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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

Table of content (only for reviews)

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]. 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 boiling 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-

80 treatment of biomass for its demineralization in order to increase the sugars yield after fast pyrolysis [19].

82 Some molecules (glutamic acid, 3-hydroxypropionic acid, isoprene, farnesene, itaconic acid, lactic acid, etc.) [20] can only be synthetized through biological conversion involving enzymes or 84 microorganisms. For instance, fermentation can convert the saccharides into various chemicals of industrial interest such as: butanol, ethanol, xylitol, sorbitol, 1,3-propanodiol, biodiesel, lactic acid, 86 succinic acid, isoprene, hydrogen, among others [21,22].

Therefore, it is of tremendous interest to combine pyrolysis of cellulose for a fast depolymerisation 88 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 90 conversion of cellulose pyrolysis oils.

92 **Insert Table 1 here.**

94 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 96 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 98 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 100 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 102 are suitable for industrial production for two main reasons: 1) its adaptability to different environments (pH, temperature, etc.) without adjusting carbon and nitrogen inputs (flow rate and 104 hydrodynamic), 2) its simplicity of homogenization in the multiphase bioreactor, and 3) the capability of monitoring cell density in a relatively short time [30].

106 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
108 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
110 important microbial adaptation and a good resistance to various inhibitors [35,36].

Another promising way is the fermentation of syngas produced by biomass gasification using
112 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
114 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
116 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
118 but it seems more favourable to convert cellulosic biomass into soluble sugars (than into a syngas) for a further fermentation process.

120 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.

122

Insert Figure 1. here.

124

Cellulose was pyrolyzed in a fluidized bed reactor using a fractional condensation train to produce
126 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
128 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).
130 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 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 bed was fluidized using 1033 g of quartz sand. The fluidization gas was nitrogen at 25 standard litres per minute (SLPM) and compressed air was used to activate the valve of the pulsating feeding system [40]. The average temperature inside the reactor was 475 °C, with a vapour residence time of 2.2 s. 0.5 kg of cellulose was pyrolyzed during 30 min. 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 -20ºC 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 200 mL of an acidic water solution (1.5 wt.% of H_2SO_4) 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

Spores of *Clostridium acetobutylicum* ATCC 824 were maintained in Difco™ Reinforced *Clostridial* Medium (RCM) at 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 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 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 KH_2PO_4, 1.5 g/L $(NH_4)_2SO_4$, 1 g/L $MgCl_2$, 0.15 g/L $CaCl_2$, 1.5 g/L yeast extract, 0.01 g/L $FeSO_4.7H_2O$, 0.01 g/L $MnSO_4.H_2O$, $4x10^{-5}$ g/L biotin. All the chemicals, yeast extract and biotin were provided by Sigma Aldrich.

2.5 Fermentation

Batch fermentations were carried out in 50 mL fermentation vials. 40 mL of the synthetic media 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 atmosphere and then inoculated with 5 mL of pre-culture. The medium pH was set to 5 before inoculation.

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 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 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 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 MultikanTM 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

206 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
208 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
210 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: 40ºC (hold 10 min), then
212 increased 5ºC/min until 200ºC (hold 21 min). The relative response factor were calculated according to the predictive method of Saint-Laumer et al. [45].

214

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

216 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
218 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
220 Europe GmbH (Dinslaken, DE). The method was presented in a previous article [46].

222 2.6.4 Analysis of sugars by HPAEC-PAD

Glucose and cellobiose were analysed by high performance anion exchange chromatography with
224 pulsed amperometric detection (HPAEC-PAD) [46]. The column used was a CarboPac PA100. The HPLC system was equipped with a quaternary solvent delivery pump. Two solvents were used
226 in this analysis: de-ionized water (A) and a 100 mM sodium hydroxide solution (B). The method uses a gradient program as follow: 5 min of stabilization at 25 % B, then from 0 to 5 min 25 % B,
228 from 5 to10 min a gradient from 25 to 100 % of B and from 10 to 25 min 100 % B.

2.6.5 Analysis of the fermentation products by HPLC-UV

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 (HPLC-RID-UV), using an Aminex HPX 87h column. The samples were filtered with a 0.2 µm filter and the injection volume was 10 µL. The oven of the column was kept at 45 °C. The mobile phase was a 25 mM sulphuric acid (H_2SO_4) solution. The analysis time was 35 min in isocratic mode.

3 Results

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 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 fast 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 dew 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. The bath temperatures of the downstream condensers were set at 5°C so that the lighter compounds were collected in the last condensers.

280 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
282 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
284 in Table 2.

286 **Insert Table 2 here.**

288 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
290 (according to the total sugars analysis) and was not suitable for this GC analysis due to its high content of water.

292 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.

294 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
296 representing 99% of produced LVG. This LVG yield is similar than that reported by Piskorz et al. [48] on a continuous fluidized bed reactor. However, it is lower than that reported by Patwardhan
298 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 shown a yield of 40 wt.% with a
300 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
302 presence of levoglucosan in fraction C3 may be explained by interactions with lighter compounds which may reduce its dew point. Levoglucosan may also come from fine mist droplets escaped
304 from the electrostatic precipitator C2. C3 is mainly composed of glycolaldehyde, 1-hydroxy-2-

propanone, 3-hydroxy-3-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-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 of the molecule, finishing in the characteristic heterocycle of furanoses. Any anhydrous end among the oligomeric compounds are potential precursors of furanosic 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 dew points than the

330 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.

332

These data show that pyrolysis with staged condensation provided a bio-oil fraction with a high 334 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 336 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 338 fractions can be used for other purposes in an integrated biorefinery for instance for biomass leaching prior to pyrolysis [59].

340 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 342 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*. 344 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 346 was implemented in order to obtain fermentable saccharides from anhydro-saccharides.

348 3.2 Hydrolysis of cellulose pyrolysis oils

The anhydro-saccharides produced by pyrolysis, collected in fractions C1 and C2, were hydrolysed 350 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 352 Figure 4.

354 **Insert Figure 4 here.**

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

358 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 360 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, 362 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 364 (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- 366 sugars with DP>2 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 368 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 370 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 372 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 374 reactor heated from ambient temperature to 120ºC, as displayed in Figure S4b. Then, during the 44 min of reaction, cellobiose decreases probably hydrolysed into glucose. A simplified scheme 376 presenting the hydrolysis of LVG and cellobiosan in real bio-oils based on this HILIC-Orbitrap MS analysis is presented in Figure 5.

378

Insert Figure 5 here.

380

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 are highly energy demanding due to the high quantity of solvent (water in this case). This also dilutes 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 in water than direct hydrothermal conversion of cellulose (without the pyrolysis stage) [65–67].

406 ## 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
408 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
410 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.*

412

Insert Table 4 here.

414

In all cases, glucose was almost completely converted with a conversion degree of around 90 wt.%.
416 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
418 (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
420 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
422 levoglucosan could be explained by levoglucosan hydrolysis at very low rate due to the relatively weak acidic environment of the fermentation media.

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

426 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
428 levoglucosan and they have not developed tools for its metabolism. Indeed, levoglucosan is a stable marker of forest fires in the environment.

430 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

508 initial cellulose. Table 5 presents a comparison between the main process for the production of butanol from different resources.

510

Insert Table 5 here.

512

Butanol mass yield from propylene is considerably higher than those of the processes that use 514 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 516 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 518 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 520 provoke socio-political issues. Second generation biomass is commonly depolymerized using enzymes that add compelling production costs to the global process [13].

522 Therefore, our approach presents potential benefits compared to other production routes of butanol. Indeed, cellulose is the most available bio-macromolecule on Earth. Its depolymerisation 524 by pyrolysis does not consume costly enzymes or toxic solvents. Moreover, the hydrolysed sugar-rich bio-oil does not require any detoxification to ensure its fermentability. Nevertheless, our 526 butanol yield could be further improved. The batch fermentation process using *C. acetobutylicum* converts approximately one third of the carbon source (sugars) in gas and another third in other 528 cells. The yields and selectivity to ABE products could be improved by a continuous fermentation process, avoiding the consumption of sugars for cell growth and a better control of fermentation 530 conditions [71,72]. Gas produced by *C. acetobutylicum* is a mixture of hydrogen (H_2) and carbon dioxide (CO_2) that can be further valorised. The hydrolysed bio-oils fractions rich in glucose could 532 be converted following this same approach by other bacteria or yeast such as glutamic acid, 3-

hydroxypropionic acid, 1,3-propanodiol, succinic acid, isoprene, farnesene, itaconic acid and lactic acid [73–75].

4 Conclusion

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 a sugar-rich fraction separated from the undesired inhibitory compounds.

Anhydro-saccharides produced by fast pyrolysis were not fermentable by *C. acetobutylicum*. 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 normal fermentation pattern producing 14 %wt. of building blocks. The fermentation productivity could be further improved notably by designing a continuous reactor.

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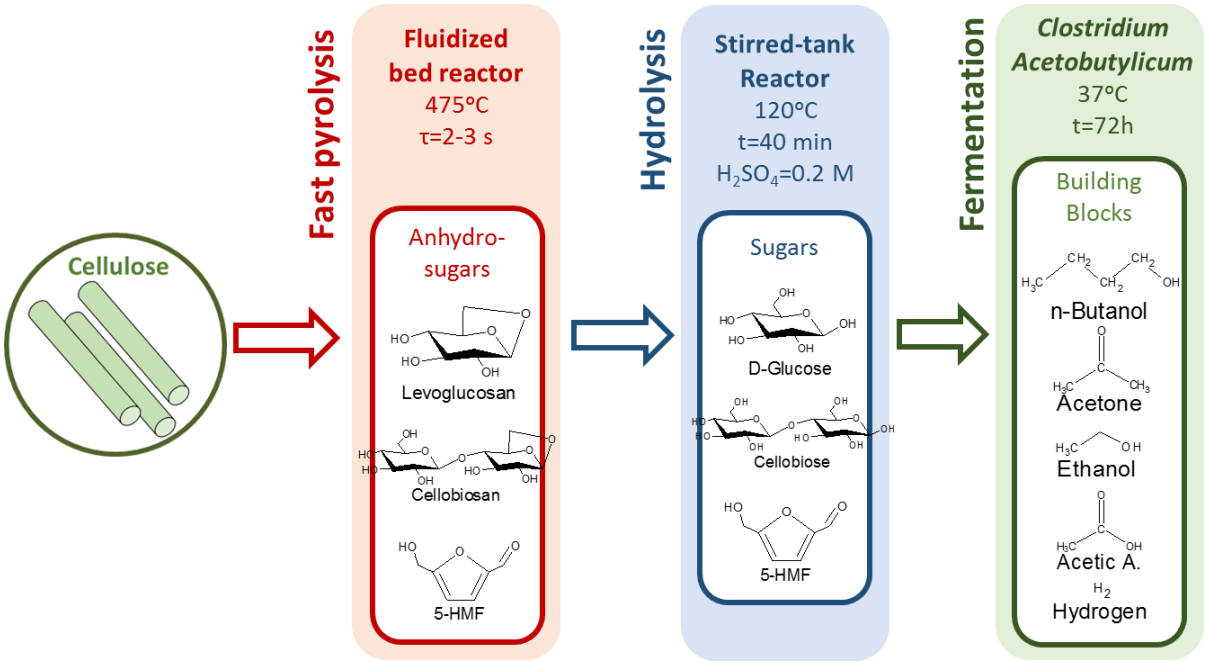

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Figure 1. Scheme of the proposed cellulose biorefinery using fast pyrolysis, hydrolysis and ABE fermentation.

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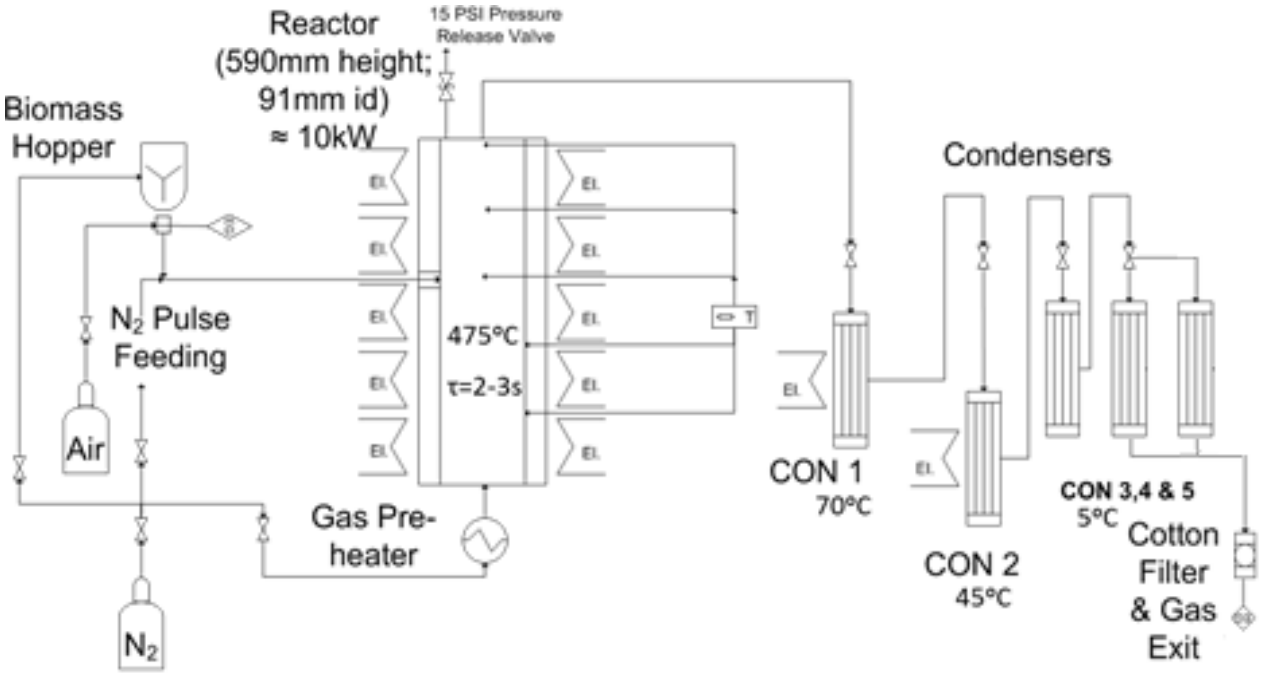

828 **Figure 2.** Schematic of the fluidized bed with staged condensation system.

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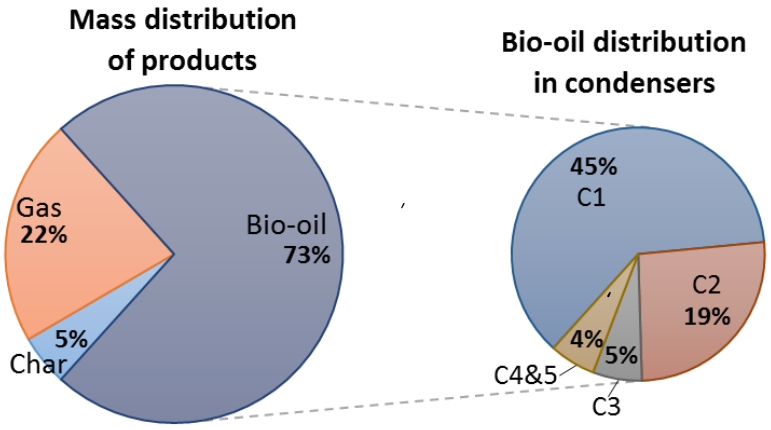

832 **Figure 3.** Mass yield of pyrolysis products and bio-oil fractions in the condensers (C1 to C5). All the yields were calculated in reference to the initial cellulose weight.

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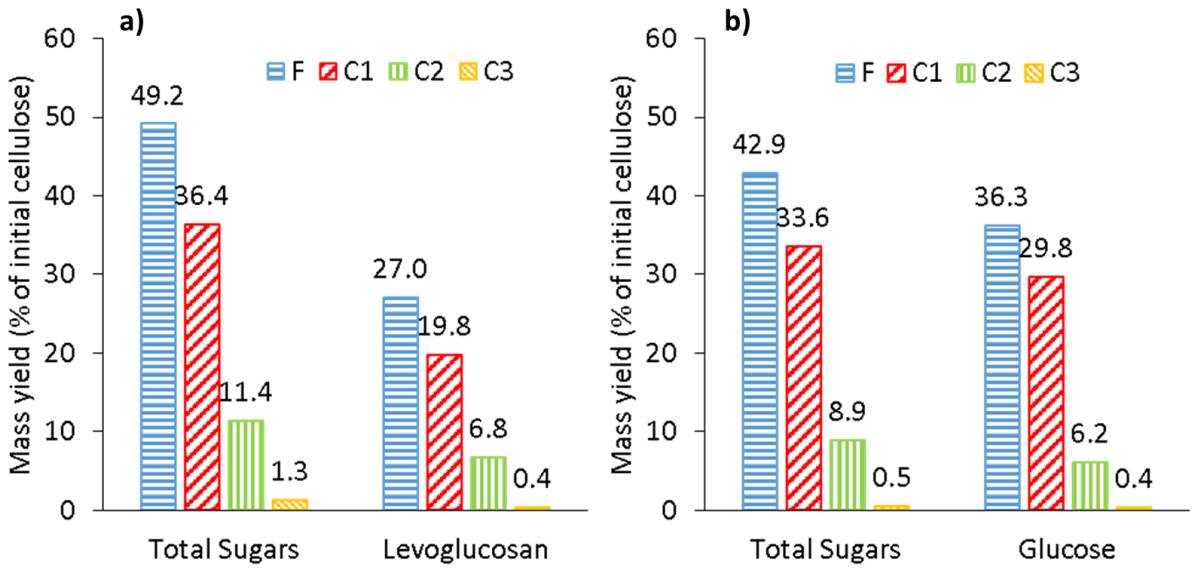

836 **Figure 4.** Total sugars, levoglucosan and glucose mass yields in the corresponding bio-oil
fractions. (F=combination of all bio-oil fractions): a) after pyrolysis; b) after pyrolysis followed by
838 hydrolysis

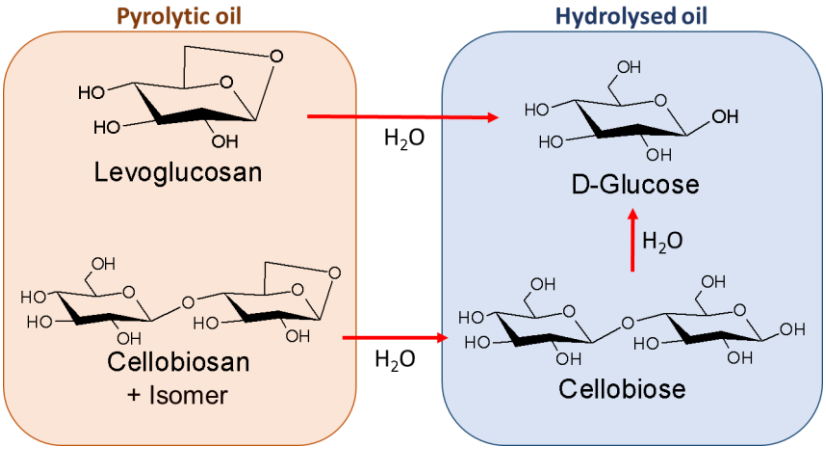

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Figure 5. Simplified mechanism of levoglucosan and cellobiosan hydrolysis in mild acidic media.

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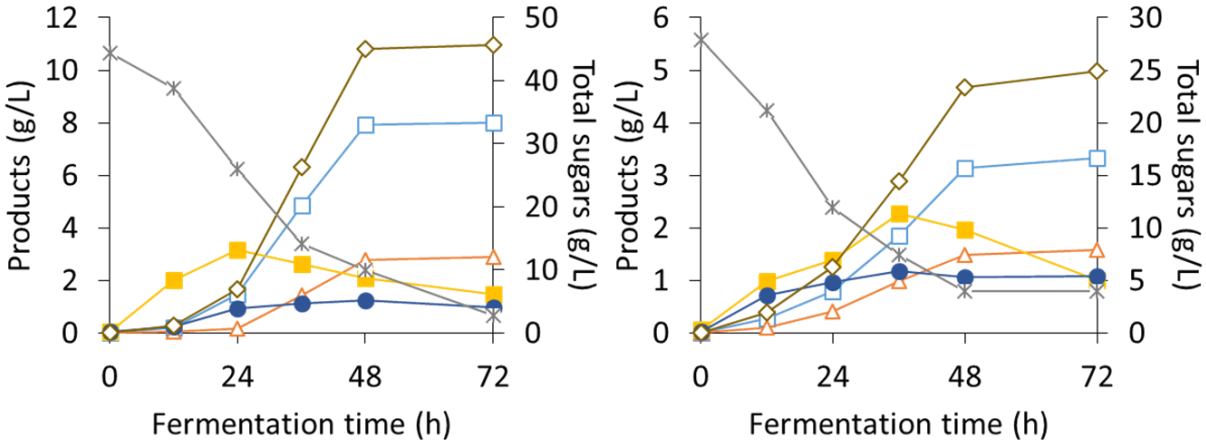
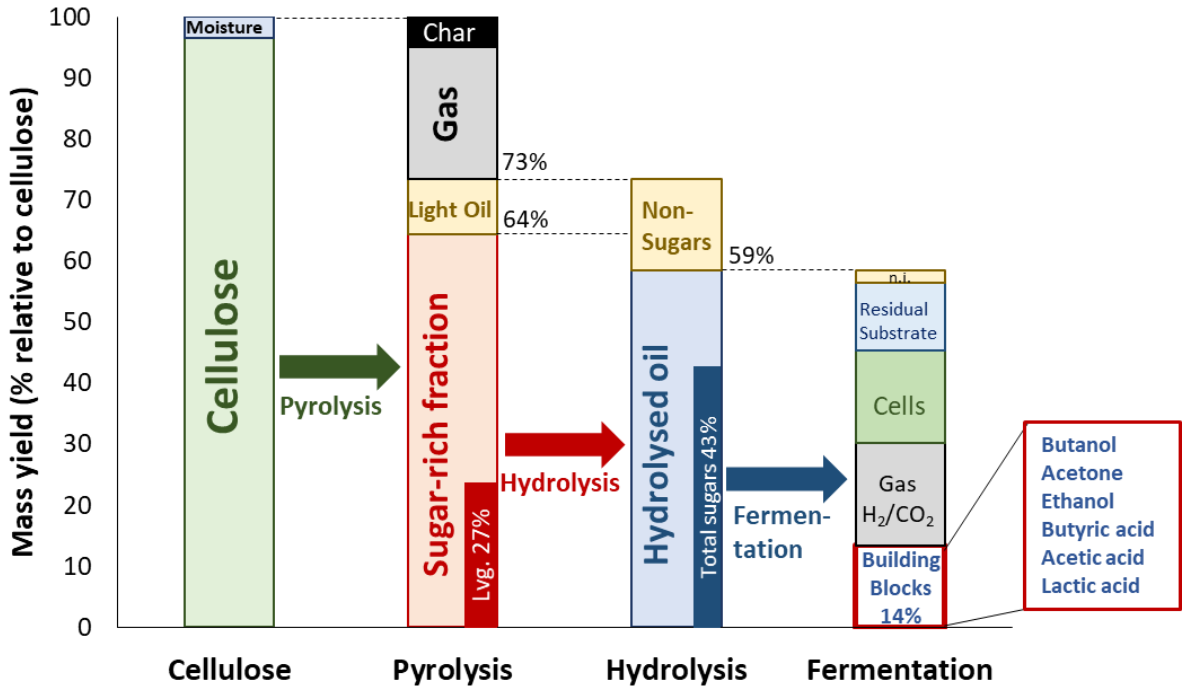


844 **Figure 6.** Kinetic of sugar consumption and products formation during the fermentation of cellulosic bio-oil obtained after hydrolysis from a) first condenser (C1) and b) second condenser
846 (C2) by *C. acetobutylicum*. Products: acetic acid (dark blue, ●), butyric acid (yellow, ■), butanol (light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

848



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

854

856 **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.

858

Type of pretreatments	Biological process	Target products (yield)	Advantages	Drawbacks	Refs.
Organosolv (organic acids, 3h, 108ºC) cellulose pyrolysis (Pyroprobe, 20 s, 550ºC at 10ºC/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, 500ºC)	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, 450ºC) Detoxification for the recovery of organic acids in the aqueous phase (evaporation, activated carbon)	Fermentation of acetic and formic acids by oleaginous yeast (*C. curvatus, R. glutinis and L. starkeyi*)	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: *Saccharomyces cerevisiae,* and lipids by: *Cryptococcus curvatus* and *Rhodotorula glutinis*	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, 400ºC for 10 min at 100ºC/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 *Saccharomyces cerevisiae*	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 E. Coli 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 *Chlamydomonas reinhardtii*	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 *Clostridium acetobutylicum*	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

860

Table 2. Saccharides quantified by GC-FID-MS and Total Carbohydrates Analysis of the bio-oil 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	Levoglucosan	33.6	43.7	19.8
	1,6-Anhydro-β-D-glucofuranose	35.8	3.1	1.4
	Total sugars	-	80.4	36.4
C2	Levoglucosenone	21.1	0.17	0.03
	1,4:3,6-Dianhydro-α-d-glucopyranose	24.3	0.41	0.08
	Levoglucosan	33.6	35.5	6.8
	1,6-Anhydro-β-D-glucofuranose	35.9	2.49	0.48
	Total sugars	-	59.7	11.4
C3	Levoglucosan	33.6	9.6	0.4
	Total sugars	-	29.0	1.3
C4	*Total sugars*	-	0	0
F	Levoglucosenone	21.1	0.04	0.03
	1,4:3,6-Dianhydro-α-d-glucopyranose	24.3	0.1	0.08
	Levoglucosan	33.6	36.9	27.0
	1,6-Anhydro-β-D-glucofuranose	35.9	2.6	1.9
	Total sugars	-	71.3	49.2

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

870

Cellulose conversion method	Reaction Time	Reaction agent	Yield (wt.%)	Comments	Ref.
Enzymatic hydrolysis 50ºC 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 220ºC 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 250ºC 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: 475ºC 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

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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

878 **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

880 *Yield expressed per gram of sugar