolysis allowed converting 70 % of these anhydro-saccharides
into sugars that were successfully fermentable. The conversion of the hydrolysed oil presented a
558 normal fermentation pattern producing 14 %wt. of building blocks.

Concerning the perspectives of this work, the fermentation of pyrolysis oil produced by lignocellulosic biomasses (like wood) will be a next interesting step forward. Woody biomass could be pre-treated by the acid fraction of the as-produced pyrolysis oil in order to promote the production of anhydrosugars. The fermentation productivity could be further improved notably by designing a continuous reactor.

Then, based on the continuous and optimized operations of the thermochemical and biological reactors, a detailed process model (for instance under Aspen Plus® software) could be developed in order to assess the mass and energy balances of the integrated process. The integrated process must also include the purification processes of chemicals. A technical-economical study of the biorefinery may consider the valorisation of other products than anhydrosugars such as the gas from fermentation and pyrolysis, chemicals in the pyrolysis oils (furans, phenols, glycolaldehyde, etc.) and biochar.

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Figure 1. Scheme of the proposed cellulose biorefinery using fast pyrolysis, hydrolysis and ABE fermentation.

Figure 2. Schematic of the fluidized bed with staged condensation system.

Figure 3. Mass yield of pyrolysis products and bio-oil fractions in the condensers (C1 to C5). All the yields were calculated in reference to the initial cellulose weight.

Figure 4. Total sugars, levoglucosan and glucose mass yields in the corresponding bio-oil fractions. (F=combination of all bio-oil fractions): a) after pyrolysis; b) after pyrolysis followed by hydrolysis

868 **Figure 5.** Simplified mechanism of levoglucosan and cellobiosan hydrolysis in mild acidic media.

Figure 6. Kinetic of sugar consumption and products formation during the fermentation of cellulosic bio-oil obtained after hydrolysis from a) first condenser (C1) and b) second condenser (C2) by *C. acetobutylicum*. Products: acetic acid (dark blue, ●), butyric acid (yellow, ■), butanol (light blue, □), acetone (orange, Δ), total ABE (brown, ◇) and total sugars (grey, x).

Figure 7. Mass yields in main products along the conversion chain: pyrolysis, hydrolysis and fermentation. Sugar-rich fraction=C1+C2 condensers

Table 1. Overview of different works dealing with the biological conversion of cellulose-derived liquid products with a focus on a pretreatment of lignocellulosic material by pyrolysis.

Type of pretreatments	Biological process	Target products (yield)	Advantages	Drawbacks	Refs.
Organosolv (organic acids, 3h, 108°C) cellulose pyrolysis (Pyroprobe, 20 s, 550°C at 10°C/ms)	Not tested (just proposed)	Levoglucosan: (relative yield by GC/MS 42.1-59.8 wt.%)	Fast depolymerization of cellulose producing high bio-oil yields	Pyrolysis technology (Pyroprobe) might not be representative of those used at industrial scale (like fluidized beds).	[26]
Biomass fast pyrolysis, detoxification by activated carbon and hydrolysis	Fermentation of the sugar-rich fraction by yeast	Ethanol (yeast: 0.02 – 0.43 g/ g of substrate)	Fast and low-cost production of sugars. Good detoxification.	Probable carbon sources not accounted. The method was not tested for bacteria.	[76]
Biomass pyrolysis (Fluidized bed reactor, 500°C)	Anaerobic digestion of aqueous pyrolytic condensate	Methane: (262 mL from 1 g/L of acetic acid condensate equivalent)	Valorisation of a pyrolysis aqueous phase by-product	Requires a long period (days) for the inoculum to adapt	[77]
Biomass pyrolysis (Rotatory reactor, 450°C) Detoxification for the recovery of organic acids in the aqueous phase (evaporation, activated carbon)	Fermentation of acetic and formic acids by oleaginous yeast (<i>C. curvatus</i> , <i>R. glutinis</i> and <i>L. starkeyi</i>)	Lipids (11 wt%) Yeast cells (40 wt%)	Valorisation of the pyrolytic aqueous phase to added-value products (lipids)	Requires a detoxification process with drawbacks at industrial level	[78]
Fast pyrolysis of acid washed poplar, acid hydrolysis and activated carbon detoxification	Fermentation producing ethanol by: <i>Saccharomyces cerevisiae</i> , and lipids by: <i>Cryptococcus curvatus</i> and <i>Rhodotorula glutinis</i>	Ethanol (50 wt.% of initial glucose) Lipids (9 - 16 wt.% of initial glucose)	Flexibility of the detoxification method working in both ethanol and lipid fermentation	Require better extraction and detoxification procedures	[79]
Biomass intermediate pyrolysis (fixed bed reactor, 400°C for 10 min at 100°C/min)	Anaerobic digestion of the aqueous pyrolysis liquid with biochar addition	Methane (0.2 mL CH ₄ /mL _{reactor} /day or 65 % of the theoretical yield)	Utilisation of biochar for the conversion of pyrolytic liquid with acceptable rate	Inhibitors slow down the conversion and thus, the methane yield. Methane is a low value product.	[80]
Biomass fast pyrolysis with fractional condensation, hydrolysis	Fermentation of the sugar rich pyrolytic fraction by <i>Saccharomyces cerevisiae</i>	Ethanol (41 % of theoretical yield based on the cellulose fraction)	Advanced fast pyrolysis combined with fractional condensation. Detoxification procedure by liquid extraction.	The fermentation substrate was blended with pure glucose	[81]
Biomass fast pyrolysis with staged condensation	Fermentation by <i>E. Coli</i> genetically modified	Ethanol (0.35 g per g of levoglucosan consumed)	Advanced pyrolysis process. Strain capable of metabolizing	Sensitive to inhibitors present in the raw aqueous pyrolytic liquid	[82]

			levoglucosan directly		
Biomass fast pyrolysis with staged condensation, alkali treatment with sodium hydroxide	Fermentation of acetic-acid rich fraction by microalgae <i>Chlamydomonas reinhardtii</i>	Lipids (only positive cell growth reported)	Effective detoxification of the aqueous bio-oil fraction	Requires a metabolically evolved strain to metabolise the bio-oil fraction	[83,84]
Cellulose fast pyrolysis with staged condensation and hydrolysis	Fermentation of the sugar-rich fraction by <i>Clostridium acetobutylicum</i>	Building blocks (23.7wt.% of the substrate). Butanol (15 wt.% of the sugars)	Simple separation of the sugar-rich fraction without further detoxification. Tolerant and robust strain.	Fermentation stage needs to be up-scaled in a continuous process	This work

888 **Table 2.** Saccharides quantified by GC-FID-MS and Total Carbohydrates Analysis of the bio-oil
 890 fractions produced by cellulose pyrolysis with staged condensation system (C1 to C4: condensers
 1 to 4+5, F: combination of all bio-oil fractions).

Fraction	Compound	Retention Time (min)	Mass yield (wt.% of the oil fraction)	Mass yield (wt.% of cellulose)
C1	Levogluconan	33.6	43.7	19.8
	1,6-Anhydro- β -D-glucofuranose	35.8	3.1	1.4
	<i>Total sugars</i>	-	80.4	36.4
C2	Levogluconanone	21.1	0.17	0.03
	1,4:3,6-Dianhydro- α -d-glucopyranose	24.3	0.41	0.08
	Levogluconan	33.6	35.5	6.8
	1,6-Anhydro- β -D-glucofuranose	35.9	2.49	0.48
	<i>Total sugars</i>	-	59.7	11.4
C3	Levogluconan	33.6	9.6	0.4
	<i>Total sugars</i>	-	29.0	1.3
C4	<i>Total sugars</i>	-	0	0
F	Levogluconanone	21.1	0.04	0.03
	1,4:3,6-Dianhydro- α -d-glucopyranose	24.3	0.1	0.08
	Levogluconan	33.6	36.9	27.0
	1,6-Anhydro- β -D-glucofuranose	35.9	2.6	1.9
	<i>Total sugars</i>	-	71.3	49.2

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Table 3. Comparison of the main methods of cellulose depolymerization into fermentable sugars,
for the same cellulose substrate (Avicel cellulose)

Cellulose conversion method	Reaction Time	Reaction agent	Yield (wt.%)	Comments	Ref.
Enzymatic hydrolysis 50°C and 1 bar	18 h	Enzyme cocktail of cellulase and β -glucosidase	~56	Glucose yield obtained after ball milling. Long cellulose pre-treatment and high cost of enzyme.	[13]
Hydrothermal liquefaction in batch reactor 220°C and 24 bar	120 min	Water	~50	Important secondary reactions occur in batch reactors. Batch process not well intensified for up-scaling. Various by-products diluted in the same water stream as glucose.	[46]
Hydrothermal liquefaction in continuous flow reactor 250°C and 100 bar	80 min	Water	80	Primary sugars obtained in a highly diluted solution under these conditions of flow reactor. Various by-products diluted in the same water stream as glucose.	[60,85]
Direct mild acid hydrolysis of cellulose (Whatman no. 1 paper, not Avicel in this case) 215 °C and 345 bar	120 min	Percolating flow of weak (5 mM) acid (H_2SO_4)	71	Glucose yield depends on complex diffusion resistances for released glucose to the bulk medium, which is a function of the reactor configuration.[65] Various oligomers and by-products produced.[12,66]	[12,65,86,87]
Fast pyrolysis in fluidised bed reactor followed by hydrolysis Pyrolysis: 475°C and 1 bar Hydrolysis: 120°C and 10 bar	Pyrolysis: few seconds Hydrolysis: 44 min	Heat then mild-acid (H_2SO_4)	43	Yield of total fermentable sugars. Requires strain capable of fermenting multiple sugars.	This work

Table 4. Levoglucosan, glucose and glucose/levoglucosan mixtures consumption by *C. acetobutylicum* ATCC 824 grown on two different culture media. (Initial pH was 5 in all cases)

Type of culture media	Initial Glucose (g/L)	Initial Lvg* (g/L)	% Glucose used	% Lvg used	Final pH
Synthetic media	0	5.1	0	1.1	5.8
	4.9	0.0	90.2	0	4.9
	4.7	5.2	100.0	3.6	4.7
	9.6	4.6	89.9	5.2	4.3
Reinforced clostridial medium (RCM)	5.7	0.0	89.3	0.0	4.8
	5.0	5.5	89.4	3.8	4.8

Table 5. Comparison of this approach with other butanol production process

Type of resource	Process	Butanol mass yield	Advantages	Drawbacks	Ref.
Crude oil (propylene)	Chemical synthesis Oxo process	75 - 95 wt.% based on propylene and not crude oil	High yield in a two-step process based on propylene after refining	Fossil feedstock. Requires low pressures and modified Rh-catalyst Mass yield integrated in multiproduct oil refinery	[88]
Ethanol	Chemical synthesis	50 – 80 wt.%	High yields. Possibility of renewable feedstock.	High production cost involving three reaction steps. Ethanol is not a primary biomass resource (production efficiency).	[89,90]
Syngas (CO and CO ₂)	Hexanol-butanol-ethanol (HBE) fermentation	~ 50 % of CO conversion. 1.6 – 2.3 g/L of butanol (in a 1.2 L reactor medium).	Industrial waste syngas as feedstock	Tar present in syngas must be managed. Mass transfer between gas and liquid (low solubility of syngas) is a major bottleneck for up-scaling syngas fermentation.	[91,92]
Alimentary biomass (as corn, starch, glucose)	Acid hydrolysis and ABE fermentation	18 – 35 wt.%*	Renewable feedstock	Feedstock in competition with alimentary feedstocks	[93,94]
Lignocellulosic biomass (switchgrass)	Enzymatic hydrolysis and ABE fermentation	23 – 24 wt.%*	Renewable and non-alimentary feedstock	High cost of enzymes. Complex detoxification procedures required.	[95,96]
Cellulose	Pyrolysis and ABE fermentation	15 wt.%*	Renewable and non-alimentary feedstock. No enzyme or solvent for the depolymerisation	Further up-scaling in continuous fermentation reactor should be tested.	This work

*Yield expressed per gram of sugar