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THÈSE

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Par

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CELLULOSE VALORIZATION IN BIOREFINERY: SYNERGIES BETWEEN THERMOCHEMICAL AND BIOLOGICAL PROCESSES

Soutenu le 27 Juin 2018 devant la Commission d'Examen

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"Not everything that counts can be counted,

and not everything that's counted truly counts"

Albert Einstein

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INTRODUCTION

I. General context

Global population is increasing continuously and requires more and more energy and chemicals which production relies on fossil resources: oil, coal and gas.

Oil reserves are decreasing and their utilization represent 38% of the global energy consumption. Renewable energies are only 18%, being biomass the biggest fraction with 14% (Figure I1).

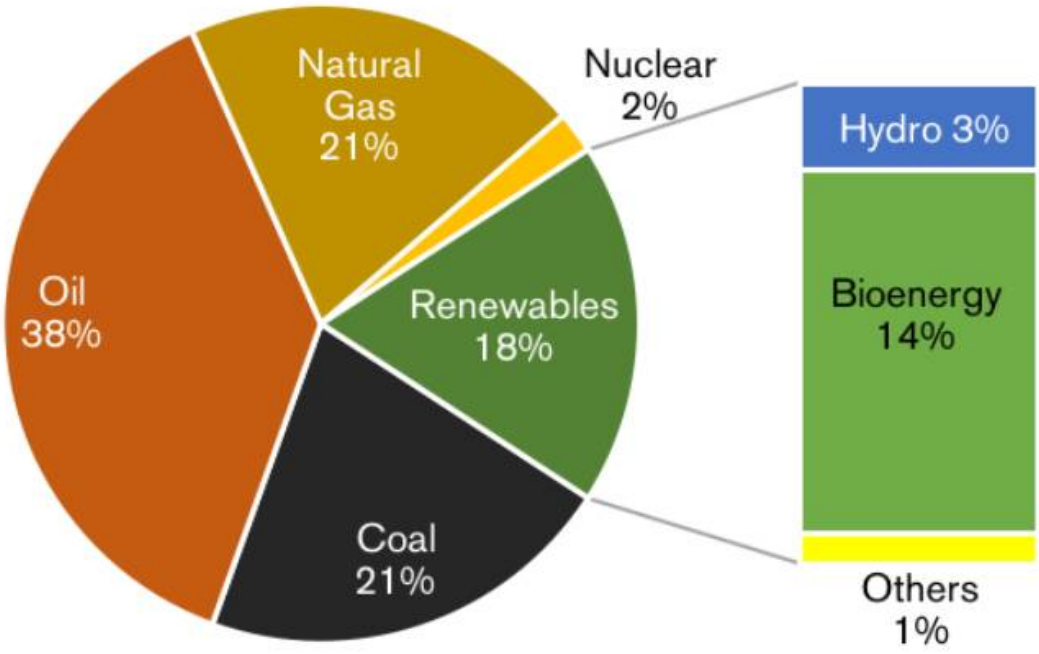

Figure I1. Global final energy consumption in 2013. (World Bioenergy Association - 2016)

Moreover, the utilization of these resources contribute significantly to the environmental pollution, notably on climate change.

Finding an alternative source for the production of fuels and chemicals is one of the major concerns of the current society.

Biomass is a source of renewable carbon that represents one of the best alternatives to fossil resources and respects the environment.

Concerning its utilization for energy supply, biomass is mostly used as a solid fuel for domestic heating in small combustion stoves (Figure I2). Additionally, the conversion of biomass into fuels and added-value chemicals represents the lowest percentages.

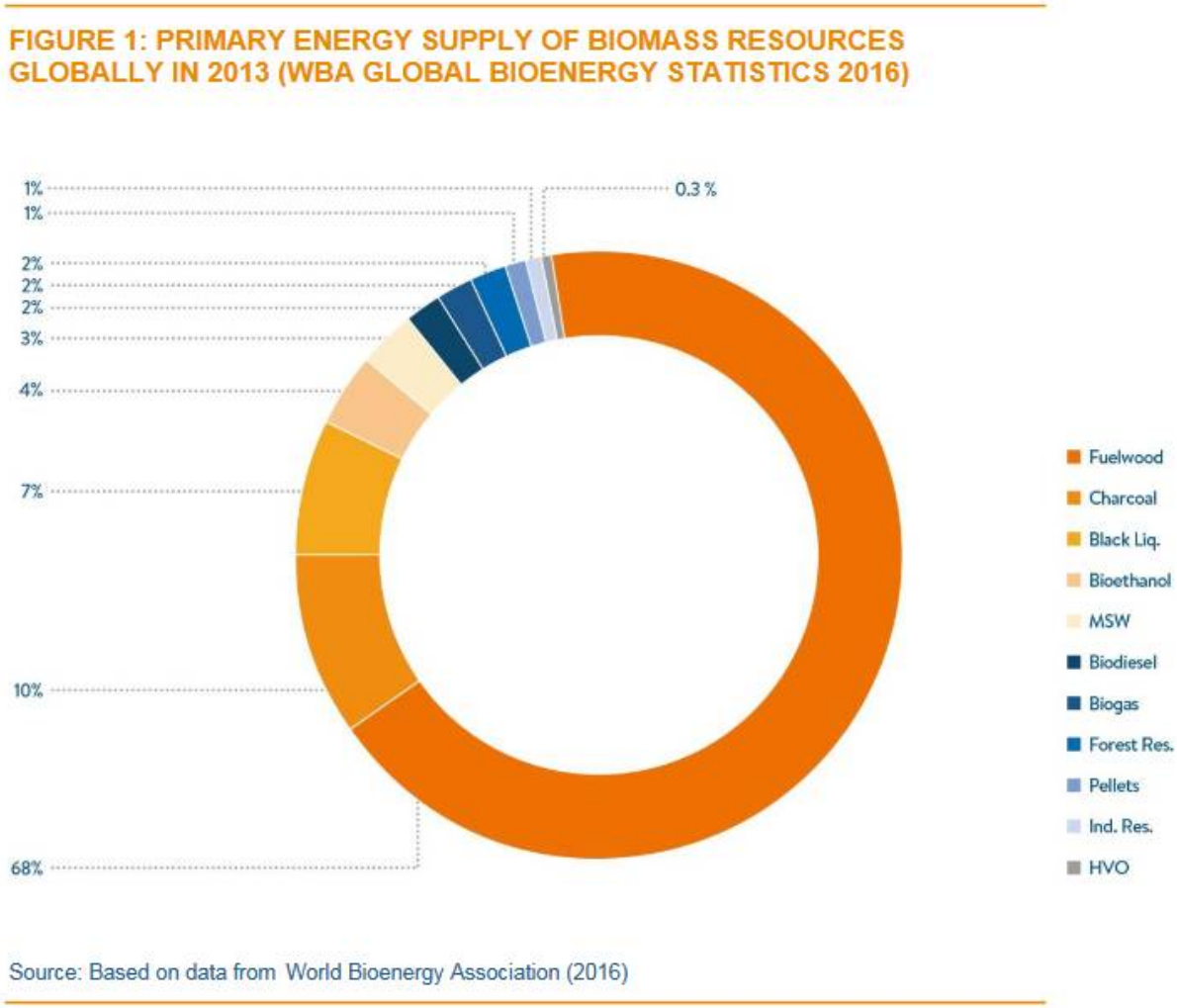

Figure I2. Global energy uses of biomass resources. (World Bioenergy Association - 2016)

Contrary to oil that is a mixture of liquid hydrocarbons that can be refined, biomass conversion into profitable chemicals requires the deconstruction of the polymers present in the solid lignocellulosic matrix, its transformation into liquid (or gas), with a higher energy density than the solid biomass, and its refining into the desired products.

This fact gives a clear idea of how challenging is to achieve the implementation of a cost-effective process for the conversion of biomass into fuels and chemicals. Moreover, the competition with the petrochemical in the industrial market is quite complicated for the biorefineries.

The most common industrial scale biorefineries for fuel production used some thermomechanical pretreatment followed by biological conversion processes (enzymatic hydrolysis and fermentation with yeast). Other type of biorefinery uses only thermochemical conversion such as gasification or pyrolysis.

Novel biorefinery strategies are required in order to overcome the technological and economic challenges of the industry. Implementation of biorefineries needs the support of policies and environmental law enforcement to guarantee the energetic transition without compromise the current economic development.

There is no industrial scale process combining both of these methods and technologies (i.e. thermochemical and biological). Besides, only few works combining thermochemical and biological processes have been reported and this might represent a promising methodology for a versatile biorefinery.

II. Scope and objective

In this work, a new strategy of biorefinery, using thermochemical and biological processes, is proposed. The main idea is to take advantage of the synergies between the strong points of each process and define a novel alternative for an efficient conversion of lignocellulosic biomass into profitable fuels and chemicals.

This biorefinery strategy employs three major stages. Firstly, a fractionation of the lignocellulosic matrix, recovering a cellulose-rich pulp. The lignin-rich stream could be further valorized but this was not studied during this Ph.D. Secondly, the cellulose-rich pulp is depolymerized by a thermochemical process (liquefaction or pyrolysis) in order to produce a mixture of soluble sugars. Finally, a bacterial fermentation using *C. acetobutylicum,* converts the sugars into fuels or building blocks.

The thermochemical process is faster than enzymatic depolymerization of cellulose. Bacterial metabolism allows producing interesting chemicals by fermentation processes.

Furthermore, in the background of this PhD, several analytical methods were implemented in order to properly characterize the product of each stage of the biorefinery and a model using Aspen Plus® was used for establishing the mass and energy balance to analyze the energetic performance of the different technologies involved in the global process.

This work presents a first methodological approach on how thermochemical and biochemical process could be combined. This methodology could be then applied to the production of other building blocks by targeting 1) the pulping method, 2) the thermochemical process and 3) the biochemical process. We believe that this approach is of interest and may be versatile. This work presents first results at laboratory scale and much development is still needed to push this concept up to pilot and then commercial scales.

III. Thesis outline

The **section A** summarizes all the general concepts about lignocellulosic biomass and its conversion through thermochemical and biological processes, highlighting hydrothermal liquefaction and bacterial fermentation, that are the processes of interest for this work. A global vision of the current biorefinery studies combining thermochemical and biological processes is briefly addressed.

The **section B** contains the results of this study presented in five articles that are divided in three chapters or sub-sections.

The **first section (I)** includes two articles presenting the results about the hydrothermal conversion of cellulosic materials. The first one is a study about the deconstruction of model cellulose (microcrystalline, Avicel) in hydrothermal media using several analytical methods and rationalizing the main reaction mechanisms. The second one concerns the hydrothermal

depolymerization of fractionated woody biomass. Two fractionation methods were used (organosolv and SC / AA) to obtain cellulose-pulps. The effect of the fractionation method on the composition and yields of hydrothermal liquefaction products was assessed.

The **second section (II)** contains two articles about the fermentation of cellulose-derived substrates by *C. acetobutylicum*. The first one presents a study about the pre-culture and culture of *C. acetobutylicum* grown on glucose / cellobiose mixtures, unraveling discontinuities in its growth and substrate utilization kinetics. The second one comprises the fermentation of cellulose-derived mixtures produced by liquefaction of microcrystalline cellulose, woody biomass and the two cellulose-rich pulps. This work allowed establishing for the first time the metabolism and an order of utilization of a mixture of cello-oligosaccharides. Building blocks yields were also assessed.

The **third and final section (III)** of the results contains one article about the process integration modeling. A simple model was developed in Aspen Plus® to establish the mass and energy balance of the global biorefinery process proposed in this work: biomass fractionation, hydrothermal liquefaction and bacterial fermentation. An energy analysis of each one of the operation units was performed.

Conclusions and perspectives are addressed in section C. Furthermore, other works performed during this PhD are presented in the appendices, in **section D**. These results are not included in the main manuscript to avoid the over extension of the manuscript and because they still need deeper assessment and discussion.

The first one is the result of an international collaboration with Pr. Hongwei Wu at Curtin University in Perth, Australia. For this purpose, I personally visit his laboratory in order to perform experiments with their fixed-bed liquefaction reactor of the cellulose-rich pulps produced in this work.

Pyrolysis was studied for the production of sugars from cellulose and cellulose-rich pulps. An international collaboration was established with Pr. Franco Berruti and Pr. Cedric Briens at the

Institute of Chemicals and Fuels from Alternative Resources (ICFAR) in London, Canada. I had the opportunity to visit their facilities to carry out experiments of fast pyrolysis of microcrystalline cellulose (Avicel) with a staged condensation system to recover fractions with high content of sugars (levoglucosan, cellobiosan). The hydrolysis and bacterial fermentation of these oils was studied as well. Besides, fast pyrolysis of cellulose-rich pulps, the same used in all the experiments, were performed using a micro-fluidized bed reactor coupled to a single photoionization (SPI) mass spectrometer (done in LRGP, Nancy).

A. LITERATURE REVIEW

This section concerns a brief literature review that will facilitate the understanding of the results reported in this work. It is divided in three sections. In the first sub-section, the general characteristics of lignocellulosic biomass are introduced. In the second sub-section, the conversion of biomass is explained highlighting hydrothermal liquefaction and fermentation.

Only general concepts are included in this part of the manuscript, since each result section features an introduction providing a specific state of the art for each specific topic studied in these sections.

I. Lignocellulosic biomass

Lignocellulosic biomass is the principal and more interesting raw material used in this work. It is the main feedstock used in an integrated biorefinery. Its nature and properties play a critical role in the development of the different processes, which allowing its fractionation and transformation into profitable products, take part in an integrated biorefinery.

Global composition, macro- and microscopic structure and its relation to its recalcitrant matrix are discussed in the next section, highlighting their influence on the conversion technologies used in this work.

1. Definition

Biomass is an organic material derived from living or recently living organism. In this general context, biomass can be all the organic material coming from a vegetal, animal, fungus or bacterial source.[1] However, the concept of “biomass” may change according to the field of study. In the

energy field, the definition of biomass corresponds to organic material that can become a source of energy by combustion, anaerobic digestion and by other chemical transformations. On the other hand, the "biomass" term is generally used to define the cell dry weight in the field of microbiology.[2] To avoid confusions, in this document, bacterial biomass will be always denominated as cell density. In this manner, lignocellulosic biomass or only biomass will correspond only to the plant based material. This renewable resource may come from different sources, the major biomass feedstocks are indicated in Table 1.

Table 1. Principal categories of biomass feedstocks. Adapted from "Biorefineries"[3]

Forest products	**Biorenewable wastes**
Wood Logging residues Trees, shrubs, leafs sawdust, bark	Agricultural wastes Crop residues Mill wood wastes Urban wood wastes Urban organic wastes
Energy crops	**Aquatic plants**
Short rotation woody crops Herbaceous woody crops Grasses Starch crops Forage crops	Algae Water weed Water hyacinth Reed
Food/sugar crops	**Others**
Grains Oil crops Sugar cane Sugar beets Molasses Sorghum	Landfill Industrial organic wastes

The most promising feedstocks for biorefinery are those that do not compete with alimentary purposes, leading to a production of second generation biofuels and chemicals. Within this vision, some forest and agricultural wastes attract an important interest due to their abundance and relatively low cost.

2. Global composition

There are millions of plant species around the world; each one with its own specific features that may even change according to environment and other external factors. However, the atomic composition, in terms of carbon (C), hydrogen (H) and oxygen (O) remains relatively constant for almost all non-processed lignocellulosic biomasses. Table 2 shows the proximate and ultimate analysis of a wide variety of biomasses.

Table 2. Summary of the proximate and ultimate analysis of different biomasses.[4]

Biomass materials name	Proximate analysis (% by mass, dry basis)			Ultimate analysis (% by mass, dry basis)			Ref.
	FC*	VM*	ASH*	C	H	O	
Rusk, hull and shell							
Rice husk	16.9	61.8	21.2	38.5	5.2	34.6	5
Sal seed husk	28.06	62.54	9.4	48.12	6.55	35.9	6
Olive husk	26.1	70.3	3.6	50	6.2	42.2	7
Peanut hull	21.1	73.0	5.89	45.8	5.5	39.6	8
Hazelnut shell	28.3	69.3	1.4	52.9	5.6	42.7	7
Brazil nut shell	22.2	76.1	1.7	49.2	5.7	42.8	8
Akhrot shell	18.8	79.9	1.2	49.8	5.6	42.94	5
Coconut shell	22.1	77.19	0.71	50.2	5.7	43.4	5
Pistachio shell	16.8	82.0	1.1	48.8	5.9	43.41	6
Groundnut shell	21.6	72.7	5.7	48.6	5.64	39.5	8
Wood, bark, chip and stick							
Chaparral wood	18.7	75.2	6.1	46.9	5.1	40.2	5
Spruce wood	29.3	70.2	1.5	51.9	6.1	40.9	7
Ailanthus wood	24.8	73.5	1.7	49.5	6.2	41	7

Beech wood	24.6	74	0.4	49.5	6.2	41.2	7
Western Hemlock wood	15.2	84.8	2.2	50.4	5.8	41.4	5
Bamboo wood	11.2	86.8	1.9	48.8	6.3	42.8	5
Red wood	19.9	79.7	0.36	50.6	5.9	42.9	6
Douglas fir wood	12.6	87.3	0.1	50.6	6.2	43	5
Casurina wood	19.6	78.6	1.8	48.5	6.2	43.1	5
Neem wood	12.2	85.9	1.9	48.3	6.3	43.5	5
Subabul wood	18.5	81.0	1.2	48.2	5.9	44.8	5
Wood bark	31.8	66.6	1.6	53.1	6.1	40.6	7
Douglas fir bark	32.8	65.5	1.8	53.1	6.1	40.6	8
Wood chips	23.5	76.4	0.1	48.1	6.0	45.7	6
Mulberry stick	22.8	75.1	2.1	44.2	6.6	46.3	6
Pit							
Peach pit	19.8	79.1	1.1	49.1	6.4	43.5	6
Olive pit	21.2	75.6	3.2	48.8	6.2	43.5	8
Needles and leaves							
Pine needles	26.1	72.4	1.5	48.2	6.6	43.7	8
Sena leaves	25.5	57.2	17.3	36.2	4.7	37.5	6
Fiber and coir							
Coconut fiber	26.6	70.6	2.8	46.4	5.5	43.8	8
Coconut coir	29.7	66.6	3.7	50.3	5.1	39.6	6

*FC= Fixed carbon, VM= volatile matter, ASH= ash content.

This data allows appreciating how lignocellulosic biomass is a renewable carbon-rich feedstock that presents a potential alternative to fossil carbon, no matter what plant species and geographic zones. Its C, H, and O content (ultimate analysis) is always similar for most of the lignocellulosic biomasses, with about 45-50%wt. C, 40-45%wt.O and 5%wt. H.

The main difference between this renewable resource and oil is the oxygen content, that is significantly important in lignocellulosic biomass and insignificant for petroleum.[9]

Contrary to the ultimate analysis, the mineral content is very variable for each kind of biomass. This feature depends strongly not only of the tree species but also of the soil where it was cultivated. The content of inorganic elements found in lignocellulosic biomasses can vary according to the essence. The inorganic composition of wood ashes from different types of lignocellulosic biomass are presented in Table 3.

Table 3. Inorganic concentration (mg / kg) in wood ashes from different tree species.[10–14]

Element	*Al*	*Ca*	*Fe*	*K*	*Mg*	*Mn*	*Na*	*P*	*S*	*Si*
Pinus banksiana	33.3	387	35.0	22.5	33.2	39.0	23.0	12.2	10.4	74.8
Pinus sylvestris	1–18	600	3–15	300	120	70.0	3–22	30.0	NG	NG
Picea abies	NG	700	NG	300	90.0	90.0	NG	20.0	NG	NG
Pinus sp	4.7	290	5.8	162.5	70.3	40.4	0.6	8.4	10.7	ND
Tsuga heterophylla	11.1	421	9.1	25.3	79.0	19.0	8.2	9.2	5.6	46.7
Betula sp	0.0	466	20.3	36.3	25.3	47.0	9.6	12.6	12.8	14.0
Betula pubescens	3.0	500	7.0	400	90.0	90.0	7.0	40.0	100	90.0
Acer sp	20.1	402	11.9	31.9	117.0	27.0	16.3	4.8	5.6	46.3
Populus tremuloides	1.4	212	2.6	112.5	35.5	1.4	0.6	11.8	7.0	1.1
Populus sp	3.5	257	3.2	79.3	90.9	4.5	23.0	9.5	10.2	ND
Quercus rubra	6.8	366	NM	60.8	52.0	14.9	0.8	15.6	18.0	ND
Quercus (white oak)	ND	314	0.9	102.5	75.7	1.4	ND	5.6	12.1	1.3

In a general way, inorganic elements found in lignocellulosic biomass are extracted by the plant from the soil, giving enormous importance to all the contaminations of the soil where it grows. However, each type of plant has its own profile of inorganic elements accumulation.

The average content of inorganic elements (ashes) in woody biomass is around 0.5 wt.% for the hardwoods and 0.4 wt.% for the softwoods.[3]

This presence of inorganics has an important catalytic effect during the thermochemical conversion of biomass. The presence of these minerals has shown significant effects on the pyrolysis mechanisms. For instance, potassium (K) and sodium (Na) presence was found to hinder anhydro-saccharides formation and to increase the phenols yields during pyrolysis.[15,16] Evidently, the opposite effect has also been observed, when inorganics are removed from biomass, anhydro-saccharides yield increases significantly.[16] Moreover, insoluble inorganic elements act as a heat sink, lowering the heating value of the biomass as a fuel.

3. Multi-scale organization of lignocellulosic biomass

According to their origin and species, each plant has unique components, set in a specific arrangement, that perform precise functions that allow them to develop in a particular environment. Lignocellulosic biomass refers more specifically to the material found in the plant cell wall. The cell wall is constituted by a complex matrix of fibers. Even if each species has unique features, all plants have a comparable cell wall structure. Figure 1 shows the multi-scale structure of lignocellulosic biomass. In this section, lignocellulosic biomass will be unraveled at different sizes: from macroscopic, passing by microscopic, to a molecular scale.

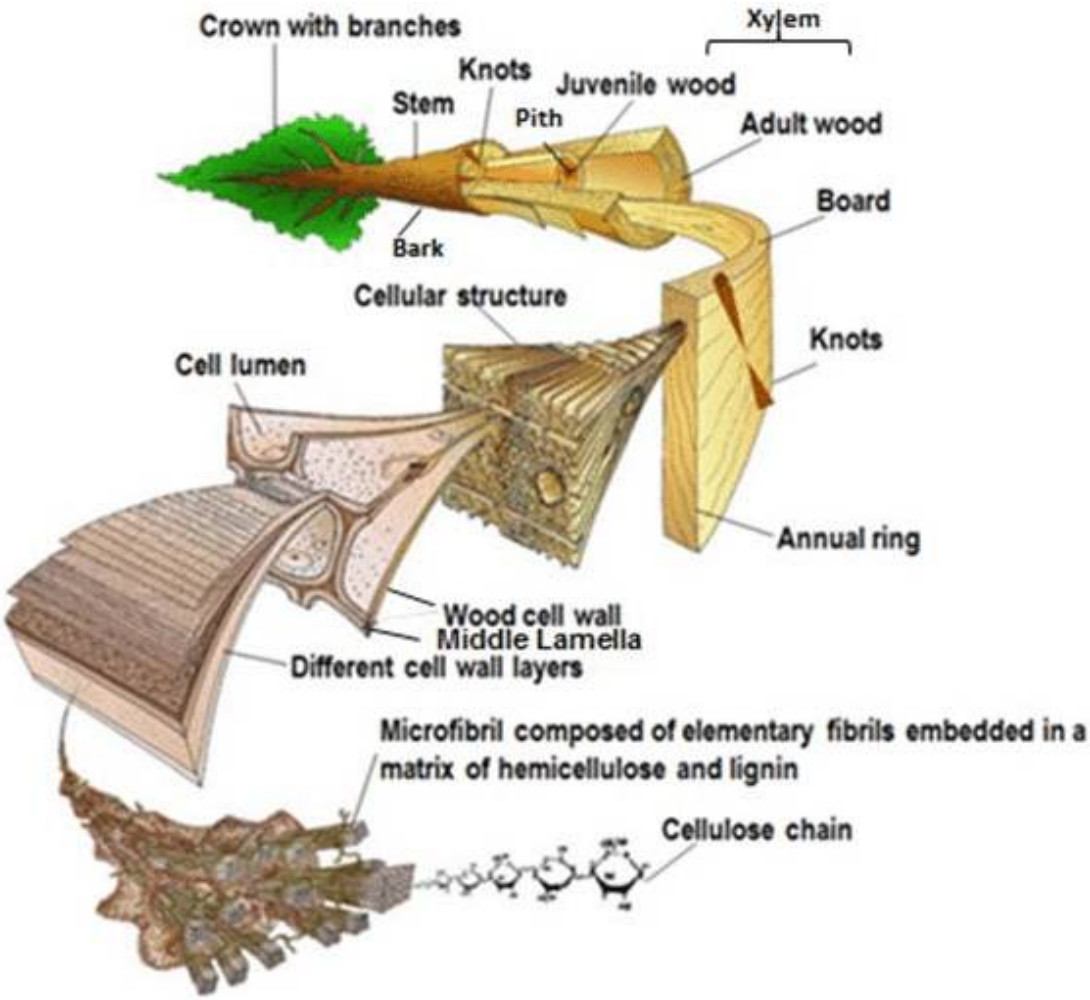

Figure 1.Representation of the multi-scale structure of lignocellulosic biomass.[17]

3.1. Macroscopic structure of biomass

There are two principal types of lignocellulosic plants, non-woody plants that are often called "grasses" and woody plants (without taking into account some algae that belong to this are also classified as plants). The main characteristics of the plant cell structure will give each biomass different mechanical properties that will play an important role in their applications and transformation.

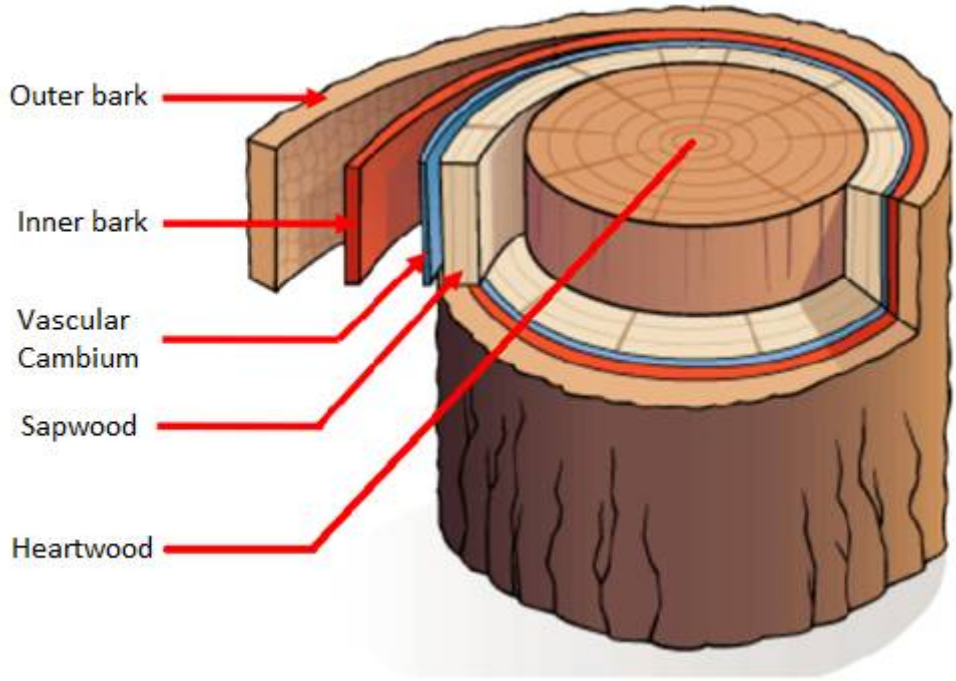

Figure 2. Schematic of wood anatomy.[18]

The anatomy of woody biomass is shown on Figure 2, where different parts, each one with specific characteristics can be identified. The central zone of a wood log is called heartwood, surrounded by the sapwood, which in turn is surrounded by the vascular cambium and the bark. The latter one can be divided into inner bark and outer bark.[18]

The composition and fiber arrangement of each one of these layers can be very variable; bark being the most different fraction relative to the rest of the wood (sapwood and heartwood).

Bark contains extractives and a different fiber arrangements that are often not suitable for the wood industry, and thus bark is removed by a process called “debarking”.[19]

3.2. Microscopic scale: Porous structure

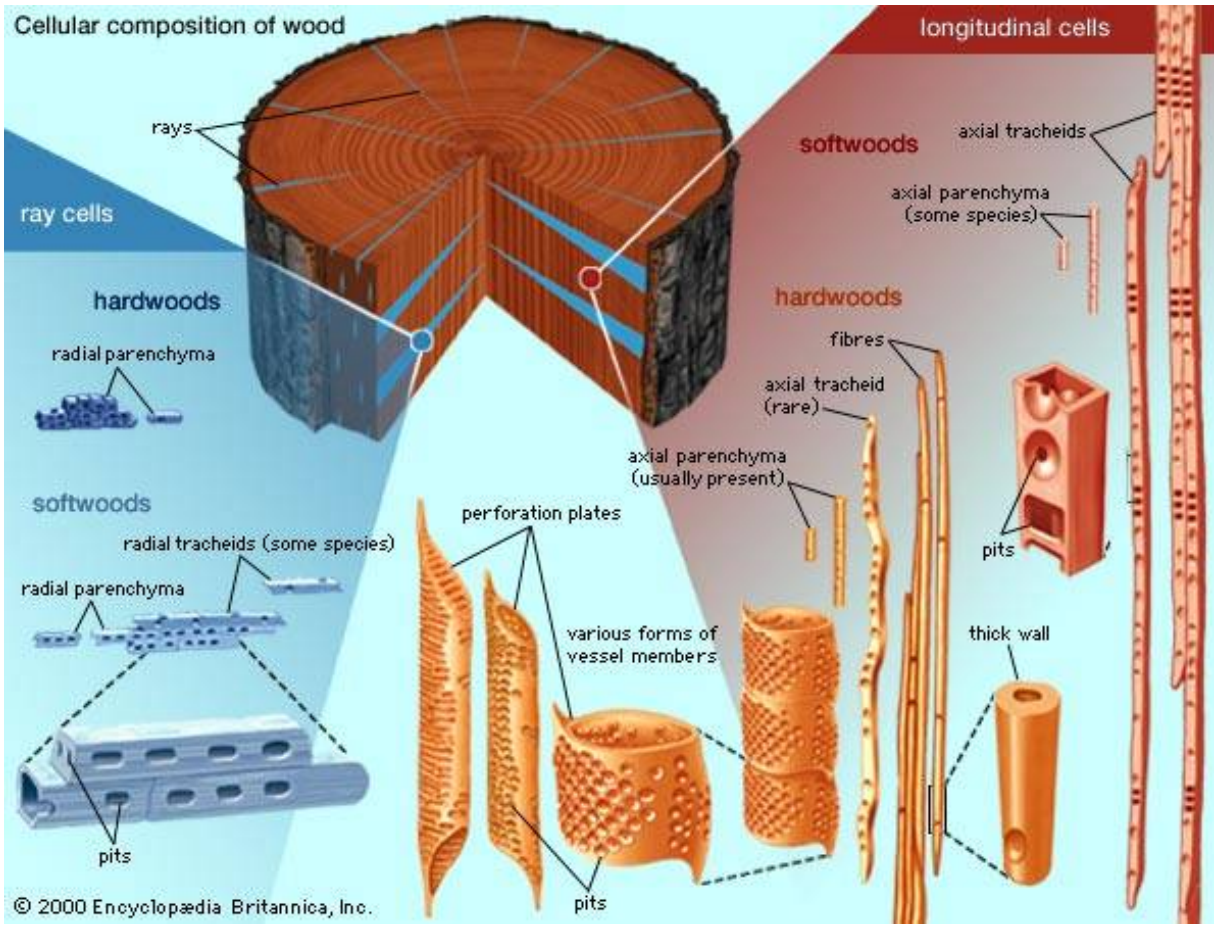

Figure 3. Composition of wood according to the plant species

Woody plants are the most abundant type of lignocellulosic biomass. It can be classified in two types: hardwood and softwood. The plant cell structure varies according to the plant type. Figure 3 illustrates the variety of components found in each type of wood. It is easily remarkable that fiber shapes and lengths are different. The softwood family has fibers of 2 to 4 mm. On the other hand, in the hardwood family, the fibers are shorter, with an average length of about 1 to 2 mm.[20] These features will give each plant type a different use in the wood industry.[21,22]

Different severities in the fractionation pretreatments will be needed according to the biomass mechanical properties that are directly dependent on the fiber length and disposition of the porous structure.

The hardwoods are known by their hierarchical porous structure, as illustrated in Figure 4.

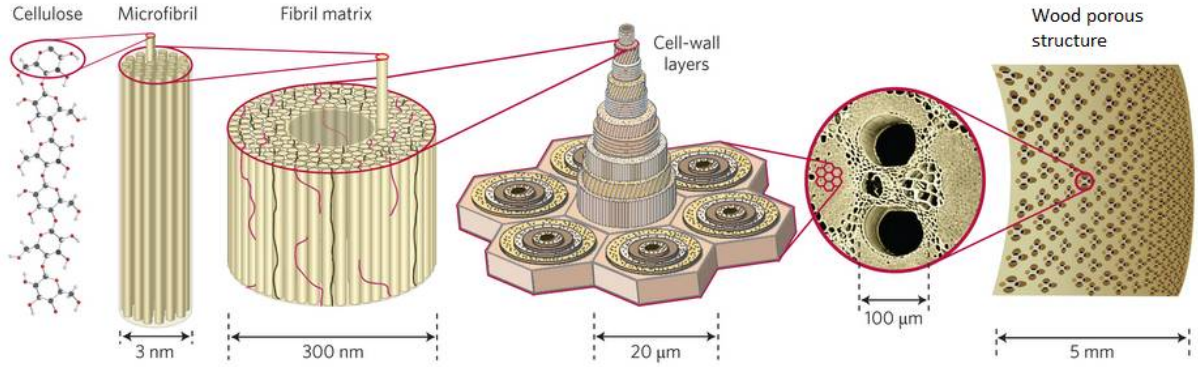

Figure 4. Schematic of wood hierarchical structure: from the pore to the molecule.[23]

This type of wood presents pores of approximately 100 µm where water and sap can flow all along the plant. Contrary to softwoods that use their tracheid cells (Figure 3) for the water and sap transport, pores are bigger in the initial wood, allowing a better fluid transport and they get smaller in the final wood, giving the plant a better hydraulic safety by easily keeping its fluids in it. The porous structure is strongly correlated with the seasons and environmental temperature.[24,25]

3.3. Biomacromolecules properties

At microscopic scale it is possible to identify different cell-wall layers that surround vegetal cells. These cell-wall layers are made of a lignocellulosic matrix, in which several cellulose microfibrils, embedded in a tridimensional arrangement with hemicelluloses and lignins, hold up the rigid structure of wood. Those three, cellulose, hemicelluloses and lignin, are the major components of woods and each one of them represents a potential renewable resource for the production of fuels, chemicals and materials.

The macromolecule composition of typical lignocellulosic biomasses is shown in Table 4. The main characteristics of these three macromolecules will be discussed in the next three sub-sections.

Table 4. Macromolecules composition of some lignocellulosic feedstocks.

Feedstock	Cellulose	Hemicelluloses	Lignin
Beech wood[26]	49.0	22.0	24.0
Corn stover[27]	37.5	22.4	17.6
Pine wood[27]	46.4	8.8	29.4
Poplar[27]	49.9	17.4	18.1
Wheat straw[27]	38.2	21.2	23.4
Switch grass[27]	31.0	20.4	17.6
Office paper[27]	68.6	12.4	11.3

3.3.1. Cellulose

Cellulose is a polymer composed exclusively of D-glucose molecules; it is a homopolysaccharide (Figure 5). Its repeating unit is the cellobiose ($C_{12}H_{22}O_{11}$). Cellulose is the most abundant organic biomolecule on Earth.

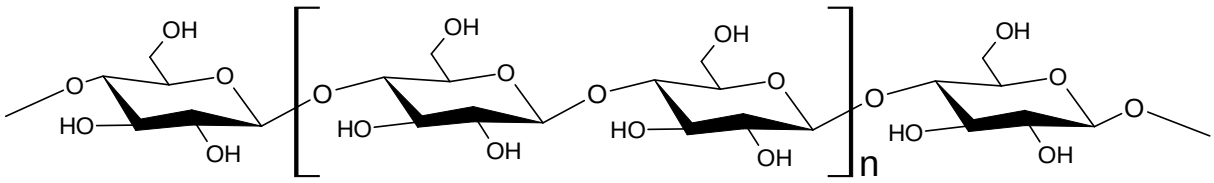

Figure 5. Chemical structure of cellulose.

The degree of polymerization varies significantly according to the origin of the cellulose; its value can vary from a few hundreds to thousands. This molecule has a semi-crystalline structure, explaining why the fibers it forms have very good mechanical properties. The glucose units in cellulose are bound by β-(1,4) glycosidic linkages, that contributes to their great resistance to chemical, biochemical and biological conversions.[28]

Cellulose is biodegradable. Some fungus and cellulolytic bacteria may be able to metabolize this polymer in solid state. However, this biological conversion can last several days / weeks, and for this reason does not represents much interest for industrial purposes.[29,30]

Moreover, cellulose is insoluble in water and most organic solvents, only ionic liquids (e.g. Schweizer reagent, cadmiumethylenediamine (Cadoxen), cupriethylenediamine (CED), etc.) are known to solubilize this polymer and even extract it from the lignocellulosic matrix.[31]

Cellulose is mainly used to produce cardboard and paper. In addition, small amounts are transformed into a wide variety of by-products such as rayon and cellophane. The thermochemical conversion of cellulose from energy crops to biofuels such as bioethanol or biodiesel is considered as a potential alternative to fossil fuel/energy.[32] One of the most industrialized processes for cellulose biorefinery is the production of bioethanol through enzymatic hydrolysis and subsequent fermentation using yeast.[33,34]

3.3.2. Hemicellulose

Hemicelluloses are the second sugar-based biomacromolecules by importance that can also be found in biomass. It is the second most abundant biopolymer on earth. Hemicelluloses are amorphous heteropolymers composed of pentoses (xylose and arabinose), hexoses (glucose, galactose, mannose and rhamnose) and carboxylic acids (aceric acid, glucuronic acid and galacturonic acid). There are different types of hemicelluloses, such as xylans, that are characteristic of hardwoods, galactoglucomannans, that are predominantly found in softwoods, among others (e.g. galactans, arabinogalactans, arabinoglycuronoxylans and glucomannans).[35]

In comparison with cellulose long and linear polymer chains, hemicelluloses exhibit shorter and branched chains. The repeating units of these polymers are shown in Figure 6. While the cellulose is crystalline, solid, and resistant to hydrolysis, hemicellulose has a random amorphous structure with low strength. It is easily hydrolyzed by a dilute acid or base, as well as multiple hemicellulase enzymes.[36]

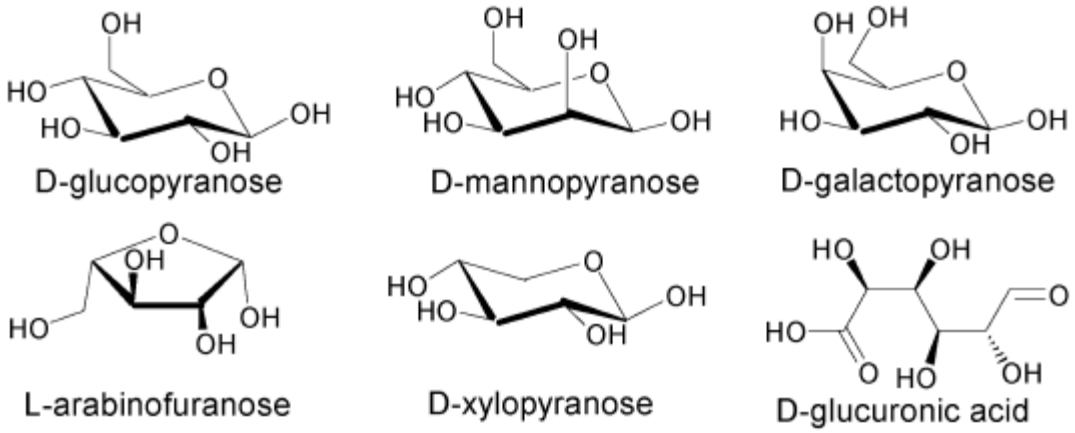

Figure 6. Monomeric units found in hemicelluloses.

Hemicelluloses represent an industrial interest for the production of platform chemicals such as: 5-hydroxymethylfurfural (5-HMF), furfural and levulinic acid.[37]

3.3.3. Lignin

Lignins are racemic, amorphous and complex heteropolymers composed of aromatic alcohols known as monolignols. Lignin is associated with cellulose and is an integral part of the secondary walls of plants by ensuring its rigidity, strength and resistance to degradation. The lignin content usually decreases from softwoods (24-33 wt.%) to hardwoods (19-28 et.%) to grasses (15-25 wt.%) (Table 4).

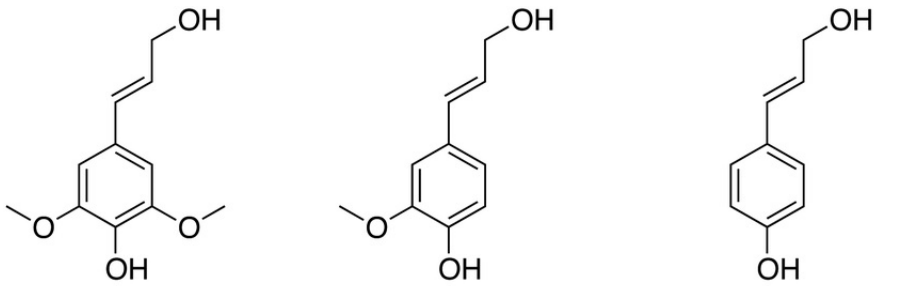

Figure 7. Principal monomeric units found in lignin.[38]

Lignin is a polyphenol-based polymer conformed of various methoxylated phenylpropane structures.[39] This biopolymer is mainly formed by coumaryl, coniferyl and sinapyl alcohol (Figure 7), which are incorporated to lignin and converted into p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units respectively.[38,39]

Because of its heterogeneity, this biopolymer does not have a defined primary structure. The main links between monolignols are C-O bonds of α- and β-arylalkyl ethers.[38] An approximate structure of lignin is shown in Figure 8.

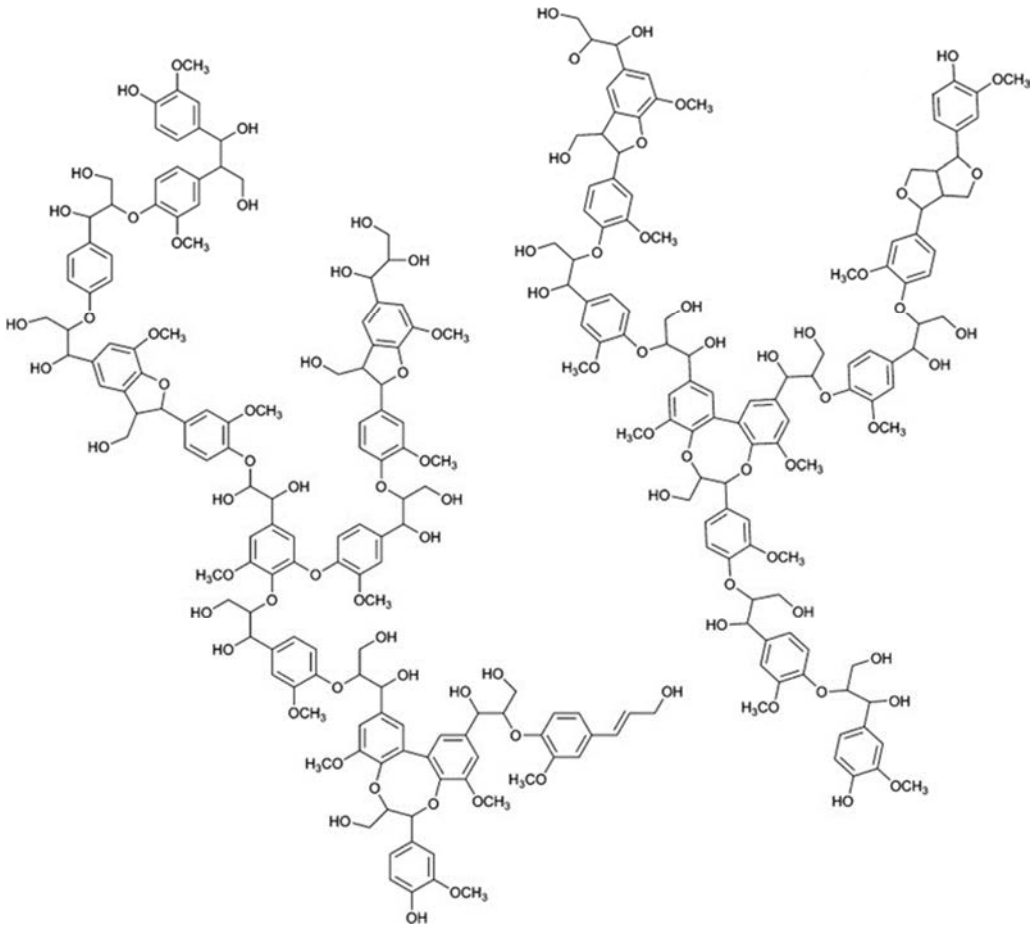

Figure 8. Representation of the lignin structure.[40]

Lignin derived products are normally undesired in biological and biochemical processes due to their strong inhibitory effect on the biocatalysts.[41] However, these lignin products (i.e. phenols, aldehydes, aromatics, etc.) have a high chemical value in the industry and their separation and

valorization represents one of the major challenges to accomplish the economic viability of an integrated biorefinery.[42,43]

3.4. Extractives

The extractives are considered as non-structural components of the lignocellulosic biomass. Their functions are to give the wood characteristics such as color, smell and natural durability.[44] Despite some of these compounds are commonly found in a wide variety of plants, the extractive compositions are rather variable according to the species and even from one part (i.e. root, stem, branches, bark, etc.) to another.[45] Normally, extractive contents are higher in bark, leaves and roots, than in the stem wood.[44] Extractives belong to different chemical families such as: resins, terpenes, terpenoids, fatty acids, phenols, flavonoids etc.[35] An example of some extractives that are commonly found in woody biomass is presented in Figure 9.

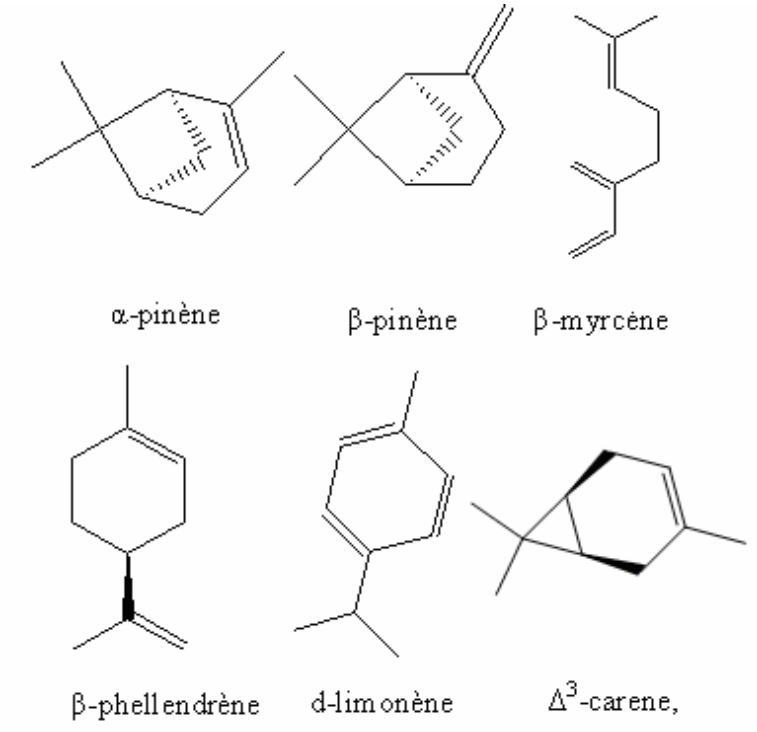

Figure 9. Examples of extractives found in lignocellulosic biomass.[44]

From the moment when a plant is harvested, the extractive content, decreases significantly, changing their compositions and their chemical properties due to airexposure.[45]

The extractive content is in general very low in comparison with the structural content of macromolecules. Nevertheless, the chemical value of these molecules is very high in the market of chemical specialties.[44]

II. The biorefinery: a deconstruction game

For several decades, the society relies on petroleum, coal and gas for the production of fuels, energy and chemicals. These are fossil resources and they are called “non-renewable” due to the long term they take to regenerate themselves during hundreds, even millions of years.

Hydrocarbons found in petroleum are fractionated and upgraded in facilities called “refineries” to produce a wide variety of chemicals that made part of current industrial market.

In an analogous sense, a “biorefinery” is a facility that combines several processes allowing to convert biomass feedstocks into fuels, energy and chemicals.[46]

However, in this case the raw material constitutes a complex polymer matrix that needs to be deconstructed to generate carbon-based compounds that can be purified or upgraded into value-added chemicals.

1. Biorefinery classification

In a conventional biorefinery, biomass feedstocks are submitted to chemical, thermochemical and biochemical process to recover simple molecules with attractive chemical properties as building blocks or platform chemicals.[3] They can be classified according to the type of process and biomass treated.[1,47]

A schematic of the main biomass valorization pathways is shown in Figure 10.

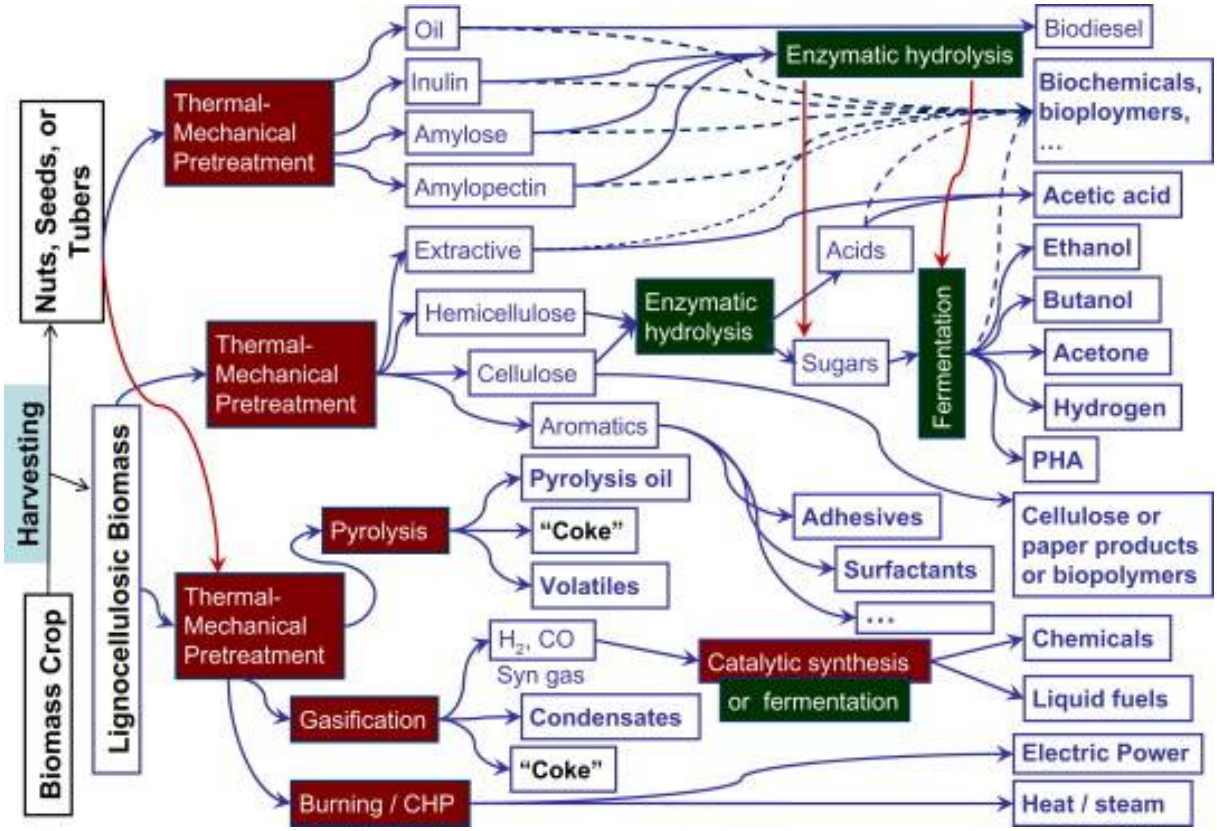

Figure 10. Schematic of biomass valorization pathways.[48]

Intensive research studies propose a wide variety of biomass conversion processes into renewable fuels such as: 1st and 2nd generation ethanol, biodiesel, pyrolytic oil, butanol, methanol, syngas liquids, biohydrogen, algae diesel, algae jet fuel and green hydrocarbons.

The most common biorefinery platforms are indicated in Table 5, including only one that uses thermochemical processes. Thermochemical based biorefineries that have been developed use gasification technologies to produce gas that can be upgraded to syngas and then to chemicals using catalytic processes (e.g. Fisher-Tropsch).[49] Another thermochemical biorefinery uses fast pyrolysis for the production of oils than can be upgrade by catalytic reactions (typically hydrodeoxygenation).[50]

Table 5. Major biorefinery platforms.[51,52,47,48,53,46]

Platform	Raw materials	Main processes	Products	Development stage
Sugar (Biochemical)	Lignocellulosic and starch biomass	Chemical and enzymatic hydrolysis, fermentation, biotransformation, chemical and catalytic processes	Added value chemicals (both from sugar and lignin), building blocks, materials (from lignocellulose, lignin or sugars) fuel ethanol, heat and electricity	Laboratory, large scale pilot plant and commercial (for sugarcane and starch based)
Syngas, bio-oil (Thermochemical)	Lignocellulosic biomass but also plastics, rubber etc.	Thermochemical processes (Gasification and pyrolysis)	Syngas, pyrolytic oil, added-value chemicals, gaseous or liquid fuels	Laboratory, large scale pilot plant
Biogas	Liquid effluents Manure	Anaerobic digestion	Methane and carbon dioxide (biogas) Added-value chemicals	Large scale pilot plant, commercial
Carbon-rich chains (Oil)	Plant oils such as soybean, rapseed, corn, palm and canola oils. Animal fats	Transesterification	Fatty acid methyl ester (biodiesel), glycerin and fatty acids as platform chemicals	Commercial

Concerning the production of biofuels, the most developed biorefining technology exists for the production of bioethanol, which is considered as the only transportation biofuel commercially available. The global process has three major steps: firstly, the fractionation of biomass into a cellulose-rich substrate, secondly, an enzymatic hydrolysis allowing to recover sugars (mostly glucose) and finally, the fermentation using yeast, that is the current microorganism giving the highest ethanol production yield.[33] This process is currently present at industrial scale in several countries (Brazil, USA, China, Germany, France, etc.).[3]

Some technologies have been developed to convert the biomass into products containing significantly less oxygen (increasing the energy content) than the initial biomass, through a process called hydrodeoxygenation.[54,50,55]

Pulp and paper industries are another traditional facilities for the valorization lignocellulosic biomass. Some of them have already integrated processes to valorize the residual lignin into chemicals and materials.[48]

In this work, we propose a biorefinery approach combining both thermochemical and biological processes; starting with the pulping of biomass, the thermochemical depolymerization of the cellulose-rich fraction and the fermentation of the sugar mixture into building blocks Figure 11.

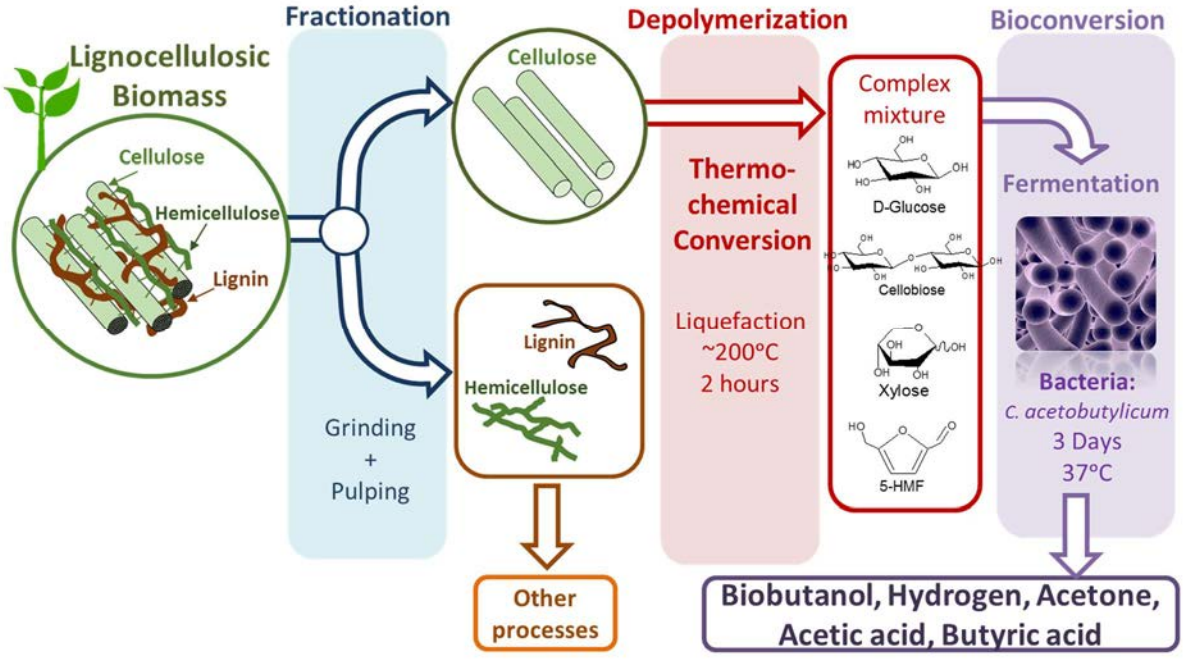

Figure 11. Scheme of the proposed biorefinery.

The main purpose of using thermochemical processes instead of enzymatic hydrolysis is to reduce the production cost of the biofuel.

Indeed, enzymes represent an important operational cost[56] and their hydrolysis kinetics are relatively slow (2-3 days). Thermochemical depolymerization instead of enzyme would considerably fastened the process (from days to hours / minutes).[57]

One of the major problems of this type of biorefinery is the slow kinetic involved in the biochemical processes and the high price and non-recyclability of enzymes.[56]

Only few works with this type of biorefinery can be found in the literature, but in a general way, this type of biorefinery has not been well studied.[58,59] These concepts of combining thermochemical and biological conversion of lignocellulosic biomass are described in the section III (Combining thermochemical and biological conversion of biomass).

2. Biomass pretreatment and fractionation

As presented in section I, lignocellulosic biomass constitutes a three-dimensional matrix making it very resistant to chemical and biological conversions. In order to produce biofuels and chemicals from biomass through biological processes, cellulose and hemicelluloses need to be converted into monomeric sugars.[60]

Lignin is not only embedded around the holocellulose (cellulose plus hemicelluloses) protecting it, but also is a source of strong inhibitors for enzymes and microorganism used in the subsequent stages of the biorefinery.[61] In order to reduce the recalcitrance of lignocellulosic biomass some pretreatments and fractionation processes have to be used.

2.1. Mechanical pretreatment

Different pretreatments of biomass can be implemented according to the final purpose and process. Commonly, biomass is initially submitted to a mechanical pretreatment (e.g. grinding, ball milling,[62] extrusion,[63] acoustic cavitation,[64] hydrodynamic cavitation,[64] etc.). The main objectives of these pretreatments are to reduce the particle size, to decrease the crystallinity of

cellulose and to "unravel" the fibers, increasing their accessibility, and thus, the reactivity during the subsequent processes.[27,63]

2.2. Pulping pretreatments

Some pretreatments are called pulping or fractionation methods. These technologies ensure an efficient valorization of each one of the constituents of the lignocellulosic matrix, which is a critical point for an economically viable biorefinery.[65–67]

Different pulping processes such as: kraft,[39,68] organosolv,[69,70] ammonia fiber expansion (AFEX),[71] alkaline,[27] chlorite/acid,[72,73] ozonolysis,[74] ammonia, [75] ionic liquids,[76] acid-steam explosion,[77] among others.[77–80]

The key factors to take into account when choosing an efficient pretreatment method for cellulose valorization are a high cellulose recovery yield regardless of the feedstock, high digestibility of the recovered cellulose (if subsequent enzymatic hydrolysis), no significant sugar degradation, no toxic compounds generated, low energy demand for downstream operations and no need of drying and efficient lignin recovery.[63]

The main advantages and drawbacks of different pretreatment and fractionation methods of biomass are indicated in Table 6.

Table 6. Advantages and drawbacks of different pretreatment methods for lignocellulosic biomass[39,63–65,79,81,82]

Pretreatment method	Advantages	Disadvantages
Milling	-Decrease of cellulose crystallinity and degree of polymerization -Reduction of particle Size to Increase specific surface area and pore Size	-High power and energy consumption

Steam explosion	-Causes lignin transformation and hemicellulose solubilization -Lower cost -Higher yield of glucose and hemicellulose in the two-step method	-Generation of toxic compounds -Partial hemicellulose degradation
Liquid hot water	-No chemicals required -No requirement of corrosion-resistant materials	-High energy and high water requirement -Formation of toxic compounds
Ammonia fiber expansion (AFEX)	-Increases accessible surface area -Less inhibitors formation -Do not require small particle size of biomass	-Not very effective for the biomass with high lignin content -High cost of large amounts of ammonia
CO2 explosion	-Increases accessible surface area -Availability at relatively low cost -Do not form inhibitory compounds -Non-flammability -Easy recovery after extraction	-Very high pressure requirements
Wet oxidation	-High degree of solubilization of hemicellulose and lignin -Avoid formation of degradation compounds	-High cost of oxygen and alkaline catalyst
Concentrated acid	-Relatively high glucose yield -Ambient temperature	-High cost of acid and need to be recovered -Corrosion-resistant equipment are required -Concentrated acids are toxic and hazardous
Diluted acid	-High recovery of sugars -Low formation of toxic products	-Concentration of reducing sugars is low -Generation of degradation products
Sodium Chlorite / Acetic acid (SC / AA)	-High recovery of cellulose and hemicelluloses -Mild conditions (~70ºC)	-Toxic chlorine-derived gases formed during the fractionation –Requires several hours for good recovery yields
Kraft	-High cellulose yields -Good fiber properties	-Manipulation of hazardous compounds -Production of malodorous compounds
Alkali	-Decrease of cellulose crystallinity and degree of polymerization -Disruption of lignin structure	-High cost -Not used for large-scale plant

Ozonolysis	-Effectively removes lignin -No toxic residues -Reaction at room temperature and atmospheric pressure	-High cost of large amounts of ozone
Organosolv	-Causes lignin and hemicellulose hydrolysis -Easy solvent recovery	-Solvents need to be drained (energy requirements)
Biological	-Low energy requirements -Delignification -Reduction in degree of polymerization of cellulose -Mild environmental conditions	-Slow process rate -High cost of enzymes -Very low treatment rate -Not very effective for commercial / industrial applications

In terms fractionation of biomass, the Kraft fractionation process is the global industrial dominant. This process uses sodium hydroxide and sodium sulfide in order to separate lignin from biomass.[39] During this method, several malodorous and toxic compounds are formed. For this reason, research in new fractionation methods draws a lot of interest in the industry.[39,63,64,66,78,79,81]

Organosolv process attracted a lot of attention due to its efficiency for biomass pretreatment at industrial scale.[63]

Different organic solvents can be used for this method: methanol, ethanol, acetone, etc. The separation and recycling of the solvent is the most important factor to reduce the operational cost.[63]

2.3. Conclusion

According to the literature, the best fractionation processes, that adapt well to the process proposed in this work, are organosolv, ammonia fiber expansion, chlorite / acid (SC / AA) and ionic liquids. This is because these methods allow a good recovery of cellulose (or holocellulose in the case of SC / AA) with a high yield of lignin removal, reducing the inhibitors quantity.

Ionic liquids were considered as a good alternative for the fractionation of biomass allowing the recovery of pure cellulose. Despite this, they were not studied because of their elevated price and availability.

Ammonia fiber expansion has the advantage of reducing the formation of toxic/inhibitory compounds. Nonetheless, it is not effective enough with high lignin content feedstocks, that is a critical factor for the subsequent fermentation stage.

Organosolv is a potential pretreatment for biomass allowing to recover a cellulose-rich pulp that is suitable for a biological conversion that is the ulterior purpose in this work.

Low molecular weight alcohols, such as methanol and ethanol, that have a low boiling point are favored for this process. For this reason, in this work ethanol-organosolv process has been selected as pretreatment method for biomass.

In addition, a comparison with a richer cellulosic fraction will be very useful for a better understanding of the role of lignocellulose constituents in the depolymerization and fermentation. Therefore, the chlorite/acid method was used to prepare a second cellulose-rich pulp that will be also subjected to the biorefinery experiments proposed in this work.

3. Thermochemical depolymerization of cellulose

The conversion of cellulose into fermentable sugars has been extensively studied for many years because it is crucial for the development of a cost-effective biorefinery.

One of the most common methods used in industrial scale biorefineries to depolymerize cellulose is enzymatic hydrolysis.[27] This method has been frequently criticized for compromising the economic viability of biorefineries[56] (this will be discussed in more details in the next sub-section) and the requirement of new processes for this purpose are now being studied.[57]

Concerning this objective, thermochemical processes are being studied as an alternative to enzymatic hydrolysis for the production of sugars from lignocellulosic feedstocks,[58,83] but also to convert biomass in other streams than sugars.

There are four types of thermochemical processes each one with different reaction mechanisms:

1. Combustion: excess of oxygen
2. Gasification: partial use of oxygen
3. Pyrolysis: absence of oxygen
4. Liquefaction: use of a solvent, commonly water, ethanol, etc.

Among these four thermochemical processes, combustion and gasification are not designed to produce liquid products. Combustion is used mostly for the recovery of energy and gasification, evidently, for the production of a syngas. Only pyrolysis and liquefaction allow recovering soluble sugars which are the targeted product for the present work.[84]

The next sub-sections will be focused on the liquefaction of (ligno)cellulose that is the most interesting method in this work. Pyrolysis will be shortly recapitulated, since it is considered as another potential alternative for sugar production.

Pyrolysis was also studied for this work, but the results are not presented in the main text for the sake of clarity and brevity. These results are briefly reported in the appendices section 2.

3.1. Hydrothermal liquefaction of cellulosic materials

Hydrothermal liquefaction is by principle the best thermochemical process to convert cellulose into fermentable sugars. The main characteristic of this process is the presence of water that guarantees a common hydrolytic mechanism and has been widely studied for the depolymerization of cellulose into sugars.[85–91] For this reason, this process has attracted significant interest for the purpose of this work.

In this section, the concept and reaction mechanisms of biomass liquefaction are explained. Then, the effect of operational conditions on the liquefaction products is explained. Finally, the reactors used for this process are briefly summarized.

3.1.1. Concept

Hydrothermal liquefaction is a thermochemical process were a substrate can be transformed using a hot, pressurized water environment sufficient time to break down its structure and recover mainly liquid components.[92,93] The main principal of this biomass conversion technology is the utilization of water, as a solvent and reactive, to cleave the β-1,4 glycosidic bonds of cellulose.[87,88] The properties of water change drastically when the temperature increases.

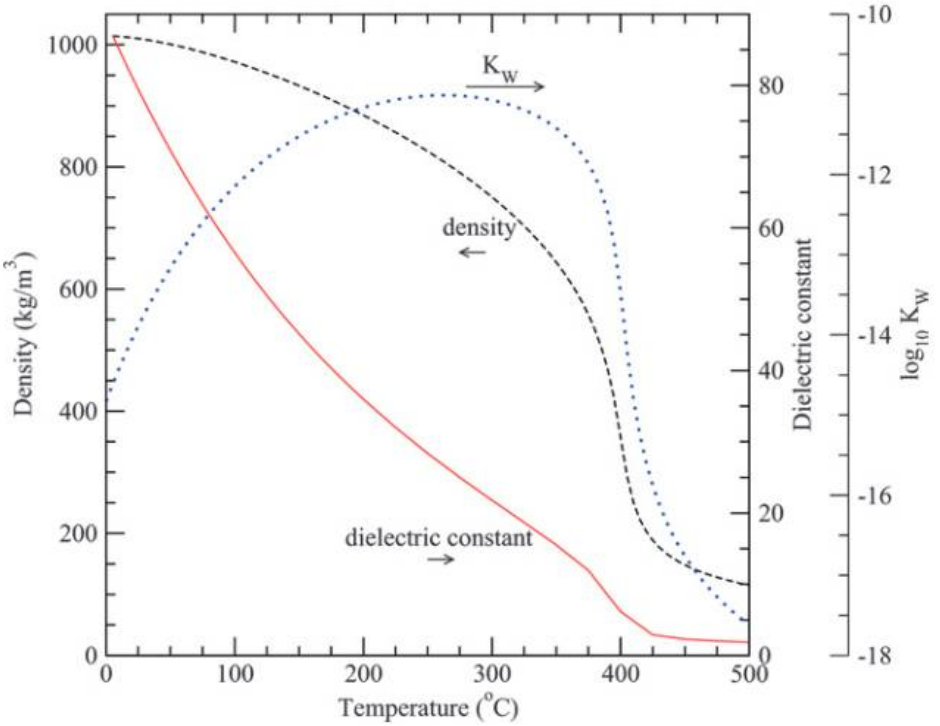

Figure 12. Density, static dielectric constant and ion dissociation constant of water at 30 MPa as a function of temperature.[88]

Figure 12 shows the variation of the density, static dielectric constant and ion dissociation constant of compressed water (30 MPa) as a function of temperature.

Density decreases from 1000 kg / m^3 at room temperature to a value around 600 kg / m^3 near the critical point (374^{o}C) and about 150 kg / m^3 (dense-like value) when the temperature is around 500^{o}C. The ion dissociation constant (K_w) reaches a maximum value around 10^{-11} for temperatures in the range of 200 to 350^{o}C and then it decreases significantly (10^{-17} to 10^{-18}) for temperatures over 450^{o}C.[88]

This behavior of water presents an important advantage due to the possibility of set conditions that provide a solvent with more or less acidic properties according to the requirements of the process and feedstock.

Changes in other properties of water, such as solvation power, degree of hydrogen bonding, polarity, dielectric strength, molecular diffusivity and viscosity are also correlated to those discussed previously and might be potentially used in other processes.[88]

Matsumura et al.[86] intensively studied the reaction mechanisms of hydrothermal liquefaction of cellulose (Figure 13). This work proposes that a combination of a thermal and hydrolytic cleavage is present during hydrothermal liquefaction of cellulosic materials.

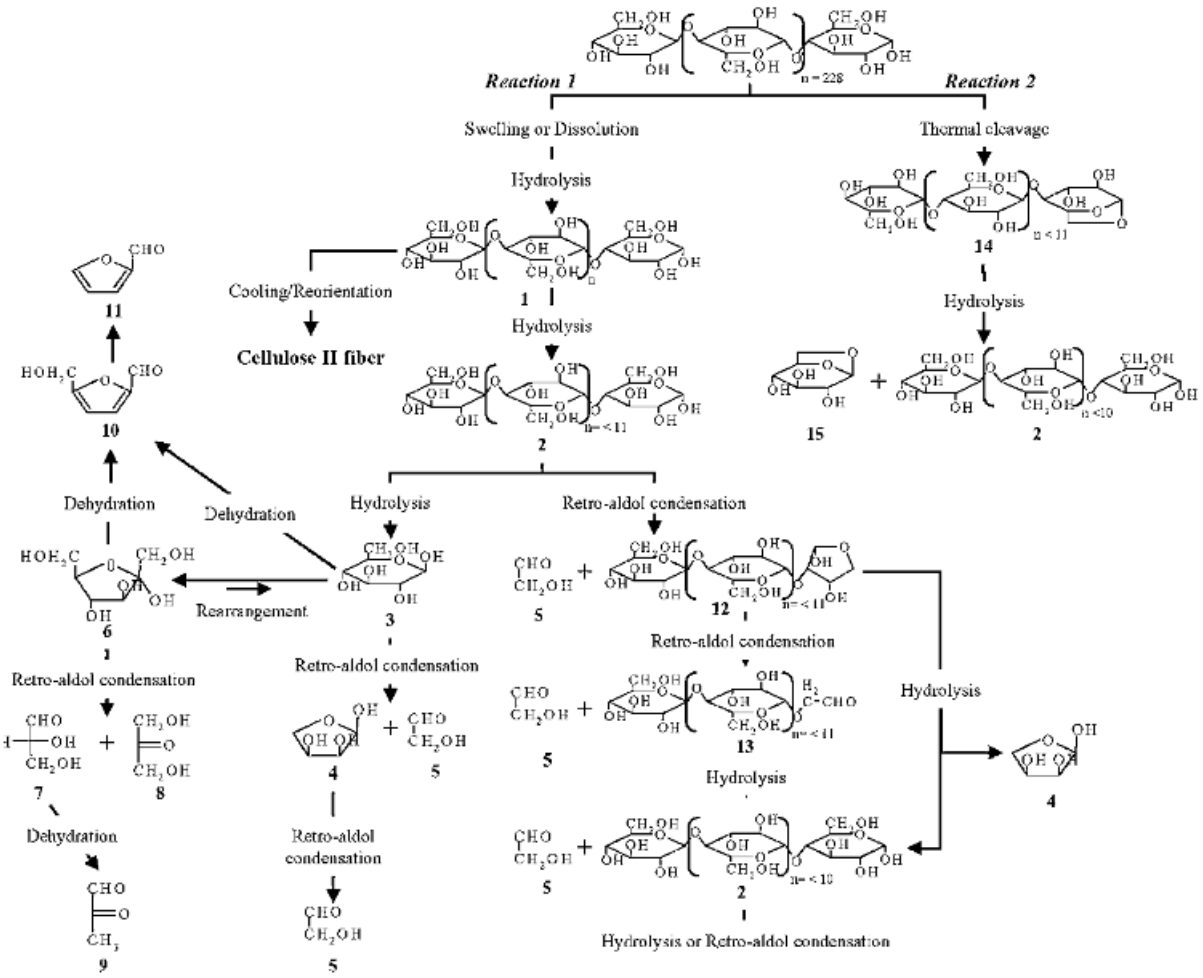

Figure 13. Proposed reaction mechanism of microcrystalline cellulose in supercritical water. 1) Swelled (or Dissolved) cellulose; 2) cellooligosaccharides; 3) glucose; 4) erythrose; 5) glycolaldehyde; 6) fructose; 7) glyceraldehyde; 8) dihydroxyacetone; 9) pyruvaldehyde; 10) 5-HMF; 11) 2-furfural; 12) glucopyranosyl-erythrose; 13) glucopyranosyl-glycol-aldehyde; 14) anhydro-cellooligosaccharides; 15) levoglucosan

The reaction mechanism that predominates in the hydrothermal liquefaction of cellulose is the beta-1,4 cleavage with a molecule of water, producing oligosaccharides and eventually glucose.[85,89]

Glucose can then undergo several other reactions, mainly dehydration, retro-aldol condensation, isomerization, decarboxylation and decarbonylation, that will produce aldehydes, carboxylic acids and eventually permanent gas.[94,95]

3.1.2. Effect of hydrolysis conditions on product distribution

The most important operating condition in hydrothermal processes is the temperature. As mentioned before, this will strongly affect the properties of water and will provide the energy required to break the linkages in lignocellulosic biomass.

Yun et al.[96] found that 150°C are required in order to break a glycosidic bond in the amorphous zone of cellulose and 180°C in the crystalline one. Higher temperature will provoke the degradation of sugars. Since the conversion kinetics of the soluble compounds are much faster than that of the solid cellulose (with sub-critical water), increasing the temperature will drastically increase the secondary reactions of the soluble sugars, transforming them into lighter compounds (e.g. aldehydes, carboxylic acids, carbon dioxide).[85,97,98,87]

There is no direct effect of the pressure on the reaction mechanisms. However, if water is partially evaporated, the depolymerization will follow similar reaction mechanisms but with different composition and the energetic requirements of the process will increase significantly.[88] It has been reported that a pyrolytic mechanism may be promoted if there is not enough water accessibility.[86]

The residence time plays a fundamental role for the selective conversion of cellulosic materials into sugars or chemicals. Long residence times will provoke secondary reactions and therefore

the degradation of the sugars.[89,96,99] A good combination of temperature and residence time are needed in order to optimize the sugar yield.

3.1.3. Reactors and processes

The reactor technology will define the range of residence times for this process. Principally, two technologies have been studied for the depolymerization of cellulose: batch,[85,86] semi-continuous[89] and continuous reactors.

Continuous reactors have been developed where a slurry of the cellulosic feedstock is pumped in a tubular reactor. A tubular reactor allows a good control of the conversion degree of cellulose and therefore of the selectivity to target products. The solid particles can exhibit a short residence time, producing a high yield of oligosaccharides with less monosaccharides. The main problem of this technology is the pumping of slurries that contain high solid contents and the continuous separation of solids (hydrochar) with soluble sugars.[85,97,100]

In semi-continuous reactors hot water flows through a fixed bed of the cellulosic feedstock. The solid remains in the reactor while the water with the soluble products is rapidly removed, giving a long residence time for the solid, and a very short one for the liquid. This technology allows recovering a high yield of mono- and oligosaccharides but it is not a continuous process.[101,102,89,89,96]

In batch reactors, the solid feedstock is mixed with the liquid, remaining in the reactor during the whole process. Both, liquid and solid residence times are relatively long.[98] Because the liquid residence time is long, a fraction of the sugars produced can be degraded into lighter compounds

(e.g. aldehydes, carboxylic acids and gases). This technology has the advantage to use less water, allowing the recovery of relatively more concentrated sugar solutions.[103,100,98,104]

The choice of the reactor will depend mostly on the selectivity requirements for the process, the final concentration of the products and the targeted quantity of water.

3.2. Pyrolysis of cellulose

Pyrolysis is considered as another good alternative to produce soluble sugars from lignocellulosic biomass.

The pyrolysis of biomass results in the production of a solid residue (called char in the case of a complete pyrolysis), a liquid product, which corresponds to water and condensable organic vapors commonly called bio-oil or tar, and a mixture of permanent gases composed of carbon monoxide, carbon dioxide, hydrogen and methane, among others.[105,106]

This thermochemical conversion takes place in the absence of oxygen at a temperature range from 200°C up to 600°C.

The operation conditions will define the type of pyrolysis: fast, intermediate or slow, as is shown in Table 7.[105]

Table 7. Operating conditions according to the type of pyrolysis.[105,107]

Process	Heating rate (°C/min)	Conversion Time of the solid	Particle size
Slow pyrolysis	<50	Dozens of minutes	Briquette / whole
Intermediate pyrolysis	200-300	Few minutes	Chopped / finely ground
Fast pyrolysis	600-1000	Few seconds	Finely ground

The conversion time of biomass varies from few seconds to hours. The main parameters controlling the pyrolysis regime are: 1) the heat flux density brought by the reactor to the surface of biomass particles, 2) the characteristic length of particles, 3) the residence time of solid and vapors in the hot zone of the reactor.

The main reaction pathways of cellulose pyrolysis are illustrated in Figure 14. Cellulose first will produce anhydro-oligosaccharides, mainly levoglucosan and cellobiosan, that will undergo a subsequent degradation into pyrans, light oxygenated and permanent gas.[106,105]

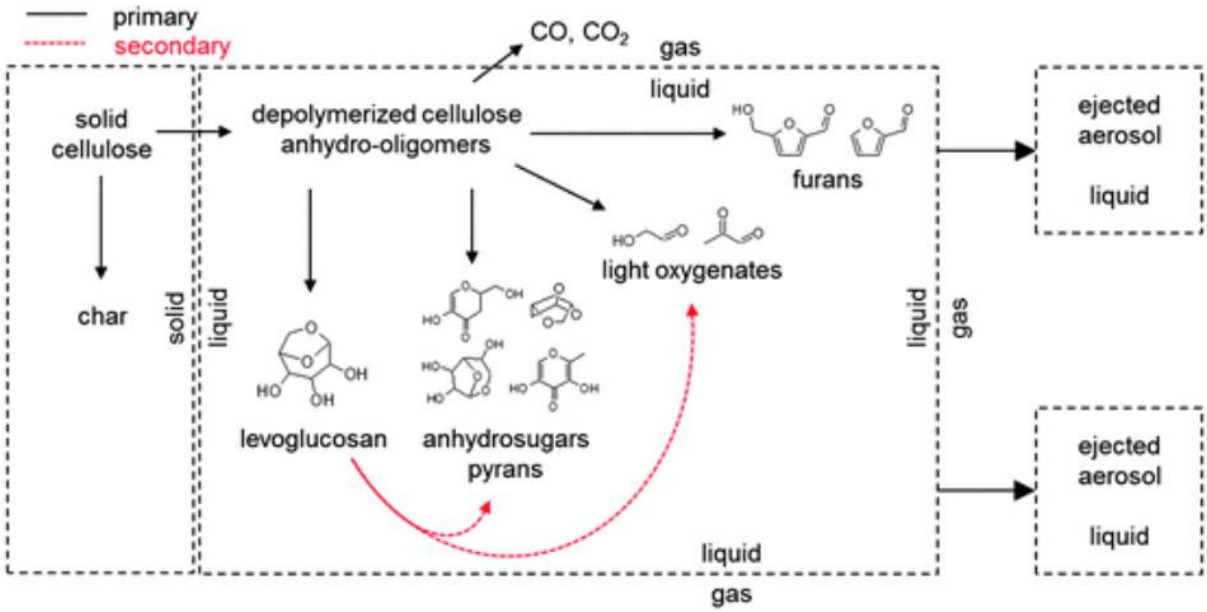

Figure 14. Simplified cellulose pyrolysis mechanism.[108]

Fast pyrolysis has shown to give the highest yields of bio-oil.[106,105]

Products derived from lignin pyrolysis have shown strong inhibitory behavior during biochemical transformations.[109] Staged condensation after fast pyrolysis is an attractive technology for the production of near-pure sugars with low content of light oxygenated, phenols and other undesired compounds for fermentation processes.[110–113]

3.3. Conclusion

For the production of soluble carbohydrates there are two possible thermochemical processes, liquefaction and pyrolysis; each one has his own advantages and drawbacks.

The use of water, characteristic of liquefaction processes, has the advantage of providing both a solvent and a reagent that stabilize the cellulose hydrolysis products into glucose oligomers instead of anhydro-oligomers that present difficulties for their biological transformation. The main drawback is the presence of secondary reactions that will cause the degradation of some sugars. Another important challenge of this technology is the elevated energy loads needed to heat water, requiring the reutilization of the heat in order to ensure its economic viability.

Pyrolysis is recently attracting more attention as a method for producing sugars. The main advantages of this technology are the relative high yields and selectivity for levoglucosan production. This method will be considered as a second alternative to hydrothermal liquefaction.

The fermentation of levoglucosan represents the main challenge of this pathway. Fermentation of pyrolytic oil was performed during this PhD but not presented in this manuscript, only briefly in the appendices section (2 and 3).

4. Biological conversion of cellulosic materials

There are different processes considered as biological and biochemical transformations, such as fermentation, anaerobic digestion, enzymatic hydrolysis, among others. In this work, fermentation was used to transform sugars obtained from (ligno)cellulosic substrates.

So, the main generalities about the fermentation and more specifically the Acetone-Butanol-Ethanol (ABE) fermentation process are briefly summarized. Then, some features of Clostridial species will be given, highlighting the carbohydrate utilization by the strain used in this work: *Clostridium acetobutylicum* ATCC 824.

4.1. Brief history of fermentation

Fermentation has existed since the beginning of society. From ancient times, during the ancient Egyptian empires, to the present day, fermented beverages had been used frequently in everyday life (Figure 15).[114,115]

Figure 15. Fermentation through history.[116–118]

Fermentation is an ancient form of food preservation, which also improves the nutritional content of foods. In many regions of the world, fermented beverages have become known for their health-promoting attributes [119].

Fermentation started to be scientifically studied since the XIX century. French microbiologist Louis Pasteur is known for his important contributions in this field proving for the first time that fermentation was initiated by microorganisms[120–122].

Regarding the carbohydrate utilization by bacteria, Jacques Monod made big contributions discovering the phenomenon of *Diauxie* that consist in a catabolite repression resulting in a biphasic growth when two carbon sources are present in the growth media [123,124].

Since then, fermentation technologies have experienced a steadily development with the assistance of advance sciences like molecular biology and genetic modification. This promotes the uses of these technologies to the production of pharmaceuticals, building blocks, chemicals and fuels.

4.2. Current definition of fermentation

Fermentation is a process in which living cells (bacteria, yeast or other microorganisms) are used for the chemical breakdown of a carbon source under anaerobic conditions [125].

Industrially, it consists in a bulk growth of microorganisms on a growth medium that are cultivated to produce a specific chemical product.

In most of the cases, the substrates are carbohydrates and the products are, for most of them alcohols and organic acids. However, microorganisms possess a unique set of enzymatic reactions that allow them to transform a wide variety of substrates that can lead to compounds which their chemical synthesis is too complicated or does not exist [125].

This process was first called fermentation by the French microbiologist Louis Pasteur, pioneer of this field, describing this phenomena as life in the absence of oxygen.

Pasteur first called this process “the life without air” [126], but nowadays, fermentation processes can be defined as those biological transformations that occur without any respiratory chain involving oxygen or other alternative final electron acceptor [127]. During this process, bacteria can

carry out a wide variety of oxidation-reduction reactions that can lead to products with industrial interest such as biofuels.

4.3. ABE Fermentation process

Solventogenic clostridia are able to produce butanol in high yields and acetone-ethanol in lesser quantities, accompanied by the production of hydrogen (H_2) and carbon dioxide (CO_2) in a process called ABE fermentation [128,129].

This fermentation process was discovered by the chemist Chaim Weizmann during world war I, using *C. acetobutylicum* [130]. The main purpose was to produce large quantities of acetone needed in the production of cordite used in the war industry and when the world war II started, ABE fermentation was given top priority [131].

The industrial interest in ABE fermentation declined rapidly during the 1960's due to the fast development of the petrochemical industry and increasing cost of the substrates [128,131].

In the recent years, solventogenic clostridia are receiving much attention due to their ability to produce profitable chemicals such as butanol, 1,3-propanediol and hydrogen.

Butanol presents an interesting alternative to fossil fuels [132]. For this reason, its production from alternative resources is taken a lot of attention and new efforts are being made for its proper development [129,133].

Butanol can also be produced by hydroformylation starting from propylene with H_2 and CO over rhodium catalyst (oxo-process) [134] or the aldol process starting from acetaldehyde [135]. However, these are not sustainable processes and for this reason production of these chemicals from renewable resources attracts a lot of interest in the present days [132,135].

Several studies have been made in order to better understand the mechanisms of sugar transport [136], regulation of butanol production [137], butanol tolerance [138], utilization of lignocellulosic biomass [139–141], bacterial inhibition [142,143] and genetic tools [138,144,145] in order to improve butanol final yield, overcome its toxicity, inhibition and other limiting factors.

A wide variety of microorganisms belonging to the genus Clostridia are available to study this process [146]. In the next section the generalities of these microorganisms will be briefly summarized, followed by an explanation of the minimum requirements needed to achieve a performant growth and solvent production by these microorganisms.

4.4. Clostridial species

The genus *Clostridium* was established for the first time by Prazmowki[147] in 1880. Later, more precise studies about these microorganisms were described by Morris[148] and Dürre.[149] It is the second largest bacterial genera, only after *Streptomyces*.[146]

They exhibit a rod-shaped morphology, gram-positive staining cell membrane, able to form heat-stable endospores and fermentative metabolism without the capacity for dissimilatory sulphate reduction.[146] Most of Clostridial species are strict anaerobes with few exceptions, like *Clostridium oroticum*, that is an aero-tolerant strain.[150]

Clostridium form an exceptionally resistant structure capable of surviving for long periods in an unfavorable environment. This dormant structure is called endospore since it develops within the cell. Under favorable conditions the spore germinates and gives rise to a vegetative cell, as is shown on Figure 16.[146,151–153]

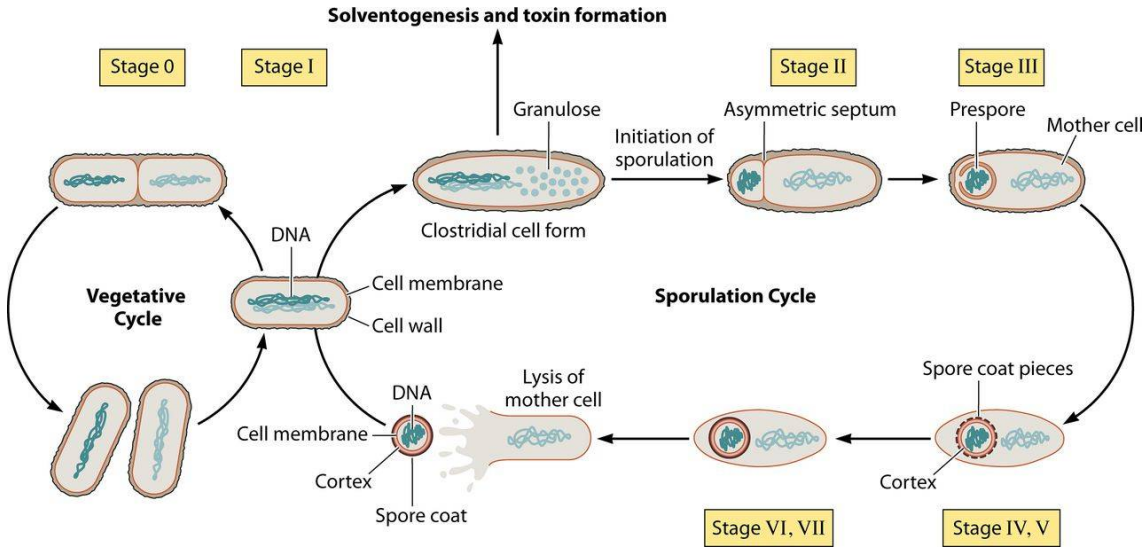

Figure 16. Sporulation cycle by endospore-forming bacteria.[153]

Clostridial species can be classified according to the substrate that they are able to metabolize. Some species possess a cellular organ called "cellulosome"[154] allowing the direct utilization of insoluble cellulose; these species are called "cellulolytic". These species have the advantage of using cellulosic substrates without requiring its depolymerization. However, their kinetics last several days and the product yields are relatively low.[155,29] The species that use only soluble carbohydrates are called "saccharolytic".[156,149] These species present much faster kinetics than the cellulolytic species. Nonetheless, they require fermentable and soluble carbohydrates as substrates, implying a depolymerization stage in the upstream process.[132]

They can be also classified according to their optimal growth temperature. For low and moderate temperatures (20 to 45°C), they are called mesophiles. At higher temperatures, between 45 and 80°C, they are called thermophiles and hyperthermophiles if they grow in temperatures higher than 80°C.[148,149,146]

4.5. *Clostridium acetobutylicum*

In this work, *C. acetobutylicum* ATCC 824 is used for the fermentation of cellulose-derived carbohydrates. The fact that this species shows fast kinetics and the ability to use a wide variety of substrates make it the best choice for this process.

C. acetobutylicum is an anaerobic, saccharolytic and mesophilic strain with an optimal growth temperature of 37ºC. It is able to produce some commercially useful products (e.g. butanol, acetone, ethanol, hydrogen, acetic acid, butyric acid). It is one of the first bacteria to be used at industrial scale.[131]

4.5.1. Metabolism

Metabolism in *C. acetobutylicum*, like in all cells, starts with the transmembrane transport. The nucleotide adenosine triphosphate (ATP) is the main carrier of biologically available energy in living cells. This is because all living processes requiring energy are coupled to the conversion of ATP to adenosine diphosphate (ADP) [157], as is shown in the equation (1).

$$ATP + H_2O \xrightarrow{Cellular\ process} ADP + P_i \qquad (1)$$

ATP can be formed either by substrate-level or electron transport phosphorylation.[158] This molecule will play an important role in cellular transport. Once the molecules is inside the cell, it will be metabolize according to its nature by different enzyme systems.[158]

A scheme showing the main metabolic pathway of hexoses by *C. acetobutylicum* is illustrated in Figure 17. Normally, only obligate anaerobes have this type of metabolism.

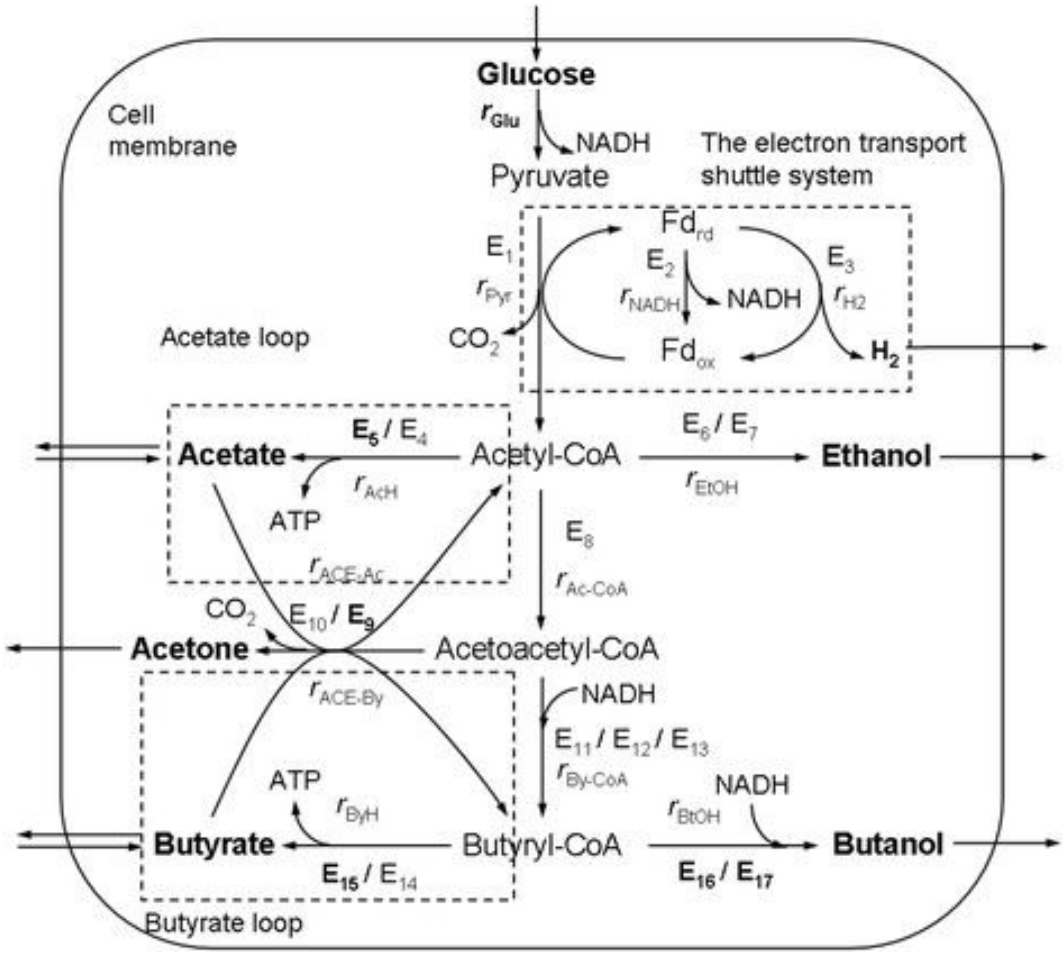

Figure 17. Main metabolic pathways of *C. acetobutylicum.* Intercellular enzymes: E1, pyruvate-ferredoxin oxidoreductase; E2, ferredoxin-NAD reductase; E3, hydrogenase; E4, phosphotransacetylase; E5, acetate kinase (encoded by ask); E6, acetaldehyde dehydrogenase; E7, ethanol dehydrogenase; E8. thiolase; E9, CoA-transferase (encoded by ctfAB); E10, acetoacetate decarboxylase; E11, β-hydroxybutyryl-CoA dehydrogenase; E12, crotonase; E13, butyryl-CoA dehydrogenase; E14, phosphotrans butyrylase; E15, butyrate kinase (encoded by buk); E16, butyraldehyde dehydrogenase (encoded by adhE); E17, butanol dehydrogenase (encoded by bdhB).[159]

This strain, like other Clostridial strains, uses phosphotransferase systems for sugar uptake and the Embden-Meyerhof-Parnas pathway for degradation of hexose phosphates to pyruvate. Then the pyruvate is converted to acetyl-CoA, involving the production of gas (hydrogen and carbon dioxide), by an enzyme system: pyruvate-ferredoxin oxidoreductase.

This enzyme system has a relatively low redox potential; similar to that of a hydrogen electrode at pH 7[160]

$H_2 / 2H^+$ E'_0= -0.41 V[158]

red. ferredoxin / ox. ferredoxin	E'_0= -0.41 V[158]
$NADH + H^+ / NAD^+$	E'_0= -0.41 V[158]

The next step of the metabolism in a batch culture of *C. acetobutylicum* occurs in two successive phases; first the acidogenesis, followed by the solventogenesis.[158]

The acidogenesis is characterized by a fast cell growth accompanied by the production of acetic acid, butyric acid, lactic acid, hydrogen and carbon dioxide. During this phase pH decreases considerably due to the acid products, this will contribute to the triggering of the solventogenesis.[161]

During the solventogenesis, the organic acids are partially consumed producing high quantities of butanol, acetone and some ethanol. Because the acid species decreases, the pH of the culture increases again. Productions of hydrogen and carbon dioxide are much lower relative to the acidogenesis, and the cell growth is very slow or completely null.[161]

Continuous culture of *C. acetobutylicum* will start with a batch stage for the acidogenesis, to be then changed to continuous mode when stabilized in the solventogenesis phase. In this work, only batch fermentation was employed for the cultivation of *C. acetobutylicum.*

4.5.2. Growth requirements

Bacterial cultivation requires specific conditions and media with specific nutrients composition. Temperature, pH, pressure, agitation, oxygen content, redox potential and nutrients are all factors playing an important role in the fermentation behavior and final yields (Figure 18).[127]

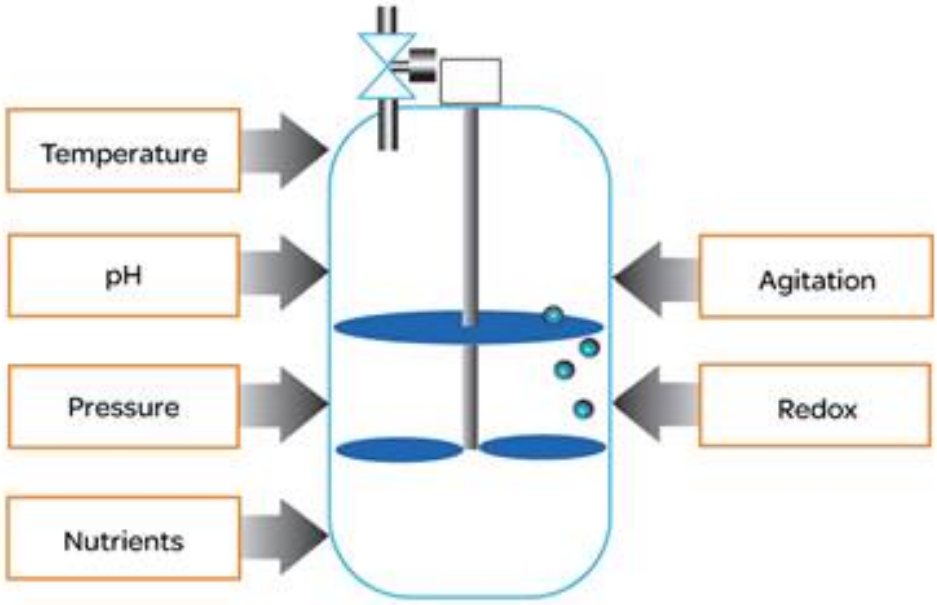

Figure 18. Schematic of a bioreactor with the variables influencing a fermentation process.[162]

The pH is one of the most important variables in the cultivation of *C. acetobutylicum.* Some studies have shown that the pH value and control can be a critical factor for the optimization of fermentative butanol production.[163–167]

pH will strongly affect the metabolic phases, more specifically, the transition from the acidogenesis to the solventogenesis. The presence of non-dissociated forms of the acidic species depends on the pH of the culture media. Non-dissociated acids will enter the cell and altered the intracellular pH, provoking the cell to recycle these acids into solvents as a detoxification strategy, that lead to the solventogenesis.[168,169,161]

As mentioned before, optimal temperature (37°C) has been deeply studied and defined for *C. acetobutylicum.* Agitation of 200 rpm and no oxygen in the culture media has been clearly establish in the literature.[170,171]

Among the chemicals of the culture media, the source of carbon represents the highest fraction. For this reason, it is the most important component for an efficient incorporation of fermentation processes in an integrated biorefinery. The most common carbon source used in fermentation processes is glucose.

Fermentation of several hexoses and pentoses by *C. acetobutylicum* has been reported in the literature, making it a potential microorganism for the fermentation of carbohydrates mixtures.[172,136,173,174]

4.5.3. Inhibition

During (ligno)cellulose conversion, is very often to found side reaction that produce undesired compounds for microorganisms. In biological conversion processes, these compounds are called inhibitors, they do not react directly with the raw material but they will interfere with the normal development of the biocatalyst (enzymes and microorganisms).[41,61]

Inhibition of Clostridial species by non-dissociated acids has an early impact on the fermentation process (see previous sub-section), affecting cell growth at concentrations starting at 0.25 g / L.[175,168]

Solvents have a different inhibition mechanism. Their hydrophobic nature allows them to disarrange the phospholipidic bilayer of the cellular membrane.[176–178] Short-chain alcohols, like ethanol, will decrease the fluidity of the cell membrane. In contrary, butanol and long-chain alcohols will increase it.[179]

Because the sources of these carbohydrates are so diverse, microbes with a high level of substrate adaptability that can tolerate toxic inhibitors in industrial media, are sought after for industrial-level fermentation.[180] *C. acetobutylicum* exhibit resistance to aldehydes such as 5-hydroxymethyfurfural and furfural,[143] that are the main inhibitors produced during liquefaction of (ligno)cellulose.[86,181,182]

4.6. Conclusion

Direct fermentation of cellulose using cellulolytic *Clostridia* have kinetics that are too slow to be economically viable. For this reason, a saccharolytic *Clostridium*, *C. acetobutylicum,* is used to convert cellulose-derived carbohydrates into profitable chemicals. Moreover, this strain has already been used at the industrial scale processes. Its use leaves the door open to high-profile projects.

Concerning the utilization of carbohydrates, *C. acetobutylicum* is able to use a wide variety of sugars (hexoses and pentoses) which gives a potential advantage to ferment the carbohydrate mixtures obtained by thermochemical conversion of (ligno)cellulose. It has been proven its resistance to some of the most common inhibitors derived from thermochemical depolymerization (e.g. 5-HMF, furfural), providing the sturdiness to endure the fermentation of complex mixtures.

Concerning the fermentation of pyrolytic oil, only few works are reported in the literature using yeast or a modified *E. coli* (explained better in the next section), but there is no report in the literature of pyrolytic oil fermentation by Clostridial strains. This approach was studied in this PhD but only presented in the appendices section 2.

III. Combining thermochemical and biological conversion of biomass

The main objective of this work is to propose a biorefinery strategy combining thermochemical and biological conversion processes. In this section some of the current studies at the interface of these two fields will be summarized. Then our proposed biorefinery concept will be explained.

1. Existing concepts

Only few works have been reported considering a thermochemical process as an entry process for biological conversion in an integrated biorefinery.[58,59,183]

Jarboe et al.[58] published a mini-review proposing fast pyrolysis as a viable non-enzymatic method for the depolymerization of cellulose. Shen et al.[59] reported a review summarizing the features, challenges and mitigation strategies for pyrolysis-fermentation and gasification-fermentation hybrid biorefinery processes. Fast pyrolysis allows producing high yields of levoglucosan. Gasification to produce syngas, followed by ethanol fermentation is commercially more advanced but has the main viability obstacle due to the low energy density of final product: ethanol.[59]

Zheng et al.[183] proposed a biorefinery strategy using organosolv fractionation, coupled to fast pyrolysis to produce levoglucosan, showing an efficient method to produce sugars. However, the main drawback of this strategy is that levoglucosan is not easily fermentable by bacteria.[184]

Only some yeast are able to ferment this anhydro-saccharide[185,186] and more recent developments using engineered bacteria have shown the utilization of small quantities of this substrate.[187–190]

2. Proposed concept in this PhD

In this work, we proposed an advance biorefinery strategy, combining 1) lignocellulosic biomass fractionation recovering a cellulose-rich pulp, 2) its depolymerization by hydrothermal liquefaction producing a mixture of carbohydrates and 3) its fermentation by the bacterial strain *C. acetobutylicum,* producing building blocks such as: butanol, acetone, ethanol, acetic acid, butyric acid and hydrogen.

Figure 19shows a scheme of the general biorefinery process proposed in this work and the structure of this work.

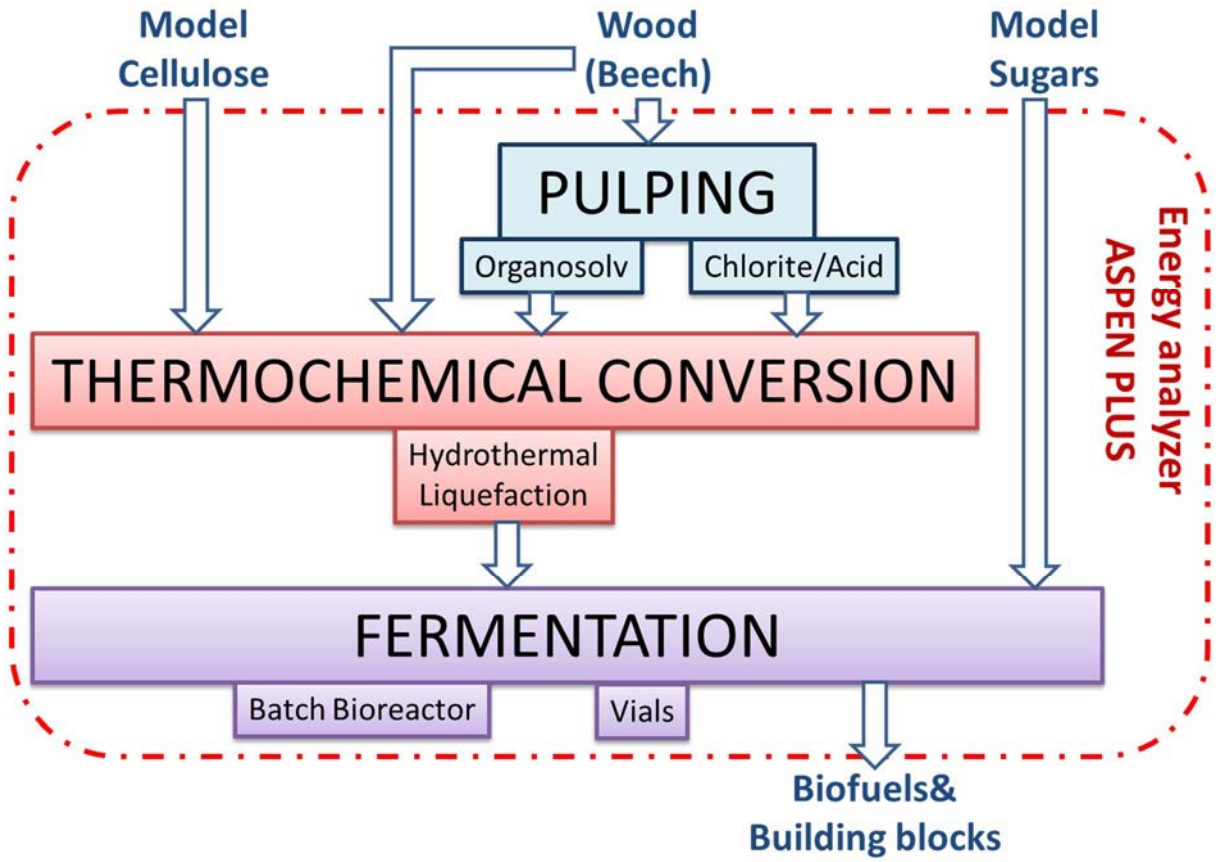

Figure 19. Scheme of the proposed biorefinery strategy.

Concerning the lignocellulosic substrate, beech wood (*Fagus sylvatica)* was chosen due to its importance and availability in our region of France. In the first part of this biorefinery strategy, fractionation (or pulping) was performed by the organosolv and chlorite/acid methods. These

methods allow recovering a cellulose-rich pulp that was characterized in macromolecules content, inorganics, cellulose crystallinity and degree of polymerization.

In the second part, cellulose-rich pulps were depolymerized by hydrothermal liquefaction. Before starting with the lignocellulosic biomass treatment and liquefaction, model cellulose (microcrystalline cellulose, Avicel) was studied in order to understand its depolymerization and to separate the effect of the side products coming from cellulose from those coming of other constituents present in pulps.

The identification and quantification of the cellulose-derived carbohydrates has required the implementation of improved analytical methods using ionic chromatography (HPAEC-PAD) and advance mass spectrometry (LTQ-Orbitrap).

Fast pyrolysis of cellulose and pulps (followed by hydrolysis of bio-oils) has been also conducted but it is not presented in this manuscript for sake of conciseness and clarity.

Then, the fermentation of the carbohydrate mixture produced by the various thermochemical processes was performed in batch mode. The development of the substrate preparation protocol (e.g. pH, concentration) was critical in order to ensure its utilization with normal fermentation patterns and yields. Results about the effect of the pH on the growth of *C. acetobutylicum* when cultivated on glucose or cellobiose are presented in the appendices section.

In the same way as for thermochemical conversion, cultures of *C. acetobutylicum* using mixtures of model substrates (i.e. glucose and cellobiose) was studied to reveal phenomena related to the utilization of carbohydrate mixtures. This experiments report for the first time the order of utilization of a complex mixture carbohydrates and its transformation to profitable chemicals.

Besides this experimental work, a simplified process model has been developed using the commercial software Aspen plus®, in order to establish mass and energy balance allowing to analyze the energetic performance and its key factors and challenges.

IV. References

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B. RESULTS

The main results found during this PhD are presented as five articles in this section. They are divided in three sub-sections: I) hydrothermal conversion of cellulosic materials, II) Biological conversion of cellulose-derived products and III) Integration of thermochemical and biological processes.

I. Hydrothermal conversion of cellulosic materials

The results about hydrothermal conversion of cellulose are presented in the following two articles. In the first one, hydrothermal liquefaction of microcrystalline cellulose was studied in a relatively wide range of temperatures to characterize its effect on the reaction mechanisms and to identify the best conditions for its depolymerization.

In the second article, lignocellulosic biomass was fractionated, by two methods: organosolv and sodium chlorite / acetic acid (SC / AA), in order to recover cellulose-rich pulps. These pulps were liquefied according to the conclusions of the precedent study on pure cellulose. The product distribution and sugars produced were characterized.

The following article presents results to get a better understanding of the effect of temperature and reaction time on the reaction mechanisms of cellulose liquefaction in hydrothermal media, under our conditions (in a batch reactor). Moreover, most of the analytical methods required for the characterization of products during this PhD were developed during this study case.

1. Article 1:

Decomposition of cellulose in hot-compressed water: detailed analysis of the products and effect of operating conditions

Article published in *Energy & Fuels*. DOI: 10.1021/acs.energyfuels.7b02994

ABSTRACT

Understanding the reaction pathways of cellulose hydrolysis in hot-compressed water (HCW) is crucial for the optimization of fermentable sugars and chemicals production. Advanced analytical strategies are required in order to better assess the wide range of products from cellulose conversion in HCW. In this work, cellulose conversion in HCW was conducted in an autoclave with sampling upon the reaction time under isothermal conditions (180°C, 220°C, 260°C, 0 to 120 min). Total water-soluble carbohydrates were quantified (phenol/sulfuric acid method). These products were first characterized by size exclusion chromatography coupled to mass spectrometry and evaporative light scattering detector (SEC-ELSD-MS). SEC is useful for screening the molecular weight distribution of soluble products. Then the chemical structure of water solubles has been attributed by hydrophilic interaction liquid chromatography coupled to a linear trap quadrupole Orbitrap mass spectrometer (HILIC-LTQ-Orbitrap-MS). This method notably evidences the formation of a cellobiose conformer under some HCW conditions. A

specific high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) method has been developed. This method allows for a selective separation of 5-hydroxy-methyl-furfural (5-HMF), glucose, fructose, mannose and oligomers up to cellopentaose. Carboxylic acids were quantified by high performance liquid chromatography with ultraviolet detection (HPLC-UV). Solid residues obtained after HCW conversion were characterized by X-ray diffraction (XRD) and permanent gas by micro-gas chromatography. The global reaction mechanism of cellulose liquefaction in HCW is rationalized based on these complementary methods. Cellulose conversion first proceeds with heterogeneous hydrolysis (fiber surface) to produce soluble oligomers in competition with pyrolysis (inner fibers with limited mass transfer of water) producing levoglucosan (promoted at higher temperature). Soluble oligomers produce glucose and isomers by homogeneous hydrolysis (liquid phase). C6 sugars can then undergo further conversion to produce notably 5HMF and erythrose.

1.1. Introduction

The depletion of fossil fuels represents one of the major concerns of our society. Moreover, their use produces high quantities of carbon dioxide emissions that are the main factor in the global warming. As a consequence, bio-fuels are developed in order to replace fossil fuels. Bioethanol is mainly produced from carbohydrates rich materials (sugarcane, sugar beet, maize) that are normally used for food production. This represents a controversy problem to society.(Berndes, Hoogwijk, and van den Broek 2003) Lignocellulosic biomass (wood, straw…) is a promising source of renewable carbon that does not compete with food production. It could be processed into second generation biofuels.(McKendry 2002; Briens, Piskorz, and Berruti 2008) This resource is composed mainly of cellulose (40-60 wt. %), hemicelluloses (20-40 wt. %) and lignin (15-25 wt. %) tightly bonded in a complex matrix.(Henricson 2000; Schweingruber 2007; Salmén 2015) Cellulose is the most abundant polymer on earth and its valorization to produce biofuels or chemical is widely studied.

Cellulose can be depolymerized into glucose and soluble oligosaccharides by a wide variety of technologies. Acid hydrolysis was developed to obtain glucose from cellulose. Several methods and technologies have been studied, reporting that high sugar yields and low reaction times can be achieved with strong acid media and high temperatures.(Torget, Kim, and Lee 2000; Neureiter et al. 2002; Rinaldi and Schüth 2009; Jiang et al. 2015) Cellulose can also be depolymerized into levoglucosan and other anhydro-oligosaccharides with high yields by fast pyrolysis.(Piskorz et al. 2000; Lédé, Blanchard, and Boutin 2002; Lédé 2012; Jia et al. 2016; Garcia-Nunez et al. 2017) The main drawback is that these anhydro-saccharides need to be hydrolyzed into saccharides in order to be suitable for fermentation, that allows to transform them into biofuels and other chemical products of interest.(Bennett, Helle, and Duff 2009) Different hydrothermal treatment technologies for cellulose / biomass have been developed. The operating conditions have a strong effect on water properties.(Toor, Rosendahl, and Rudolf 2011) The ionization of water (H_2O) into OH^- and H_3O^+ increases with temperature allowing the water to become the acid catalyst for cellulose hydrolysis. That is why the solvation power is different, and water at high temperatures near the critical point can dissolve organic compounds, unlike the ambient temperature water that will only dissolve the polar compounds.(Peterson et al. 2008; Kumar and Gupta 2009) Supercritical water treatment ensures a fast solubilisation of cellulose and a subsequent hydrolysis of the primary products into simple molecules.(Sasaki et al. 1998, 2000; Tolonen et al. 2015) Nevertheless, working with supercritical water will increase considerably both the operational and reactor cost.(Sasaki et al. 1998; Yu and Wu 2010b; Abdullah, Ueda, and Saka 2014) Supercritical water is highly corrosive and expensive materials will be needed for the reaction system. This is because of the high concentration of acidic hydroniums H_3O^+ generate by the autoionization of water at this temperature.(Peterson et al. 2008) Another alternative technology is liquefaction in hot-compressed water (subcritical conditions), that normally operates at conditions between 150-300°C at around 10 MPa. Even if water at these conditions has a reactivity that is considerably lower than at supercritical conditions, this technology presents an interesting

alternative to perform cellulose depolymerization into profitable carbohydrates for subsequent fermentation into biofuels and/or chemicals.(Matsumura et al. 2006; Yu, Lou, and Wu 2008) Significant progress has been made in cellulose/lignocellulose pretreatment by hot-compressed water (HCW). Several reaction systems in continuous,(Sasaki et al. 1998, 2000; Ehara and Saka 2002) semi-continuous(Yu and Wu 2009; Sasaki, Furukawa, et al. 2002; Matsunaga et al. 2008) and batch(Ehara and Saka 2002; Kumar and Gupta 2009) mode have been developed.

Cellulose hydrolysis products are often characterized by HPLC methods using conventional ion-exchange or aqueous size exclusion columns that allow the separation of monomers but not oligomers.(Sasaki et al. 1998; Kabyemela et al. 1999; Sasaki et al. 2000) High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) have been studied and used as a powerful tool for the analysis of carbohydrates.(Townsend et al. 1988; Lee 1990; Paskach et al. 1991; Rohrer and Townsend 1995) Several methods have been developed according to the targeted compounds and now is currently the most common and efficient analytical method for the quantification of cellulose derived compounds.(Yu and Wu 2009, 2010b; Corradini, Cavazza, and Bignardi 2012; Tolonen et al. 2013; Yu, Shafie, and Wu 2013; Cao et al. 2016) Yu et al.(Yu et al. 2016) and Tolonen et. al(Tolonen et al. 2015) have developed interesting methods coupling HPAEC-PAD with mass spectrometry (MS), allowing the identification of isomers and cellulose derived compounds with a high degree of polymerization, for which there is no standard available in the current market. MALDI-TOF is commonly used for the analysis of molecules with a high molecular weight.(Mechref, Novotny, and Krishnan 2003; Ren et al. 2005) An accurate and comprehensive analysis of liquid products is fundamental for the understanding of the reaction mechanisms of cellulose decomposition in HCW. In the same way, the latter is fundamental for improving efficiency of this technology. Sasaki et al.(Sasaki et al. 1998, 2000; Sasaki, Furukawa, et al. 2002; Sasaki, Goto, et al. 2002; Sasaki, Adschiri, and Arai 2004) have intensively studied the hydrolysis of cellulose in HCW. These studies concluded that cellulose depolymerization follows two major reaction pathways: 1) dehydration of reducing-end glucose

via pyrolytic cleavage of the glycosidic bond in cellulose (or partly retro-aldol reaction as described later) and 2) hydrolysis of the glycosidic bond via swelling and dissolution of cellulose. Several other studies on cellulose hydrolysis in HCW confirm that the main products of cellulose depolymerization in HCW are: glucose, fructose, 5-hydroxymethyfurfural (5-HMF), glycolaldehyde, dihydroxyacetone, erythrose, levoglucosan, cello-oligosaccharides (cellobiose, cellotriose, cellotetraose, cellopentaose, etc.).(Antal Jr., Mok, and Richards 1990; Ehara and Saka 2002; Kruse and Gawlik 2003; Sasaki, Adschiri, and Arai 2004; Kamio et al. 2006; Matsumura et al. 2006; Peterson et al. 2008; Yu, Lou, and Wu 2008; Lu et al. 2009; Yu and Wu 2010b, 2010c; Lü and Saka 2012; Yu, Shafie, and Wu 2013; Abdullah, Ueda, and Saka 2014; Tolonen et al. 2015; Yu, Long, and Wu 2015) The main reaction pathways have been summarized by Matsumura et al. This work highlights that a heterogeneous hydrolysis occurred at the surface of the cellulose fibers while pyrolysis takes place inside the fibers. Then the soluble products undergo secondary reactions designated as “homogeneous hydrolysis” (Figure 20). Despite all the extensive studies on cellulose conversion in HCW, there is still an important lack of a comprehensive characterization of the products using various analytical techniques on the same liquid sample in order to account for the global mechanism of cellulose conversion.

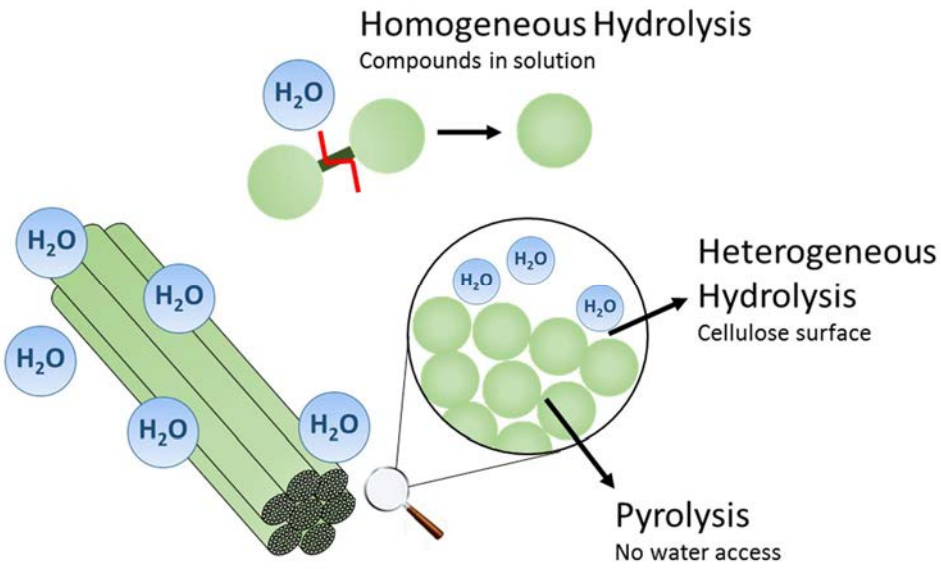

Figure 20. Schematic view of cellulose thermochemical depolymerization in hot-compressed water (adapted from Matsumura et al.)

The last two decades have witness important advances in analytical chemistry that can be used as a powerful tool in the characterization of cellulose hydrolysis products. In this work, an advanced methodology is proposed in order to characterize the products from cellulose conversion in HCW. Solid, liquid (water-soluble compounds) and gaseous products were recovered to determine the mass balance. Liquids were characterized for the first time by a combination of:

1) Size exclusion chromatography with evaporative light scattering detection and mass spectrometry (SEC-ELSD-MS) for assessing the distribution of molecular weight of species soluble in water;

2) Hydrophilic interaction liquid chromatography with linear trap quadrupole Orbitrap mass spectrometry (HILIC-LTQ-Orbitrap MS) for identifying the chemical structure of some important products (a cellobiose isomer has been identified);

3) A specific high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) method. This HPAEC-PAD method allows the characterization of both monomeric and oligomeric saccharides and is able to discriminate glucose isomers (mannose and fructose) and 5-hydroxymethyfurfural that often escape detection or are poorly quantified with regular chromatographic methods.

The predominant products and reactions as evidenced by all these complementary methods are discussed. This work provides a better understanding of the evolution of the products of cellulose liquefaction in HCW as a function of temperature and reaction time.

1.2. Materials and Methods

1.2.1. Reactants

Microcrystalline cellulose Avicel PH-101 (particle size < 5µm, Sigma Aldrich, San Luis, USA) was used for all the experiments. Glucose (99.5 %), 5-hydroxymethyl furfural (5-HMF, 99 %), glycolaldehyde (99 %), fructose (99 %), mannose (99 %), erythrose (75 %) standards were purchased from Sigma-Aldrich. Levoglucosan (98 %), cellobiose (98 %), cellobiosan (95 %), cellotriose (95 %), cellotetraose (95 %) and cellopentaose (95 %) standards were purchased from Carbosynth (Compton, United Kingdom).

1.2.2. Liquefaction in hot-compressed water

The overall experimental and analysis procedure is illustrated in **Figure 21**. A batch reactor system was used to study the cellulose depolymerization.

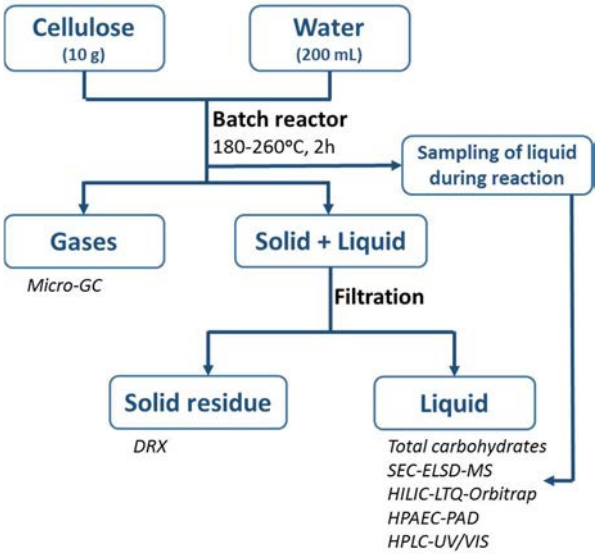

Figure 21. Experimental procedure and conducted analysis

A stirred reactor (stainless steel 316, Parr Instrument Company) of 300 mL was filled up with 200 mL of de-ionized water and 10 g of cellulose. Reactor was purged 2 times with nitrogen. Then 10 bar of nitrogen atmosphere was used to maintain the water in liquid phase and as a tracer for the quantification of permanent gases. Reactor was heated to the target temperature (180°C, 220°C or 260°C) at 5 K / min, with a final pressure of 14 bar, 24 bar and 50 bar respectively. When the target

temperature was reached, reaction time started and 7 samples were taken every 20 min for the liquid phase analysis (from initial time to 120min). The sample volume was 3 mL. 2 purges were first sampled followed by the sample for further analysis (leading to 9 mL of total sampled volume per sampling). The sampling of this small volume does not impact the pressure and temperature inside the autoclave (pressure and temperature constant within 1bar and 3°C respectively thorough the test). At the end of each experiment (2h), the reactor was rapidly quenched (with cold water) to ambient temperature (3-4min.). Specific experiments were conducted without sampling in order to quantify mass balances.

The solid / liquid mixture was filtered using a glass microfiber filter (Whatman®, pore size: 0.7 µm) with a Büchner under vacuum suction. The solid residue was washed with de-ionized water and subsequently dried at 105°C for 24h. Then it was cool down in a desiccator to be weighted and it was finally stored for further XRD analysis. The recovered liquid was immediately analyzed by liquid chromatography and total carbohydrates determination. Then it was stored in a freezer at -80°C. Once the liquid is completely frozen, the water was removed by freeze drying at -40°C, <0.04 mbar for 24 h. The solid residue was weighted and defined as the "total water-solubles". Analysis by liquid chromatography has been conducted before and after freeze drying to verify that there was no significant change in the composition of soluble products.

1.2.3. Analysis of the crystalline structure of solid residues by XRD

The washed and dried solid residues have been manually crushed (with a mortar) to be analyzed by XRD analysis. XRD has been performed using an INEL XRG-3000. This X-ray diffractometer uses a copper radiation (CuKα) having wavelength λ (Kα1) =1.5406 nm generated at 30 kV and 25 mA. The diffraction intensities were measured in a range of 0 to 82° (2θ) using a step size of 0.029°. This analysis allows determining if the solid is amorphous or crystalline (crystallinity index) and also allows characterizing changes in the crystalline structure of solid residues. Crystallinity index (CI) values were calculated for each solid as the ratio between the height of

the (002) reflection peak and the minimum between the (021) and (10$\bar{1}$) reflection peaks, corresponding to $2\theta=18.9°$ (Park et al. 2010).

1.2.4. Soluble products analysis

a) Total carbohydrates determination

The total carbohydrates (sugars) determination was performed as in Rover et al.(Rover et al. 2013) using the phenol/sulfuric acid assay (DuBois et al. 1956). 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 total water-solubles (weighted after lyophilisation) and total carbohydrates.

b) Analysis of the molecular weight distribution of soluble products by SEC-ELSD-MS

The separation of soluble products was performed with two SEC PL aquagel-OH 20 columns (Agilent) using 0.4 mL/min of a methanol/water (50:50 v/v) mixture as mobile phase. Ammonium acetate (100 µM) and formic acid (0.1%) were also added to the mobile phase in order to improve ionization (Le Brech et al. 2016). The oven containing the columns was kept at 40°C. The system was equipped with an evaporative light scattering detector (ELSD, Shimadzu) and mass spectrometry (MS, Shimadzu), using an electrospray ionization (ESI) system.

c) Analysis of the chemical structure of main carbohydrates by HILIC-LTQ-Orbitrap-MS

The separation was made using a hydrophilic interaction liquid chromatography (HILIC) column (YMC-Pack Polyamine II (250mm×4.6mm) composed of a polymeric support (silica base) with mixed secondary and tertiary amino derivative (particles size are 5µm and pores size are 12 nm).

The pre-column is a 7.5mm×4.6mm cartridge. Both were obtained from YMC Europe GmbH (Dinslaken, DE). This analytical method was previous develop by Ricochon et al.(Ricochon et al. 2011) for the analysis of mono- and disaccharides. In this work, its selective ionization for carbohydrates, without the interference of aldehydes, was confirmed.

The HILIC is coupled to a hybrid system of mass spectrometry consisting in a linear ion trap mass spectrometer with an Orbitrap analyzer (LTQ-Orbitrap, Thermo Electron Corporation, Waltham, MA, USA). LTQ is equipped with an atmospheric pressure chemical ionization (APCI) interface operating in negative ion mode. Xcalibur 2.0 SR2 software is used for computer control and data process. For this study, the detection was performed in SIM mode.

d) Quantification of main soluble products by HPAEC-PAD

Soluble products were quantified by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). All the major compounds were identified and quantified using the respective calibration curve for each compound. The column used was a CarboPacPA100. A CarboPacPA10 was also tested but no satisfactory results were found. The HPLC system is 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 uses a gradient program as follow: 5 min of stabilization at 25% B, then from 0 to 5 min 25% B, from 5 to10 min a gradient from 25 to 100% of B and from 10 to 25 min 100% B.

e) Analysis of carboxylic acids by HPLC-UV

Carboxylic acids were analyzed by HPLC-UV using an Aminex HPX-87H column (Biorad®). The detection was made using an ultraviolet (UV) detector at 254 nm. This wavelength has been proven to be specifically sensitive for carboxylic acids. The injection volume was 10 µL. The oven kept the column at 45°C, the mobile phase was a 25mM sulfuric acid (H_2SO_4) solution. The analysis time was 30 min in isocratic mode. Chromatograms are shown in Figure S6 (supporting information).

1.2.5. Permanent gas analysis

The permanent gases were collected in a Tedlar bag when the reactor was cooled down to ambient temperature. Permanent gas was characterized by a µGC-Varian 490 equipped with four modules, composed of two molecular sieves 5A, a PoraPlot U and a CP-Wax 52CB columns. The µGC was calibrated using standard bottles (Air Liquide, France). Hydrogen (H_2), carbon monoxide (CO) and carbon dioxide (CO_2) were quantified using N_2 as a tracer (known volume and pressure of N_2 introduced in the closed reactor).

1.3. Results and Discussion

1.3.1. Mass balance

Mass yields of solid residue, water soluble products and permanent gases are presented as a function of the reaction temperature (after 120min of reaction time under isothermal conditions) in Figure 22.

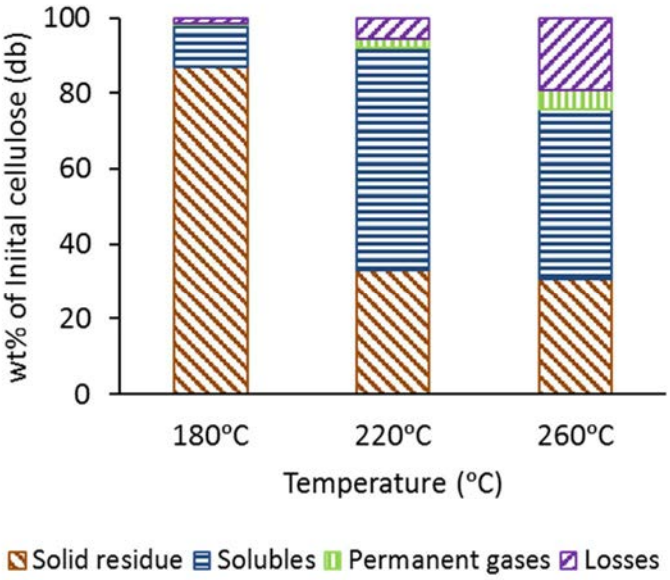

Figure 22. Mass balance for cellulose liquefaction in HCW as a function of the reaction temperature (after 120min at isothermal condition)

The mass balances are between 81 and 98 wt.%. The quantified products decrease with temperature due to a more important loss most probably in the liquid phase during filtration and lyophilisation. High percentage of loses (19.1 wt%) at 260°C is probably due to the high amount of volatile species that are produced at this temperature and the lack of analytical methods to measure them. These species are not easily recoverable because they are often evaporated during the experiment and/or the products recovery. On the other hand, all permanent gases were completely analyzed.

The mass balance is based on the initial cellulose. However, water also takes part as a reactant during cellulose hydrolysis in hot-compressed water. Therefore, a fraction of the initial water mass can be found in the products. The determination of the reacted water with cellulose is a difficult task and this was not taken into account.

Solid yield decreases as temperature increases. It decreases sharply from 86.9 wt.% at 180°C to 33.1 wt.% at 220°C. The decrease is less pronounced from 220°C to 260°C, changing only from 33.1 wt.% to 30.9 wt.%.

Depolymerization of microcrystalline cellulose in water soluble compounds is known to start at 180°C (Yu and Wu 2010c), that is why this is the lowest temperature chosen for this study. At 180°C, cellulose depolymerization rate is relatively slow resulting in a soluble products yield of 11.1% and gas yield of only 0.3%. Both yields increase significantly to 58.9% for the soluble products and 2.4% for the gases at 220°C. Gas yield still increases to 5.2% at 260°C. However, soluble products yield decreases to 44.8%. This is because of high decomposition rate of the soluble products into lighter molecules predominant at this temperature. This can explain also the increase in gas yield.

1.3.2. XRD of the solid residues

After each experiment of cellulose liquefaction in HCW, the solid residue has been analyzed by XRD. Figure 23 shows the diffractograms obtained for the raw cellulose and the solid residues obtained after liquefaction in HCW at three different temperatures (180, 220 and 260°C).

The purpose of this analysis was to illustrate the changes on the crystallinity index and also to look for some possible changes in its crystalline structure and / or evidence of its recrystallization into other crystal networks like cellulose II (Ago, Endo, and Hirotsu 2004) or hydrochar (Guo et al. 2015).

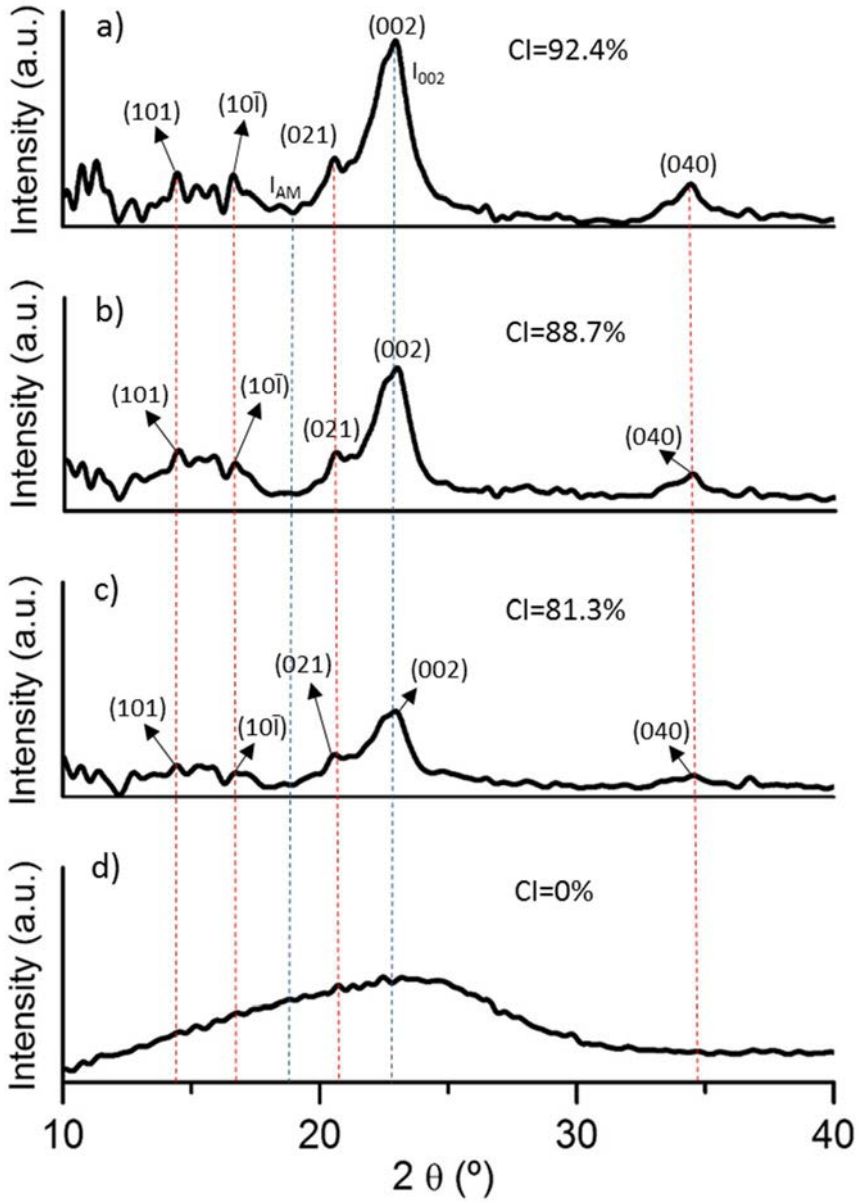

Figure 23. XRD patterns of cellulose Avicel PH-101 and solid residues after cellulose liquefaction in HCW during 2h at different temperatures. a) reference Avicel PH-101, b) 180°C, c) 220°C and d) 260°C. Crystallinity index (CI) for each sample are also indicated.

The crystallinity index (CI) for the Avicel cellulose was 92%, that is in agreement with the value of 91% found in (Park et al. 2010). The CI values determined for the solid residues after cellulose liquefaction in HCW were 91, 84 and 0 for the temperatures 180, 220 and 260°C respectively. These results indicate that at temperatures lower than 220°C it is still possible to recover a cellulosic material from the liquefaction process. Nonetheless, at 260°C its structure presents no crystalline phases. CI values calculated for the other reflection peaks (always relative to I_{AM}) are shown in Table 1.

Table 8. Crystallinity index (%) for all the reflection peaks of crystalline cellulose. Calculated relative to I_{AM} and for λ (Kα1)=1.5406

Reflection peak	*Angle 2θ (°)*	*Spacing d (Å)*	*Temperature*			
			Untreated	*180°C*	*220°C*	*260°C*
(002)	22.7	3.9	92	89	81	0
(101)	14.4	6.1	76	68	66	0
(10$\bar{1}$)	16.6	5.3	75	61	60	0
(040)	34.5	2.6	79	72	59	0
(021)	20	4.4	66	56	1	0

The Figure 23a shows the typical XRD pattern of raw cellulose (Avicel PH-101) indicating the characteristics reflections that serve as guide to identify changes in the crystalline structure.

Cellulose residue after treatment in HCW at 180°C presented a crystalline structure that remained relatively unchanged in comparison to the raw Avicel cellulose. However, the reduction in the intensity of the reflection (002) makes more evident another nearby peak at 2θ=22.4°. This peak becomes more detectable as the reaction temperature increases from 180 to 220°C (Figure 23b-c).This is a slight change in the regular pattern of Avicel cellulose but it is an interesting proof of some change in the crystalline structure of the initial cellulose. Moreover, the reflection (040) is barely recognizable, showing that during hydrolysis in HCW at this temperature the whole crystalline structure has been affected without being completely converted to an amorphous material.

The X-ray diffraction pattern at 260°C does not depict a pronounced crystalline phase (Figure 23d). The presence of a big broad peak is characteristic of the amorphous carbonaceous structure

in hydrochar (Xie et al. 2011; Yuan, Xu, and Zhang 2011). The major reflections (002, 021, 101 and 10$\bar{1}$) and the new peak are no longer sharp and intense.

1.3.3. Characterization of liquids

f) Total carbohydrates determination

One of the main interests of cellulose hydrolysis is the sugar recovery. For this reason, we have first conducted the quantification of total carbohydrates as glucose equivalent by the phenol/sulfuric acid method.

The total sugars yields (wt. %) of the recovered liquid phase after 2 h of cellulose liquefaction in HCW for each temperature studied are shown in Figure 24. From 180°C to 220°C the sugar yield increases from 6.2 to 59.3 wt. %. At 180°C, total sugars represent approximately one third of the total soluble products. At 220°C, total sugars represent more than 80% of all the soluble products. Therefore, at this temperature the decomposition rate into lighter compounds was slower enhancing the selectivity into soluble sugars.

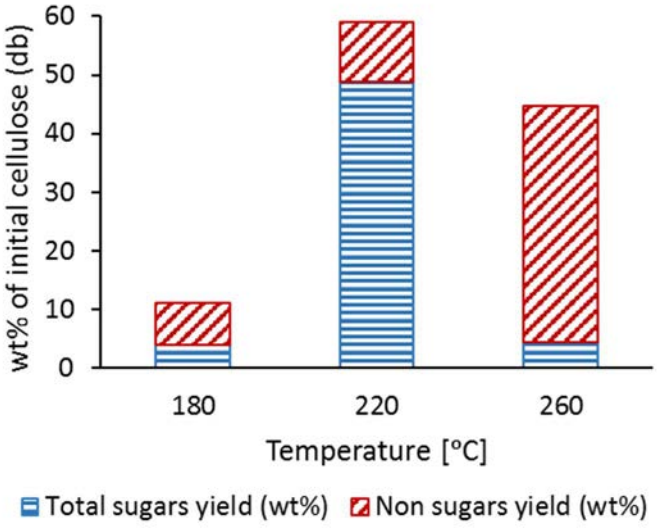

Figure 24. Total sugars and non-sugars fractions in the liquid phase as a function of the reaction temperature (after 120 min of isothermal reaction in HCW)

At 260°C the homogeneous hydrolysis rate is very fast (Sasaki, Furukawa, et al. 2002; Sasaki, Adschiri, and Arai 2004; Yu and Wu 2011) and most of the sugars produced are rapidly degraded into low molecular weight species (MW<170 g/mol) like aldehydes and carboxylic acids (Kabyemela et al. 1999; Sasaki, Goto, et al. 2002), as it will be further evidenced by HPLC analysis in a next section.

g) Analysis of the molecular weight distribution of soluble products by SEC-ELSD-MS

The quantification of total sugars is important to present an overall selectivity of the process but it is also of interest the molecular weight distribution of the products because their valorization depends on their molecular weight (and chemical moieties).

For this reason, all the liquid samples were first analyzed by SEC-ELSD-MS. Figure 25 illustrates the total ion current (TIC) spectra as a function of the retention time for different liquefaction reaction times. This analysis gives an overall qualitative fingerprint of products distribution. The ESI ionization source allows the screening of a wide range compounds derived from cellulose depolymerization: from oligomers to lighter compounds.

At 180°C, the major detected compounds correspond to oligosaccharides. Lines indicating the *m/z* key species like: glucose (*m/z* 180), levoglucosan (*m/z* 162) and erythrose (*m/z* 120) are presented in Figure 25. At 220°C the production of monomeric saccharides and anhydrosaccharides becomes more important compared with 180°C. At 260°C, the presence of oligosaccharides is almost null. The peaks appearing between 50 and 55 min do not have m/z values corresponding to anhydro-oligosaccharides. Other peaks appearing between 55 and 60 min retention time have not yet been identified but their *m/z* signals could correspond to furans and aldehydes.

On the other hand, ELSD is complementary to ESI-MS because this detection method allows identifying the presence of a macromolecule (that cannot be ionized and identify by ESI-MS) eluted during the first 10 min of analysis, as presented in the supporting information in Figure

S1. The signals of ELSD showing the evolution of this macromolecule(s) during the reaction at 260°C are shown in Figure S2. The size of these compounds can hardly been determined but their elution at the beginning of the two SEC columns indicates that they are very big molecules (but soluble).

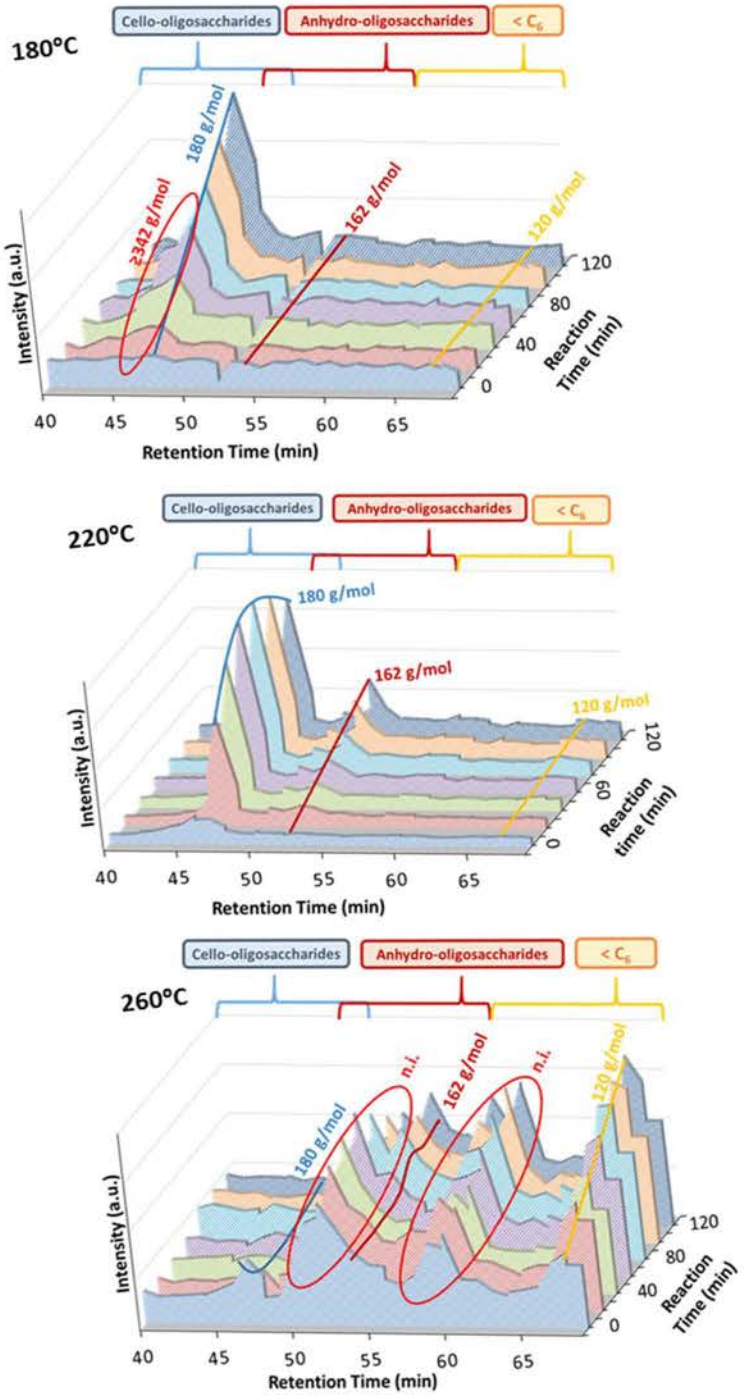

Figure 25. TIC obtained by SEC-ESI-MS of the liquid phase during cellulose liquefaction in HCW as a function of the retention time for the 3 temperatures of cellulose conversion studied (180, 220 and 260°C) and as a function of liquefaction reaction time (7 samplings from initial isothermal time to 120min)
(n.i.=not identified product)

h) Analysis of the chemical structure of main carbohydrates by HILIC-LTQ-Orbitrap-MS

This analysis is achieved in order to properly identify the carbohydrates present among the water-soluble products and differentiate them from other chemical families like aldehydes and carboxylic acids that might hinder the interpretation of the ESI-MS analysis. The APCI source used within the LTQ-Orbitrap analytical method selectively ionizes the carbohydrates without having the interference of aldehydes and carboxylic acids(Ricochon et al. 2011). Some major compounds were assigned by running the corresponding standards and samples on SIM mode in the HILIC-LTQ-Orbitrap (see supporting information, Figure S3).

Anhydro-saccharides like levoglucosan and cellobiosan were also identified by SIM mode in the HILIC-LTQ-Orbitrap. There are two main possibilities to explain the presence of these anhydro-saccharides; either by dehydration of glucose and cellobiose(Minowa et al. 1998; Kabyemela et al. 1999; Sasaki, Goto, et al. 2002; Yu and Wu 2011) producing levoglucosan and cellobiosan respectively or by cellulose transglycosylation,(Matsumura et al. 2006; Yu, Lou, and Wu 2008) producing firstly anhydro-oligosaccharides that will be hydrolyzed into levoglucosan and cellobiosan. This phenomenon will be discussed later. The presence of erythrose among the water-soluble products was also successfully confirmed thanks to this method.

Moreover, a cellobiose isomer was identified at *m/z* 377.5 and 379.5 as presented in Figure 26. The nature of this isomer has not been verified but according to previous studies(Lomax et al. 1991; Yu, Shafie, and Wu 2013; Soisangwan et al. 2016), it is probably epicellobiose or cellobiulose (Figure S4).

The hydrolysis of this isomer will produce one molecule of glucose and one of mannose (in the case of epicellobiose) or fructose (in the case of cellobiulose) (Yu, Shafie, and Wu 2013). This can explain another reaction pathway for the production of these two monomeric sugars.

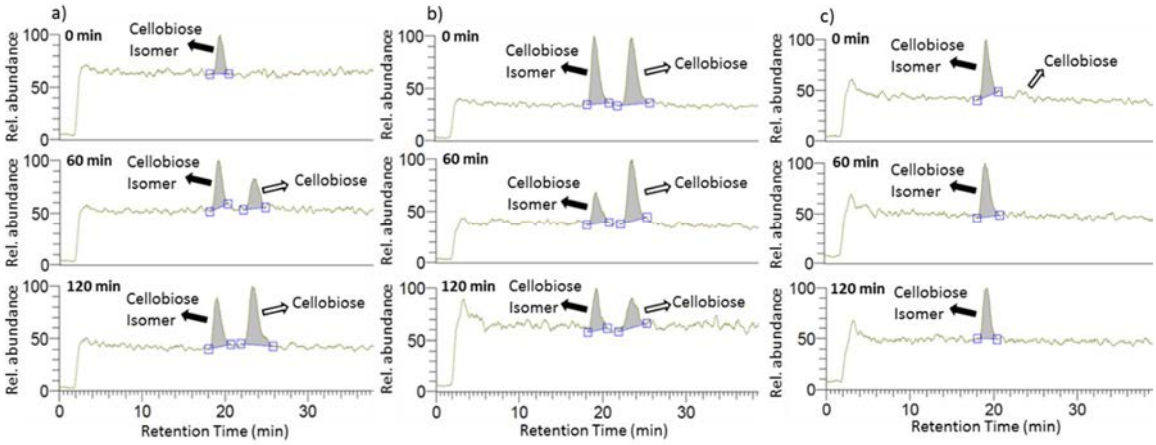

Figure 26. SIM mode chromatogram following the chlorine adducts $[M+35]^-$ and $[M+37]^-$ of cellobiose (M=342.3) at reaction temperatures a) 180°C, b) 220°C and c) 260°C. The cellobiose isomer is most probably cellobiulose or epicellulose (see supporting information).

Figure 26a and b show that at 180 and 220°C both dimers are present among the water-soluble products during the 2 hours of reaction. On the other hand, at 260°C only the cellobiose isomer was detected all along the test. Cellobiose is detected only at the beginning of the reaction (Figure 26c).

In the case of monomeric isomers, the separation was good enough to perform the identification (by SIM mode in a HILIC-LTQ-Orbitrap), but it was not possible to be quantitative. For this reason, the chromatographic system chosen for the quantification of these monomers was HPAEC-PAD.

i) Quantification of main soluble products by HPAEC-PAD

Besides monomeric and oligomeric saccharides, the major products of cellulose liquefaction in HCW include also some anhydro-saccharides like levoglucosan and cellobiosan. Other light aldehydes such as 5-HMF and glycolaldehyde are also present. HPAEC-PAD was found to be an effective analytic system to quantify all these products. The effect of the eluent on the response factor is presented in Figure S5 (supporting information). Figure 27 shows the effect of mobile phase NaOH concentration on the HPAEC-PAD analysis of the recovered liquid from cellulose liquefaction in HCW for 2 h at 180°C.

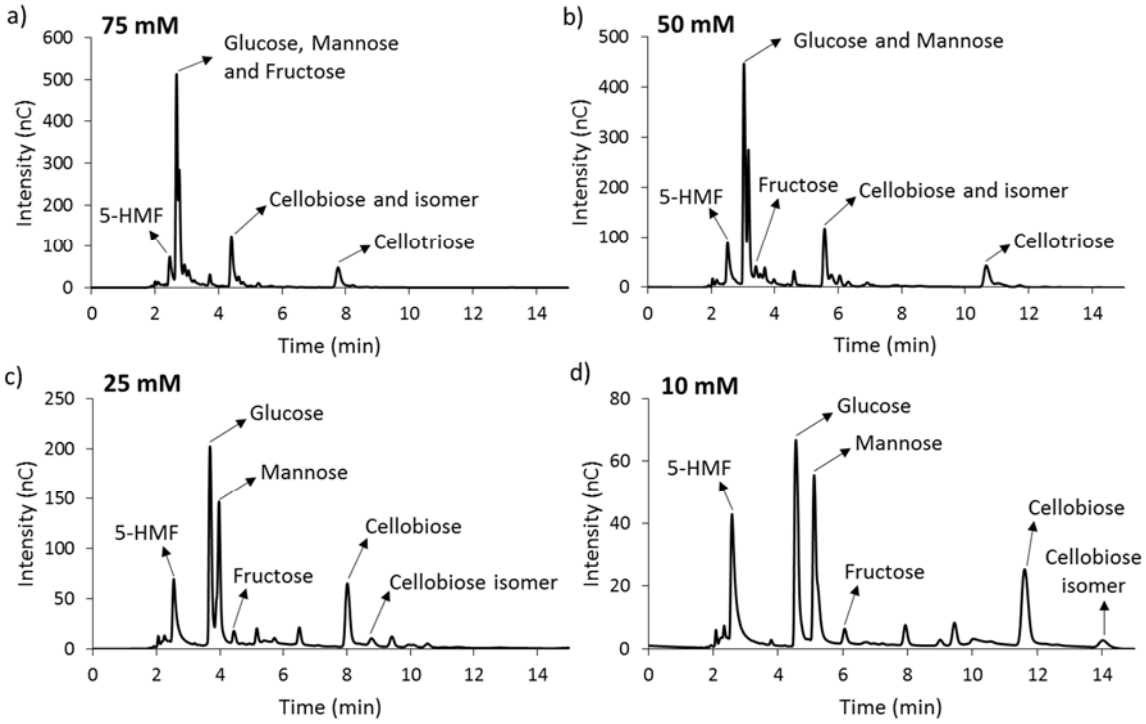

Figure 27. HPAEC-PAD chromatograms of a recovered liquid from cellulose liquefaction in HCW for 2 h at 180°C using different mobile phase concentrations (mM of NaOH, indicated on the upper left side of each panel). The peaks were assigned according to the retention time of pure chemicals for each mobile phase concentration.

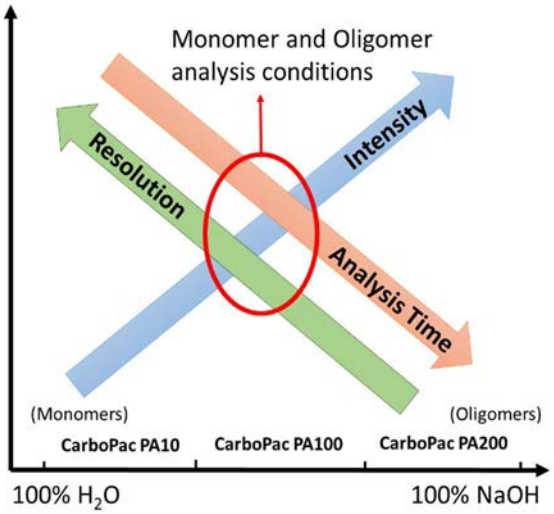

Figure 28. Schematic of the HPAEC performance for monomer and oligomers analysis indicating the effect of the eluent on the resolution, intensity and analysis time for a given CarboPac column. Three different columns are presented according to its most appropriate use: CarboPac PA10: monomers, CarboPac PA100: mono- and oligomers and CarboPac PA200: high-DP oligomers.

Moreover, Figure 28 shows a schematic summarizing the influence of the columns used and the eluent program on the resolution, intensity and analysis time. A full explanation of the development of this method related to Figure 8 and 9 can be found in the supporting information. Then the major soluble species during the cellulose batch liquefaction in HCW has been quantified by this optimized method (5 min of stabilization at 25mM NaOH, then from 0 to 5 min 25mM NaOH, from 5 to10 min a gradient from 25 to 100mM of NaOH and from 10 to 25 min 100mM NaOH).

Figure 29 presents the yields of the major compounds quantified by the optimized HPAEC-PAD method as a function of reaction time for the temperatures studied. All the products quantified by HPAEC-PAD at 180, 220 and 260°C, represent 5.2, 29.8 and 8.9 wt% of the initial cellulose and 46.9, 50.6 and 19.8 wt% of the total soluble products respectively. This result highlights that there is still an important fraction of not quantified compounds by this method, such as the macromolecules evidenced by SEC-ESI-MS, the isomer detected by HILIC-LTQ-Orbitrap, and oligosaccharides with DP>5 that have no standard available. Small species (aldehydes, ketones, carboxylic acids) produced at 260°C were not quantified by this method (but some of them by the HPLC-UV method as presented hereafter).

Glucose yield increases from 180 to 220°C. So the quantity of glucose produced by the hydrolysis of cellulose is more important than its degradation at this range of temperature (Figure 29 a and d). The highest glucose yield (92.7 mg/g of cellulose) was measured after two hours at 220°C as is shown in Figure 29d. On the contrary, at 260°C, glucose yield decreases rapidly, from 4 mg/g of cellulose to its complete consumption during the first 20 minutes of isothermal reaction. This result indicates that the glucose produced from the primary hydrolysis of cellulose is rapidly converted at 260°C into light oxygenated compounds like aldehydes and carboxylic acids (Kabyemela et al. 1999; Yu and Wu 2011).

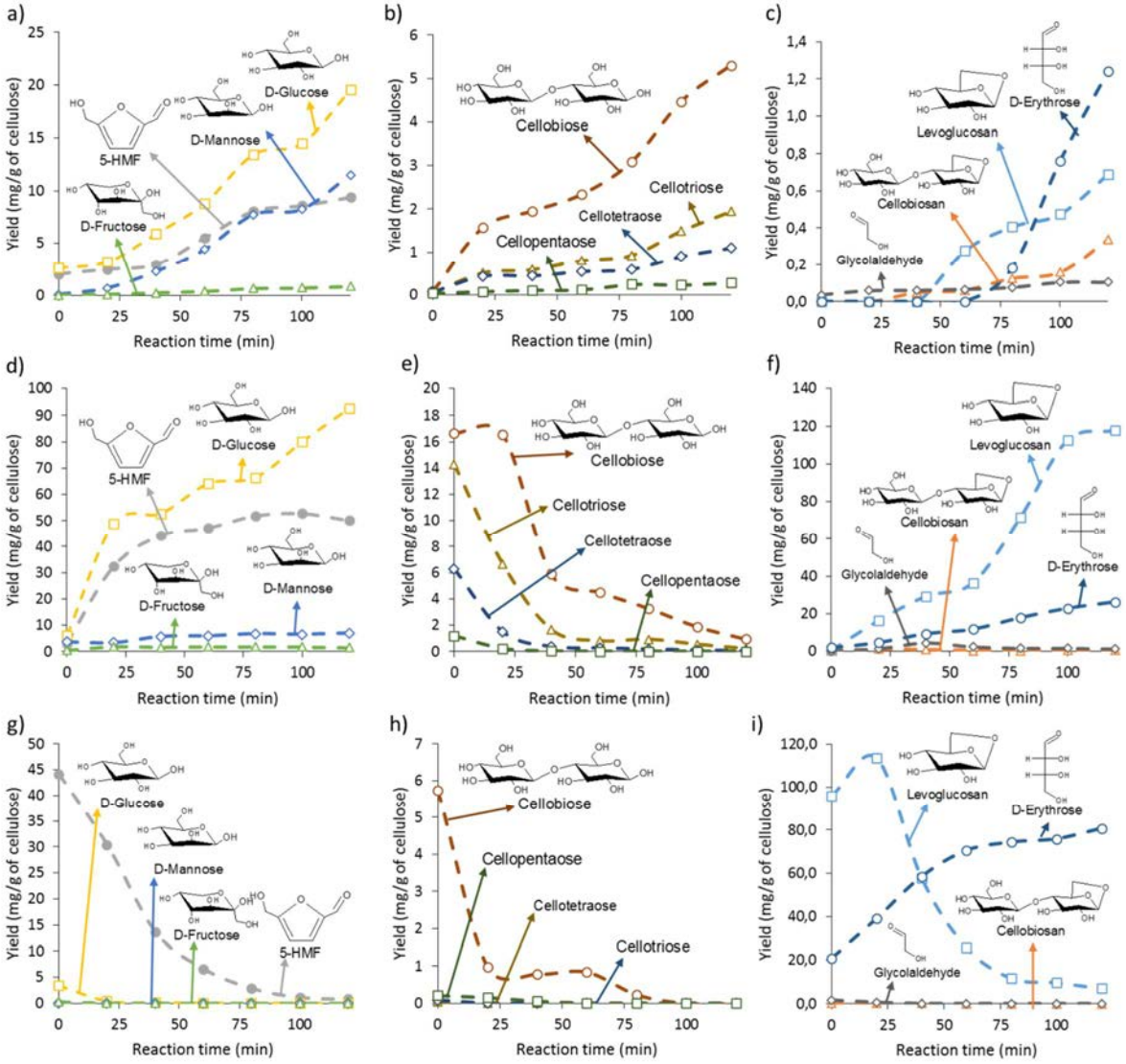

Figure 29. Mass yields evolution for the major compounds quantified by HPAEC-PAD at different reaction temperatures: panels a-c at 180°C, d-f at 220°C and g-i at 260°C.

Contrarily to glucose, mannose and fructose showed their maximum yield after 2 h in HCW at 180°C. These yields are slightly lower at 220°C. There are two probable reasons to explain the decrease of the yield of these monomers. The first reason is that fructose is known to be a precursor of 5-HMF formation (Antal Jr., Mok, and Richards 1990). At 220°C this reaction was more prevalent than that at 180°C and so fructose yield is very low while 5-HMF is an important product (Figure 29 d). Mannose may be involved in a similar degradation pathway not yet identified. Another reason is that the isomerization (Bilik 1972; Kolarić and Šunjić 1996) reaction ends up in glucose as the more stable form, causing that the yields of fructose and mannose to be limited by the equilibrium of this reaction(Bilik 1972; Delidovich and Palkovits 2016).

At 260°C, the yield of mannose and fructose was barely detectable during the first 20-40 minutes of reaction, and then it was not detectable for the rest of the reaction, similarly to glucose. For the same reason as glucose, these monomeric sugars should have been rapidly converted to produce mostly 5-HMF and other aldehydes and carboxylic acids at this temperature(Antal Jr., Mok, and Richards 1990; Kabyemela et al. 1999).

Oligosaccharides of DP 2 to 5 (cellobiose, cellotriose, cellotetraose and cellopentaose) were also quantified by HPAEC-PAD. Their yields as a function of the reaction times at 180, 220 and 260°C are shown in Figure 29b, e and h respectively. The production of these oligomers by cellulose depolymerization in HCW have been extensively studied(Sasaki et al. 1998, 2000; Yu and Wu 2010b, 2010c, 2010a; D. Liu, Yu, and Wu 2013; Tolonen et al. 2015; Yu, Long, and Wu 2015). As it is shown in Figure 29b, oligosaccharide yields increase with reaction time at 180°C. Cellobiose yield is the higher of them, followed by cellotriose, cellotetraose and cellopentaose. This can be explained because the secondary hydrolysis reactions of primary polysaccharides (high DP) will produce oligosaccharides (lower DP) that will react at the same time producing saccharides with DP<6. In consequence, the lower the DP of saccharides is, the higher the number of reactions producing it, resulting in a higher yield of the DP=2. Conversely, all oligosaccharides of DP 2 to 5 present a decreasing yield over time when the reaction temperature is 220°C. Figure 29e shows that the disappearance of oligosaccharides is as fast as the DP is high, starting by cellopentaose at 20 minutes, followed by cellotetraose at 60 minutes, then cellotriose and cellobiose. Cellobiose yield is stable during the first 20 minutes of reaction, then it exhibits a fast decrease during the subsequent 20 minutes, followed by a slower decrease for the rest of the reaction. This is because there are no more high-DP oligomers to produce more cellobiose. Moreover, cellobiose reacts to produce monomeric sugars(Yu and Wu 2010a; Yu, Shafie, and Wu 2013; Tolonen et al. 2015).

The degradation of oligosaccharides was faster at 260°C than for the previous reaction temperature (Figure 29h). Cellobiose starts with a sharp decrease in the first 20 minutes, then its yield becomes constant up to 60min and then decreases further. Oligosaccharides of DP 3-5

present a much lower yield than cellobiose during the first 60 minutes, to be then almost undetectable for the rest of the reaction. At this temperature the conversion rates increase considerably and the studied species are no longer the major compounds within the soluble products. Moreover, the competition to form anhydrosaccharides through a pyrolytic mechanism becomes more important. In both cases, these compounds are most likely degraded into lighter compounds such as carboxylic acids, aldehydes and permanent gases(Sasaki et al. 1998; Kabyemela et al. 1999; Watanabe et al. 2005; Yu and Wu 2011).

Levoglucosan and cellobiosan were quantified by HPAEC-PAD. At 180°C, levoglucosan was detected only after 40 min of reaction (Figure 29c), then its yield increased continuously until the end of the experiment. On the other hand, at 220°C levoglucosan yield increases up to 120mg/g of cellulose after 100 min (being the major quantified product).

At 260°C, levoglucosan is the major product at the beginning of the reaction. It increases during the first 20 minutes of reaction and then decreases continuously until the end of the experiment. At this temperature levoglucosan is probably degraded into aldehydes and carboxylic acids for the same reason as monomeric sugars.

Several light oxygenated products from cellulose liquefaction in hot-compressed water, including: glycolaldehyde, glyceraldehyde, dihydroxyacetone, pyruvaldehyde, 5-(hydroxymethyl)furfural, furaldehyde, acid formic, acid acetic, acid lactic and levulinic acid, have been reported in the literature as products of the degradation of the primary monomeric sugars(Sasaki, Goto, et al. 2002; Matsumura et al. 2006; Yu, Shafie, and Wu 2013). According to the analysis performed in this work, 5-HMF is the most representative of the species mentioned before. Many of these compounds have a high volatility and their accurate quantification is complicated.

j) Analysis of carboxylic acids by HPLC-UV

Formic, acetic and lactic acid were quantified by HPLC-UV. Their presence among the soluble products agrees the results found by Holgate et al.(Holgate, Meyer, and Tester 1995), that studied the degradation of glucose into carboxylic acids and Sasaki et al.(Sasaki et al. 2000) who reported that acetic and formic acid are the major primary products from glucose degradation in HCW. Figure S6 and Table S1 show the identification and quantification of formic, acetic and lactic acid. At 220°C, mainly lactic acid (57mg/g initial cellulose) is formed. Yields of carboxylic acids are relatively low at 180°C, in relation to the latter temperature. At 260°C, lactic and acetic acid yields are relatively low and formic acid is not detected, probably because they are rapidly converted to permanent gas.

1.3.4. Permanent gas composition

Table 2 reports the permanent gases yield for each temperature studied. As it was expected, permanent gases yield increases with the temperature. The results indicate that carbon monoxide (CO) and carbon dioxide (CO_2) are produced by the decarbonylation and decarboxylation of water soluble compounds in agreement with analogous studies found in the literature(Williams and Onwudili 2006; Elliott 2008; Goodwin and Rorrer 2008; Onwudili and Williams 2009).

Table 9. Permanent gases yields in mg/g of cellulose

Compound	Temperature		
	180°C	220°C	260°C
Hydrogen	0	0.0013	0.092
Carbon monoxide	0.014	1.58	5.13
Carbon dioxide	0.50	20.24	43.56

At 220°C, CO_2 and CO yields increased 40 and 115 times respectively, in comparison to 180°C. However, the yield increment from 220-260°C was only 2 and 3 times for CO_2 and CO respectively, being much less important than that from 180-220°C. This is probably because at

260°C, cellulose depolymerization produces other more stable light species like erythrose that may not be directly decomposed into carboxylic acids. Gas formation corresponds to the co-production of water insoluble products related to the decrease of polarity caused by the loss of the carboxylic functional groups.

1.3.5. Mechanism of cellulose conversion in HCW

The reaction mechanism of cellulose under subcritical (HCW comprised) and supercritical water have been widely studied(Antal Jr., Mok, and Richards 1990; Kabyemela et al. 1999; Matsumura et al. 2006; Yu, Lou, and Wu 2008).

Taking into account the previous mechanisms proposed in the literatures(Sasaki et al. 1998; Matsumura et al. 2006; Yu, Lou, and Wu 2008) and including some results obtained in this present work based on our various analytical methods, the main reaction pathways undergone by cellulose in HCW have been rationalized and are presented in Figure 30.

The effect of temperature on the different reaction pathways is highlighted. The reactions are numbered in order to better describe the reference work and to discuss them based on our analysis.

Globally, low temperature (180°C) promotes the production of oligosaccharides by [R1]. This is because at this temperature the kinetic rate of homogeneous hydrolysis [R3] is not that high and high-DP oligomers can be recovered in the liquid fraction. Intermediate temperature (220°C) promotes the formation of glucose [R3] and levoglucosan [R4, R11] under our reactions conditions. High temperature (260°C) promotes erythrose that is the most stable of the studied species under our conditions.

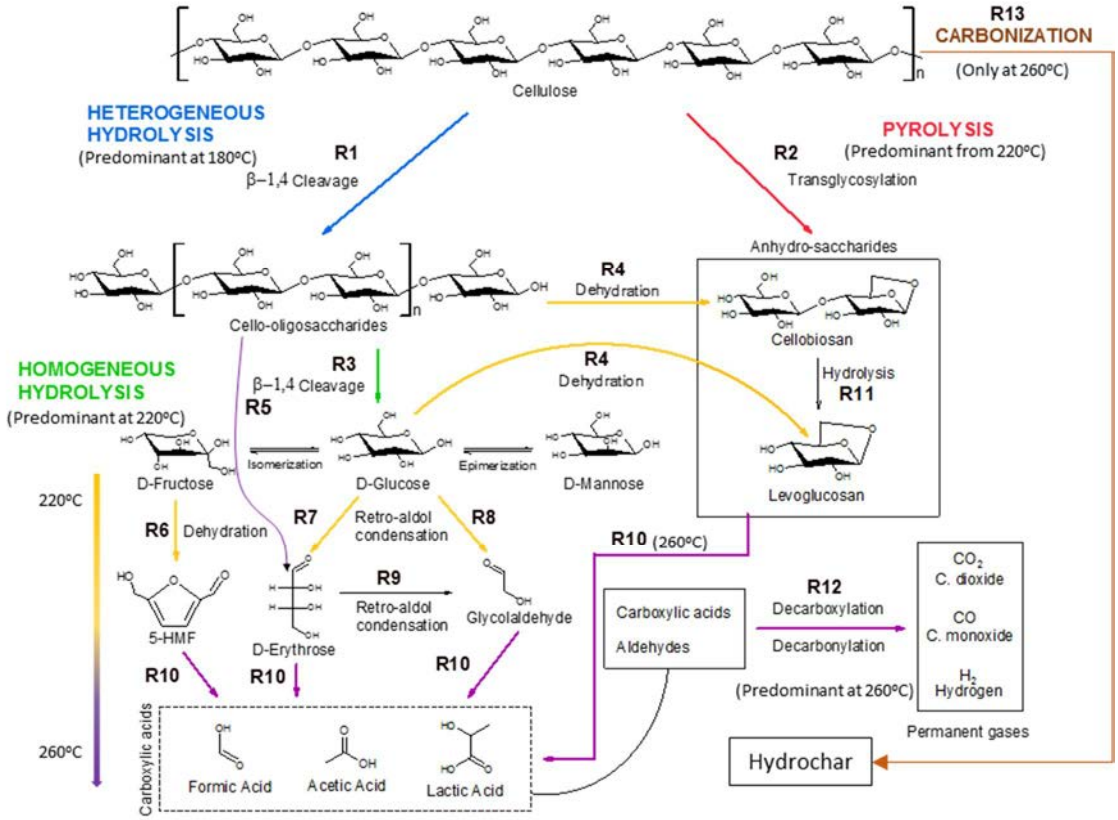

Figure 30. Schematic of the main reaction pathways of cellulose liquefaction in HCW adapted from (Matsumura et al. 2006) and based on this work, highlighting the temperature that promotes each reaction (R) under our investigated conditions. R1, R3=(Sasaki et al. 1998), R2=(Matsumura et al. 2006), R4, R6=(Kabyemela et al. 1999; Yu and Wu 2011), R5=(Sasaki, Furukawa, et al. 2002; Yu, Shafie, and Wu 2013), R7, R8, R9=(Sasaki, Furukawa, et al. 2002; Yu, Shafie, and Wu 2013), R10=(Watanabe et al. 2005; Yu and Wu 2011), R11=(Bilik 1972; Román-Leshkov et al. 2010), R12=(Williams and Onwudili 2006) and R13=(Guo et al. 2015; Y. Liu et al. 2017).

The cellulose is completely insoluble in hot-compressed water at the conditions studied in this work: 180-260°C and 10 bar. Therefore, two major primary mechanisms occur during cellulose liquefaction in hot-compressed water: heterogeneous hydrolysis and pyrolysis, in agreement with Matsumura et al.

Our analysis demonstrates that these 2 primary mechanisms [R1 and R2] occur concurrently because both oligosaccharides and anhydrosaccharides are analyzed under our conditions.

On one hand, cellulose will experience a primary heterogeneous hydrolysis [R1] on the fibers surface that has direct contact with water to react, forming high-DP oligosaccharides, and on the other hand, a pyrolytic reaction mechanism occurs in the inner fibers that are not exposed to

water; so they can not react by a hydrolytic mechanism but by thermal cleavage [R2] (Figure 20), in agreement with Matsumura et al.

All of the primary products are further converted until they are completely soluble in water. These soluble products can also undergo secondary reactions of dehydration, retro-aldol condensation, decarbonylation, decarboxylation, isomerization. All these "secondary" reactions are encompassed as "homogeneous hydrolysis" because they occur in the liquid (water) phase. Moreover, the oligomeric and anhydro-oligomeric compounds (e.g. cellopentaose, cellotetraose, cellotriose, cellobiose, cellobiosan…) are depolymerized to monomers by hydrolytic cleavage [R3 and R11], producing high quantities of glucose and levoglucosan. For this reason, other type of reactor with low liquid residence time, as one developed by (Yu and Wu 2010a), would be needed to better study primary reactions and to minimize secondary reactions.

Glucose undergoes a reversible keto-enol isomerization *via* LBAE (Lobry de Bruyn, Alberda van Ekenstein) transformation to produce fructose(de Bruyn and van Ekenstein 1895; Speck 1958). On the other hand, glucose can also be converted into mannose by an epimerization reaction *via* enendiol anion(Bilik 1972; Román-Leshkov et al. 2010; Delidovich and Palkovits 2016).

The monomeric saccharides can undergo a subsequent degradation reaction of dehydration, decarbonylation and decarboxylation. In that way, glucose can produce one molecule of erythrose and one of glycolaldehyde by a retro-aldol condensation mechanism [R7], in agreement with (Kabyemela et al. 1999; Sasaki, Goto, et al. 2002; Watanabe et al. 2005). Another possible mechanism for the production of erythrose is by the formation of glucosyl-erythrose [R5] from cellobiose as was previously studied(Sasaki, Furukawa, et al. 2002; Yu, Shafie, and Wu 2013). However, glucosyl-erythrose has not been studied in this work due to the lack of a commercial standard to perform quantitative analysis.

Then erythrose can produce two molecules of glycolaldehyde by retro-aldol condensation [R9] (Sasaki, Goto, et al. 2002) or endure a further degradation to light carboxylic acids [R10](Kabyemela et al. 1999; Peterson et al. 2008). However, among the chemical species studied,

erythrose was found to be the most stable product at 260°C. Levoglucosan and other anhydro-saccharides could also be converted to carboxylic acids (formic, acetic and lactic acid)(Kabyemela et al. 1999; Ehara and Saka 2002) and permanent gases[R10 and R12](Goodwin and Rorrer 2008). The homogeneous hydrolysis reactions are highly sensitive to temperature and it was found that their rates increase significantly in the range of 180 to 260°C. They are so fast at 260°C that the majority of the primary products were already degraded at the first sampling time chosen for this study. Moreover, at this temperature (260°C) the solid residue was carbonized forming "hydrochar" [R13].

1.4. Conclusion

The main focus of this work is a better understanding of cellulose depolymerization in hot-compressed water using different analytical techniques. The reactions were studied using a batch reactor system during 2 h for three different temperatures: 180, 220 and 260°C. The solid, liquid and gas products were characterized. We have shown in this work how various complementary LC methods are needed in order to give a detailed characterization of the soluble products. HPAEC-PAD has been shown as a powerful tool for the characterization of the major liquefaction water-soluble products.

The reaction temperature presents a strong effect on the distribution of products and different predominant reaction pathways. The presence of levoglucosan and cellobiosan is a good evidence of the existing pyrolytic mechanism that inner cellulose fibers may undergo during the liquefaction in HCW. Under our conditions temperatures around 220°C lead to high yields of levoglucosan (12 wt.%) and glucose (9 wt.%) after 2 hours. Higher reactions (260°C) promotes the production of levoglucosan. After less than 20 minutes at 260°C, secondary reactions become important and lead mainly to erythrose and acids formation. Moreover, the global hydrolysis mechanism taking into account the permanent gases and major soluble compounds from different chemical species have been rationalized.

Acknowledgements

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The objectives of the previous article were 1) to better understand the mechanisms of cellulose conversion water; 2) to find the best liquefaction temperature for our purpose, i.e. the production of a mixture of oligosaccharides from lignocellulosic materials. Now that the main reaction pathways of cellulose liquefaction are understood in our, a the range of temperatures from 180 to 220°C has been chosen for the hydrothermal liquefaction of cellulose-rich pulps and biomass. This second article deals with the conversion of real cellulosic materials which are produced from wood.

In this work, fractionation of woody biomass (beech) is studied in order to remove lignin that is the source of inhibitory compounds. The recovery of hemicelluloses is another important factor, since it is another source of sugars. The effect of the fractionation method on hydrothermal liquefaction of lignocellulosic substrates is studied.

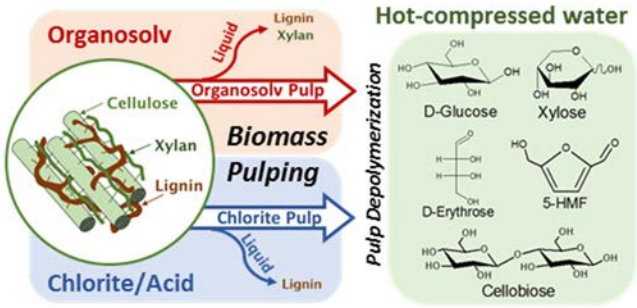
2. Article 2:

Production of soluble sugars: Coupling of fractionation and hydrothermal depolymerization of woody biomass

Article submitted to ACS Sustainable Chemistry and Engineering

ABSTRACT

Lignocellulosic biomass is a promising source of renewable carbon. Holocellulose can be extracted from its matrix and depolymerized to produce soluble carbohydrates. In this



work, beech has been delignified either by ethanol organosolv or by sodium chlorite / acetic acid (SC / AA) treatment, obtaining delignification yields of 50 and 91 wt. % respectively. Cellulose, hemicelluloses and lignin content of untreated beech, organosolv and SC / AA pulps were determined. The holocellulosic pulps were then submitted to liquefaction in hot-compressed water (HCW). Three temperatures were studied: 180, 200 and 220°C during 2 h with sampling every 30 min. The same experiments were conducted on untreated beech and model cellulose (Avicel PH-101). Product yields and total carbohydrates were determined. The liquefaction products, i.e. solid residue, water soluble compounds and permanent gas, were analyzed respectively by X-ray diffraction (XRD), high performance anion-exchange chromatography with pulsed amperometric detector (HPAEC-PAD) and gas chromatography (GC). SC / AA treatment allowed a high recovery of hemicelluloses and for this reason, xylose content was significantly higher for the SC / AA pulp. The maximum yields of total carbohydrates, i.e. 13.7 and 36.8 wt.% from the organosolv and SC / AA pulp respectively, were found when the liquefaction temperature was 220°C. At this temperature, liquefaction of the organosolv pulp produced glucose with the lowest 5-HMF content among the water-soluble fraction, in comparison to raw beech and SC / AA pulp.

2.1. Introduction

Fossil fuels are being depleted and finding an alternative source is one of the major current concerns of society. Lignocellulosic biomass represents a potential renewable alternative source of carbon(Ragauskas, Williams, et al. 2006; Caes et al. 2015). It is composed principally of cellulose, hemicelluloses and lignin. Hemicelluloses and cellulose (holocellulose) can be used in biotechnological routes to produce biofuels, chemicals and building blocks(Desvaux, Guedon, and Petitdemange 2000; Johnson 2006; Ezeji, Qureshi, and Blaschek 2007; Luque et al. 2014;

Morone and Pandey 2014; Maurya, Singla, and Negi 2015). However, lignocellulose constitutes a complex matrix that is highly recalcitrant to chemical and biological transformation.

Several pretreatments have been studied in order to increase the accessibility of enzymes, producing glucose in high yields, that can be lately fermented(Mosier et al. 2005; Brosse, Sannigrahi, and Ragauskas 2009; Hendriks and Zeeman 2009; Alvira et al. 2010; Harmsen et al. 2010; Chen et al. 2011; Mante et al. 2014; Maurya, Singla, and Negi 2015; Jönsson and Martín 2016; C. K. Nitsos et al. 2016; Wu et al. 2017).

The presence of lignin derived compounds will strongly inhibit the enzymes and microorganisms used in the later biological processes(Qureshi et al. 2012; Sivagurunathan et al. 2017; Yoo et al. 2017). A cellulose-rich pulp can be extracted from the lignocellulosic matrix by several technologies like organosolv,(Caes et al. 2015; Erdocia et al. 2016; Hosseinaei et al. 2016; C. Nitsos et al. 2016; C. K. Nitsos et al. 2016; Sadeghifar et al. 2017) chlorite fractionation,(Siqueira et al. 2013) ionic liquids,(Zhu et al. 2006; Lee et al. 2009; Yang et al. 2013) among others.(Katahira et al. 2014; Liu et al. 2017) Each one of these fractionation (or pulping) methods has its own advantages and drawbacks. The choice of the fractionation technology needs deep techno-economic and environmental viability studies.(Viell et al. 2013)

There are two important reasons to perform this delignification process. The first one is that recovering lignin allowing its valorization by a side process into chemicals and/or materials.(Yuan, Xu, and Sun 2013; Galkin and Samec 2016; Smichi, Messaoudi, and Gargouri 2017) This will increase the economic viability of the overall biorefinery process. The second reason is the removal of lignin-derived products (phenols), which exhibit strong inhibitory properties on micro-organisms used in the subsequent fermentation processes.(Palmqvist and Hahn-Hägerdal 2000a, 2000b; Jönsson and Martín 2016; Yoo et al. 2017)

The organosolv treatment is a promising technology that allows recovering a cellulose-rich pulp much less recalcitrant to biological conversion (e.g. enzymatic hydrolysis, fermentation). Studies for the fractionation of Poplar(Pan et al. 2006) and Miscanthus(Brosse, Sannigrahi, and Ragauskas

2009) reported several study cases with delignification yields between 60 and 70 wt.%, obtaining substrates with good enzymatic digestibility.

Lignin can also be removed by sodium chlorite / acetic acid (SC / AA) treatment. This method can removed lignin up to 60 wt. % without generating cellulose loses and up to 99 wt. % with significant cellulose loses.(Siqueira et al. 2013) The main advantage of this method is the high yield of hemicelluloses recovery that leads to a higher sugar yield after saccharification as well. In both cases, it is possible to recover a large amount of lignin that can be valorized by another side process.

Liquefaction can be an interesting process for the production of sugars from lignocellulosic biomass. Saccharification of cellulose is often made by enzymatic hydrolysis. This process leads to glucose in high yields, but kinetics are slow and enzymes, that can not be recycled, are very expensive.

Liquefaction in hot-compressed water (HCW) is an economic, flexible and environmentally friendly technology that uses only water as solvent and reactive. This process allows converting (holo)cellulose into soluble carbohydrates that can be transformed into biofuels and chemicals.(Peterson et al. 2008; Y. Yu, Lou, and Wu 2008; Zhao et al. 2009) Studies using hot-compressed water in batch and continuous reactors have been promising for this purpose.(Y. Yu, Lou, and Wu 2008; X. Lu et al. 2009; Y. Yu and Wu 2009, 2010a; Tolonen et al. 2011; Abdullah, Ueda, and Saka 2014; Shi et al. 2014; Tolonen et al. 2015) But to the best of our knowledge, there is still a lack of work combining both the fractionation and liquefaction on the same biomass in order to compare the effect of the fractionation method on the sugar yields produced after the hydrothermal depolymerization of the pulps.

In this work, beech wood (*Fagus sylvatica*) is delignified by organosolv and SC / AA processes, obtaining a cellulose-rich pulp. These pulps are depolymerized by liquefaction in HCW using a batch system at 180, 200 and 220°C during 2h with sampling every 30 min.

Product yields and detailed analysis of the solid residue, water soluble compounds and permanent gas are performed. Total carbohydrates content is determined by the phenol / sulfuric acid assay, in order to compare the performance of the two fractionation methods in terms of sugar production. Moreover, the quantification of all the main sugars is conducted by HPAEC-PAD. The liquefaction products of organosolv and SC / AA pulps are compared with the products obtained from untreated beech and a model cellulose (Avicel PH-101). We describe the effect of delignification treatments on depolymerization reactions in HCW conducted in a batch reactor. Moreover, the effect of reaction temperature and time on product distribution and composition have been studied for all the substrates.

2.2. Materials and Methods

2.2.1. Biomass and reactants

The lignocellulosic biomass chosen for this study was beech (*Fagus sylvatica*), because of its abundance and special interest for this species in the North East region of France. The wood was grinded and sieved.The fraction between 250 and 500 µm was recovered for this work. The reactants: ethanol (95 %, C_2H_6O), sulfuric acid (98%, H_2SO_4), sodium chlorite ($NaClO_2$) and acetic acid (99.5 %, CH_3COOH) were purchased from Sigma Aldrich (San Luis, USA).

2.2.2. Biomass delignification

a) Organosolv treatment

Organosolv conditions were taken at the optimal yield reported by (Pan et al. 2006) with some modifications. For this treatment 200 g of dried beech was loaded in a 2L reactor with 1.4 L of an ethanol / water mixture 50:50 v / v at 1.25 wt. % H_2SO_4 (relative to the dried biomass). The final liquid / solid ratio was 7:1 v / w. The fractionation was conducted at 180°C for 60 min. After fractionation the solution was vacuum filtered in a Büchner, washing the solid pulp twice with

1L of a mixture ethanol / water 50:50 v / v at 60°C. The solid residue (the pulp) was dried at 105°C for 24 h, then weighted and stored.

b) Sodium chlorite/acetic acid (SC/AA) treatment

The SC / AA treatment has been performed according to that reported by (Siqueira et al. 2013) with some modifications. 100g of beech were treated with sodium chlorite / acetic acid solution for 6h. Initially, for each gram of dried beech, 0.33 g of sodium chlorite ($NaClO_2$), 0.33 mL of acetic acid (CH_3COOH) and 40 mL of deionized water were added to a 7L stirred reactor at 70°C. Every 2h the same amount of sodium chlorite and acetic acid was added to the reactor (two times: at 2h and 4h). After 6h of treatment the sample was vacuum filtered and washed with 2L of water. The solid was dried at 105°C for 24h, then weighted and stored.

2.2.3. Liquefaction in HCW

Liquefaction in HCW was performed according to our previous study of microcrystalline cellulose reported in section B, I, 1.(Buendia-Kandia, Mauviel, et al. 2017) A batch reactor system was used to study the cellulosic pulps depolymerization. An Hastelloy (C-276) stirred reactor (Parr Instrument Company) of 300 mL was filled up with 200 mL of de-ionized water and 10 g of substrate (beech, organosolv pulp, SC / AA pulp and Avicel cellulose). Reactor was purged 2 times with nitrogen. Then, 10 bar of nitrogen atmosphere was used as a tracer for the quantification of permanent gases. Reactor was heated to the target temperature (180°C, 200°C or 220°C) at 5 K / min. At the end of each experiment (2h), the reactor was rapidly quenched (with cold water) to ambient temperature.

The solid / liquid mixture was filtered using a glass microfiber filter (Whatman®, pore size: 0.7 µm) with a Büchner under vacuum suction. The solid was dried for 24h in an oven at 105°C, then weighted and stored for further analysis. The recovered liquid was immediately analysed by liquid chromatography and total carbohydrates; then it was stored in a freezer at -80°C. Once the

liquid is completely frozen, the water was removed by freeze drying at -40°C, <0.04 mbar for 24 h. The solid residue was weighted and taken as the total water solubles. The analysis by liquid chromatography was made before and after freeze drying to verify that there was no significant change in the composition.

2.2.4. Chemical analysis

a) Cellulose, hemicelluloses and lignin content

Macromolecules composition were determined using the Klason lignin method and high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) to quantify the cellulose and hemicelluloses according to its monomeric sugars equivalents. The Klason lignin method was perform according to National Renewable Energy Laboratory (NREL) method, precipitating lignin using sulfuric acid and recovering the monomeric sugars in solution.(Sluiter et al. 2012) Thereby, cellulose was quantified as glucose and hemicelluloses as the sum of arabinose, xylose, mannose and galactose.

b) Total carbohydrates analysis

Total carbohydrates were determined by the phenol/sulfuric acid assay as reported in the previous work (section B, I, 1). The absorbance was measured in a spectrophotometer MultikanTM GO (Thermo Scientific™) at 490 nm. The total sugars were quantified as glucose-equivalent.

c) Analysis by HPAEC-PAD

Soluble products were quantified by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) with the method developed in our previous work (section B, I, 1). All the major compounds were identified and quantified using the respective calibration curve for each compound. The column used was a CarboPacPA100 (Dionex, Thermo

Fisher Scientific). The HPLC system is 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 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.

d) Analysis by micro-GC

The permanent gases were collected in a Tedlar bag when the reactor was cooled down to ambient temperature. Permanent gas was characterized by a µGC-Varian 490 equipped with four modules, composed of two molecular sieves 5A, a PoraPlot U and a CP-Wax 52CB columns. The µGC was calibrated using standard bottles (Air Liquide, France). Hydrogen (H_2), carbon monoxide (CO) and carbon dioxide (CO_2) were quantified using N_2 as a tracer (known volume and pressure of N_2 introduced in the closed reactor).

e) Analysis by XRD

The washed and dried solid residues have been manually crushed (with a mortar) to be analyzed by XRD analysis. XRD has been performed using an INEL XRG-3000. This X-ray diffractometer uses a copper radiation (CuKα) having wavelength λ (Kα1) =1.5406 nm generated at 30 kV and 25 mA. The diffraction intensities were measured in a range of 0 to 82° (2θ) using a step size of 0.029°. This analysis allows determining if the solid is amorphous or crystalline (crystallinity index) and also allows characterizing changes in the crystalline structure of solid residues. Crystallinity index (CI) values were calculated for each solid as the ratio between the height of the (002) reflection peak and the minimum between the (021) and (10$\bar{1}$) reflection peaks, corresponding to 2θ=22.7 and 18.9° respectively.(Park et al. 2010)

2.3. Results and discussion

In the first part of this work the fractionation of woody biomass is studied. Two methods were implemented: organosolv and chlorite/acid to remove lignin from biomass and recover a cellulose-rich pulp.

In the second part, the liquefaction in HCW from the recovered pulps was performed at different temperatures (180, 200 and 220°C) in order to depolymerize the holocellulose into fermentable sugars. The overall process is presented on Figure 31 indicating the typical yields found in this work.

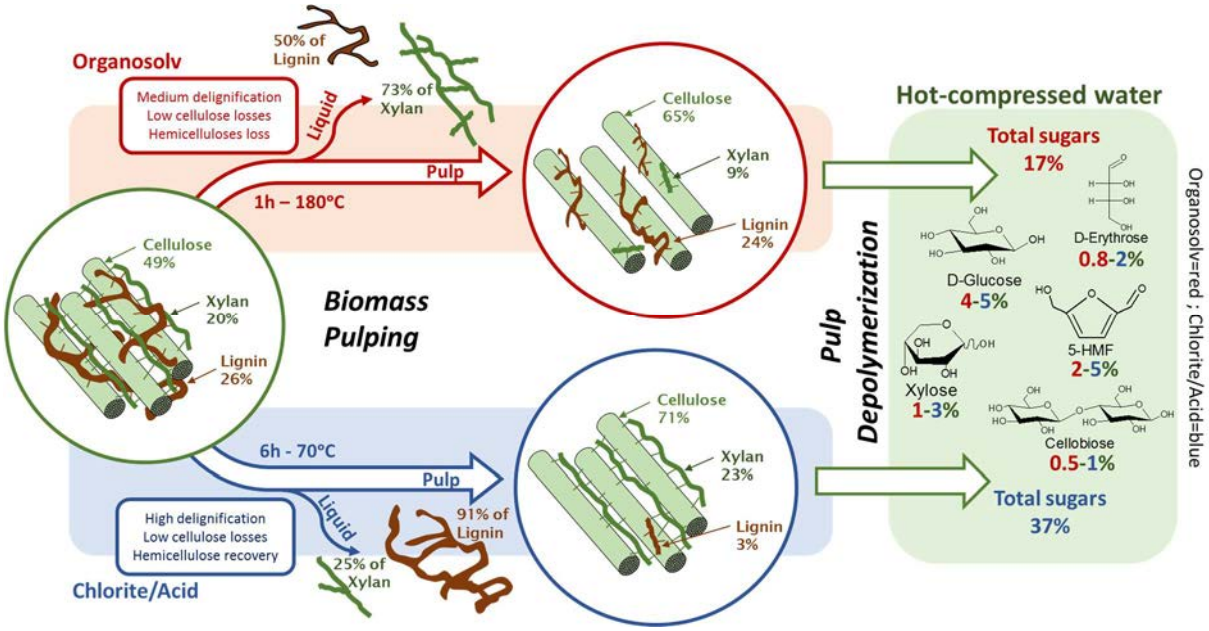


Figure 31. Schematic representation of the fractionation process coupled to a depolymerization stage in hot-compressed water. The typical yields obtained in this work are indicated for the most representative compounds. The yields of liquefaction products are calculated in relation to the initial pulp.

2.3.1. Biomass fractionation

The performance (cellulose recovery, pulp and delignification yield) of biomass delignification by the organosolv and SC/AA processes has been studied. These two methods present different approaches concerning the fractionation process; while SC/AA method removes lignin with high selectivity,(Siqueira et al. 2013) the economic viability of the organosolv process represents high interest for industrialization purposes.(Viell et al. 2013; Budzinski and Nitzsche 2016).

The fractionation yields for the pulp, lignin, hemicelluloses and cellulose are presented on Table 10. The delignification of beech was possible with both methods, organosolv and SC / AA, obtaining delignification yields (quantity of lignin removed relative to the initial lignin) of 51.6 and 91.4 wt.% respectively. The delignification yield for the SC / AA method was much higher relative to the organosolv method. However, it is necessary to take into account that SC / AA method required 6h (at 70°C) instead of 1h (at 180°C) for the organosolv fractioning. In both cases the recovery of a cellulose-rich pulp is possible; with cellulose recovery yields of 70.7 wt.% for organosolv and 90.6 wt.% for SC / AA. Figure S1 illustrates the yields of cellulose, hemicellulose and lignin relative to the initial beech.

Table 10. Solid and macromolecules fractionation yields of beech pulps obtained by organosolv and SC / AA processes. Yields are calculated relative to the initial content of the respective initial lignin, cellulose and hemicellulose content of the raw beech.

(wt. %)	*Organosolv*	*SC/AA*
Pulp yield	53.3	62.4
Lignin removed	51.6	91.4
Hemicelluloses recovered	26.3	73.6
Cellulose recovered	70.7	90.6
Holocellulose recovered	58.3	85.8

The pulps recovered after organosolv and SC / AA fractioning represented 53.3 and 62.4 wt.% of the initial raw beech. SC / AA method presents higher pulp yield thanks to the preservation of hemicelluloses (73.6 wt.%) in the recovered pulp. On the contrary, the organosolv method is not suitable to recover this hemicellulosic fraction (26.3 wt.%). These results are confirmed by the sugar composition presented on Table 11, showing that xylose, arabinose, mannose and galactose concentration decreased significantly for the organosolv pulp but not so much for the SC / AA pulp. This species contains a high quantity of glucuronoxylans in the hemicellulosic fraction. This is normal for the angiosperm plants.(Brosse, Sannigrahi, and Ragauskas 2009) Mannose and

galactose present low yields for the SC/AA process and a complete loss for the organosolv process.

Table 11. Composition of the raw and fractionated beech. Mass yields (dry basis) were calculated relative to the respective pulp. Cellulose was taken as glucose. *Oxygen was calculated by difference.

(wt. %)	*Raw beech*	*Organosolv*	*SC/AA*
Moisture	5.6	3.9	4.1
Minerals	0.5	0.1	1.1
Macromolecules			
Lignin	26.5	24.0	3.7
Hemicellulose	19.0	9.4	22.5
Cellulose	48.9	64.9	71.0
Holocellulose	67.9	74.3	93.5
Sugars			
Glucose	48.7	64.9	70.9
Xylose	13.7	8.6	19.2
Arabinose	2.7	0.8	2.2
Mannose	2.5	0.0	0.9
Galactose	0.1	0.0	0.1
Elements			
C	42.3	47.5	35.2
H	5.3	5.4	4.8
O*	40.4	38.1	49.6
N	<0.1	0.6	<0.1

These results highlight that the recovery of hemicelluloses is one of the most important differences between these two methods. This can be explained by the fact that SC/AA is an oxidative method using an organic acid as buffer, partially preventing the hydrolysis of hemicellulose but guaranteeing a pH high enough to remove lignin.

In the case of the organosolv process, the high temperature provokes a direct hydrolysis of hemicelluloses. This hemicellulosic fraction can be separated (precipitating the lignin at acid pH) and recovered for its further valorization. Moreover, the sulfuric acid used as catalyst will promote this reaction. The hemicelluloses must be taken into account as a source of carbohydrates for the production of chemicals and fuels and the choice of the most appropriate technology can

be directly imposed by the desired use of the hemicellulosic fraction. Ultimate analysis (Table 11) revealed that hydrogen/carbon ratio (H/C) remains similar for the biomasses after the fractionation processes by organosolv and SC/AA. However, oxygen/carbon ratio (O/C) decreases by 15.8% for the organosolv pulp but increases by 48.4% for the SC/AA pulp, relative to the initial O/C for the raw beech measured in this work. This is because the efficient removal of lignin, that has the lowest O/C ratio in lignocellulosic biomass,(Nhuchhen, Basu, and Acharya 2014) causes the increase of the O/C ratio of the recovered pulp in the case of SC/AA treatment. The major inorganics were quantified for the raw beech and the recovered pulps from organosolv and SC/AA treatments. The inorganics composition obtained is shown on Table S1.

Most of the inorganic species decreased after both organosolv and SC/AA treatments. The exception was sodium (Na) that increased considerably for the pulp recovered from SC/AA process. This is evidently due to the sodium salt used for this fractionation process.

Hardwood is generally less recalcitrant to fractionation than softwood.(Z. Yu et al. 2011) However, this specific type of hardwood has a relatively high lignin content in comparison to other hardwoods and some softwoods.(Ragauskas, Nagy, et al. 2006) Therefore, its high lignin content represents a major concern that generally presents some potential implications for the effectiveness in the delignification process.(Hallac et al. 2009)

The presence of lignin in the lignocellulosic matrix hinders the effects of the acidic hydrolysis and oxidation cleavage of the cellulose chains during the acetic acid/sodium chlorite (SC/AA) delignification.(Hubbell and Ragauskas 2010)Therefore lignocellulosic materials with high lignin content will have the more preserved cellulose chains.(Hubbell and Ragauskas 2010) The desired severity for the delignification method will depend on the final use of the recovered pulp.

SC/AA conditions have been widely studied for a long time and the procedure has been tailored according to the specifications of the biomass and reactor.(Campbell and McDonald 1952; Ahlgren and Goring 1971; Hubbell and Ragauskas 2010; Siqueira et al. 2013)

If the depolymerization of the cellulose chains is too important, the saccharides will be partially soluble and lost in the liquid phase. On the contrary, if the cellulose fibers were partially deconstructed, this will probably ease their further hydrolysis in HCW.

XRD patterns are shown on Figure 32. This analysis puts in evidence the changes in cellulose structure after organosolv and SC / AA processes. Comparison with reference cellulose (Avicel) was made to follow the characteristic reflection pattern of cellulose I (combination of cellulose Iα and Iβ).(Park et al. 2010; Mukarakate et al. 2016)

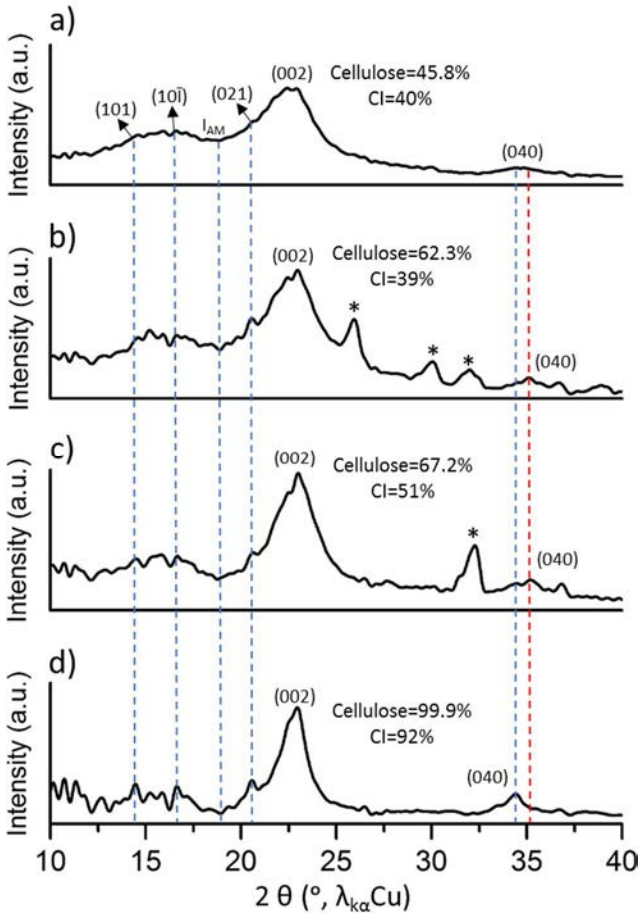


Figure 32. XRD patterns of a) untreated beech, b) organosolv beech pulp, c) sodium chlorite / acetic acid beech pulp and d) microcrystalline cellulose (Avicel PH-101). Crystallinity index (CI) and cellulose content are indicated for each panel. * Non-identified peaks.

Crystallinity index (CI) presented on Figure 32 indicate that the crystalline region is higher for the SC / AA pulp in relation to the initial beech, but remains more or less the same for the organosolv pulp, implying that there is no change in the amorphous and crystalline proportions in the pulp. However, the width of the main cellulose reflection, for the plane (002), decreases

from beech to organosolv pulp to SC / AA pulp. This result indicates that in fact the order of the cellulosic fibers in the organosolv pulp is higher to that of the initial beech. This is due to the hydrolysis of less ordered fractions (such as amorphous cellulose, hemicellulose and lignin).

Some unknown peaks were found in the XRD pattern of the delignified pulps. These peaks agree with the ones reported by Ago et al.(Ago, Endo, and Hirotsu 2004) as contaminations, most probably minerals. Nevertheless, ICP analysis did not show any atypical augmentation of inorganic species (Table S1).

2.3.2. Liquefaction of the beech-extracted pulp in HCW

The recovery of a cellulose-rich pulp allows a thermochemical depolymerization largely avoiding the lignin-derived compounds for which subsequent separation represents high difficulty. In this subsection, the liquefaction in HCW of beech and the recovered pulps from the organosolv and SC / AA processes are studied in the context of fermentable sugars production. The effect of the reaction temperature (180, 200 and 220°C) on the product distribution and the composition of the aqueous phase is reported below. Raw beech, organosolv and SC / AA pulps represent real feedstocks of carbohydrate, which are compared with Avicel cellulose, under the same conditions, due to its frequent use as a model substrate of the cellulosic fraction in biomass. Detailed results of the depolymerization of Avicel cellulose can be found in section (B, I, 1).(Buendia-Kandia et al. 2017) Other experiments of model cellulose were performed for this work according to the temperature chosen.

The lower temperature studied was chosen as the minimum one needed to break the glycosidic bonds of crystalline cellulose.(Y. Yu and Wu 2010b) The maximum temperature was chosen according to previous results (section B, I, 1), showing that temperatures higher than 220°C will increase significantly the degradation of sugars into light oxygenated compounds (carboxylic acids, aldehydes, etc.).

Mass balances after 2 h of liquefaction in HCW of the four biomasses are reported in Figure 33. For all the biomasses treated, the solid residue decreases when the temperature increases. Moreover, the soluble and gas products always increased as the temperature increased from 180 to 220°C.

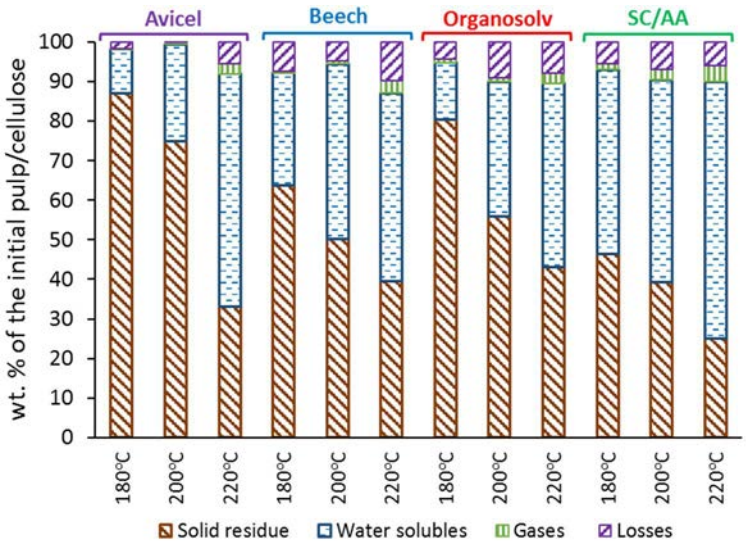


Figure 33. Product distribution after liquefaction in HCW of avicel cellulose, beech, organosolv and sodium chlorite / acetic acid (SC / AA) beech-extracted pulp as function of the reaction temperature after 2h reaction time.

The water-solubles fraction was higher for beech and SC / AA pulp than that for Avicel and organosolv pulp. This can be explained by the high content of hemicelluloses in beech and SC / AA that are easily hydrolysable at moderate temperatures. At 220°C, water-solubles maxima of 47 and 65 wt.% were found for organosolv and SC / AA pulps respectively.

The XRD patterns of the solid residues after 2h of liquefaction in HCW are illustrated in Figure 34. This analysis allows identifying if there is some reorganization of the crystalline structure or carbonization reactions that lead to disordered solids (hydrochar). The XRD patterns for beech, organosolv and SC / AA pulps exhibit the characteristic reflections of crystalline cellulose. For all three substrates, the crystallinity increases with the temperature in the range of 180 to 220°C. It has been shown that higher temperatures lead to hydrochar formation.(Kang et al. 2012; Guo et al. 2015; Buendia-Kandia et al. 2017)

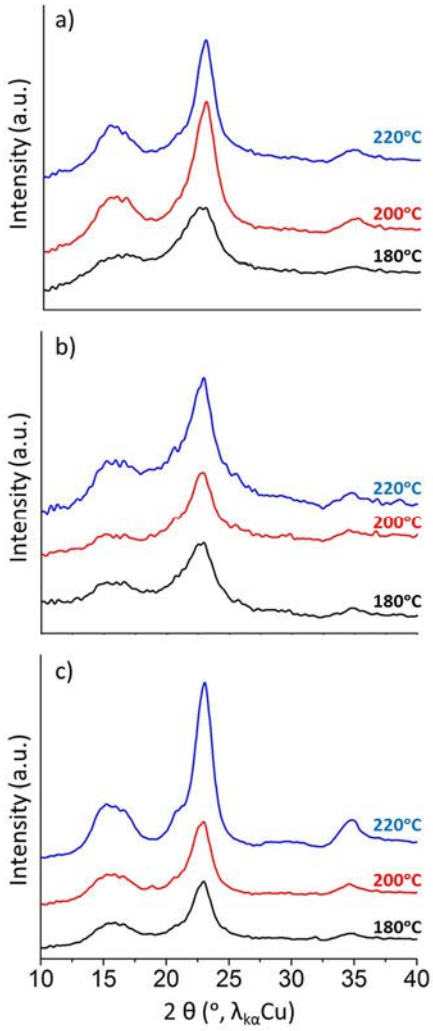


Figure 34. XRD pattern of the solid residues of a) Beech. b) organosolv pulp and c) SC / AA pulp after liquefaction in HCW under isothermal conditions**.**

One of the main objectives of this work is the production of sugars that can be transformed into biofuels and building blocks by fermentation processes. The total carbohydrates analysis by phenol / sulfuric acid provides a simple and rapid method to quantify all the sugars present in the water-soluble fraction of the liquefaction in HCW.(Rover et al. 2013) The mass yields of total sugars and non-sugars as function of the liquefaction temperature for each pulp are shown in Figure 35.

For the raw beech, the water-solubles yield is relatively high but the total sugars represent only 2 to 6 wt.%. This is probably because the lignin is depolymerized producing high quantities of non-sugar compounds. In the same way, the organosolv pulp keeps an important fraction of lignin, explaining why the non-sugars yield is higher than for the others substrates.

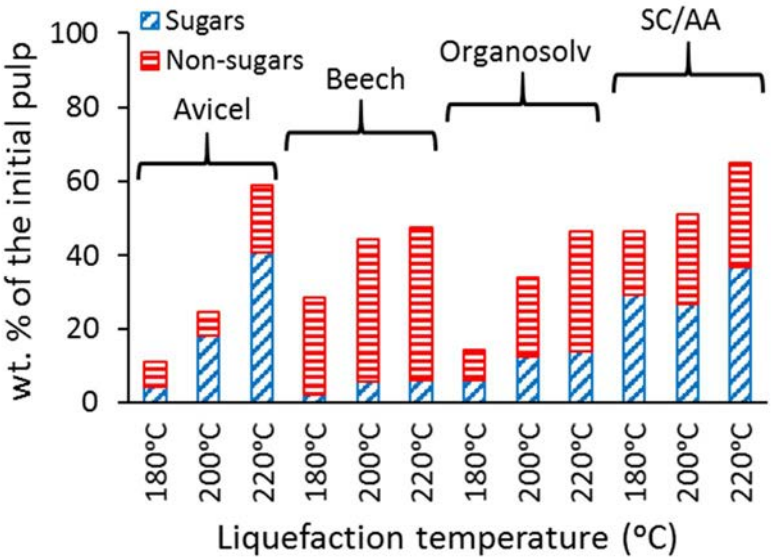


Figure 35. Water soluble sugar content for each temperature and extraction method.

The sugar yield for the organosolv pulp did not present significant change between 200 and 220°C. However, the non-sugar yield increased considerably. This is probably because during the organosolv process, the operating conditions promote the accessibility to the lignocellulosic matrix, making the production and degradation of sugar compounds much easier during the further liquefaction in HCW.

The highest yields, for all the temperatures studied, were found when the substrate was the SC / AA pulp and the highest yield for each pulp was found when the temperature was 220°C. It was expected that the sugar yield of the SC / AA pulp was higher than that of the organosolv pulp because in the first delignification method a high portion of hemicelluloses are recovered in the pulp, while in the latter method they are lost during the fractionation process.

Total sugars content allows meaningful comparisons between the different experiments. However, not all the sugars are fermentable and the identification of the main sugar species is crucial for the selection of the proper microorganism involved in the subsequent fermentation process. In order to identify the nature of the soluble carbohydrates produced, the liquid fractions were analyzed by HPAEC-PAD.

The main species identified and quantified are levoglucosan, glycolaldehyde, 5-hydroxymethylfurfural, glucose, mannose, xylose, fructose, erythrose, cellobiose, cellotriose, cellotetraose and cellopentaose.

The yields calculated in relation to the initial grams of substrate are presented on Figure 36Figure 37. The effect of the liquefaction temperature on the composition of the liquid fraction follows the same trend for the four substrates tested with one exception; levoglucosan yield increases significantly with temperature only for Avicel cellulose.

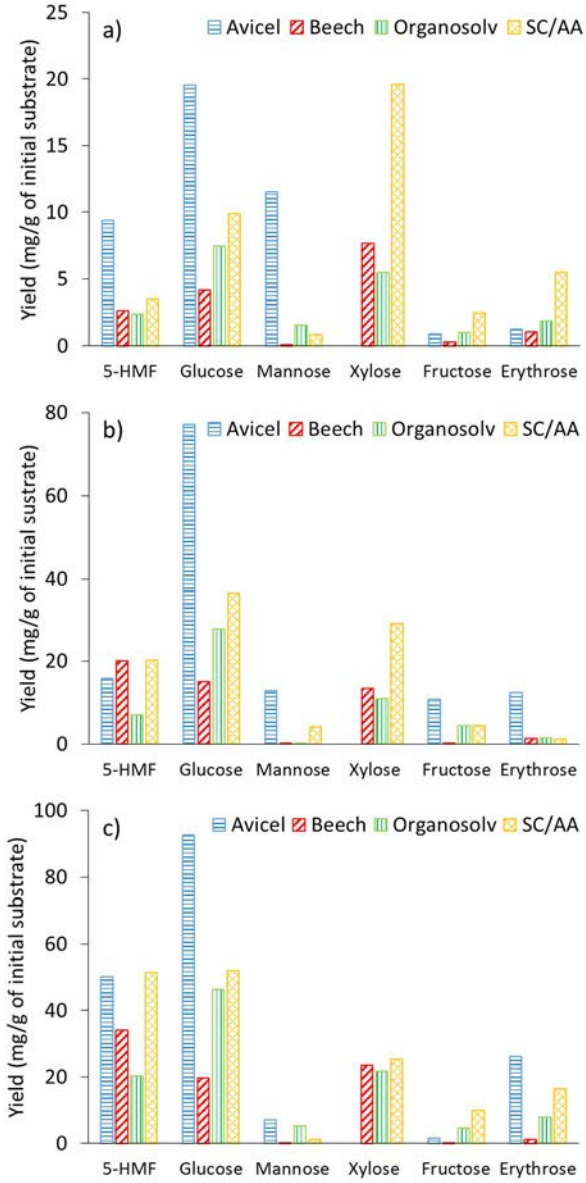


Figure 36. Mass yield of glucose, mannose, xylose, fructose, erythrose and 5-HMF obtained by liquefaction of lignocellulosic pulps in HCW at a) 180°C, b) 200°C and c) 220°C for 2h under isothermal conditions.

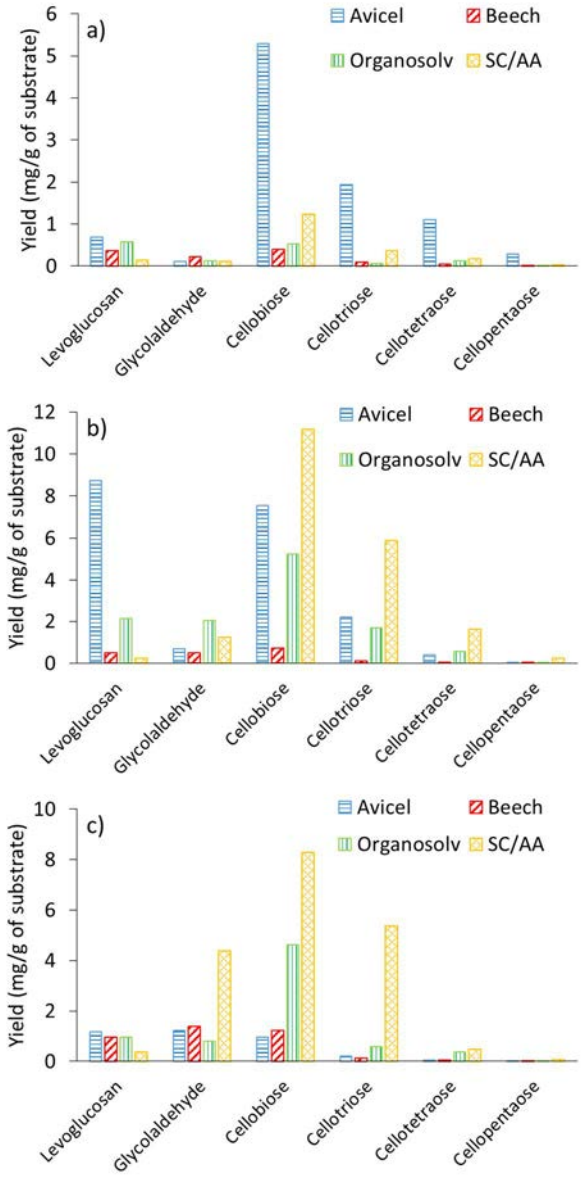


Figure 37. Mass yield of levoglucosan, glycolaldehyde, cellobiose, cellotriose, cellotetraose and cellopentaose obtained by liquefaction of lignocellulosic pulps in HCW at a) 180°C, b) 200°C and c) 220°C for 2h under isothermal conditions.

Monosaccharides are produced in relatively high yields for liquefaction temperatures in the range of 180 to 220°C for all the substrates. Complementary experiments showed that higher liquefaction temperatures (>220°C) will promote the degradation kinetics rates that will convert saccharides into aldehydes, carboxylic acids and eventually permanent gases, meaning that the carbohydrates species yield will decrease among the soluble products. On the other hand, all monomeric species showed to be quite stable at 180°C, this is in good agreement with other

studies found in the literature. The maximum yield for each monosaccharide occurred generally at 220°C.

The highest glucose yield for beech, organosolv and SC / AA pulps were 19.7, 46.3 and 52.0 mg / g respectively. It always occurred at 220°C for the reaction times studied (2h). Xylose was the major product derived from hemicelluloses. The maximum yield for the organosolv pulp was 21.6 mg / g, found when the liquefaction temperature was 220°C. For this pulp, xylose yield increases always with the reaction temperature. On the other hand, SC / AA pulp showed a different behavior. Xylose yield increased significantly when the temperature increases from 180 to 200°C, leading to a maximum xylose yield of 29.0 mg / g. Then, xylose yield slightly decreased when the temperature was increased to 220°C. Fructose and mannose yields did not present the same behavior in relation to the reaction temperature. This can be explained by their instability and constant conversion due to isomerization and degradation reactions. Previous studies(Antal Jr., Mok, and Richards 1990; Kabyemela et al. 1999) showed that fructose is an intermediate compound in the fast reaction that produces 5-HMF, explaining why this monosaccharide is never found in high quantities.

In our previous work,(Buendia-Kandia, Mauviel, et al. 2017) levoglucosan yield obtained using Avicel cellulose (117.9 mg / g) was very high in comparison with the values obtained with the organosolv and SC / AA cellulose-rich pulps. When the cellulose fibers are highly organized as in Avicel cellulose, inner cellulose chains do not have contact with water. Consequently, they will undergo thermal cleavage producing an anhydrous end that gives place to levoglucosan and other anhydro-saccharides.(Shafizadeh et al. 1979, 19; Antal, Várhegyi, and Jakab 1998; Lédé, Blanchard, and Boutin 2002; Q. Lu et al. 2011; Bridgwater 2012; Lédé 2012; Mukarakate et al. 2016) On the contrary, native cellulose fibers are much less organized. In comparison with the raw beech, Avicel cellulose is already more crystalline that cellulose in biomass. Moreover, the fiber network was already deconstructed thanks to the delignification process, that gives more access to water promoting an hydrolytic reaction mechanism instead of a pyrolytic one. This explains

why liquefaction of native cellulose does not produce high quantities of levoglucosan as with Avicel cellulose. In this case, Avicel cellulose is not a good model of cellulose in biomass. Because even if cellulose in the lignocellulosic matrix is protected by lignin, it has less mass transfer limitations that the microcrystals of Avicel cellulose.

Besides, most of bacteria are not able to metabolize anhydro-saccharides. Its biological conversion is more common in fungi and yeast.(Kitamura, Abe, and Yasui 1991; Prosen et al. 1993; Layton et al. 2011) For this reason, promoting glucose instead of levoglucosan increases the fermentable fraction among the water-solubles.

Concerning oligosaccharides (Figure 37) yields increase in the temperature interval from 180 to 200°C. When the temperature changes from 200 to 220°C, oligosaccharides' yields decrease slightly. This result indicates that at the latter temperature the hydrolysis of oligosaccharides in solution competes with the hydrolysis of insoluble cellulosic chains. The optimal temperature to depolymerize cellulosic materials into soluble carbohydrates will depend on the targeted DP of products. Around 200°C more oligosaccharides will be recovered but at 220°C the major species will be monosaccharides. The DP distribution of the carbohydrates mixture will have a significant effect on the downstream processes.

Aldehydes are produced by degradation of monosaccharides. The two major species found in the liquid phase were glycolaldehyde (Figure 37) and 5-HMF (Figure 36). Their yields increased when the temperature increased from 180 to 220°C. 5-HMF was one of the major products of cellulose liquefaction with maximum yields of 34.2, 20.2 and 51.4 mg/g for beech, organosolv and SC/AA biomasses. This compound represents the major inhibitor derived from cellulose liquefaction (also pyrolysis) that will affect the subsequent fermentation process.(Qureshi et al. 2012) Its removal, with other lignin derived compounds is now the object of several studies.(Palmqvist and Hahn-Hägerdal 2000a; Moreno et al. 2015; Gao and Rehmann 2016) Glycolaldehyde was produced in relatively low quantities in all cases. Its presence is an indicator

of monosaccharides degradation.(Sasaki et al. 1998; Kabyemela et al. 1999; Sasaki et al. 2002; Y. Yu and Wu 2010a)

Erythrose is another major liquefaction product that often escapes detection and quantification. Our tailored HPLC method allows for an accurate quantification of this product. Its yield was more important for the SC/AA pulp (16.5 mg/g) than for the beech (1.1 mg/g) and organosolv pulp (7.8 mg/g). It is the only sugar with four carbons detected. Similar to oligosaccharides, its biodegradability has not been well studied. Further analysis of the fermentation inhibitors derived from lignin will be needed to establish the optimal operating conditions of the processes taking part in an integrated biorefinery.

Table 12. Composition of the permanent gas obtained by liquefaction in HCW

Material / Temperature	Yield in mg/g of cellulose		
	H_2	CO_2	CO
Avicel			
180°C	n.d.	0.50	0.01
200°C	n.d.	4.1	0.2
220°C	0.001	20.3	1.6
Beech			
180°C	n.d.	1.3	0.06
200°C	n.d.	6.1	0.5
220°C	0.009	28.2	2.10
Organosolv			
180°C	n.d.	7.4	n.d.
200°C	n.d.	9.6	0.1
220°C	n.d.	22.1	0.3
SC/AA			
180°C	n.d.	14.2	n.d.
200°C	n.d.	23.3	0.05
220°C	0.02	39.7	0.8

Permanent gas composition is presented in Table 12. Carbon dioxide (CO_2) is the major species quantified among the permanent gas. Decarbonylation and decarboxylation reactions increase with the temperature in all cases, as it is evidenced by the augmentation of (CO_2) and carbon monoxide (CO). Hydrogen is detected only when the liquefaction temperature is 220°C and in

very low yields relatively to CO_2 and CO. The gas composition between native cellulose (beech and pulps) and microcrystalline cellulose (Avicel) is comparable, showing that CO and CO_2 are not representative markers for the different depolymerization mechanisms between pure cellulose and pulps under our conditions.

2.4. Conclusion

Wood (beech)fractionation was possible by the organosolv and SC / AA processes with delignification yields of 50 wt.% and 91 wt.% respectively. Using the SC / AA method, 75 wt.% of the hemicelluloses are recovered in the pulp, whereas the organosolv method allows to recover only 27 wt.%.

Depolymerization of fractionated pulps by liquefaction in HCW showed that the maximum quantity of water soluble sugars, ~14 wt.% for organosolv and ~37 wt.% for SC / AA, are produced when the reaction temperature is 220°C. Liquefaction of the organosolv pulp achieved glucose yields similar to the SC / AA pulp, but with much less 5-HMF formation. This can be significantly positive for the subsequent fermentation processes. Even if glucose is the most abundant sugar, there are several others saccharides among the water-soluble fraction (i.e. xylose, fructose, cellobiose, etc.) that are potentially fermentable. Besides, this study concerns only the valorization of the cellulosic fraction, the lignin and hemicellulosic fractions can be valorized into added-value chemicals increasing the economic viability of this biorefinery approach.

2.5. References

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II. Biological conversion of cellulose-derived products

This section deals with the study of the fermentation of cellulose-derived sugars by *C. acetobutylicum.* Two articles present the results found for this purpose.

In the first article, model sugars were used to study the response of *C. acetobutylicum* when the carbon source is a mixture of glucose and cellobiose, that represent mixtures obtained from cellulose hydrolysis.

In the second article, the fermentation of cellulose-derived sugar mixtures by *C. acetobutylicum* is studied. Firstly, sugar mixture obtained by microcrystalline cellulose hydrothermal liquefaction is studied to identify the order of utilization of the different carbohydrates. Moreover, the yields in building blocks obtained with substrates produced from the liquefaction of real biomasses (i.e. beech, organosolv and SC / AA pulps) are reported.

In this article the utilization of glucose and cellobiose mixtures by *C. acetobutylicum* was studied. The main objective is to identify the behavior of *C. acetobutylicum* when the carbon source is a mixture of glucose and cellobiose and its fermentation pattern. The effect of the pre-culture substrate on the cell growth was also studied. This work provides useful information to establish the best conditions for the fermentation of cellulose-derived sugars.

1. Article 3:

Diauxic growth of *Clostridium acetobutylicum* ATCC 824 when grown on mixtures of glucose and cellobiose

Article accepted to *Applied Microbiology and Biotechnology Express (AMB Express).*

ABSTRACT

Clostridium acetobutylicum, a promising organism for biomass transformation, has the capacity to utilize a wide variety of carbon sources. During pre-treatments of (ligno) cellulose through thermic and/or enzymatic processes, complex mixtures of oligo saccharides with beta 1,4-glycosidic bonds can be produced.

In this paper, the capability of *C. acetobutylicum* to ferment glucose and cellobiose, alone and in mixtures was studied. Kinetic studies indicated that a diauxic growth occurs when both glucose and cellobiose are present in the medium

In mixtures, D-glucose is the preferred substrate even if cells were pre grown with cellobiose as the substrate. After the complete consumption of glucose, the growth kinetics exhibits an adaptation time, of few hours, before to be able to use cellobiose. Because of this diauxic phenomenon, the nature of the carbon source deriving from a cellulose hydrolysis pre-treatment

could strongly influence the kinetic performances of a fermentation process with *C. acetobutylicum.*

Key words: Biomass, cellulose, cellobiose, diauxie, fermentation, *Clostridium acetobutylicum.*

1.1. Introduction

Lignocellulosic biomass represents an interesting alternative to fossil carbon resources (McKendry 2002; Mosier et al. 2005; Wyman et al. 2005; Briens et al. 2008; Wettstein et al. 2012; Nanda et al. 2014).

Indeed, lignocellulosic biomass can be transformed into energy and fuels through a variety of chemical, thermo-chemical and biological conversion processes. , Among biological conversion processes, anaerobic digestion and more specifically fermentation processes are the most implemented in industries (Lin and Tanaka 2005; Azman et al. 2015), because it offers the most important biological conversion ways to transform a wide variety of organic materials mainly coming from agroindustry such as by-products into fuels and chemical products with higher values (McKendry 2002; Briens et al. 2008, Turon et al. 2016). However, fermentation processes need an efficient monitoring and control to ensure optimal conditions for productions and yields (Lin and Tanaka 2005; Azman et al. 2015).

Bioethanol, one of the main fermentation products, is predominantly produced using yeast through the fermentation of easily degradable carbohydrate substrates, such as corn starch and sugar cane (Henstra et al. 2007). Nonetheless, the utilization of these kinds of substrates to produce biofuels threatens food supplies and biodiversity. For this reason, the production of new generation biofuels using bacteria may offer an interesting alternative to this problem (Ranjan and Moholkar 2012; Morone and Pandey 2014). As an alternative, some *Clostridium* species can produce Acetone-Butanol-Ethanol (ABE) from renewable resources, such as biomass and derivatives (Lee et al. 2008a; Tracy et al. 2012; Jang et al. 2012; Gu et al. 2014). Butanol can

be blended with gasoline or used directly as a fuel for transportation (Qureshi and Ezeji 2008; Pfromm et al. 2010; Ranjan and Moholkar 2012). For this purpose, a diversity of *Clostridium* strains coming from the butyric and butylic groups has been studied (*C. saccharoperbutylacetonicum, C. acetobutylicum, C. beijerinckii, C. butyricum)*(Lee et al. 2008a; Jang et al. 2012; Gu et al. 2014).

C. acetobutylicum is one of the most studied species thanks to its capability of producing butanol and hydrogen by anaerobic fermentation in high yields, from a wide range of substrates (Qureshi and Ezeji 2008; Survase et al. 2011b; Napoli et al. 2011; Li et al. 2012; Jurgens et al. 2012; Gao et al. 2014; Aristilde et al. 2015; Raganati et al. 2015). This strain was largely used in the ABE fermentation process during the 1910s until the end of 1950s, because the petrochemical industry development led to the decline of this fermentation as an industrial process (Jones and Woods 1986). An important feature of the ABE fermentation is its biphasic development leading to two distinct groups of metabolic products. The first phase is the acidogenesis, which is characteristized by acids (mainly butyric, acetic and lactic acid) and hydrogen production. During acidogenesis, the cells will usually display an active growth including an exponential growth phase (Anderschet al. 1983; Hartmanis and Gatenbeck 1984). The second phase is the solventogenesis, which is characteristized by organic acids re-assimilation and solvent production, with butanol, acetone and ethanol as the major products (Monot et al. 1984). The transition between the two phases is the result of strong gene expression changes (Grupe and Gottschalk 1992; Girbal et al. 1995).Nowadays, with the depletion of oil, renewable processes to replace fossil carbon need to be developed. But, a major factor that determines the viability of this process is the cost of feedstock. In this regard, lignocellulosic biomasses are considered as interesting feedstocks for fermentation (Ezeji et al. 2007; Jang et al. 2012), Whereas its complex structure composed of cellulose embedded in a complex hemicellulose and lignin matrix may hinder biological conversion due to a high

resistance to most chemical and biological pretreatments (Wyman et al. 2005; Kumar et al. 2009; Rinaldi and Schüth 2009; Hendriks and Zeeman 2009; Alvira et al. 2010; Jurgens et al. 2012).

To enhance the fermentability of cellulose a preliminary treatment is frequently used. Chemical, thermal and enzymatic pre-treatments allow to produce a mixture of carbohydrates and other organic compounds that are soluble and accessible to bacteria (Kumar et al. 2009; Rinaldi and Schüth 2009; Alvira et al. 2010; Ibrahim et al. 2015). The conversion of each one of these compounds into added-value products and building blocks is crucial to increase the efficiency of an integrated bio-refinery, and the understanding the metabolism of individual carbohydrates and mixtures is very important to lower the cost of fermentation process. This and the ability of saccharolytic clostridia to use a wide range of carbohydrates have prompted research dedicated to the production of a cheaper substrates (Jones and Woods 1986; Lee et al. 2008a; Tracy et al. 2012; Gu et al. 2014).

In addition, the growth of *C. acetobutylicum* on a mixture of substrates has been already studied using different combinations of carbon sources (glucose and xylose, glucose and mannose, D-glucose and glycerol, …) and different metabolic responses were obtained according to the nutritional environment (Mes-Hartree and Saddler 1982; Ounine et al. 1985; Fond et al. 1986; Vasconcelos et al. 1994; Mitchell et al. 1995; Survase et al. 2011a). Recently, the interest in *C. acetobutylicum* as un effective biofuel producer of butanol instead of ethanol from lignocellulose derivated substrates (glucose, cellobiose and xylose) has increased, resulting in numerous studies privileging mainly butanol production from xylose alone or in mixture with glucose rather than with cellobiose (Patakova et al., 2013; Nogué and Karhumaa, 2014; Raganati et al., 2015; Zhao et al., 2016) whereas this last substrate with glucose and other cello-oligosaccharides in mixture would be also expected to be major products of lignocellulose degradation.

Indeed, despite a recent proteomic study describing the influence of lignin in the metabolic behavior of *C. acetobutylicum* ATCC 824 with cellobiose as the substrate (Raut et al.,

2016), the capability of *Clostridium* strains to ferment mixtures of cellobiose and glucose in synthetic medium is not well studied yet. Furthermore, bacteria have developed mechanisms that allow them to use selectively mixtures of different carbon sources (Mitchell et al. 1995).

In this study, kinetics of *C. acetobutylicum* cultivated with glucose and / or cellobiose, two substrate models representatives of cellulose hydrolysis products were compared. Only the acidogenic phase in which the active growth phase and substrate consumptions could be studied was considered. Transitions and re-assimilation of acids mechanisms were excluded from this work.

1.2. Materials and Methods

1.2.1. Microorganism and media

Spores of *Clostridium acetobutylicum* ATCC 824 were maintained in Difco™ Reinforced Clostridial Medium (RCM) at ambient temperature. Whereas precultures were different for each culture experiment, they were prepared rigorously 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 12h and then transferred into 30 mL (pre-culture tubes) of a synthetic medium. In fact, the volume of preculture was prepared depending of the final volume of culture in order to get a ratio of 1 / 4. The synthetic medium was composed of 20 g / L glucose, 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 $MnSO_4.H_2O$, 3 g / L $CaCO_3$, $4x10^{-5}$ g / L biotin. All the Chemicals, yeast extract and biotin were provided by Sigma Aldrich.

1.2.2. Fermentation

Batch fermentations were carried out in a bioreactor controlled by an Applikon ADI 1030 bio controller (Applikon Biotechnology). Throughout all fermentation experiments, temperature was maintained at 37°C and pH at 5.5 by the automatic addition of 3N NaOH, 1N HCl using the biocontroler and a pH probe (Mettler Toledo). The bioreactor was initially purged with nitrogen to ensure an anaerobic atmosphere and then inoculated with 6% (v/v) active growing pre-cultures into 1.5 L of synthetic media with the same composition of the pre-culture media (without $CaCO_3$ to prevent interference with DO measurements). The glucose concentrations for the fermentation studies were comprised between 25 - 35 g/L (138 – 195 mM), unless otherwise indicated. Other experiments with cellobiose were carried out with the same glucose-equivalent mass concentration. However, controlling residual substrate from pre-culture was not possible. For this reason, the initial substrate concentration was not exactly the same. These variations did not alter the study case and the desired results. Each experience was carried out until complete substrate consumption. All the experiments were performed at least in duplicate.

1.2.3. Analyses

Cell density was measured at 600 nm using a spectrophotometer (HITACHI U-2000). The relationship between the cell dry weight and the optical density was established thanks to a calibration made by triplicate using spectrophotometer at 600 nm. The correlation factor found was 0.346 g/L cell dry weight per unity of absorbance. This value was in agreement with others related in the literature (Kim et al. 1984)

Glucose, cellobiose, acetone, ethanol, butanol, acetic acid, lactic acid and butyric acid concentrations were measured by high-performance liquid chromatography equipped with a refractive index detector and an ultraviolet-visible spectroscopy (HPLC-RID-UV) using a

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

1.2.4. Calculations

The main products of glucose or cellobiose fermentation by *C. cellulolyticum* were acetate, Butyrate, ethanol, butanol, acetone, lactate, H_2, and CO_2, as previously described (Vasconcelos et al., 1994) Carbon recoveries were calculated from the production of metabolites, and biomass, present in the supernatant. Biomass was taken into account on the basis of the cell dry weight and a mean biomass formula of $C_4H_7O_2N$ (Guedon et al. 1999). According to the metabolic scheme (Vasconcelos, et al.,1994),the conversion of glucose to products can be written as follows:

glucose + 2 ADP + 2 NADH → 2 ethanol +2 ATP + 2 NAD^+ + 2 CO_2 + 2 H_2

glucose + 4 ADP + 2 NAD^+ → 2 acetate + 4 ATP + 2 NADH + 2 CO_2 + 2 H_2

glucose + 3 ADP → butyrate + 3 ATP + 2 NADH + 2 CO_2 + 2 H_2

glucose + 2 ADP + 2 NADH → butanol + 2 ATP + 2 NAD^+ + 2 CO_2 + 2 H_2

glucose + 2 ADP + 2 NAD^+ → acetone + 3 ATP + 2 NADH + 3 CO_2 + 2 H_2

For example, from the last stoichiometric equation, each acetone molecule produced is associated to the formation of 3 CO_2 molecules. Therefore, the CO_2 production was calculated on the basis of product formation, as the sum of [acetate], 2*[butyrate], [ethanol], 2*[butanol], and 3*[acetone] concentrations.

CO_2 production was calculated as the sum of [acetate], 2*[butyrate], [ethanol], 2*[butanol], and 3*[acetone] concentrations.

1.3. Results

1.3.1. General growth and metabolic features of Clostridium acetobutylicum ATCC 824 cultivated with glucose or cellobiose

kinetics of growth and metabolism of *C acetobutylicum* with glucose or cellobiose as the carbon and energy source were performed with precultures carried out with either glucose or cellobiose (figure 1). Pre cultures and cultures performed with the same carbon source were compared with precultures and cultures performed with the switched substrates. The pH was set at 5.5 in order to favor acidogenesis, resulting In butyric, acetic and lactic acids, as the main products whatever the substrate used

The carbon balances for these experiments were comprised between 90 and 98%, indicating that most of the carbon substrates and products have been taken into account. Therefore, resulting kinetics can be fully interpreted.

Figure 1a shows the growth kinetic of *C. acetobutylicum* on glucose inoculated with a preculture grown with the same substrate. Taking into account the residual substrate coming from the preculture, the initial glucose concentration was 136 mM (25 g/L). After approximately 13 h of culture, all the glucose was completely consumed and no lag phase was observed since the culture rapidly entered in exponential phase. In these experimental conditions, a maximal specific growth rate (μ_{max}) of 0.26 h^{-1} was reached after 3 hours of culture whereas a maximal cell concentration of 2.36 g/L (dry cell weight) was obtained when glucose was entirely depleted. Glucose fermentation by *C. acetobutylicum* was accompanied by the accumulation of acidic products (figure 1d) such as butyric, acetic and lactic acid up to 74mM, 40 mM and 9 mM respectively, whereas butanol was a minor product with a production of only 8 mM.

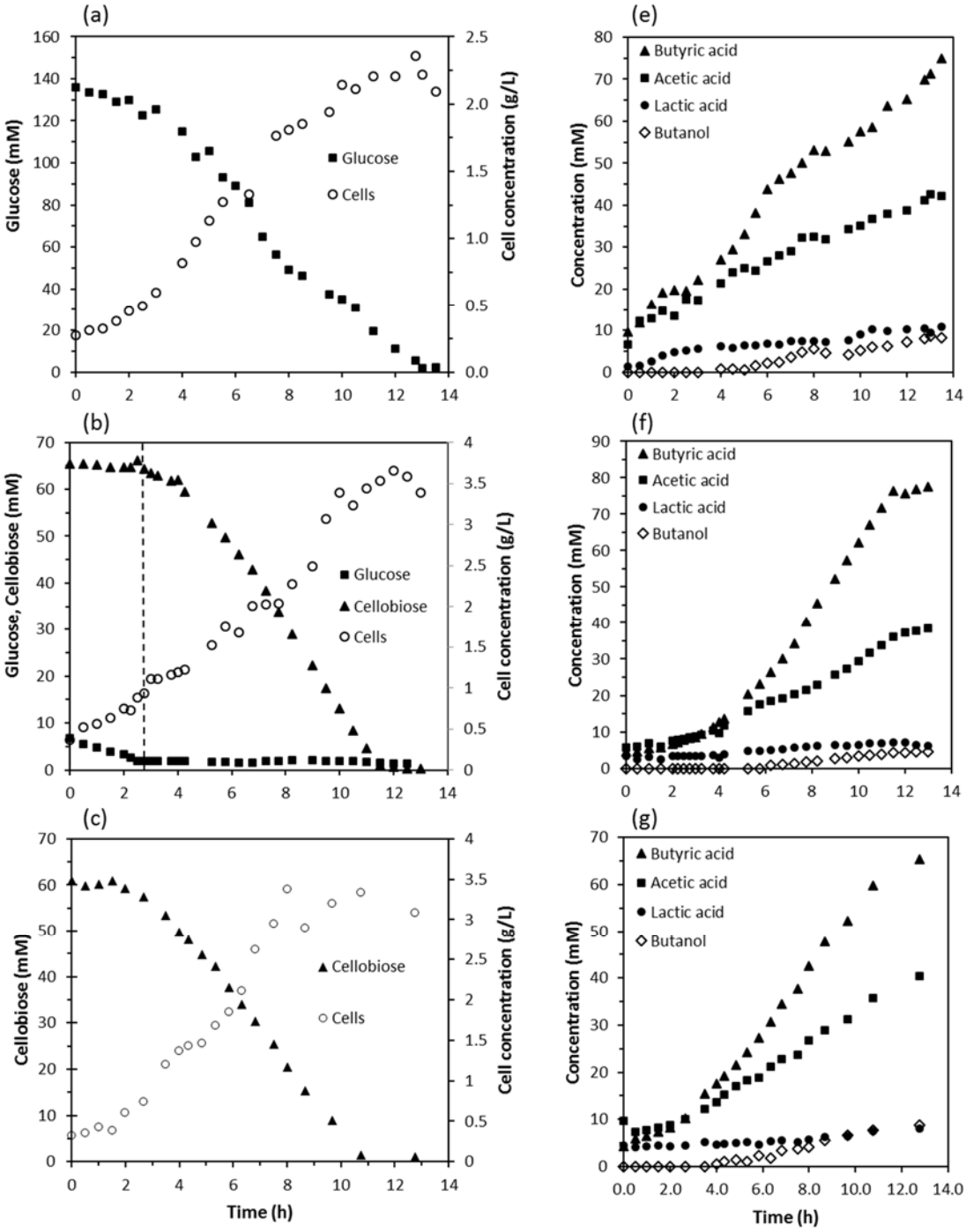

Figure 38. Kinetics of growth and metabolism of glucose or cellobiose as the substrate by *C. acetobutylicum*. (a) Glucose as the substrate (136 mM) and glucose-pregrown cells as the inoculum. (b) Cellobiose as the substrate (67 mM) and glucose-pregrown cells as the inoculum. The dashed line represents le beginning of the lag phase after glucose was exhausted. (c) Cellobiose as the substrate (67 mM) and cellobiose-pregrown cells as the inoculum. (d), (e) and (f) are the respective product kinetics of (a), (b) and (c). Each graph is representative of at least three independent experiments (n = 3).

Figure 38b and c report the growth kinetics of of *C. acetobutilycum* with cellobiose (67 and 61 mM respectively) as the carbon and energy source, and with glucose or cellobiose as the respective preculture substrates.

When inoculated with a preculture cultivated with glucose, an immediate and short growth phase occurred during the 3 first hours of culture (Figure 38b). During this phase, no cellobiose was used, whereas glucose coming from preculture (7.2 mM) was completely exhausted. Then, the consumption of cellobiose by *C. acetobutylicum* occurred until exhaustion and was associated with an active cell growth.

Interestingly, as indicated by the dashed line (Figure 38b), the use of cellobiose was accompanied by a short lag phase during approximately 2 hours after glucose exhaustion. Then the growth of *C. acetobutylicum* started and reached a μ_{max} of 0.21 h^{-1} and maximal biomass concentration of 3.7 g / L after 12 hours of culture.

As observed during fermentation kinetics performed with glucose in Figure 38a, the product pattern obtained with cellobiose as the carbon source and with glucose as the preculture substrate (Figure 38e) is similar since butyric (76 mM), acetic (35 mM) and lactic acids (5.5 mM) were the main products at the end of the culture whereas butanol accumulated at a low concentration.

Figure 38c displays the growth kinetic of *C. acetobutylicum* with cellobiose as the substrate (61 mM) after an inoculation with a preculture grown with cellobiose. In less than 11 hours, all the cellobiose was fully consumed by *C. acetobutylicum*, resulting in an active cell growth without apparent lag phase. In these conditions, a μ_{max} of 0.23 h^{-1} and a maximal cell concentration of 3.4 g / L after 8 hours of culture were observed. Similarly to the previous kinetics (Figure 38a, b), the product pattern obtained with cellobiose as the carbon source in the fermentation broth and in precultures (Figure 38f) are similar (Figure 38d, e), indicating that neither the carbon source in precultures, nor the carbon source in cultures have an influence on the carbon distribution during fermentation.

However, when pre-cultures were grown with glucose as the substrate (Figure 38b), an adaptation stage of *C. acetobutylicum* seems to be important before to be able to use cellobiose as the main carbon and energy source.

1.3.2. Fermentation of glucose and cellobiose mixtures by C. acetobutylicum:

In order to get a better understanding of the lag phase observed during the cellobiose culture inoculated with pre-cultures performed with glucose (Figure 38b), experiments using mixtures of glucose and cellobiose were performed with either glucose and cellobiose pre-cultures as the inoculum. These experiments were carried out in similar conditions to the previous ones. Glucose and cellobiose were entirely consumed and typical acidogenesis product profiles were observed in batch fermentations. In these experimental conditions, the carbon recoveries were a bit lower than previously and comprised between 85 and 87%.

Figure 39a reports sequential consumption of glucose and cellobiose as well as growth kinetics observed when pre-cultures were performed with glucose. In this experiment, the culture medium contained a glucose and cellobiose mixture with respective initial concentrations of 47 and 43.3 mM.

During this fermentation process, no lag phase was observed and glucose was the first substrate to be consumed by cells. In these experimental conditions, a μ_{max} of 0.28 h^{-1} was measured; resulting in a cell concentration of 2 g / L. Cellobiose began to be consumed by *C. acetobutylicum* in a second time and only when glucose was completely exhausted after 4.5 hours of culture. During the first 2 hours of cellobiose utilization, a temporary growth cessation was observed. Then, cellobiose was fully consumed, in association with a second growth, resulting in a maximal cell concentration of 3.2 g / L, and a μ_{max} of 0.22 h^{-1}. However and as initially observed in Figure 38b, a lag phase was observed when glucose was exhausted suggesting that an adaptation phase may be required before cellobiose utilization *by C. acetobutylicum*.

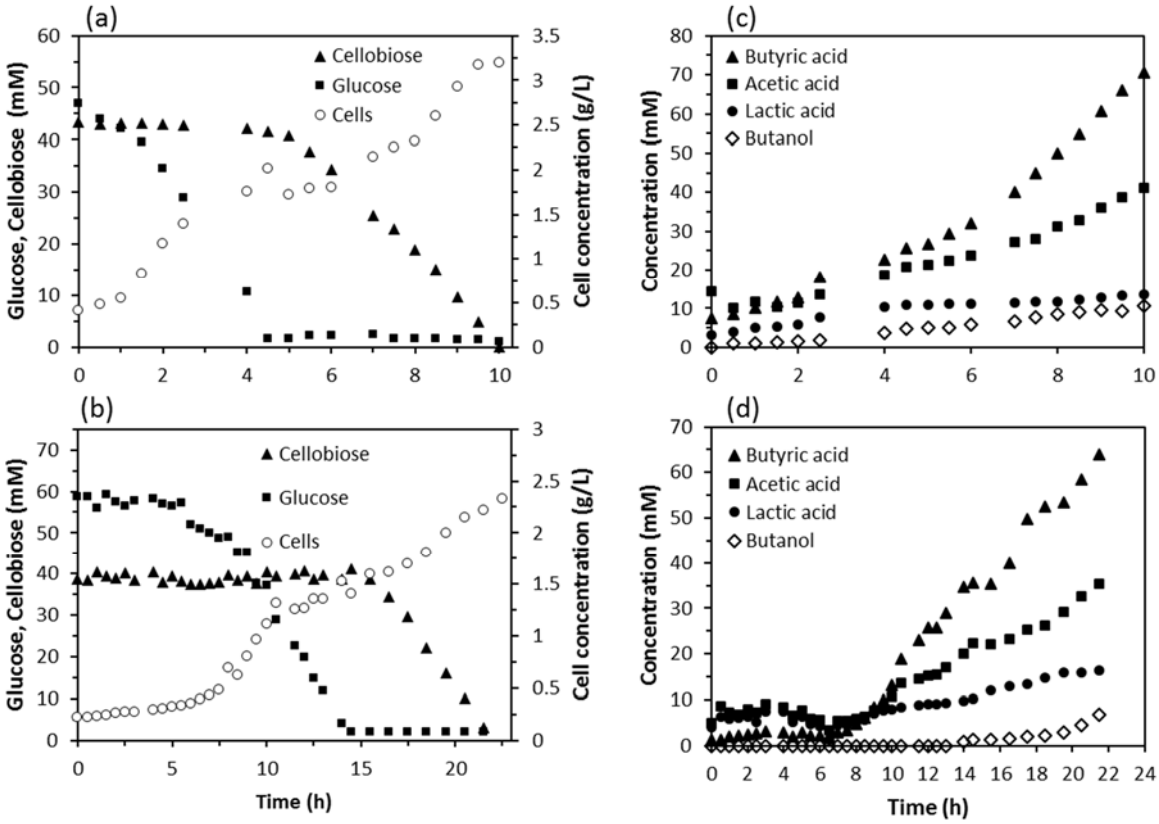

Figure 40. Kinetics of growth and metabolism of D-glucose / cellobiose mixtures by *C. acetobutylicum*. (a) glucose-pregrown cells as the inoculum, initial glucose or cellobiose concentrations were 46.97 and 43.30 mM respectively. (b) cellobiose-pregrown cells as the inoculum, initial glucose or cellobiose concentrations were 58.7.97 and 38.8 mM respectively. (c) and (d) are the respective product kinetics of (a), (b). Each graph is representative of at least three independent experiments (n = 3).

Figure 41b reports sequential consumption of glucose and cellobiose as well as growth kinetics observed when the fermentation process was inoculated with cellobiose pregrown cells. The respective initial concentrations of glucose and cellobiose in the bioreactor were 58.7 and 38.8 mM. Interestingly, a lag phase of 4 hours without any glucose or cellobiose consumption was observed. Then cells started to use glucose until exhaustion. Then cellobiose utilization started about 2 hours after glucose was entirely depleted. However, the maximal growth rate was barely slower with cellobiose (μ_{max} of 0.22 h^{-1}), compared to glucose (0,27 h^{-1}). Product patterns (Figures 42c,d) obtained with mixtures of glucose and cellobiose were similar to those previously observed in Figure 38e,f,g whatever the culture conditions.

In this study, results strongly suggest that glucose is definitely the preferred substrate, since it is always used in first place, even if pre-cultures were performed with cellobiose are exposed to a mixture of glucose and cellobiose. However, glucose consumption does not start immediately a lag phase is always observed when carbon substrates in pre cultures and in cultures are different, suggesting that a physiological adaptation may occur, resulting in a growth cessation. In fact, glucose and cellobiose are able to influence the consumption of each other by *C. acetobutylicum*.

1.4. Discussion

In this study, fermentations of glucose and/or cellobiose by *C. acetobutylicum* were performed in batch mode. Mono-substrate cultures showed that *C. acetobutylicum* is able to grow with cellobiose as efficiently as with glucose.

With glucose as the substrate, kinetics of growth, substrate consumption and products formation were in good agreement with previous studies (Jones and Woods 1986b; Girbal et al. 1995). Interestingly, no major difference was observed when cellobiose was the sole carbon substrate: specific growth rates measured with both substrate were almost similar, and no significant change in fermentation patterns (acidogenic phase) was observed. In fact, little was known about the cellobiose metabolism by *C. acetobutylicum* and such a study was never deeply investigated so far. Indeed, only studies dedicated to enzymatic activities expressed by *C. acetobutylicum* during the fermentation of cellobiose were reported in the literature (Allcock and Woods 1981; Mes-Hartree and Saddler 1982; Lee et al.1985; López-Contreras et al. 2000

However, and contrary to C*. thermocellum* (Weimer and Zeikus 1977), cultures grown on glucose/cellobiose mixtures demonstrated that *C. acetobutylicum* was unable to co-utilize both substrates at the same time and glucose was consistently the preferred carbon source. In the present work, growth cessations were always observed after glucose exhaustion and before cellobiose utilization, resulting in a second growth phase.

This phenomenon was reported for the first time by (Monod 1942), and was called "diauxie". It is nowadays better known under the generic term of catabolic repression since some substrates have the ability to repress the expression of genes encoding catabolic enzymes and / or protein transporters, as already described in bacterial species (Magasanik 1961; Brückner and Titgemeyer 2002; Deutscher, Francke, and Postma 2006; Deutscher 2008; Görke and Stülke 2008) but these mechanisms are not the same for each strain and their complete characterization is still being studied.

Besides, the effect of carbon source on cell growth and fermentation products by *C. acetobutylicum* has been previously studied for different mixtures (Vasconcelos et al. 1994). In continuous cultures, *C. acetobutylicum* grown with glucose at neutral pH, produced only acids. In the same conditions, but with substrate mixtures of glucose and glycerol, alcohols were produced in higher yields but no catabolic repression was observed with these carbon sources. However, the repression of lactose transport system by glucose (Yu et al. 2007) as well as diauxic growths of *C. acetobutylicum* cultivated on mixtures of xylose and glucose (Jiang et al. 2014) have been reported, demonstrating that these mechanisms are of a great importance and participate to the control of carbon catabolic fluxes of cells depending on the nature of carbon sources.

In this study, similar mechanisms could be involved according to our results. Indeed, two putative PTSs (phosphotransferase systems) operon genes, strongly induced by cellobiose with functions connected to cellobiose metabolism, were reported by Servinsky et al. (2010), suggesting that *C. acetobutylicum* displays multiple mechanisms to import, phosphorylate and hydrolyse B-glucosides for entry into glycolyse. Besides, the similarity of the *C. acetobutylicum* PTS to PTSs found in other well characterized low GC gram positive bacteria, has led to the suggestion that they may also play a role in carbon catabolite repression (Behrens et al., 2001; Saier and Reizer, 1992; Singh et al., 2008; Tangney et al., 2003).This hypothesis was reinforced by the fact that a recent proteomic study revealed that such PTS operons, especially those involved in cellobiose uptake in *C. acetobutylicum* was shown to be down regulated in the presence of lignin

residues, in mixture with cellobiose (Raut et al., 2016). In this study, our results suggests that an analogous phenomenon may exist that was never reported for *C. acetobutylicum* when cultivated with mixtures of glucose and cellobiose, and therefore is an original contribution to the comprehension of carbohydrate metabolism by Clostridial sp.

Besides, diauxic growths observed during the kinetic studies may have a great importance for ABE fermentation processes of pretreated (ligno) cellulosic biomass. From a technological point of view, this observation may be of critical importance if industrial substrates mixes were to be used for hydrogen and / or other chemical precursor productions by *C. acetobutylicum*, since substrate consumption discontinuities could occur and may affect production performances and productivities of processes

More studies with other complex mixtures of cellulosic derivatives as well as with various operating conditions will help to understand how *C. acetobutylicum* can manage its carbohydrate metabolism in order to perform an efficient ABE production process.

List of abbreviations

ATCC: American Type Culture Collection

ABE: Acetone-Butanol-Ethanol

PTS: Phosphotransferase System

RCM: Reinforced Clostridial Medium

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In this article, the fermentation is studied starting from the cellulose liquefaction products, which less inhibitors. Then the cellulose-rich pulps that have some residual lignin that might be detrimental for the bacterial growth and finishing with the raw biomass hydrolysates that will present more problems due to the high quantity of lignin-derived products. In order to establish the fermentation conditions, the effect of the pH on the utilization of glucose and cellobiose (Appendice 5) was a crucial point to achieve a successful ABE fermentation.

2. Article 4:

From wood to building blocks: ABE fermentation of carbohydrates produced by hydrothermal depolymerization of wood pulps

Article in preparation to be submitted.

Highlights:

-First integration of pulping, liquefaction and fermentation.

-Delignification of lignocellulosic biomass was good enough to allow butanol production. Raw-beech hydrolysates were not fermentable.

-*C. acetobutylicum* was able to utilise several carbon substrates from the raw material source, establishing an utilization order.

-First time reported cellotriose. cellotetraose and cellopentaose utilization by *C. acetobutylicum*.

ABSTRACT

Lignocellulosic biomass is a potential source of renewable carbon. The development of effective biorefinery processes for the valorization of biomass into fuels and chemicals is a major

challenges for the current society. Traditional saccharification of cellulosic materials uses enzymes that are expensive and have long reaction times (2-3 days). Hydrothermal depolymerization of cellulosic materials is a potential alternative for the production of fermentable substrates. In this work, sugar mixtures coming from cellulosic materials depolymerized by hydrothermal liquefaction are fermented by *Clostridium acetobutylicum* for the production of building blocks (butanol, acetone, acetic acid, butyric acid). Microcrystalline cellulose (Avicel) was depolymerized by hydrothermal liquefaction at 180, 200 and 220°C and then fermented by *C. acetobutylicum* to study the utilization of a sugar mixture. An order of utilization of the different sugars present in mixtures was observed. Because valorization of all the components of biomass is a key factor for a viable biorefinery, cellulose-rich pulps coming from beech wood fractionation were liquefied at the same temperatures and then used as substrates for the production of building blocks by *C. acetobutylicum.* The sugar and non-sugar content of all the liquefied mixtures were quantified and correlate to itheir fermentability. The kinetics of the total sugars utilization and building blocks production by *C. acetobutylicum* were assessed.

2.1. Introduction

Energy sources are essential for human development. With the steady increase of global population, energy availability coming from non-renewable sources is getting more and more limited and their exploitation and use impact negatively the environment. Finding renewable sources capable to replace fossil carbon derived energy and chemicals is now a major concern of society.(OPEC, 2016) Lignocellulosic biomass is a renewable source of carbon that can be transform into fuels and chemicals. Its abundance and distribution around the world make this resource a promising alternative to fossil carbon.(Harmsen et al., 2014; H. Isikgor and Remzi Becer, 2015; Ragauskas et al., 2006b)

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, lignin and some extractive compounds. In order to ensure an efficient valorization of all three components, a fractionation process is normally needed, allowing to separate the (holo)cellulosic fraction from lignin.(Kumar et al., 2009; Ragauskas et al., 2006a) Biomass is the only renewable resource that can be directly transformed into chemicals and fuels.

Conversion of biomass can be achieved by different types of technology that can be divided in two major groups: thermochemical and biological. Thermochemical conversion of biomass by fast pyrolysis allows a fast conversion with high liquid yields but no much selectivity.(Briens et al., 2008; Jia et al., 2017; Lédé, 2012) On the other hand, biological conversion is characterized by its high selectivity with slow kinetics that often last days.(Alvira et al., 2010) Traditionally, saccharification of cellulosic materials is performed by enzymatic hydrolysis using a wide variety of cellulases, β-glucosidases, endo- and exoglucanases.(Ibrahim et al., 2015; Obama et al., 2012) This process allows to produce glucose with yields around 80-90%. The main drawback of such approachis the high cost of enzymes and the inability to recycle them.(Jiang et al., 2016) Additionally, the presence of the some aldehydes and phenols will have a detrimental effect on the enzymatic efficiency.(Jönsson and Martín, 2016; Palmqvist and Hahn-Hägerdal, 2000)

Clostridium acetobutylicum is able to produce acetone, butanol and ethanol (ABE) in high yields. Butanol is a promising renewable fuel because its energy content (27 MJ/mol) is very similar to that of gasoline (32 MJ/mol)(Liu et al., 2015).

Several studies reporting the utilization of a wide variety of carbon sources by *C. acetobutylicum* can be found in the literature.(Aristilde et al., 2015; Bruder et al., 2015; Girbal and Soucaille, 1994; Grimmler et al., 2010; Mes-Hartree and Saddler, 1982; Raganati et al., 2015; Survase et al., 2011) However, most of these studies made use of model compounds (or model mixtures) cultivating *C. acetobutylicum* with only one carbon source. On the contrary, the real feedstock in an industrial biorefinery represents a much more complex mixture and its utilization has not been yet well studied.

The objective of this work is to study the valorization of lignocellulosic biomass for the production of acids and solvents by fermentation using *C. acetobutylicum.* In our previous work, beech was fractionated by two methods: organosolv and SC / AA, in order to recover a cellulose-rich material with a low quantity of lignin in order to limit detrimental effect on micro-organisms used in fermentation processes.

The two pulps and a model cellulose (Avicel PH-101) were liquefied in order to obtain complex mixtures of sugars that can be used in a fermentation process. The Avicel cellulose was studied as a model representation of lignocellulosic biomass.

This work is focused on the fermentation process of the hydrolysates recovered from the liquefaction of these cellulosic materials.

The utilization order of a complex mixture of cellulose-derived carbohydrates has been studied, reporting for the first time the utilization by *C. acetobutylicum* of cellotriose, cellotetraose and cellopentaose, three majors cello oligosaccharides present in cellulose hydrolysis mixtures. Kinetics of acid and solvent production by *C. acetobutylicum* are also reported. The substrate utilization and fermentation products patterns and yields were assessed.

2.2. Materials and Methods

Figure 43 shows a schem of the general procedure follow in this work.

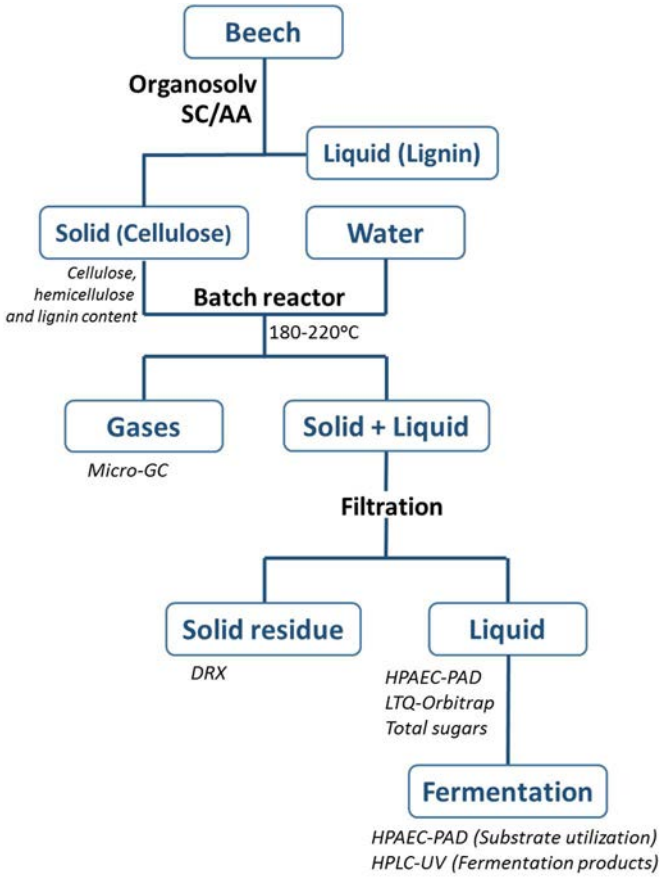

Figure 43. Schematic of the full process: biomass fractioning. liquefaction in HCW and fermentation.

2.2.1. Substrate preparation

Beech wood (*Fagus sylvatica)* was fractionated by organosolv and sodium chlorite/acetic acid methods to recover a cellulose-rich pulp. Both pulps were then liquefied at 180, 200 and 220°C to depolymerize the holocellulose, obtaining complex mixtures of carbohydrates. Specific details of the fractionation process yields and composition of the pulps can be found in section B, I, 2.

2.2.2. Fermentation

Culture media containing 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 $MnSO_4.H_2O$, 3 g/L $CaCO_3$, $4x10^{-5}$ g/L biotin was prepared and transferred to 50 mL vials that were then sterilized in an autoclave. All the Chemicals, yeast extract and biotin were provided by Sigma Aldrich.

The sugar mixtures were lyophilised and added in a 10 mL aqueous solution to the fermentation vials. pH of the culture media containing the sugars mixture was adjusted to 5.5. Finally the vials were inoculated with 5 mL (10 %) of active cells pre-grown in RCM media. Fermentation experiments were carried out for 72 h at 37°C. Samples were taken every 12 h for chemical analysis.

2.2.3. Analysis

Total soluble sugars before and during the fermentation were quantified by the phenol / sulfuric acid method. For the study of the utilization order of carbohydrates, a method using a high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used to identify and quantify these compounds. A detailed explanation of the development and parameters of this method are reported in the section B, I, 1.(Buendia-Kandia et al., 2017)

Fermentation products were analysed by high performance liquid chromatography with ultraviolet detection as explained in the section B, II, 1.

2.3. Results and discussion

The aim of this work is to study the production of biofuels or building blocks from lignocellulosic biomass. Beech wood has been delignified, by the organosolv and SC / AA processes, to remove microbial inhibitors. Microcrystalline cellulose (Avicel PH-101) was initially depolymerized by liquefaction in HCW (from 180°C to 260°C) to set up the best conditions to produce carbohydrates and limit the side reactions that lead to potent inhibitors. The cellulose-rich pulps were recovered from both processes to be then depolymerized by liquefaction in HCW at 180°C, 200°C and 220°C under isothermal conditions. The water-soluble compounds recovered from the liquefaction of Avicel, organosolv and SC / AA pulps were then fermented by *C. acetobutylicum* in order to produce acetone, butanol and ethanol (ABE).

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2.3.1. Fractionation and hydrolysis of beech wood

Valorisation of lignocellulosic biomass in a biorefinery includes often several pretreatments in order to ensure the bioconversion of this recalcitrant resource. Preliminary tests of direct fermentation of beech wood hydrolysates presented no growth. This can be explained by two main reasons: the first one is that lignin protects the lignocellulosic matrix from hydrolysis, making the liquefaction in HCW (under our conditions) unable to release enough fermentable sugars allowing a successful fermentation. The second reasons, is that lignin is depolymerised much faster than cellulose and the water soluble fraction contains is composed mostly of lignin-derived compounds that have often a detrimental effect on microbial growth. For this reasons, beech wood was previously fractionated by the organosolv and SC / AA processes

Organosolv process is an interesting fractionation process due to its economic viability and feasible industrialisation.(Brosse et al., 2009; Pan et al., 2006) SC / AA process is most common at laboratory scale but its interest is on the high delignification yields that can be achieved with this method.(Campbell and McDonald, 1952; Siqueira et al., 2013)

One of the main purpose of this work was to identify the fermentability of the carbohydrates produced by liquefaction of lignocellulosic materials. Figure 44 shows the different chemical families identified among the complex substrates that will strongly affect their fermentability and so the final ABE yields. According to the literature, the water soluble fraction was classified in five families: fermentable sugars (glucose, fructose, mannose, xylose, cellobiose, cellotriose, cellotetraose, cellopentaose),(Aristilde et al., 2015; Mes-Hartree and Saddler, 1982; Raganati et al., 2015; Survase et al., 2011) non-fermentable sugars (erythrose, levoglucosan),(Kitamura et al., 1991) non-identified sugars, aldehydes (5-hydroxy-methyl-furfural, glycolaldehyde)(Qureshi et al., 2012) and non-sugars.

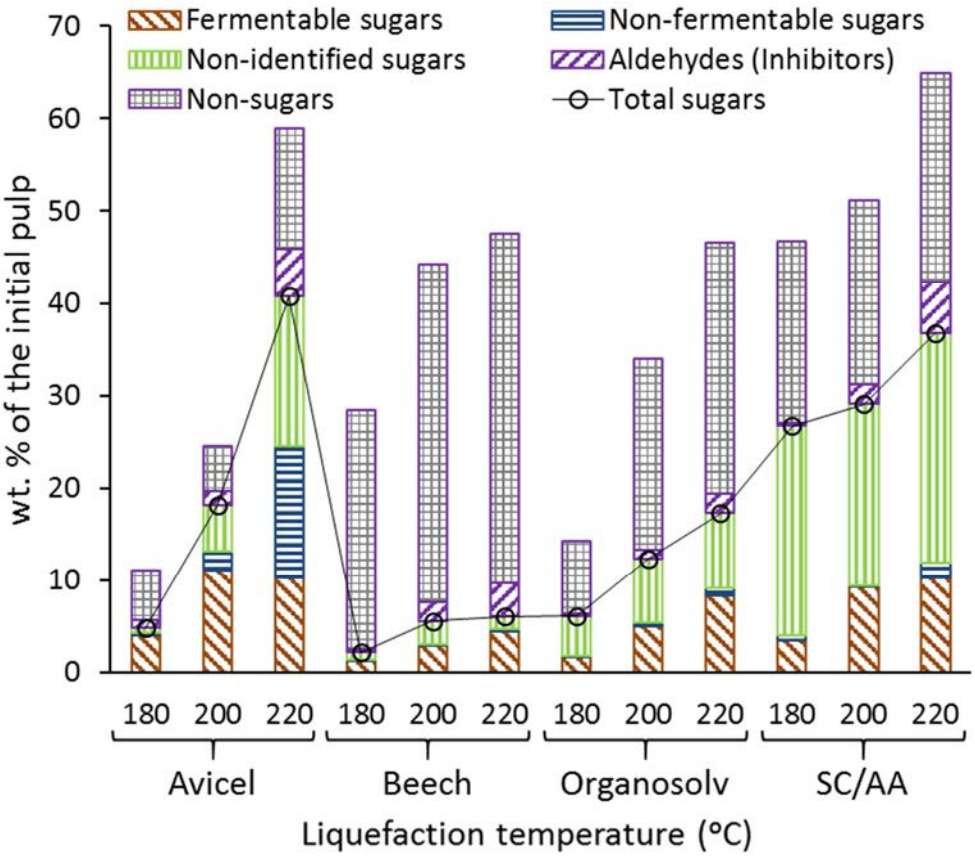

Figure 44. Mass yields of the different chemical families according to its fermentability. All the values are calculated relative to the initial mass of biomass used for the liquefaction in HCW under isothermal conditions.

The sugars whose fermentability is not known were classified as non-fermentable. This will be better discussed in the next subsection. This classification provides important information of the potential of the fractionation and liquefaction conditions as an entry point into biorefineries that include fermentation processes.

Liquefaction of Avicel cellulose produces 4.8 wt. % of total sugars when the reaction temperature is 180ºC. This value increases up to 18.1 wt. % at 200ºC and the maximum is 40.7 wt. % at 220ºC. The latter value was the highest found among all the cellulosic samples. This was expected since the initial mass was pure cellulose, contrary to the other biomasses in which lignin represents 20-25 wt. % of the material.

However, not all of the sugars produced are fermentable. At 220ºC, the production of levoglucosan was the preferred pathway (public avicel) and the fermentable sugars were only

10.2 wt. %. This can be explained by the highly ordered structure of microcrystalline cellulose that prevents the access of water to the inner fibers and does not favour the hydrolytic reaction pathway but the pyrolytic one instead. For this reason, this material produces high quantities of levoglucosan and other anhydro-saccharides that, despite being carbohydrates, are not fermentable by Clostridial strains.

In the case of lignocellulosic materials, total sugars and fermentable sugars increase with the liquefaction temperature for all the three biomasses. For a given liquefaction temperature, the higher the lignin contents are, the lower the total sugars yield are. As was mentioned before, lignin prevents cellulose to react and with the hemicelluloses, both are the first compounds to be depolymerized. Concerning the non-sugar fraction of the beech, organosolv and SC / AA pulps must contain a high content of lignin-derived products that are not suitable for bioconversion processes.

2.3.2. Fermentation of a cellulose-derived mixture of sugars

Cellulose hydrolysis ends up in a mixture of saccharides that are often converted by enzymatic hydrolysis into glucose to be then fermented by yeast or bacteria. For this reason, several works have been consecrated to the study of glucose-based fermentation without regarding other high-degree of depolymered (DP) cello-oligosaccharides as potential carbon sources. Besides, there is a lack of knowledge in the fermentability of a mixture of carbohydrates coming from cellulose depolymerization. In fact, the utilization of cello-oligosaccharides with DP>2, has not been studied.

In this work, a complex mixture of cellulose-derived compounds (produced by cellulose liquefaction in HCW) was used in order to establish the fermentability of the different carbohydrates by *C. acetobutylicum*. For this purpose, pure cellulose was depolymerized in hot-compressed water under mild conditions (180°C) allowing to produce the substrate mixture for this purpose, due to the low quantities of inhibitors (degradation products: aldehydes and

carboxylic acids), with a high polydispersity and an ease of characterization (89 wt% quantified). Figure 45 shows the utilization by *C. acetobutylicum* of this carbohydrate mixture used as the carbon source.

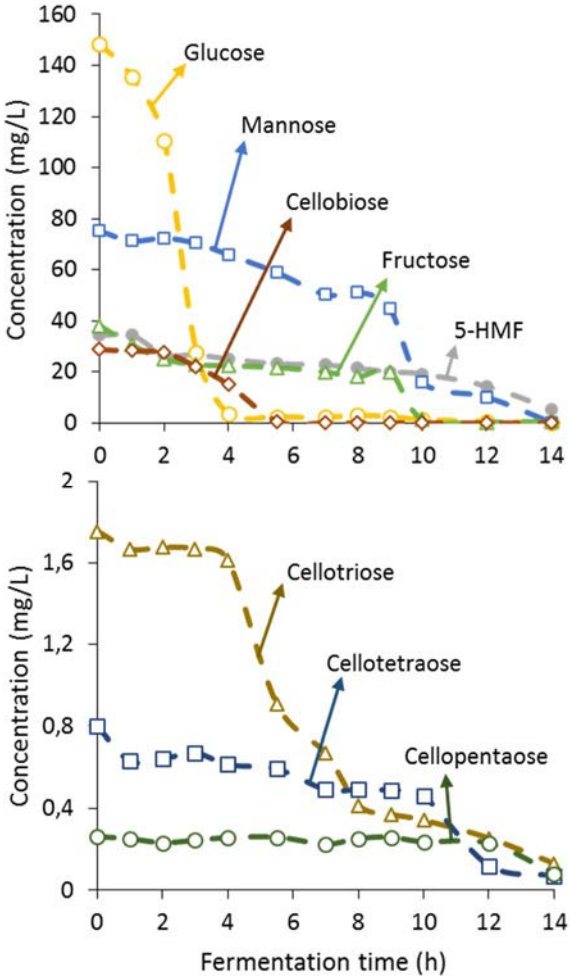

Figure 45. Utilization of a cellulose-derived carbohydrates by *C. acetobutylicum*. Microcrystalline cellulose (Avicel PH-101) was hydrolysed in HCW at 180°C for 2 hours at isothermal conditions.

Monitoring the fermentation of various carbohydrates is a difficult task due to the different uptake rate, adaptation phases and variety of compoudns found in the culture media. For this reason, a relatively diluted solution of this mixture was used to put in evidence the utilization of the major carbohydrates in a single fermentation. In that way, the fermentability of the different cellulose hydrolysis products was studied and an order in the utilization of cello-oligosaccharides have been observed.

As is shown in Figure 45, glucose is the first carbohydrate to be completely depleted after 4 h of fermentation. Then, cellobiose was depleted after around 5 h fermentation,. This result is in

agreement to our previous work showing the diauxic growth exhibited by *C. acetobutylicum* when is cultivated on glucose / cellobiose mixtures (presented in section B, II, 1).

Cellotriose was sh the third substrate to be used utilization started after 4 h of fermentation, being used at the same time that cellobiose, suggesting that s there is no catabolic repression of cellobiose on cellotriose utilization by *C. acetobutylicum* when these two carbohydrates are present in the culture media. Cellotriose utilization continued steadily for 4 h, decreasing significantly around 8 h of fermentation. Utilization of mannose was relatively low between 3 and 9 h of fermentation. After 9 h of fermentation mannose was rapidly co-utilized with fructose by *C. acetobutylicum.*

5-HMF is the major inhibitor detected among the liquid fraction produced by cellulose liquefaction. This results show that this compound has been metabolized by *C. acetobutylicum*, in agreement with what has been already reported by Qureshi et al.(Qureshi et al., 2012) and Zhang et al.(Zhang et al., 2012). On the contrary, erythrose (four carbons sugar) is not utilized by *C. acetobutylicum* under the conditions studied in this work.

According to the experimental conditions, the preferred substrate was glucose, followed by cellobiose / cellotriose; then fructose / mannose were used when cellotriose reached aklow residual concentration. Finally cellotetraose, cellopentaose and 5-hydroxylmethyfurfural (5-HMF) were used at the end of the fermentation. This data suggest that *C. acetobutylicum* exhibits some preferences in substrate utilisation when grown in a carbohydrate mixture under our conditions.

+This results put in evidence that *C. acetobutylicum* has the ability to utilize complex carbohydrate mixtures as the sole carbon source. Moreover, this is the first evidence of the utilization of cellotriose, cellotetraose and cellopentaose by *C. acetobutylicum.*

2.3.3. Fermentation of cellulose hydrolysates

As observed in our experimental conditions, an utilization order of the different carbohydrates by *C. acetobutylicum* can be proposed. In our previous work, the influence of the temperature on the composition of the liquid fraction produced by liquefaction of Avicel cellulose was studied (section B, I, 1).(Buendia-Kandia et al., 2017) In order to study the effect of the liquefaction temperature on the production of fermentable sugars from cellulosic materials, hydrolysates produced in the range of 180 and 260°C from microcrystalline cellulose were used as the carbon source to cultivate *C. acetobutylicum*. These temperatures were chosen according to the conditions reported in the literature for the production of oligo-saccharides from cellulose.(Yu and Wu, 2010, 2009)

Kinetics of substrate utilization and fermentation products of *C. acetobutylicum* cultivated on microcrystalline cellulose hydrolysates as the sole carbon source are shown in Figure 46.

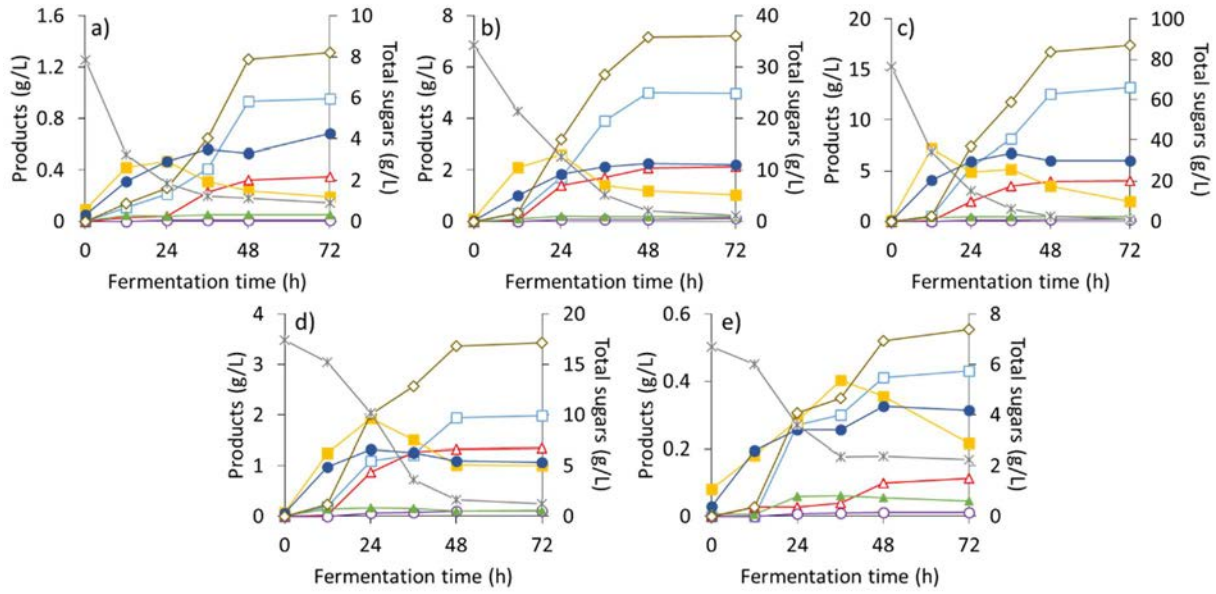

Figure 46. ABE fermentation from hydrolysates of microcrystalline cellulose (Avicel PH-101) produced by liquefaction in HCW at different temperatures: a) 180°C, b) 200°C, c) 220°C, d) 240°C and 260°C, under isothermal conditions. Products: acetic acid (dark blue, ●), lactic acid (green, ▲), butyric acid (yellow, ■), ethanol (purple, ○), acetone (red, △), butanol (light blue, □), ABE (brown, ◇) and total sugars (grey, **x**).

The total sugars in each substrate varied according to the liquefaction temperature (Figure 44). All the soluble sugars produced by liquefaction were used to cultivate *C. acetobutylicum*; and their initial concentrations in the fermentation vial were 8.0, 36.0, 82.0, 19.7 and 8.9 g / L for the cellulose liquefied at 180, 200, 220, 240 and 260°C respectively.

C. acetobutylicum was able to metabolize all the carbohydrate mixture to produce acids (i.e. butyric acid, acetic acid and lactic), solvents (butanol, acetone and ethanol) and gas (hydrogen and carbon dioxide).

In all cases, sugars were rapidly used by *C. acetobutylicum*, producing a typical ABE fermentation product patterns, characterized by an initialacidogenic phase, followed by a solventogenic phase. Residual sugars of 0.9, 1.1, 0.8, 1.2 and 2.3 g / L from the samples prepared at 180, 200, 220, 240 and 260°C respectively, quantified after 72 h of fermentation. Residual sugars are evidences that there are non-fermentable carbohydrates among the liquefaction water-soluble products. In this work, only erythrose was identified as a non-fermentable sugars. Moreover, high-DP oligomers (not quantified in this work) might be another potential species among this substrate residue.

The highest yields for all the fermentation products were obtained when the cellulose depolymerization was performed at 220°C. This is obviously due to the high concentrations of sugars (~80g / L) produced at this temperature. This initial concentration of substrate was higher than the normal tolerated by *C. acetobutylicum*, that is around 60g / L when the substrate is a single monomeric sugar, commonly glucose, as was reported in the literature.(Ezeji et al., 2007)

However, the maximal concentration of carbon substrate that *C. acetobutylicum* can used in one single batch fermentation might be different when cultivated using a complex mixture of carbohydrates as the sole carbon source. This is due to the extra substrate used during adaptation phase that the cells undergo when they move from using one specific sugar to another one, as it is explained in our previous work (section B, II, 1).

During the first 24h of fermentation, butyric acid was the major product in all cases, with the only exception of the fermentation with the carbon mixture prepared at 220°C, as shown in Figure 46c.

This is probably due to the fast initiation of the solventogenesis where butyric acid is consummed by *C. acetobutylicum* to produce butanol.

At 260°C, the maximum concentration of butyric acid was reached after 36 h of fermentation, whereas it was reached after 24 h in the other conditions excepted at the temperatureof 220°C (12 h).

As a result, a delay in the initiation of the solventogenic phase can explain lower final butanol yield than those obtain with the temperature of 220°C..

After 72h of fermentation, final acetic acid concentrations were 0.7, 2.2, 6.0, 1.1 and 0.3 g/L for the substrates prepared by cellulose liquefaction at 180, 200, 220, 240 and 260°C respectively. This compound, often considered as an undesired by-product, has an important world marked as a building block for the chemical industry. Lactic acid production was very low in comparison with the other carboxylic acids.

Total solvent productions of produced 1.3, 7.2, 16.7, 3.4 and 0.5 g/Lby *C. acetobutylicum* were obtained, when it was cultivated with substrates obtained by cellulose liquefaction at 180, 200, 220, 240 and 260°C respectively.

Figures 4 shows that the concentration of products decrease considerably when the liquefaction temperatures used to prepare the mixtures increases from 220°C to 260°C. This can be explained by the fact that the mixtures prepared at temperatures higher than 220°C promote the degradation of water-soluble sugars into aldehydes and carboxylic acids that will decrease significantly the fermentability of substrate mixtures.

In all cases, butanol production was very low during the first 12 h of fermentation (acidogenic phase), then when butyric acid reached its maximum concentration and started to be consummed, butanol production increased steadily between 12 and 48 h of fermentation. Final concentrations of butanol after 72 h of fermentation were 0.9, 5.0, 13.2, 2.0 and 0.4 g/L when the substrate were prepared by cellulose liquefaction at 180, 200, 220, 240 and 260°C respectively.

Acetone behavior was similar to that of butanol for the substrates obtained at 200, 220 and 240°C. Acetone production was relatively low during the first 24 h and 36 h for the substrates prepared at 180°C and 260°C respectively. Ethanol production was always low whatever the temperature of liquefaction used.

These results showed that fermentable sugars increased with the temperature in the range of 180 to 220°C. On the contrary, the sugar yields decreased from 220 to 260°C, probably because degradation reactions (dehydration, decarboxylation, decarbonylation, etc.) of monomeric sugars become more important at these temperatures.

2.3.4. Fermentation of hydrolysates from delignified beech

Fermentation of carbohydrates coming from a real feedstock was also studied in this work, , beech wood coming from the current region of France was selected.

The results obtained from the fermentation of cellulose (Avicel) hydrolysates allowed determining that substrates produced at liquefaction temperatures higher than 220°C were not suitable for the production of building blocks by fermentation using *C. acetobutylicum.* For this reason, liquefaction of wood pulps were performed at temperatures between 180 and 220°C, obtaining fermentable carbohydrate mixtures. These mixtures were used as the sole carbon source to cultivate *C. acetobutylicum.* To the best of our knowledge, the fermentation of hydrolysates coming from organosolv and SC / AA pulps was never reported..

Fermentation of hydrolysates produced directly from the raw beech exhibited no cell growth nor solvent production. Concluding that these mixtures are not fermentable, confirming that a fractionation process will be needed in order to remove the majority of lignin. For this reason, there are no other relevant results for the experiments with the raw beech.

Beech fractionation was performed by the organosolv and SC / AA treatments. The recovered pulps were then depolymerised by liquefaction to recover a mixture of sugars that will serve as substrate for the fermentation experiments. Figure 47 shows the kinetics of sugar utilization and

fermentation products by *C. acetobutylicum* when its cultivated using beech pulps hydrolysates as the sole carbon source.

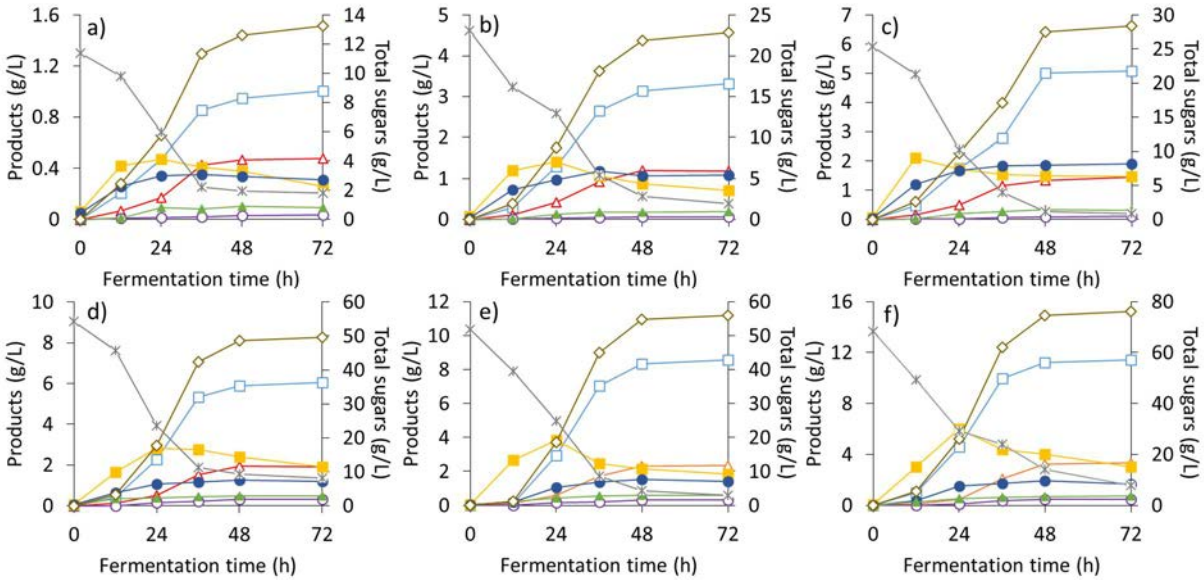

Figure 47. ABE fermentation from hydrolysates of delignified beech (*Fagus sylvatica*). Two different delignification processes were used: Organosolv and SC / AA, then the pulps were liquefied in HCW at different temperatures: Organsolv pulp a) 180ºC, b) 200ºC, c) 220ºC and SC / AA pulp d) 180ºC, e) 200ºC, f) 220ºC, under isothermal conditions. Products: acetic acid (dark blue, ●), lactic acid (green, ▲), butyric acid (yellow, ■), ethanol (purple, ○), acetone (red, Δ), butanol (light blue, □), ABE (brown, ◇) and total sugars (grey, **x**).

Hydrolysates from organosolv pulp were used to cultivate *C. acetobutylicum* giving initial sugar concentrations of 53.4, 58.2 and 73.6 g / L when the liquefaction temperatures were 180, 200 and 220ºC respectively. Carbohydrates have been rapidly used by *C. acetobutylicum* in all the cases, and after 72 h of fermentation, sugar residues were 1.8, 1.9 and 0.8 g / L for torganosolv pulps liquefied at 180, 200 and 220ºC respectively.

In the case of the SC / AA pulps, the initial sugar concentrations were 54.2, 51.9 and 68.4 g / L and the sugar residues after 72 h of fermentation were 8.2, 3.0 and 7.9 g / L for the pulps liquefied at 180, 200 and 220ºC respectively. One fraction of the sugar residues after fermentation of beech-pulp hydrolysates correspond to erythrose, a four carbon sugar that is one of the major liquefaction products, was found to be not fermentable in all cases.

The rest of the compounds present in the final residue have not been identified, but it is highly probable that these compounds are high-DP oligosaccharides, partially soluble and present in

low concentration. However, commercial standards of these compounds are not available, making therefore their analyses difficult..

The substrate residue after 72h of fermentation was higher when the liquefaction temperature was 180°C for both organosolv and SC / AA pulps. This can be explained by the fact that at this temperature, hydrolysis reactions were very mild and there awere high-DP oligomers remaining in the solution. These oligomers are probably slowly or not metabolized by *C. acetobutylicum* under the conditions studied in this work.

In all cases, the fermentation products correspond to the typical patterns of *C. acetobutylicum:* starting with the production of carboxylic acids (i.e. butyric acid, acetic acid and lactic acid), followed by the production of solvents (i.e. butanol, acetone and ethanol).

Butanol productions increased significantly when butyric acid concentration reached their maximal values and started to be consummed. This happened after 24 h of fermentation for all cases with only one exception: the organosolv pulp liquefied at 180°C. In the same way, total ABE concentrations increased steadily between 12 and 48 h of fermentation and then remained stable until the end of the experiment at 72 h. This result shows that under these experimental conditions, no significant production of solvents could be obtained after 48 h of fermentation. For this reason, extend the fermentation more than 48 h will only decrease the global ABE productivity.

Table 13. Productivities of total fermentation products after 48 and 72 (indicated in the brackets).

	Productivity (mg/L/h)		
	Avicel	Organosolv	SC / AA
180°C	26.3 (18.2)	30.0 (21.1)	169.7 (115.3)
200°C	149.4 (100.4)	91.3 (63.5)	228.1 (155.6)
220°C	348.5 (242.1)	133.7 (92.1)	310.3 (211.5)

Table 1 shows productivities of fermentation products obtained after 48 and 72 h. Maximal productivities of 348.5, 133.7 and 310.3 mg/L/h, were obtained when the liquefaction temperature was 220°C after 48 h of fermentation for the Avicel cellulose, organosolv and SC/AA pulps respectively. In all cases, the ABE productivity was higher after 48 h of fermentation than after 72 h. Our results indicate that there is no significant production of solvents during the last 24 h of fermentation and that this will only increase the operational cost of this process.

Table 14 presents a recompilation of fractionation, liquefaction and fermentation products yields obtained for the different cellulosic materials (i.e. avicel cellulose, organosolv and SC/AA pulps) treated in this work.

Table 14. Mass yields of fractionation, liquefaction and fermentation products from wood-pulps (*Fagus sylvatica*). Yields are based on the last conversion stage.

		Yield based on last conversion	
(wt. %)	**Avicel**	**Organosolv**	**SC/AA**
Beech wood	100	100	100
Pulping (solid)	63.6	53.3	55.2
Total sugars	41	17.3	36.8
Total building blocks	32.5	30.3	29.3
Butanol	15.3	14.5	15.2
Acetone	4.8	3.8	4.4
Ethanol	0.24	0.2	0.6
Acetic acid	4.4	4.3	5.5
Butyric acid	7.3	5.4	2.6
Lactic acid	0.5	1.0	0.9
Hydrogen	1.3	1.1	1.0

The overall yield of this process can still be improved by optimizing the fractionation, liquefaction and fermentation processes. In this work, the main objective was to set an efficient methodology that can be easily implemented and that respects the environment.

2.4. Conclusion

Fractionation and liquefaction were found to be fast and efficient processes to produce fermentable substrates that can be used as building blocks. On the contrary, raw beech hydrolysates were not metabolized by *C. acetobutylicum.* In addition, an utilization order of the major cellulose-derived sugars was also observed, highlighting the first evidence of cellotriose, cellotetraose and cellopentaose utilization by *C. acetobutylicum*. The fermentability of the sugars produced by cellulose liquefaction was found to be maximum at 220°C. The fermentation experiments were performed during 72 h but the maximum ABE productivities were found after 48 h of fermentation. In general, the fermentation of beech-pulp hydrolysates may represent an interesting approach for a building block biorefinery.

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III. Integration of thermochemical and biochemical processes

This section presents one article on a model developed in Aspen Plus® showing the integration of all the processes of the proposed biorefinery: organosolv fractionation of beech wood, hydrothermal liquefaction of the cellulose-rich pulp and fermentation of the sugar mixture by *C. acetobutylicum*. The recovery of lignin and hemicellulose are also included in this model. This model facilitates the establishment of the mass and energy balances. A simple energy analysis is assessed for the operation units used in this biorefinery strategy, in order to identify the critical points to optimize in terms of energetic performance.

1. Article 5:
Process integration modeling for a wood biorefinery: Pulping, Liquefaction and Fermentation to produce Building Blocks

Article in preparation.

1.1. Introduction

One of the major concerns of the actual society is the necessity of fossil resources to produce fuels and building blocks.(Ragauskas, Williams, et al. 2006; Ragauskas, Nagy, et al. 2006; P. F. H. Harmsen, Hackmann, and Bos 2014; Choi et al. 2015) Lignocellulosic biomass is now considered

one of the most promising sources of renewable carbon in order to replace the fossil-based ones. Biomass is mainly composed of cellulose (40-60wt.%), hemicellulose (20-40wt.%) and lignin (15-35wt.%). These macromolecules form a tridimensional matrix that protects biomass from chemical and biological conversion, impelling different pretreatments processes to valorize this renewable resource effectively.(Mosier et al. 2005; Alvira et al. 2010; P. Harmsen et al. 2010)

Each one of these macromolecules is a potential resources to produce a variety of chemicals with different functionalized groups of interest in different sectors of the industrial market.(Briens, Piskorz, and Berruti 2008; García et al. 2011; Amidon et al. 2011)

The conversion of biomass into fuels and chemicals requires several operation units that vary according to the industrial and chemical market needs.

Fractionation (or pulping) processes, such as: Kraft,(Chakar and Ragauskas 2004) organosolv,(Brosse, Sannigrahi, and Ragauskas 2009; García et al. 2011) chlorite / acid,(Campbell and McDonald 1952; Siqueira et al. 2013) ionic liquids,(Lee et al. 2009) ozone,(Alvira et al. 2010) among others,(Mosier et al. 2005; Alvira et al. 2010; Moreno et al. 2015) allow separating these 3 macromolecules. Then each fraction will be subjected to the most convenient process for its valorization.

Raw or fractionated biomass can be converted into chemicals of industrial interest by different processes: 1) chemical (acid / basic impregnation, hydrolysis, catalysis),(Rinaldi and Schüth 2009; Jiang et al. 2015) 2) thermochemical (pyrolysis, hydrothermal liquefaction, gasification)(Peterson et al. 2008; Elliott 2008; Bridgwater 2012) and 3) biological (enzymatic hydrolysis, fermentation, anaerobic digestion)(Chen et al. 2015; Moreno et al. 2015) processes.

The development of a multifunctional biorefinery integrating different of these technologies is required for a cost-effective valorization of biomass. Process modeling is a powerful tool to analyze and optimize the design and energy performance of the combined technologies used in biorefineries. (García et al. 2011; Jones et al. 2014; Duque, Cardona, and Moncada 2015)

Numerous authors have reported models able to represent technologies used in a biorefinery using Aspen Plus®,.(Nikoo and Mahinpey 2008; Abdelouahed et al. 2012; François et al. 2013; Liu, Zhang, and Bao 2016) a commercial simulation software. NREL has reported a model of an integrated biorefinery for the production of bioethanol from corn stover. In this model the main sections are pretreatment of biomass, enzymatic hydrolysis, fermentation and production of enzymes.("Biorefinery Analysis Process Models | NREL" n.d.; Wooley and Putsche 1996)

However, only few works have yet reported an integrated biorefinery process based on experimental data. Furthermore, no biorefinery model has been yet presented combining biomass pulping, depolymerization of the pulp by a thermochemical process and followed by fermentation of the as-produced sugars.

In this work, a model representing the valorization of beech wood in biorefinery was assessed.
The proposed biorefinery process is divided in three sections. Firstly, biomass pulping by organosolv process recovering separated fractions rich in cellulose, hemicellulose and lignin respectively.

Secondly, the cellulose-rich pulp is depolymerized by hydrothermal liquefaction allowing producing water soluble sugars.
Finally, the sugars obtained are used as substrate in a fermentation process to produce building blocks such as: butanol, acetone, ethanol, acetic acid, butyric acid and hydrogen.
Experimental data was collected by the authors for all three sections. Operating conditions, yields and concentrations were simulated according to this data.

The model obtained allows establishing an energy and mass balance from raw biomass to targeted building blocks. The purpose of this work is to build a simplified (but realistic) process model of an integrated biorefinery based on various experimental results.

1.2. Presentation of the Aspen Plus® model

1.2.1. Overview of the process

The proposed biorefinery process includes three stages: pulping, liquefaction and fermentation. Experimental studies of all three stages have been already performed and reported in results section I and II. In those sections there is more information about the set-ups used for those experimental works.

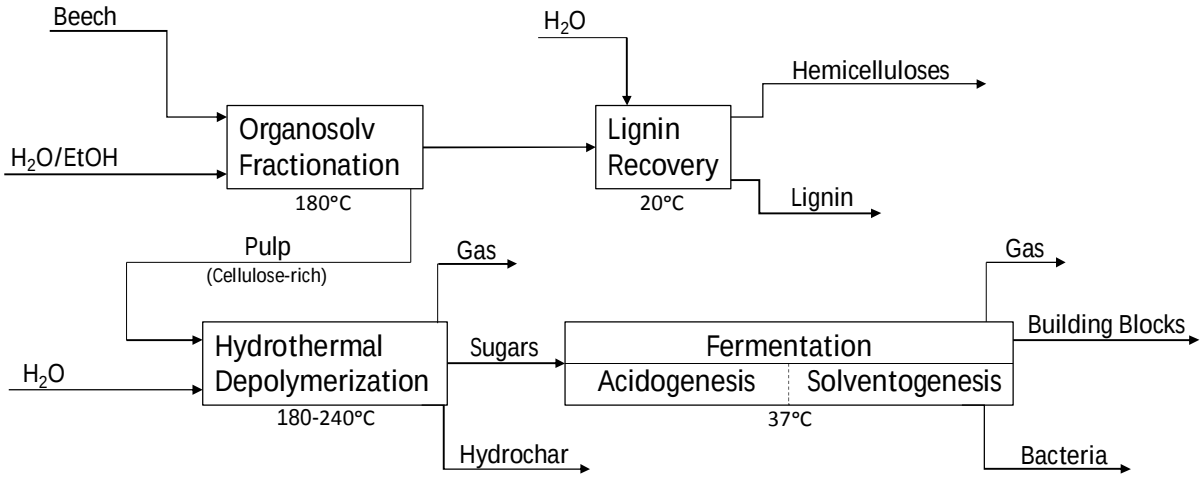

Figure 48. Scheme of the biorefinery process

The biorefinery process, including pulping, liquefaction and fermentation, was developed using the commercial simulation software Aspen Plus®. An overview of the biorefinery process proposed is shown in Figure 48.

1.2.2. Definition of compounds

Table 15. List of compounds used in the model with the corresponding formula and molecular weight.

No.	*Component name*	*Component ID*	*Type*	*Formula*	*MW (g/mol)*
1	Water	H2O	Conventional	H_2O	18.02
2	Cellulose	CELLULOS	Solid	$C_6H_{10}O_5$	162.14
3	Xylan	XYLAN	Solid	$C_5H_8O_4$	132.11
4	Arabinan	ARABINAN	Solid	$C_5H_8O_4$	132.11
5	Galactan	GALACTAN	Solid	$C_6H_{10}O_5$	162.14
6	Mannan	MANNAN	Solid	$C_6H_{10}O_5$	162.14
7	Lignin	LIGNIN	Solid	$C_{10}H_{13.9}O_{1.3}$	154.92
8	Glucose	GLUCOSE	Conventional	$C_6H_{12}O_6$	180.16
9	Xylose	XYLOSE	Conventional	$C_5H_{10}O_5$	150.13
10	Arabinose	ARABINOS	Conventional	$C_5H_{10}O_5$	150.13
11	Galactose	GALACTOS	Conventional	$C_6H_{12}O_6$	180.16
12	Mannose	MANNOSE	Conventional	$C_6H_{12}O_6$	180.16
13	Fructose	FRUCTOSE	Conventional	$C_6H_{12}O_6$	180.16
14	Erythrose	ERYTHROS	Conventional	$C_4H_8O_4$	120.11
15	Levoglucosan	LEVOGLUC	Conventional	$C_6H_{10}O_5$	162.14
16	Bacteria	BACTERIA	Solid	$C_6H_{12}O_6$	180.16
17	Cello-saccharides	CELLOSAC	Conventional	$C_6H_{12}O_6$	180.16
18	Soluble lignin	LIGNOSOL	Conventional	$C_{10}H_{15.9}O_{2.3}$	172.93
19	Manno-saccharides	MANNOSAC	Conventional	$C_6H_{12}O_6$	180.16
20	Galacto-saccharides	GALASAC	Conventional	$C_6H_{12}O_6$	180.16
21	Arabino-saccharides	ARABISAC	Conventional	$C_5H_{10}O_5$	150.13
22	Xylo-saccharides	XYLOSAC	Conventional	$C_5H_{10}O_5$	150.13
23	Acetic Acid	ACETIC	Conventional	$C_2H_4O_2$	60.05
24	Lactic Acid	LACTIC	Conventional	$C_3H_6O_3$	90.08
25	Butyric Acid	BUTYRIC	Conventional	$C_4H_8O_2$	88.11
26	Acetone	ACETONE	Conventional	C_3H_6O	58.08
27	Ethanol	ETHANOL	Conventional	C_2H_6O	46.07
28	Butanol	BUTANOL	Conventional	$C_4H_{10}O$	74.12
29	Oxygen	O2	Conventional	O_2	32.00
30	Nitrogen	N2	Conventional	N_2	28.01
31	Hydrogen	H2	Conventional	H_2	2.02
32	Carbon monoxide	CO	Conventional	CO	28.01
33	Carbon dioxide	CO2	Conventional	CO_2	44.01
34	Cellobiose	CELLOBIO	Conventional	$C_{12}H_{22}O_{11}$	342.30
35	Glycolaldehyde	GLYCOALD	Conventional	$C_2H_4O_2$	60.05
36	Hydroxymethylfurfural	HMF	Conventional	$C_6H_6O_3$	126.11
37	By-products	CHO	Nonconventional	-	-
38	Solid residue	HYDCHAR	Nonconventional	-	-

38 compounds declared for this model (29 conventional, 7 solid and 2 non-conventional compounds) are presented in Table 15. All the parameters required for the properties calculations were taken from NREL and previous works.(François et al. 2013, 2014)

Composition of the initial biomass was taken from our previous work, reported in section B, I, 2. Two non-conventional compounds containing carbon (C), hydrogen (H) and oxygen (O) were declared for this model.

The first was the "hydrochar", corresponding to the solid residue after hydrothermal liquefaction and the second was called "CHO", that corresponds to the soluble fraction that was not identified in the experimental work. The elemental composition of both compounds was determined in model through elemental conservation balances. The C, H and O content of both compounds is presented in Table 16.

Table 16. Elemental composition of the non-conventional compounds.

%wt.	Hydrochar	CHO
C	44.4	54.1
H	6.2	8.1
O	49.4	37.8

1.2.3. Process modeling

a) Modeling of pulping

Organosolv pulping of beech wood was carried out to obtain a cellulose-rich pulp. The model corresponding to this process stage is illustrated in Figure 49.

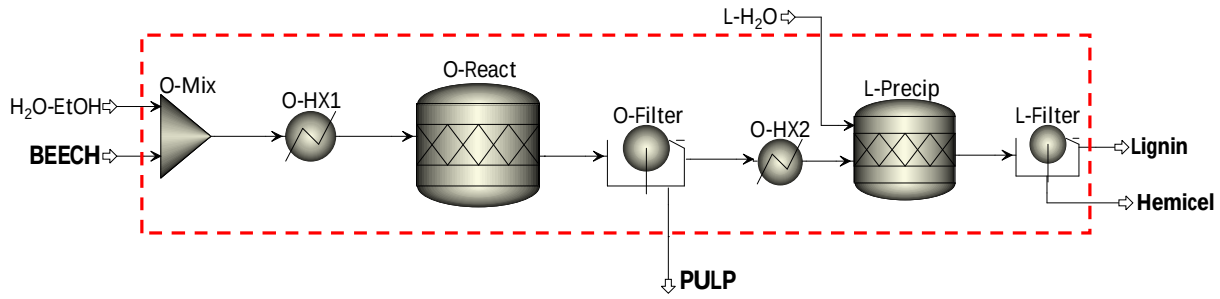

Figure 49. Aspen plus flowsheet for the organosolv pulping process zone.

In this stage water and ethanol were combined with beech wood using a mixer (O-Mix). Then the mixture is heated to 180°C and goes into a stoichiometric reactor (O-React) where the organosolv pulping takes place under adiabatic condition. The solid pulp rich in cellulose is separated from the soluble lignin and hemicellulose by filtration (O-Filter). At this point, the cellulose-rich pulp is already recovered, the remaining solution containing lignin and hemicelluloses is cooled down to recover the heat. Lignin precipitation (L-Precip) and filtration (L-Filter), was modeled only to complete the data about the lignin and hemicellulosic fractions, that can be valorized elsewhere.

b) Modeling of liquefaction

The model of the hydrothermal liquefaction of the as-produced pulp is shown in Figure 50.

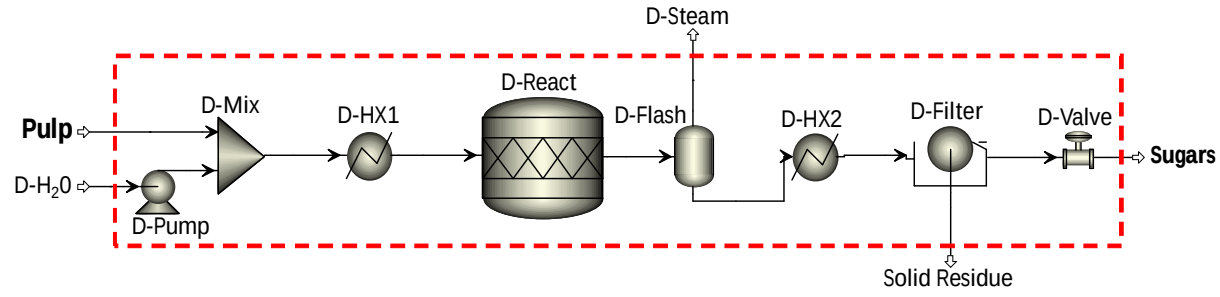

Figure 50. Aspen plus flowsheet for the liquefaction process zone.

A pump (D-Pump) is used to generate pressured water and then a mixer (D-Mix) is used to combine the water (D-H_2O) and the cellulose-rich pulp (Pulp) recovered from the organosolv pulping. Then a heat exchanger (D-HX1) is used to bring the mixture to the liquefaction temperature. A stoichiometric reactor (D-React) with 47 reactions was used to simulate the depolymerization of the cellulose pulp.

The liquefaction products are then submitted to a flash (D-Flash) in order to concentrate the solution of sugars and to recover steam carrying an important heat load. The sugaric solution is also cooled down with a second heat exchanger (D-HX2) and then filtered (D-Filter). Finally, a relief valve (D-Valve) allows recovering the solution of sugars at atmospheric pressure.

c) Modeling of ABE fermentation

The model of the fermentation section of the sugars obtained by liquefaction is presented in Figure 51.

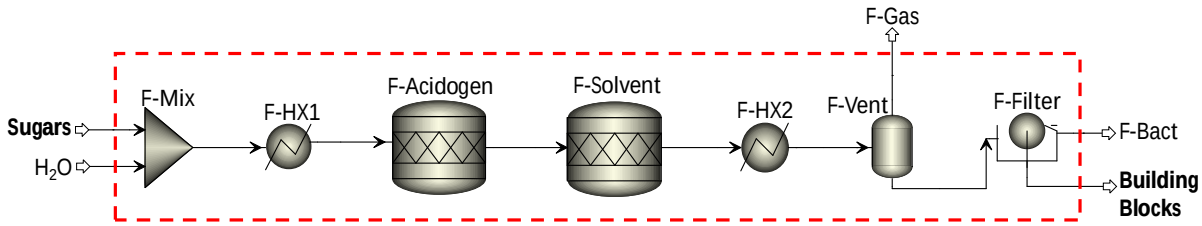

Figure 51. Aspen plus flowsheet for the ABE fermentation process zone.

For this section a mixer (F-Mix) was used to combine the sugars solution that is the carbon source for the fermentation with the right amount of water needed for the fermentation.

The inoculum and the culture media needed for bacterial growth were not included in this model.

The mixture is heated (F-HX1) to 37°C, that is the optimum temperature for this fermentation process. The fermentation process using *C. acetobutylicum* was modeled using two stoichiometric reactors, one for the acidogenesis (F-Acidogen) and one another for the solventogenesis (F-Solvent). This allows studying separately the energy involved in each one of the two fermentation steps characteristic of *C. acetobutylicum.* The output stream from the bioreactor is cool down with a second heat exchanger (F-HX2) and the reactor vent was represented with a flash (F-Vent) in order to recover the fermentation gas (i.e. H_2 and CO_2).

Finally the solid bacteria are separated from the liquid phase containing the building blocks by filtration (F-Filter).

Table 17. Conditions used in the different blocks integration the proposed biorefinery process.

Process	Equipment	Block name	Input
Pulping	Mixer	O-MIX	T=20ºC; P=1 bar
	Heat exchanger	O-HX1	T=180ºC; P=18 bar
	Organosolv reactor	O-Reactor	T=180ºC; P=18 bar, 6 Reactions
	Filter	O-Filter	Isothermal, P=1 bar
	Heat exchanger	O-HX2	T=20ºC; P=1 bar
	Precipitation reactor	L-Precip	T=20ºC; P=1 bar, 1 Reaction
	Filter	L-Filter	Isothermal, P=1 bar
Liquefaction	Mixer	D-Mix	Isothermal, 25 bar
	Heat exchanger	D-HX1	T=220ºC; P=25 bar
	HCW reactor	D-Reactor	T=220ºC; P=25 bar; 48 Reactions
	Flash	D-Flash	T=220ºC; P=22 bar
	Heat exchanger	D-HX2	T=40ºC; P=22 bar
	Filter	D-Filter	Isothermal, P=22 bar
	Relief valve	D-Valve	P=1 bar
Fermentation	Mixer	F-Mixer	Isothermal
	Heat exchanger	F-HX1	T=37ºC; P=1 bar
	Fermenter-Acidogenesis	F-Acidogen	T=37ºC; P=1 bar; 46 Reactions
	Fermenter-Solventogenesis	F-Solvent	T=37ºC; P=1 bar; 38 Reactions
	Heat exchanger	F-HX2	T=20ºC; P=1 bar
	Flash	F-Vent	T=20ºC; P=1 bar
	Filter	F-Filter	Isothermal, P=1 bar

1.3. Results and discussion

Experimental studies about pulping, liquefaction and fermentation have been reported in results sections I and II. Mass balances of these works have been used to develop the Aspen Plus model. The main objective is the integration of the biorefinery process and to analyse the energy performance of the different process units.

1.3.1. Mass balances

A calculation basis of 100 kg/h of beech wood was used for sake of simplification of numerical value but this flow rate does not represent the industrial scale suitable for a biorefinery (which should be much higher). The mass flowrates (for 1 hour basis) are shown in Figure 52. All the

mass yields, except pulping C5 and C6 sugars, were measured experimentally and reported previous sections.

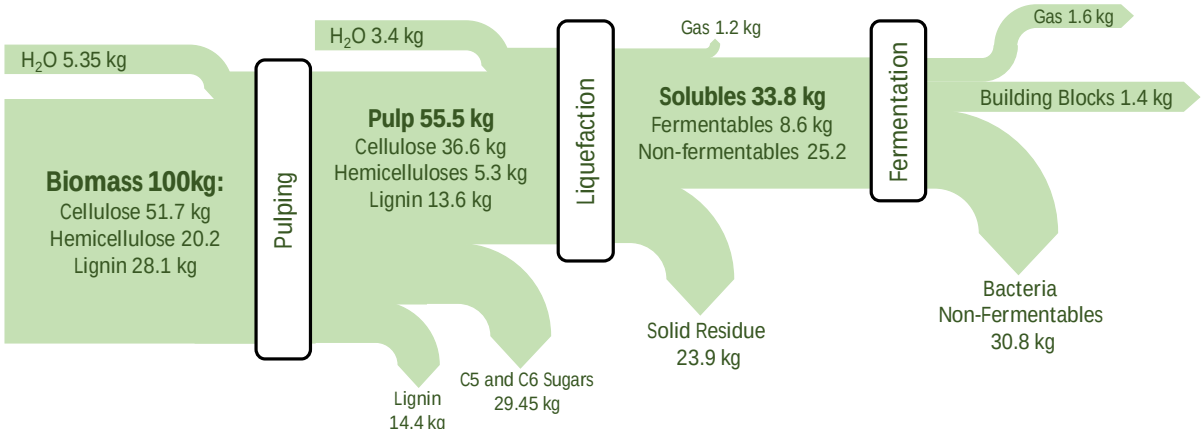

Figure 52. Sankey diagram of the mass balance through the global process.

The water indicated corresponds to the water that reacts with the biomass through hydrolytic reaction mechanisms. The water that does not react with the biomass is considered to be recovered and reutilized in a loop.

Beech pulping by the organosolv process allowed recovering 55.5 wt.% of cellulose-rich pulp. In this process 0.054 kg of water / kg of biomass reacted with the beech to produce the pulp according to the reactions proposed in this model. Lignin and hemicellulosic sugars are also recovered from this process. These two fractions should be valorized into profitable chemicals through different process and are not considered as losses in an integrated biorefinery. Their valorization is not included in the present work.

The cellulose-rich pulp was liquefied in hot-compressed water, producing 60.9 wt.% of water-soluble products. According to the reactions proposed, 0.06 kg of water / kg of pulp reacted within this process.

Among this fraction, 15.5 wt.% (8.6 wt.% of the initial biomass) are fermentable sugars and the other 45.4 wt.% are “non-fermentable compounds” such as levoglucosan, erythrose and 5-HMF. Potential inhibitors may be found among the non-fermentable compounds.

Detailed information about the water soluble fraction after liquefaction can be found in section B, I, 1.

In the final stage, the water-soluble fraction was fermented using *C. acetobutylicum* where 16.3 wt.% of the fermentable sugars (1.4 wt.% of the original biomass) are transformed into building blocks (i.e. butanol, acetone, ethanol, butyric acid, acetic acid, lactic acid) which is a very small mass yields. Therefore, this type of biorefinery could not be of interest if optimized and notably if all the streams (lignin, hemicelluloses, not sugaric products) not yet considered in this work are also converted into added-value products.

Gas produced in the ABE fermentation process is a mixture of hydrogen (H_2) and carbon dioxide (CO_2). The gas mass and composition were calculated according to the reactions proposed in the model, giving 4.7 wt.% (relative to the fermentable sugars) of gas with a composition of 92.5 wt.% of CO_2, 3.1 wt.% of H_2 and 4.4 wt.% of H_2O that evaporates in the flash module.

The H_2/CO_2 molar ratio is 1.35 that is in agreement with those found in the literature for the ABE fermentation using clostridia sp.((Vasconcelos, Girbal, and Soucaille 1994; Girbal et al. 1995)

The yield of each one of the three stages is in agreement with the literature.(Ragauskas et al. 2006; Brosse, Sannigrahi, and Ragauskas 2009; Yu and Wu 2010; Gao et al. 2014; Raganati et al. 2015) However, to the best of our knowledge, this is the first work combining these three processes including pulping, hydrothermal liquefaction and ABE fermentation.

Several other processes allow the recovery of similar yields of building blocks. Nevertheless, these processes use complicated pretreatments requiring hazardous chemicals and high quantities of energy.(Mosier et al. 2005; Alvira et al. 2010) After biomass pretreatment and/or fractionation, an expensive enzymatic hydrolysis that lasts 2 or 3 days is used for its depolymerization(Chum et al. 1988; Jiang et al. 2016), in comparison with the sugar production by liquefaction proposed here that may last 2 or 3 hours.

1.3.2. Energy balances

The energy flow of the integrated biorefinery process is shown in Figure 53. These values correspond to the enthalpy of each stream and not the combustion heat or any representation of the energy density of these materials. So this data serves mostly for the design of a heat exchanger network.

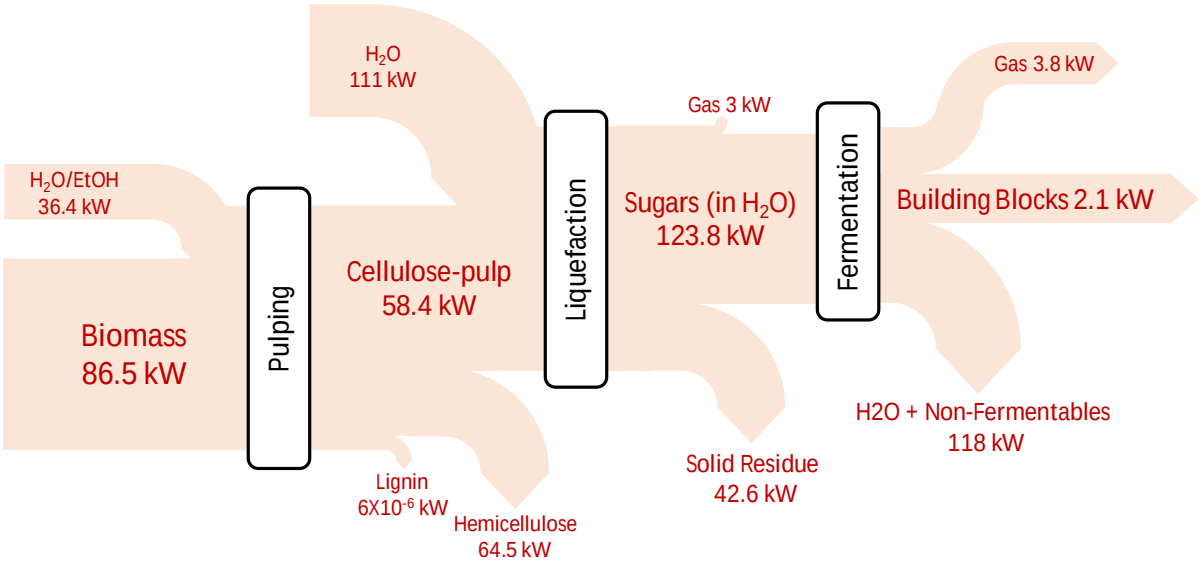

Figure 53. Sankey diagram of the energy flow through the integrated wood biorefinery process.

The energy associated with the solvent (water and ethanol) in the pulping process and the water in the liquefaction process represents 2008 and 4895 kW respectively. Assuming no energy loss, most of this energy can be recovered, therefore these streams are not presented.

The values indicated in the Figure 53 correspond to the energy that was not recovered or that made part of the products, water being the solvent and reactive in both processes. All the energy associated to water that is constantly recirculating inside the process was taken was not taken as an energy inlet. However, the energy of the water that reacts with the biomass/cellulose is the one presented here. The high quantities of energy needed to heat the water (and ethanol in the

case of the pulping) that can be recovered impels the utilization of an integrated heat exchangers network.

The reactor for the organosolv fractionation was set in adiabatic mode instead of isothermal mode. In adiabatic mode temperature increases only 5°C, that are not detrimental for this process. Moreover, this will avoid an additional heat exchanger needed if it was isothermal, only to recover a relatively low quantity of energy.

Initial biomass and solvent (water / ethanol) used in the pulping process represent 86.5 and 36.4 kW respectively. Most of the energy used in the pulping stage is needed to heat the solvents. Most of this energy is recovered after the pulp which is separated by filtration.

A cellulose-rich pulp, solid lignin and a hemicellulosic solution with enthalpic 58.4, $6x10^{-6}$ and 64.5 kW are obtained from this fractionation process. The lignin and hemicellulosic sugars can be valorized in other processes.

Cellulose-pulp is then liquefied in hot-compressed water. The water used for this process carries an energy flow of 111 kW. This is the most energetically expensive stage due to the heating of the water but mostly to the energy needed to evaporate and concentrate the sugar solution after liquefaction. A solid residue partially carbonized with 42.6 kW can be recovered to be valorized in another process or to produce heat. Small amount of permanent gas with 3 kW are also produced.

The water solubles are then fermented producing building blocks and permanent gas containing 2.1 and 3.8 kW. The remaining water, bacteria and non-fermentable compounds represent 118 kW, that is most of the energy. Most of the enthalpy associated with compounds that are not metabolized during the fermentation can be separated in order to improve the energy performance of this stage.

The net heat duties of the different blocks used in the biorefinery process model developed in Aspen Plus are shown in Table 18. The negative values indicate energy produced and the positive ones indicate energy needed for the block.

Table 18. Net heat duty of the different blocks of the biorefinery process.

Equipment	Block	Duty (kW)	Temperature (C)
Heat exchanger 1	O-HX1	124.5	180
Organosolv reactor	O-Reactor	0	185
Heat exchanger 2	O-HX2	-123.2	30
Precipitation reactor	L-Precip	-8.6	30
Pump	D-Pump	-2.5	20
Heat exchanger 1	D-HX1	361.0	220
HCW reactor	D-Reactor	-21.4	220
Flash	D-Flash	501.2	220
Heat exchanger 2	D-HX2	-5.9	40
Heat exchanger 1	F-HX1	1.9	37
Fermenter-Acidogenesis	F-Acidogen	5.3	37
Fermenter-Solventogenesis	F-Solventogen	-0.1	37
Heat exchanger 2	F-HX2	-2.4	20

In the organosolv stage only the heat exchanger (O-HX1) used to heat the reactive mixture requires a heat entry of 124.5 kW. The rest of the blocks are exothermic and thus some heat can be recovered. However, if the recovery of relatively small energy flows demands the implementation of an extra heat exchanger the economic gain can be affected. A full economical and energy analysis will be needed to better integrate the energy flows.

The liquefaction stage has the most energetically expensive blocks, being the Flash (D-Flash) the maximum with 501.2 kW. This flash reduces the pressure isothermally at 220ºC in order to evaporate most of the water and then to recover a concentrated solution of sugars.

Moreover, the heat exchanger (D-HX1) use to heat the reactive mixture entering the liquefaction reactor has the second biggest energy demand, converting the liquefaction stage in the critical point to optimize the energy performance of the global process.

The energy need of the fermentation stage is significantly lower in comparison to the other stages. The acidogenic phase of the fermentation requires only 5.3 kW, being a hundred times lower than the flash used during the liquefaction process.

Sugar yield at 180°C is relatively low, and energy load used in the HCW reactor at 240°C is relatively high. For this reason, temperatures around these values are not attractive for the production of sugars by hydrothermal liquefaction.

1.4. Conclusion

Process modeling is a powerful approach to analyse the energy and mass balances of the process. The integrated model presented here demonstrate that the mass yield in ABE products is very small (1.4wt.%) and that the other streams should be converted into added-value products. Therefore, many work is still needed to improve the mass yield of biorefineries if bacteria are used or other process scheme should be looked for (for instance combining thermochemical depolymerization with chemical catalysis instead of biochemical conversion by bacteria).

The quantity of water used in the hydrothermal liquefaction is the most important variable affecting the energy efficiency of this biorefinery process and thus the profitability. Using less water for this stage or the implementation of a technology producing a more concentrated sugaric solutions (as pyrolysis) are the most important points to optimize this process.

Moreover, the fractionation reactor (organosolv) was found to be more energetically efficient in adiabatic mode than in isothermal mode, avoiding the requirement of an extra heat exchanger to regulate its temperature. Aspen Plus modelling as to be combined with techno-economic analysis in order to assess better biorefineries integrated processes.

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C. CONCLUSIONS AND PERSPECTIVES

In this work, a novel biorefinery approach combining thermochemical and biological processes was proposed. In order to assess the main features and challenges of this methodology, experimental studies about each one of the stages (fractionation of biomass, hydrothermal liquefaction and fermentation) were performed in order to assess their combination in an integrated biorefinery process. A simplified model using Aspen Plus has been developed in order to assess the global mass and energy balances of this concept.

In the following paragraphs, the general conclusions of these studies are given and they complement the conclusion presented in each sub-sections. These conclusions are accompanied with some perspectives indicating the potential pathway for an optimal development of the biorefinery proposed.

Organosolv and chlorite / acid (SC / AA) fractionation methods allowed recovering cellulose-rich pulps with low lignin content. These pretreatments not only allow the removal of lignin, but also the deconstruction of the complex lignocellulosic matrix, lowering the cellulose crystallinity.

SC / AA method gave a pulp with high cellulose and hemicelluloses content. This is an important difference with the organosolv process. This former pulping method separates the hemicelluloses from cellulose and hemicelluloses are then recovered with the lignin in the liquid phase.

These processes can be optimized in order to reduce the cellulose loses. Establishing an optimal organosolv method with ethanol recycling is one of the most important point for its implementation in industrial processes.

Concerning the study on the hydrothermal depolymerization of the microcrystalline cellulose, several analytical methods were implemented in order to identify and quantify carboxylic acids, carbohydrates, aldehydes and permanent gas. An improved analytical method using HPAEC-PAD was developed for the analysis of complex mixtures of mono- and oligosaccharides. These results allowed identifying the predominant reaction pathways involved during the hydrothermal liquefaction of cellulose according to the temperature of reactions. In that way, it was found that 220°C was the best temperature for the production of soluble sugars and that temperatures higher than 220°C will provoke almost a full decomposition of the sugars (in an autoclave) by various secondary reactions (e.g. dehydration, retro-aldol condensation, decarboxylation, decarbonylation). The best temperature range, for 2h of cellulose liquefaction in a batch reactor, is between 180 and 220°C.

Depolymerization of beech, organosolv and SC/AA pulps was assessed according to the best conditions obtained during the study with microcrystalline cellulose.

Both pretreatments allowed significant improvement for the sugar production relative to the raw beech. Since lignin serves as a barrier that increases cellulose recalcitrance, the lower its content is, easier the depolymerization of cellulose is.

For this study, a batch reactor was used for the hydrothermal depolymerization of the substrates. However, the sugars that are firstly produced endure secondary reactions that entails to significant losses of fermentable material. Hydrothermal liquefaction using a fixed-bed reactor allowed recovering the primary products. However, this technology uses high quantities of water, which has to be heat up with high quantities of energy and the sugars produced will be highly diluted.

For these reasons, the best technology for this purpose will require avoiding secondary reactions as much as possible and the utilization of less water to decrease the energy consumption.

The development of a technology able to do this attracted a lot of interest for our team. Hereby, 5-6 months of this PhD were consecrated to develop a biomass conversion technology able to handle concentrated slurries in continuous mode with a good control of the conversion of the slurry. This subject is confidential and for this reason no details are given.

This technology will have to be compared more in depth with fast pyrolysis processes (followed by hydrolysis of the pyrolysis oils). The Aspen Plus model will be refined in order to compare these 2 types of processes. A technical-economical assessment has to be also conducted.

Several cultures of *C. acetobutylicum* with model molecules of glucose, cellobiose and glucose / cellobiose mixtures were studied in order to gather information about its behavior with cellulose-derived compounds. This study revealed that *C. acetobutylicum* exhibits a diauxic growth when the sole carbon source was a mixture of glucose and cellobiose. More studies about the effect of pH on the induction of cellobiose and the transition from glucose to cellobiose utilization allowed settling optimal conditions to cultivate *C. acetobutylicum* in a mixture of carbohydrates.

The fermentation of sugar mixtures coming from biomass liquefaction by *C. acetobutylicum* was very challenging and required exhaustive care in order to achieve the desired fermentation products pattern.

Since *C. acetobutylicum* was not able to utilize the liquefaction products coming from the raw beech, the fractionation processes presented a crucial stage to achieve a successful fermentation. The cultures growth on sugars from both pulps showed the normal distribution of products of *C. acetobutylicum.* The yields were comparable to others found in the literature. The effect of the inhibitory compounds was not studied in this work, but its comprehension is a critical point for the development and scaling-up of this process. Moreover, the implementation of other fermentation technologies presents interesting features to improve their efficiency. Indeed,

fermentation seems to be interesting only if the targeted products cannot be produced by conventional chemical catalysis and if their added-value is very high.

In my opinion, the production of the world's top building blocks according to the chemical industry market is a good target. Here we give some interest exemples of the top biobased molecules that include a biological conversion in their production and most representative companies that produce them (in the brackets): glutamic acid, succinic acid (Myriant, BASF, Bioamber and Reverdia), 3-hydroxypropionic acid (Perstorp), isoprene (Amyris), farnesene (DuPont and Goodyear), lactic acid (many companies), itaconic acid (Lucite and Mitsubishi). [1] These are important building blocks for the current industry that include fermentation processes and can integrate analogous approches to the one proposed in this work.

A simple model of the global process integration has been developed using Aspen Plus®. A brief energy analysis of each operation units of the process was assessed. These results have pointed out that the most important energy carrier component of the whole process is water, mostly used during the hydrothermal liquefaction process.

This model allowed a better understanding of the impact of the operating mode (i.e. isothermal, adiabatic, isenthalpic) of the different operation units on the product yields and energetic efficiency. The most representatives are: 1) operating the fractionation reactor in adiabatic mode, instead of isothermal mode that will require an extra heat exchanger and 2) keeping the flash after liquefaction in isothermal mode, to remove high quantities of water without losing significant quantities of sugars.

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[1] Choi, Sol, Chan Woo Song, Jae Ho Shin, and Sang Yup Lee. 2015. "Biorefineries for the Production of Top Building Block Chemicals and Their Derivatives." *Metabolic Engineering* 28 (Supplement C): 223–39. https://doi.org/10.1016/j.ymben.2014.12.007.
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Pursuing a complete energetic analysis with a heat exchanger network optimization will be the most useful evolution for this study. In order to compare these results with enzymatic hydrolysis and with pyrolysis, a proper techno-economic analysis is needed.

In general, this work provides a new methodology for the valorization of cellulosic materials in a biorefinery by combining a thermochemical and biochemical treatments. The yields obtained so far do not seem competitive enough for the current industrial market.

In the biorefinery strategy proposed in this work, it has been shown that it is possible to produce fermentable substrates from lignocellulosic biomass using thermochemical processes, since the resulting substrates can be metabolize by *C. acetobutylicum*. Producing chemicals such as butanol, acetone, acetic acid, butyric acid have not a price high enough to make this process cost-effective and competitive in the market. However, an analogous method can be used with other biomass and microorganism to produce other chemical molecules with higher added-value.

One of the best scenarios will be for the molecules that cannot be produced by a chemical route and need a biological catalyst in order to be synthetized.

Since lignocellulosic biomass has different components with different chemistry, its potential valorization cannot be evaluated for one process with only one final product. The viability of an integrated biorefinery needs the study of the valorization of all the components of this material: cellulose, lignin, extractives, hemicelluloses and even minerals. The development of biorefineries will be analogous the crude-oil refineries which are very well integrated with an extensive valorization of all the streams. This development could start with the diversification of pulp and paper mills, by producing high added-value chemicals from cellulose, aromatics or carbons from lignin, furans from hemicelluloses.

The development of an efficient biorefinery needs much more work in all the fields, but more than anything, an open mind to create new strategies combining elements that maybe did not seem to go well, but that in the end, can bring something positive.

D. APPENDICES

I. Appendice 1:
Article 6 (in development): Hydrothermal conversion of cellulose-rich pulps in a fixed-bed reactor. Performed at Curtin University in Perth, Australia.

Same pulps used in our previous work were taken to this project at Curtin University. The main objective is to recover the primary products from cellulose depolymerization in hydrothermal media. A fixed bed of cellulose was used converted with pre-heated water. Experimental procedure has been conducted as in Yu et al.[2] In that way, secondary reactions are mostly avoided. Figure D1 presents again the macromolecules composition of beech and the pulps.

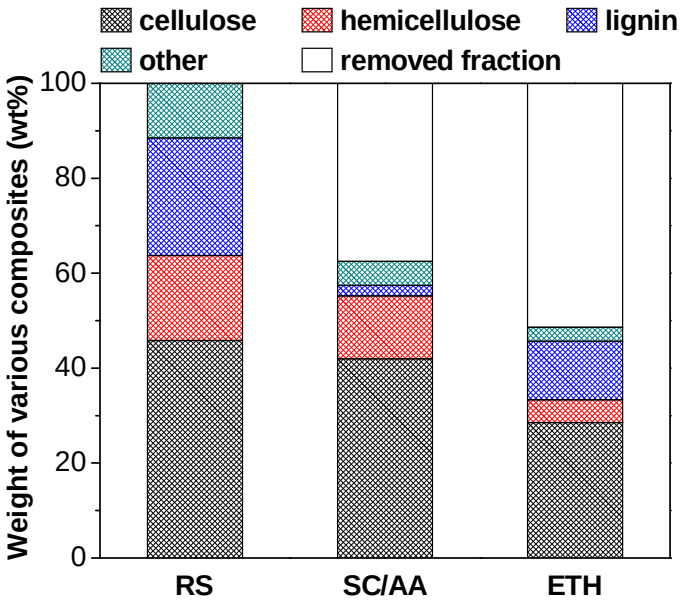

Figure D1. Composition of various components of raw beech wood and two pretreated samples. (The weight percentage are all present based on the primary weight of the raw samples.)

[2] Yu, Yun, and Hongwei Wu. 2010. "Understanding the Primary Liquid Products of Cellulose Hydrolysis in Hot-Compressed Water at Various Reaction Temperatures." *Energy & Fuels* 24 (3): 1963–71. https://doi.org/10.1021/ef9013746.

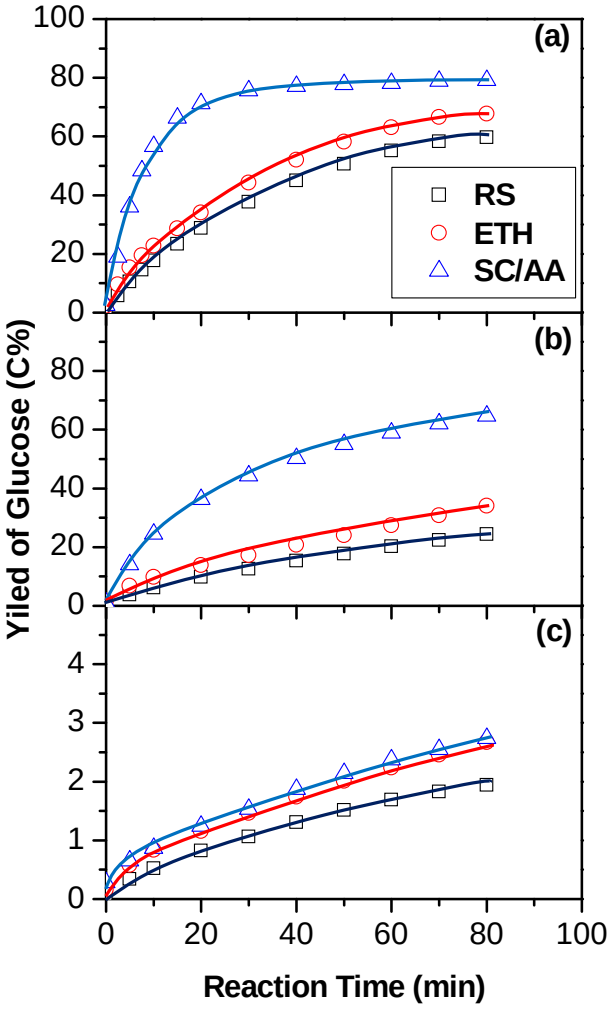

Figure D2. Yield of glucose for beech (RS), organosolv (ETH) and sodium chlorite / acetic acid (SC / AA) pulps hydrothermal liquefaction in a fixed-bed reactor at (a) 250 °C; (b) 230 °C; (c) 150 °C.

Yields of glucose recovered at three temperatures (250, 230 and 150°C) are reported in Figure D2. A maximum of ~80 wt.% (of the theoretical yield) of glucose recovery from the SC / AA pulp was achieved when the temperature was 250°C after approximately 30 minutes of reaction. At 150°C, significantly lower yields were found for all three biomasses.

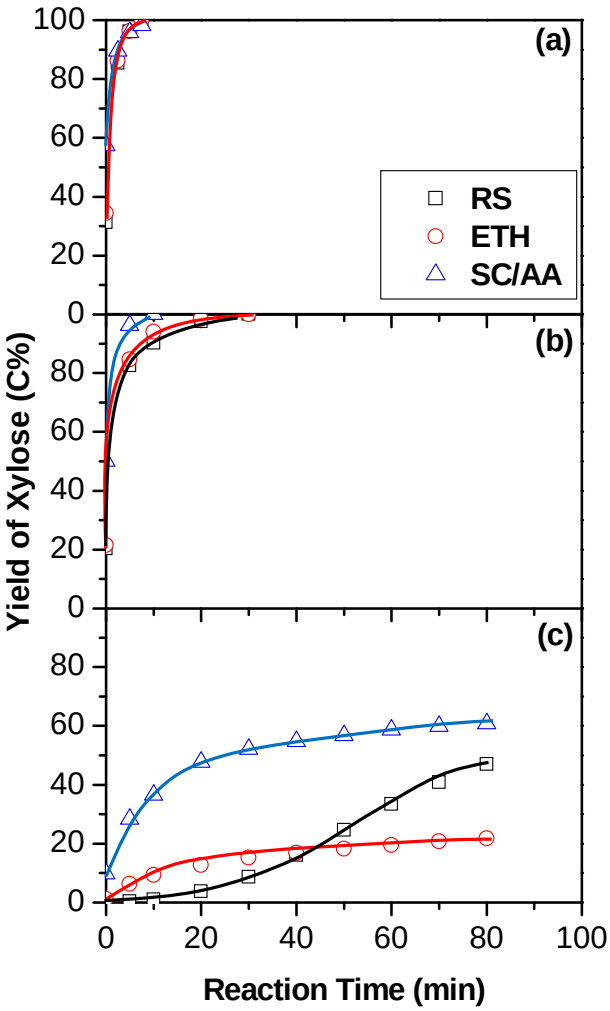

Figure D3. Yield of xylose for beech (RS), organosolv (ETH) and sodium chlorite/acetic acid (SC/AA) pulps hydrothermal liquefaction in a fixed-bed reactor at (a) 250 °C; (b) 230 °C; (c) 150 °C.

Xylose recovery yield was maximum when the temperature was 230 or 250°C (figure D3). This is because hemicelluloses are more easily hydrolysable than cellulose. At 150°C, a maximum yield of ~60 wt.% found was for the SC/AA pulp. This result confirms that the hydrolysis of hemicelluloses is much faster and requires less severe conditions in comparison to cellulose. If the interest is xylose recovery, temperatures between 150 and 230°C will allow high recovery yields using hydrothermal liquefaction in a fixed-bed reactor. Other results will complement this article but are being treated by both laboratories.

II. Appendice 2:
Article 7 (in development):
Fast pyrolysis of cellulose with a stage condensation system for the recovery of bio-oil fractions.

(Performed in collaboration with the Institute for Chemical and Fuels from Alternative Resources (ICFAR) in London, Canada)

In this work, we proposed to combine fast pyrolysis and fermentation in order to valorize cellulose into building blocks. This biorefinery strategy has three stages (Figure D4): pyrolysis, hydrolysis and fermentation. Fast pyrolysis of cellulose was performed in a fluidized bed reactor at ICFAR (Canada), then it was hydrolyzed in diluted acidic media to produce fermentable sugars. These sugars were used as substrate for an ABE fermentation process using *C. acetobutylicum.*

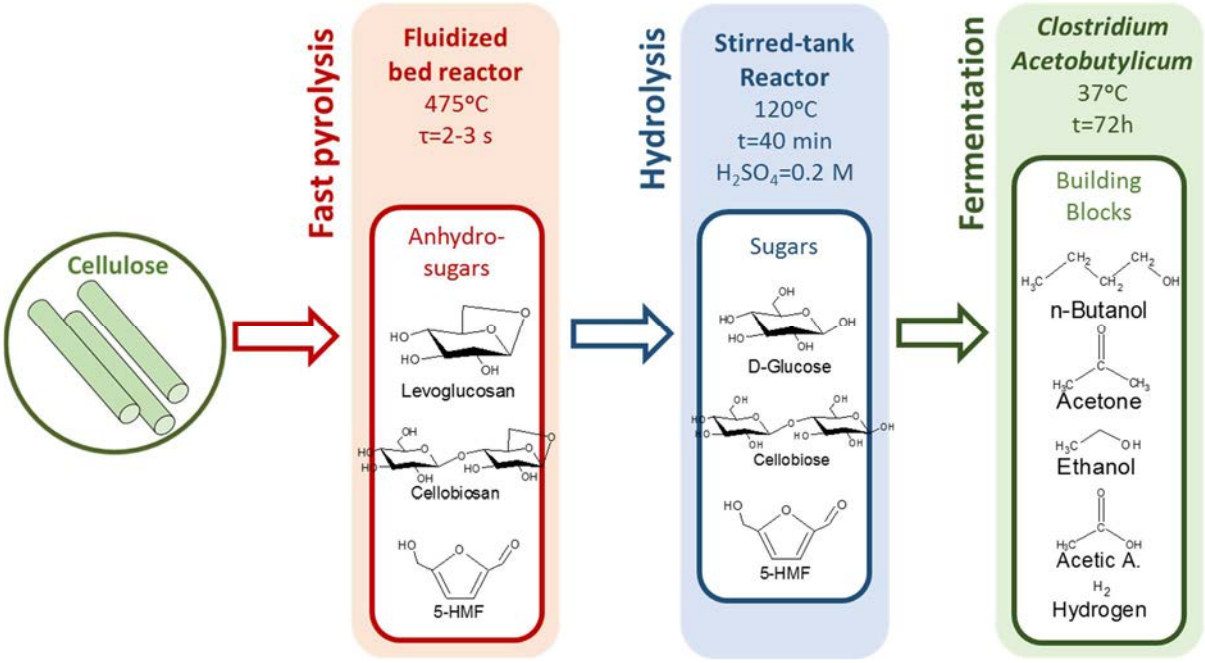

Figure D4. Scheme of the proposed cellulose biorefinery using fast pyrolysis, hydrolysis and ABE fermentation.

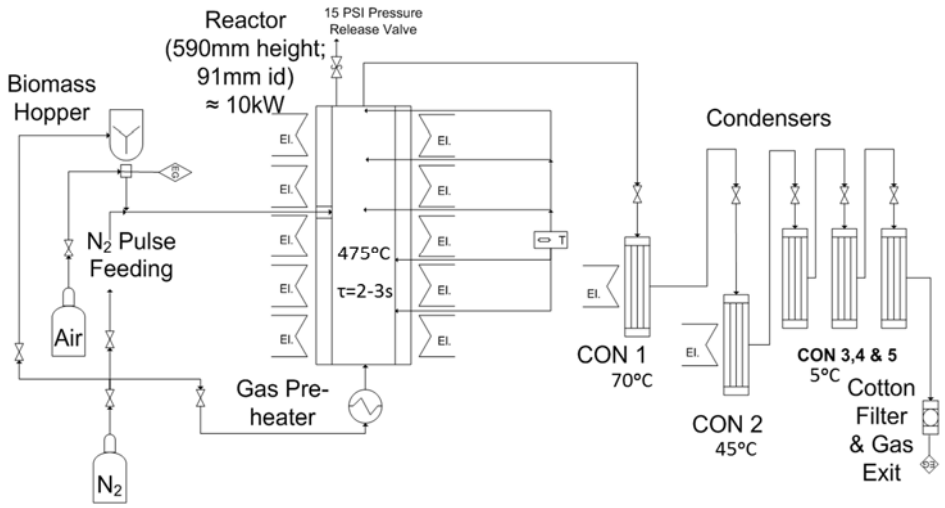

Figure D5. Schematic of the fluidized bed with staged condensation system

Fast pyrolysis of cellulose allows recovering high yields of anhydro-sugars, mainly levoglucosan. In this work, fast pyrolysis was performed in a gas fluidized bed reactor with a feeding system allowing an efficient distribution of cellulose in the fluidized zone of the reactor. Mass yields of products from cellulose pyrolysis and the oil fractions along the staged condensation system are presented on Figure D5. Fast pyrolysis allows to recover the highest oil yield among the thermochemical conversion processes. The main interest is recovering soluble sugars that can be transformed into building blocks through fermentation processes.

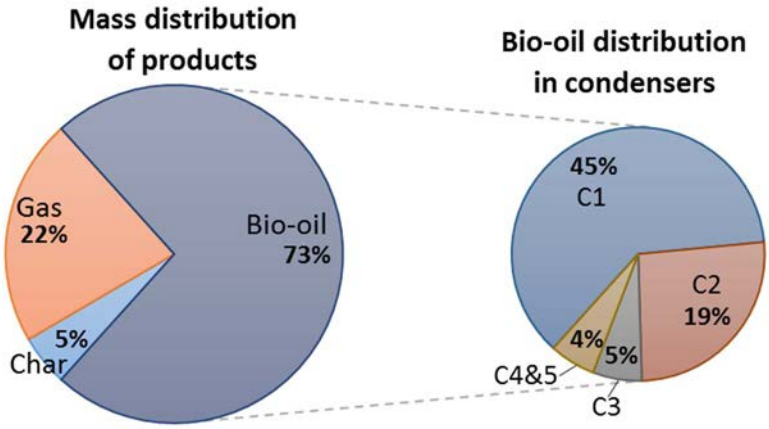

Figure D6. Mass yield of pyrolysis products and bio-oil fractions in the condensers. All the yields were calculated in reference to the initial cellulose.

Bio-oil, permanent gas and char yields were 73, 22 and 5 wt.% respectively (figure D6). Bio-oil was recovered in five condensers, with mass yields (of initial cellulose) of 45, 19, 5 and 4 for the first, second, third and fourth (includes also the fifth) condensers respectively. First (C1) and second (C2) condensers allowed recovering sugar-rich oil and both were called the heavy fraction. Condensers 3, 4 and 5 trapped the rest of the oil which is rich in light compounds, such as acetic acid and aldehydes, that wish to be avoided in biological conversion processes due to its inhibiting nature.

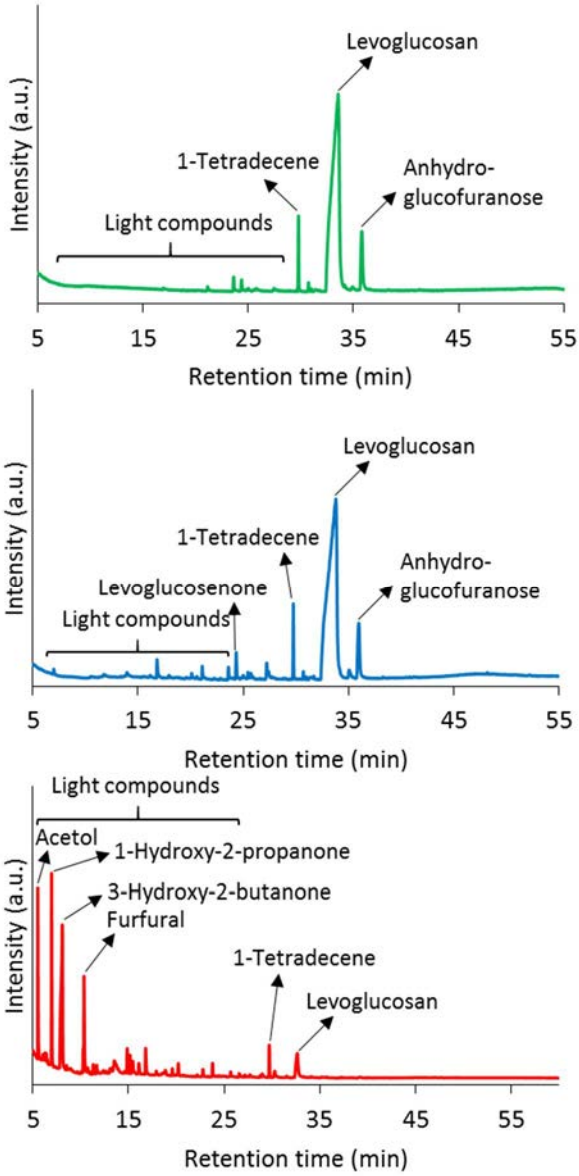

Figure D7. GC-MS total ion current chromatogram for different bio-oil fractions recovered on a) condenser 1, b) condenser 2 and c) light fraction.

. Chromatograms obtained by GC-MS showed the successful separation of levoglucosan in the first two fractions from the lighter compounds that are recovered in the last three fractions (figure D7). This data shows that pyrolysis with staged condensation allowed recovering a fraction of bio-oil with a high concentration of levoglucosan avoiding light compounds that are potential inhibitors of the microorganisms used in the fermentation process.

Table D1. Compounds detected and quantified by GC-FID-MS of the sugar-rich fractions.

Fraction	Compound	RT (min)	Mass yield (wt.% of the fraction)	Mass yield (wt.% of cellulose)
Condenser 1	Levoglucosan	33.56	43.7	19.8
	1,6-Anhydro-β-D-glucofuranose	35.80	3.1	1.4
Condenser 2	Levoglucosenone	21.10	0.17	0.03
	1,4:3,6-Dianhydro-α-d-glucopyranose	24.34	0.41	0.08
	Levoglucosan	33.81	35.48	6.78
	1,6-Anhydro-β-D-glucofuranose	35.99	2.49	0.48

Table 1 shows the major compoudns quantified by GC-FID-MS. Condenser number two (C2) still hodls an important quantity of levoglucosan. For this reason, this fraction was also considered as a potential substrate for fermentation.

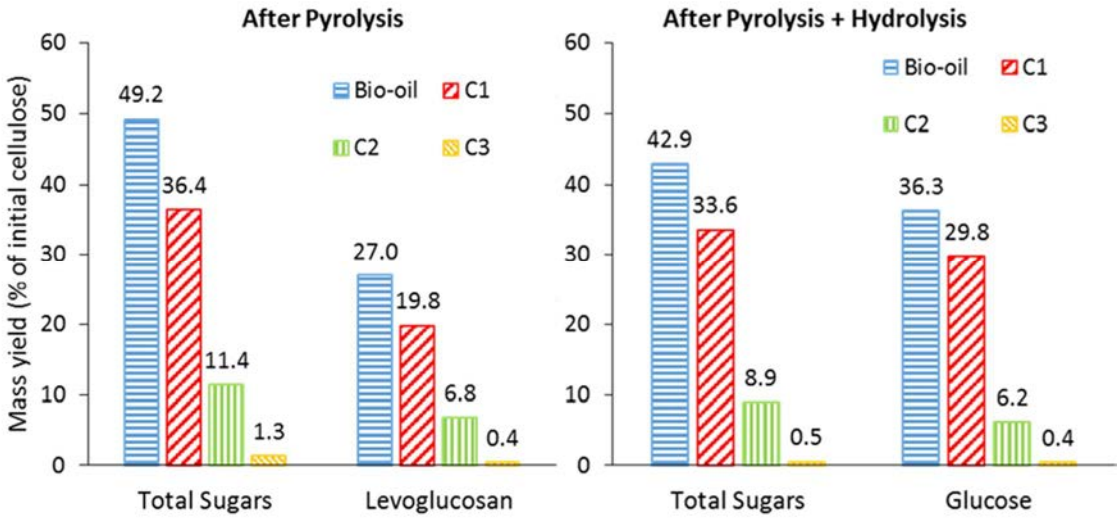

Figure D8. Total sugars, levoglucosan and glucose mass yields in the corresponding bio-oil fractions.

Hydrolysis of the bio-oil fractions allowed a successfully recovery of sugars. Figure D8 shows the yields obtained before and after hydrolysis of the pyrolytic oil. One advantage of performing this hydrolysis stage, is the conversion of anhydro-sugars with DP>2 into regular sugars. These sugars are mostly fermentable and can be more easily identified and characterized by our analytical methods.

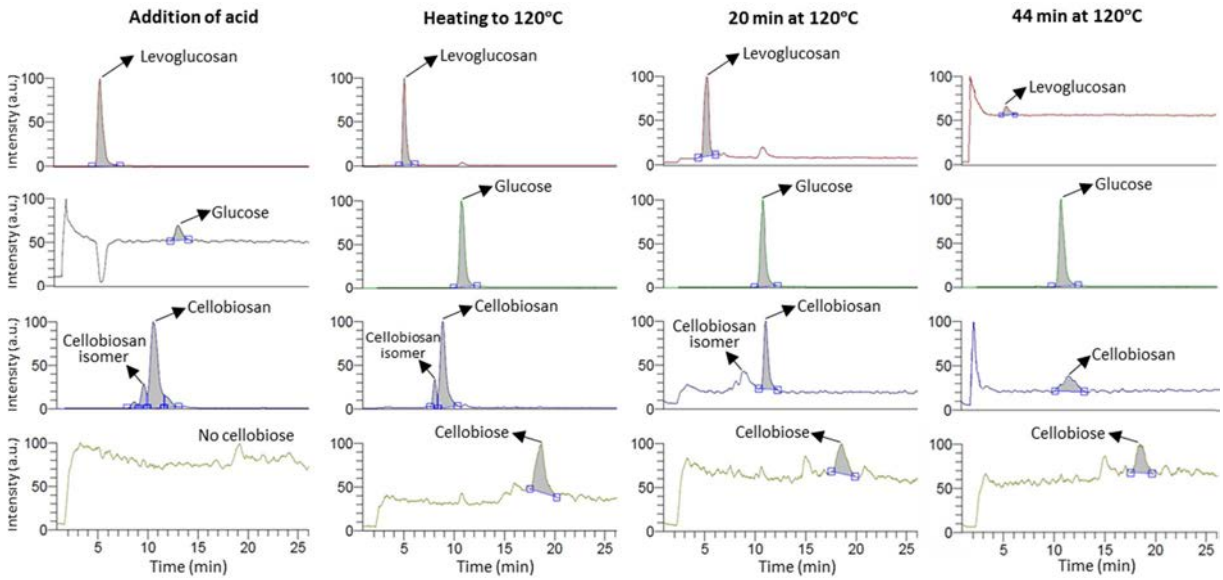

Figure D9. Levoglucosan and cellobiosan analysis by LTQ-Orbitrap during hydrolysis under acidic media.

Analysis by GC are limited to light compounds and other major products from cellulose pyrolysis, such as cellobiosan, are not yet fully studied. In this sub-section, cellobiosan produced by pyrolysis and its hydrolysis in acidic media, were analyzed by HILIC-LTQ-Orbitrap MS.

Figure D9 shows the SIM mode spectra of cellobiosan and cellobiose after pyrolysis and during hydrolysis of the bio-oil fraction recovered in the first condenser.

This analysis allowed to identify an isomer of cellobiosan and no presence of cellobiose just after pyrolysis, as is shown in the Figure D9 during the addition of acid. Cellobiose formation was important during the heating of the reactor from 20 to 120°C.

Fermentation of levoglucosan and bio-oil fractions

Table D2. Levoglucosan and glucose / levoglucosan mixture utilization by *C. acetobutylicum* grown on two different culture medias.

	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

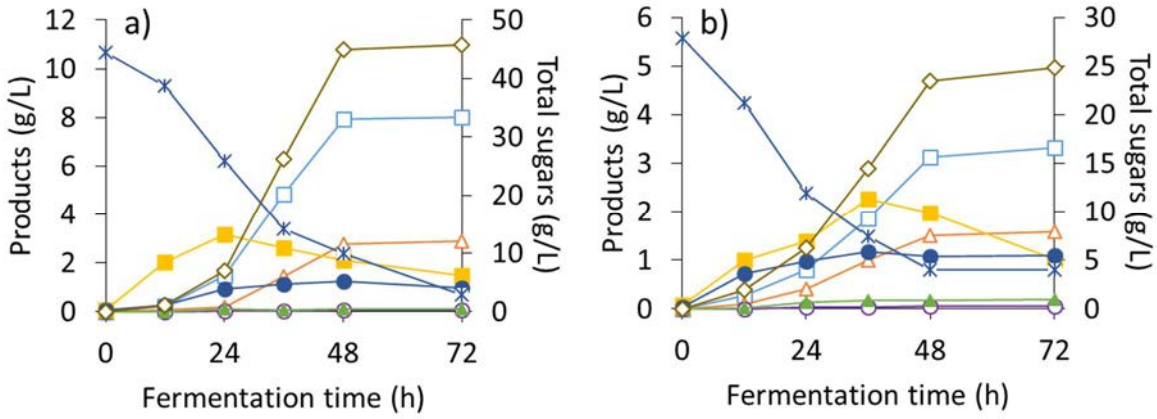

Figure D10. Sugar utilization and fermentation products kinetics during the fermentation of upgraded cellulosic bio-oil from a) firs condenser and b) second condenser by *C. acetobutylicum.* Products: acetic acid (dark blue, ●), lactic acid (green, ▲), butyric acid (yellow, ■), ethanol (purple, ○), acetone (red, Δ), butanol (light blue, □), ABE (brown, ◇) and total sugars (grey, x).

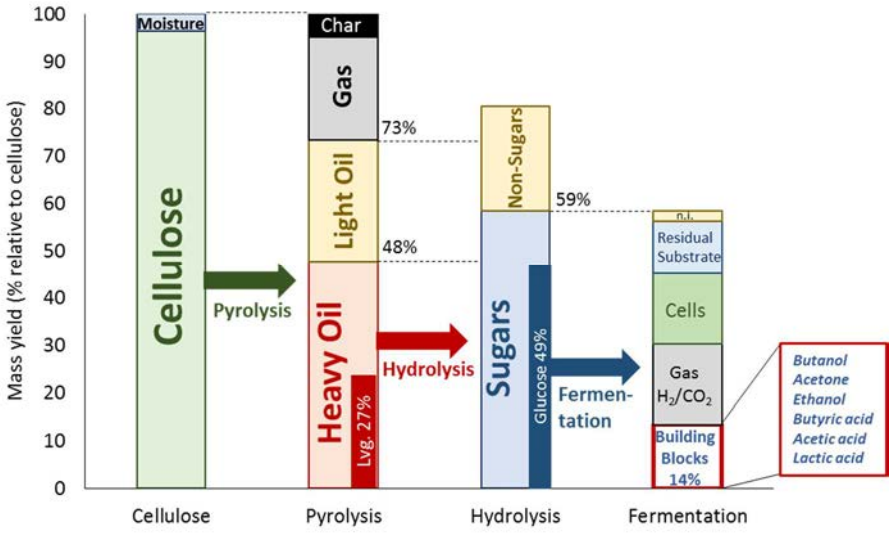

Figure D11. Mass yields obtained along the integrated process combining pyrolysis, hydrolysis and fermentation.

Figure D11 shows the yields obtained by pyrolysis of cellulose, then the hydrolysis of the bio-oil and finally the fermentation of the sugars obtained by *C. acetobutylicum.*

III. Appendice 3:
Fast pyrolysis of cellulosic materias performed in a micro-fluidized bed reactor coupled to SPI-TOF MS

(Performed at LRGP in Nancy, France)

Experiments of pyrolysis of Avicel, pulps and beech were also performed in microfluidized bed as in Jia et al.[3] This reactor is combined with photoionization mass spectrometer which allows for a soft ionization of vapours, reducing their fragmentation and therefore a fast analysis method in order to screen the main products exiting the microfluidized bed. The attribution of main m/z markers is in progress and is based on our previous work (figure D12).[3,4]

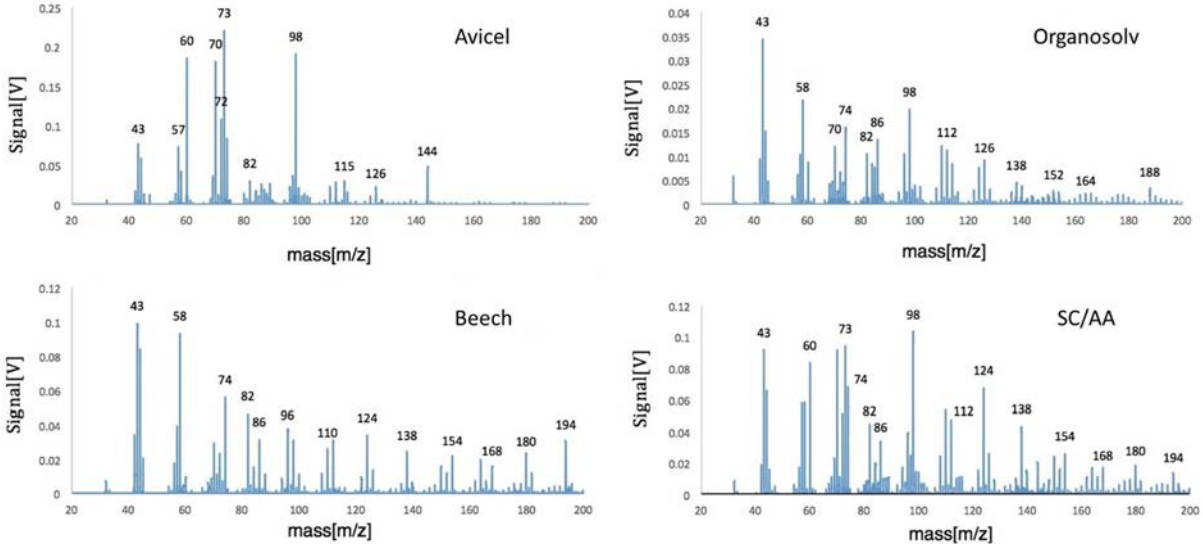

Figure D12. Mass spectra of the volatile products of fast pyrolysis of avicel cellulose, organosolv pulp, SC/AA pulp and beech wood.

[3] Jia, Liangyuan, Yann Le-Brech, Binod Shrestha, Matthias Bente-von Frowein, Sven Ehlert, Guillain Mauviel, Ralf Zimmermann, and Anthony Dufour. 2015. "Fast Pyrolysis in a Microfluidized Bed Reactor: Effect of Biomass Properties and Operating Conditions on Volatiles Composition as Analyzed by Online Single Photoionization Mass Spectrometry." *Energy & Fuels* 29 (11): 7364–74. https://doi.org/10.1021/acs.energyfuels.5b01803.

[4] Jia, Liangyuan, Felipe Buendia-Kandia, Stephane Dumarcay, Helene Poirot, Guillain Mauviel, Philippe Gerardin, and Anthony Dufour. 2017. "Fast Pyrolysis of Heartwood, Sapwood and Bark: A Complementary Application of On-Line Photoionization Mass Spectrometry and Conventional Py-GC/MS." Energy & Fuels, March. https://doi.org/10.1021/acs.energyfuels.7b00110.

IV. Appendice 4:
Main metabolic pathways of cellobiose utilization by *C. acetobutylicum*

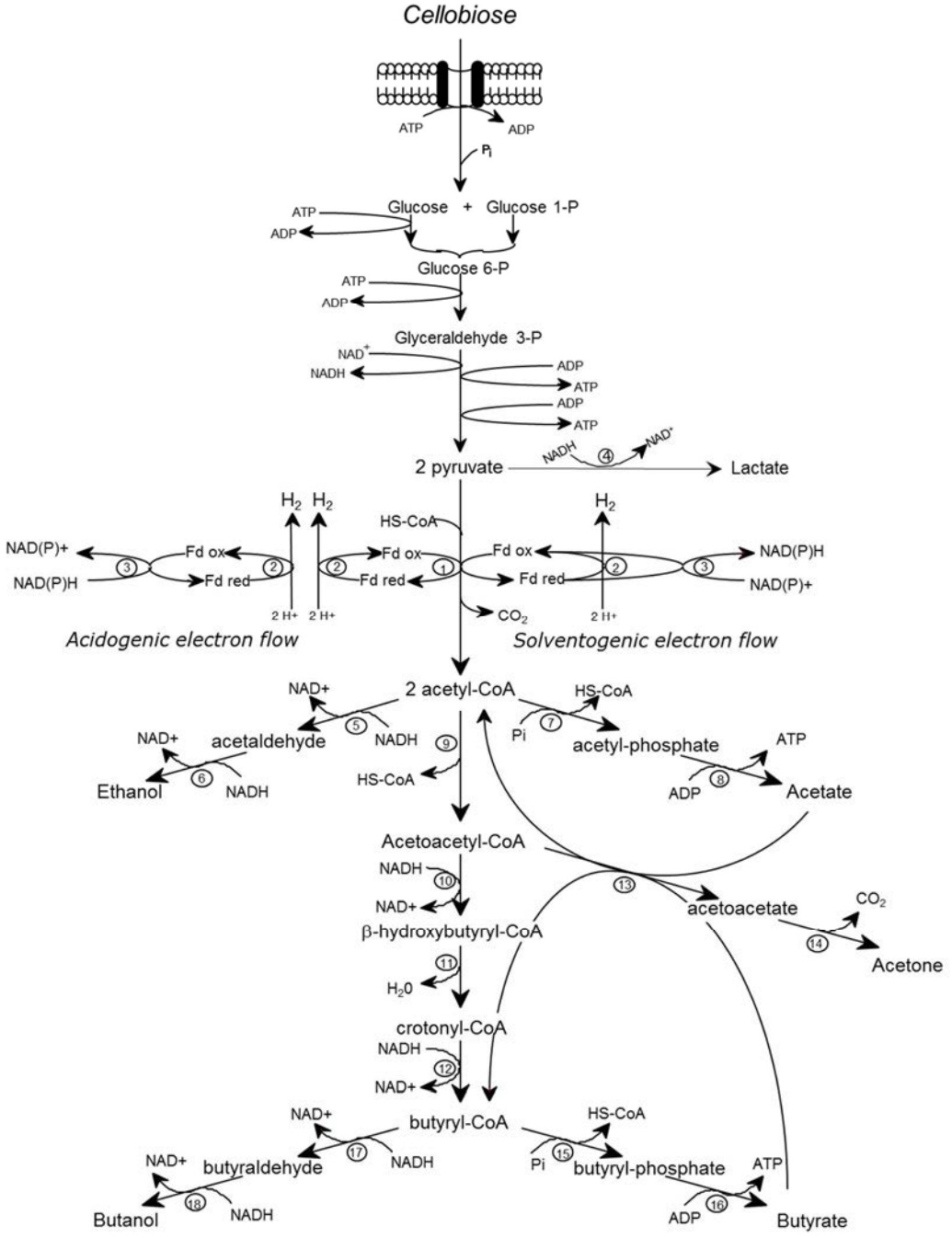

Figure D13. Metabolic pathways of *C. acetobutylicum*. 1: Pyruvate ferredoxin oxidoreductase (EC 1.2.7.1); 2: Hydrogenase (EC 1.18.99.1); 3: NADH-ferredoxin-oxidoreductase (EC 1.18.1.3) or NADPH-ferredoxin-oxidoreductase (EC 1.18.1.2); 4: Lactate dehydrogenase (EC 1.1.1.27); 5: Acetaldehyde dehydrogenase (EC 1.2.1.10), 6: Ethanol dehydrogenase (EC 1.1.1.1); 7: Phosphotransacetylase (EC 2.3.1.8); 8: Acetate kinase (EC 2.7.2.1); 9: Acetoacetyl-CoA thiolase (EC 2.3.1.9); 10: b-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157); 11: Crotonase (EC 4.2.1.55); 12: Butyryl-CoA dehydrogenase (EC 1.3.99.2); 13: Acetoacetyl-CoA:acyl-CoA transferase (EC 2.8.3.8); 14: Acetoacetate decarboxylase (EC 4.1.1.4); 15: Phosphotransbutyrylase (EC 2.3.1.19); 16: Butyrate kinase (EC 2.7.2.7), 17: Butyraldehyde dehydrogenase (EC 1.2.1.57); 18: Butanol dehydrogenase (EC 1.1.1.1 or 1.1.1.2).

V. Appendices 5:
Effect of the pH on the acidogenic phase of *C. acetobutylicum* when grown on glucose or cellobiose in a batch bioreactor.

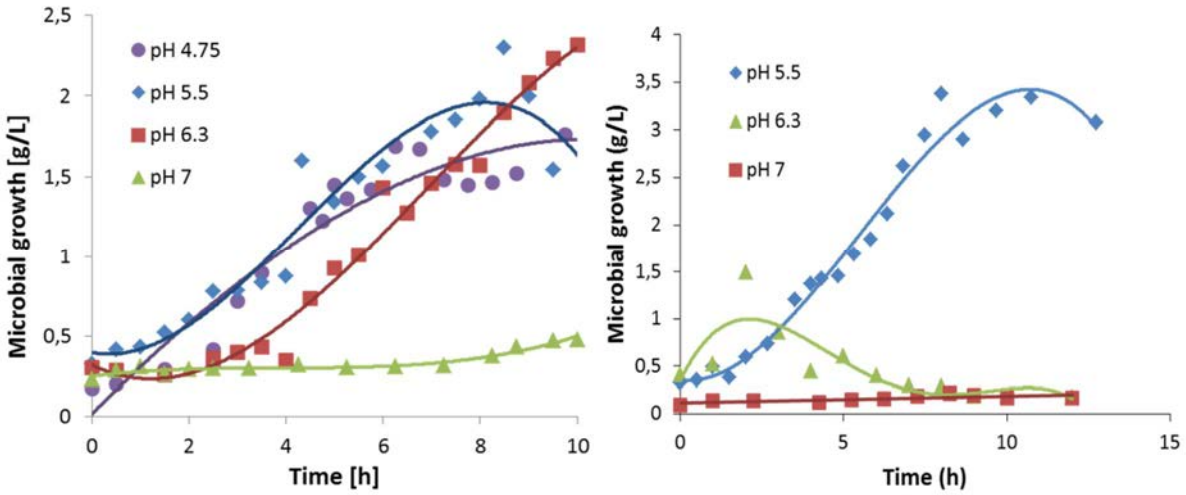

Figure D14. Effect of the pH on the growth of *C. acetobutylicum* on glucose (left) and cellobiose (right).

The effect of the pH on the growth of *C. acetobutylicum* when the substrate is glucose or cellobiose is shown in Figure D14. The pre-culture substrate was glucose in all the cases. These results showed that *C. acetobutylicum* was not able tu utilise cellobiose when the pH was higher than 5.5. For this reason, the pH value of 5.5 was chosen for the fermentation experiments on cellulose-derived sugars.

E. RESUME DE LA THESE EN FRANÇAIS

I. Objectif

La demande mondiale de pétrole n'a cessé d'augmenter ces dernières années (OPEC 2016). Cette ressource fossile est devenue indispensable pour la société. Pour cette raison, trouver un remplacement durable est un défi important. La biomasse lignocellulosique représente une alternative intéressante en tant que source de carbone renouvelable respectueuse de l'environnement. Les principaux composants de la biomasse sont la cellulose (40-45%), l'hémicellulose (20-25%) et la lignine (25-30%). La cellulose et l'hémicellulose sont des polymères à base des sucres qui peuvent être dépolymérisés pour être ensuite transformés à travers des procédés fermentaires pour produire des biocarburants et des synthons qui peuvent remplacer ceux provenant de l'industrie pétrochimique. Il est nécessaire de réaliser une étape de fractionnement de la biomasse lignocellulosique afin de séparer ses trois principaux constituants, la cellulose, les hémicelluloses et la lignine. De cette façon, chaque fraction peut être valorisée dans les différents procédés d'une bioraffinerie intégrée. La cellulose séparée de la matrice fibreuse de la biomasse est très récalcitrante pour être valorisée par fermentation directe ; en raison de son insolubilité, de sa cristallinité et des liaisons d'hydrogène entre les fibres qui diminuent fortement sa réactivité. Il est donc important de trouver une technique de dépolymérisation de la cellulose permettant d'améliorer le rendement ultérieur de la fermentation.

L'objectif de cette thèse est de coupler un procédé de traitement thermique de la cellulose, pour la dépolymériser, à un procédé de transformation microbienne pour produire des solvants, des

acides et des gaz (butanol, éthanol, acétone, acide acétique, acide butyrique, hydrogène) qui suscitent un fort intérêt dans l'industrie des carburants ou de la chimie verte.

II. Bibliographie

1. De la biomasse aux sucres

La figure R1 montre les principales filières de production de biocarburants en France. Actuellement, la production de biocarburants (éthanol) à partir de la biomasse lignocellulosique a besoin d'une ou deux étapes de prétraitement pour déconstruire le réseaux de la matrice lignocellulosique (Yoo et al. 2011; Bensah and Mensah 2013; C. Liu et al. 2013). Ensuite, la lignine est séparée par un traitement chimique, normalement à l'aide de l'hydroxyde de sodium et du sulfure de sodium (procédé Kraft) (Chakar and Ragauskas 2004). Ensuite une étape de dépolymérisation de la cellulose est nécessaire pour obtenir la plus grande quantité de sucres fermentescibles possibles en évitant certains coproduits qui peuvent empêcher le développement des bactéries / levures fermentaires.

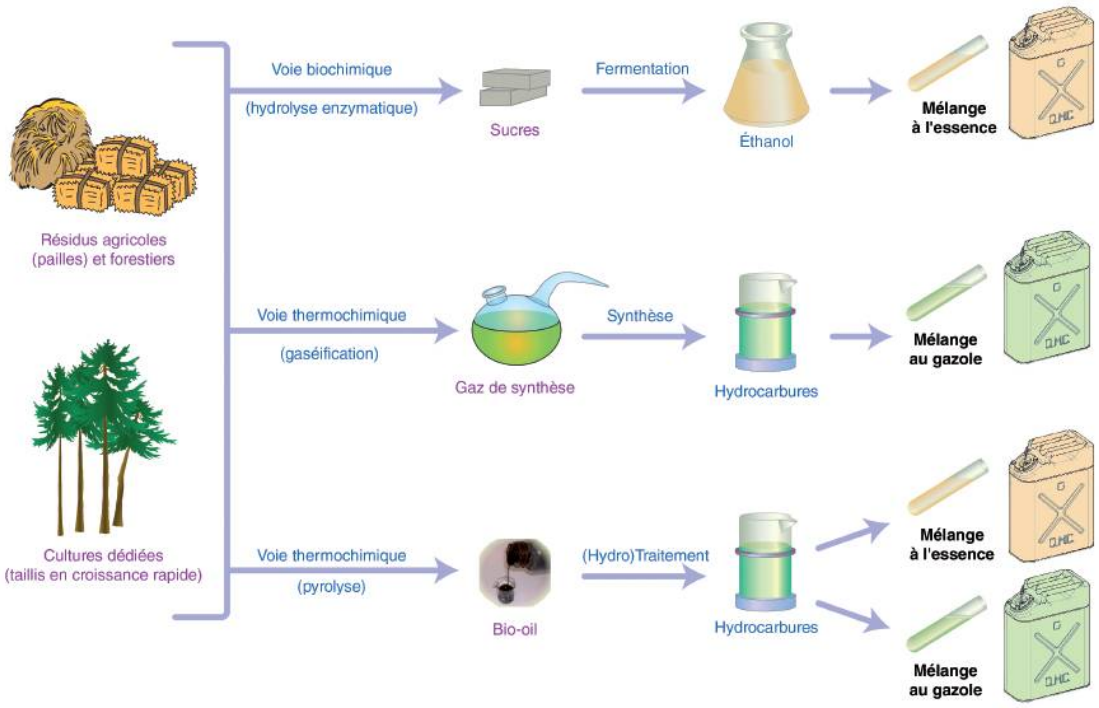

Figure R1. Schéma simplifié du procédé de production de biocarburants (IFPEN, n.d.)

La dépolymérisation de la cellulose extraite se fait communément par hydrolyse enzymatique (Yang et al. 2011). Un procédé qui permet de produire du glucose à haute sélectivité. Cependant, le prix des enzymes, l'impossibilité de les recycler et la cinétique d'hydrolyse relativement lente, ont un effet très négatif sur la viabilité économique du procédé global (Jiang et al. 2016). Une autre voie est l'hydrolyse acide, qui permet d'obtenir des sucres de façon relativement rapide (1-4 heures)(Rinaldi and Schüth 2009). Néanmoins, ce procédé nécessite des équipements construits avec des matériaux compatibles avec les acides forts et n'est plus trop utilisé actuellement.

La pyrolyse est une voie étudiée durant cette thèse pour la partie du « prétraitement de la cellulose ». Cette technique de conversion thermochimique se déroule en absence d'oxygène. Le temps et la température de réaction varient selon le type de pyrolyse (lente, traditionnelle, rapide). Des études précédentes ont montré qu'il était possible de dépolymériser la cellulose par pyrolyse à hautes températures (entre 700 et 900 K) et avec des courts temps de séjour (entre 0,1 et 0,2 s) (Lédé, Blanchard, and Boutin 2002). C'est la raison pour laquelle la pyrolyse rapide est la

technique la plus appropriée pour ce travail. Par ailleurs, cette méthode permet d'obtenir des cello-oligosaccharides solubles (45%) (Piskorz et al. 2000).

Néanmoins, pendant la pyrolyse de la biomasse/cellulose, certains composés inhibiteurs du métabolisme des microorganismes sont produits. Ces inhibiteurs peuvent être classés en trois groupes : les acides faibles, les dérivés du furane et les composés phénoliques (Palmqvist and Hahn-Hägerdal 2000).

L'utilisation de l'eau sous-critique et supercritique a été également étudiée pour la production des monomères à partir de la cellulose avec des rendements inférieures aux technologies précédents mais à faible prix et facilité d'utilisation (Yu, Lou, and Wu 2008; D. Liu, Yu, and Wu 2013; Yu, Long, and Wu 2015). L'optimisation de ce type de procédés de saccharification de la cellulose et la compréhension des mécanismes réactionnels est actuellement l'objet de plusieurs études, dont ce travail de thèse.

2. La fermentation de dérivés de la cellulose

Une fois que la biomasse est sous la forme de sucres fermentescibles, l'étape suivante est une fermentation qui permet de fabriquer une grande variété de produits chimiques d'intérêt. Ce procédé se fait à l'aide de microorganismes du règne des bactéries et des mycètes unicellulaires tels que les levures. Le métabolisme de ces microorganismes est très sélectif et peut produire une vaste gamme de produits chimiques valorisables dans plusieurs secteurs. Dans la nature, un faible pourcentage de microorganismes peut dégrader la cellulose directement (Guedon, Desvaux, and Petitdemange 2000; Desvaux, Guedon, and Petitdemange 2001), grâce à des cellulases, mais la lenteur de ce métabolisme est un facteur critique qui affecte sa rentabilité au niveau des bioprocédés (Gutiérrez-Rojas, Moreno-Sarmiento, and Montoya 2015). Pour cette

raison, le choix du substrat et de sa nature est critique pour la valorisation des produits de fermentation.

Il existe une grande variété de configurations possibles de procédés de fermentation de la biomasse prétraitée. La valorisation de cette ressource renouvelable par voie fermentaire suscite de l'intérêt grâce à la vaste gamme de substrats que ces microorganismes sont capables de consommer : hexoses, pentoses, glycérol, cellulose, amidon, gaz (Tracy et al. 2012). En outre, ces microorganismes peuvent fabriquer différents types de produits selon les conditions opératoires de la fermentation.

L'utilisation de *Clostridium spp.* pour la production d'acétone -butanol-éthanol (ABE) et d'hydrogène a été largement étudiée (Ezeji, Qureshi, and Blaschek 2007)(Gupta et al. 2014). Il y a 30 ans, la production industrielle d'acides, de solvants ou de gaz utilisant *Clostridium spp.* a connu une forte croissance(Jones and Woods 1986). Actuellement, la plupart de ces molécules est produite par voie pétrochimique, ayant un effet néfaste pour l'environnement. C'est pourquoi, il est essentiel d'éliminer les obstacles de la fermentation afin d'augmenter sa productivité et donc sa compétitivité.

III. Méthodologie développée durant cette thèse

Le travail de cette thèse se déroule en deux étapes réalisées dans deux équipes du LRGP. La première étape concerne la conversion thermochimique de la (ligno)cellulose et est effectuée dans l'équipe GREENER (site ENSIC). La deuxième étape porte sur la transformation des produits

issus de la transformation thermochimique par voie fermentaire ; réalisée dans l'équipe BioProMo (site ENSAIA).

1. Préparation de la matière première

La biomasse lignocellulosique étudiée dans ce travail est le hêtre. Cette biomasse a été choisie dû à son abondance et son manque de débouché notamment en Lorraine. La cellulose microcristalline (Avicel PH-101) a été également étudiée pour des raisons de comparaison entre une cellulose commerciale pure et les maières plus complexes issus du bois de hêtre. Le hêtre a été délignifié par deux méthodes : organosolv (Pan et al. 2006; Brosse, Sannigrahi, and Ragauskas 2009) et chlorite / acide acétique (SC / AA) (Ahlgren and Goring 1971; Siqueira et al. 2013). Le hêtre brut et les deux pâtes récupérées des procédés de fractionnement ainsi que la cellulose microcristalline ont été utilisés pour l'étude de la conversion thermochimique. Dans ce travail, l'appellation « (ligno)cellulose » désigne l'ensemble de ces matières.

2. Conversion thermochimique de la (ligno)cellulose

Dans la partie concernant la conversion thermochimique de la (ligno)cellulose deux approches ont été étudiées afin de produire des sucres fermentescibles : la liquéfaction (en milieu hydrothermal) et la pyrolyse rapide.

La liquéfaction de la ligno(cellulose) a été étudié dans un réacteur batch sous pression (14-50 bar) et en régime isotherme (180-260°C). Les produits solide, liquide et gazeux ont été récupérés, quantifiés et caractérisés par différentes techniques analytiques. Les solides ont été caractérisés par diffraction de rayons-X (DRX) et les gaz permanents par micro-chromatographie gazeuse. Les produits hydrosolubles, qui font l'objet principal de ce travail, ont été analysés par chromatographie d'échange anionique avec détection ampérométrique pulsée (HPAEC-PAD), chromatographie d'exclusion stérique couplée à la spectrométrie de masses (SEC-MS), spectrométrie de masse à haute résolution (LTQ-Orbitrap), chromatographie liquide avec détection UV (HPLC-UV) et analyse des carbohydrates totaux (phénol/acide sulfurique). La fraction liquide a été lyophilisée pour récupérer les produits hydrosolubles qui serviront ensuite comme substrat pour la fermentation. Des essais en lit fixe de biomasse ont été conduits à Curtin University (Prof. HW. Wu) pour étudier la dépolymérisation de la biomasse en milieu liquide et avec un plus faible temps de séjour de la phase liquide qu'en réacteur batch.

La pyrolyse de la ligno(cellulose) a été effectuée dans un micro-lit fluidisé couplé à l'analyse par spectrométrie de masses à temps de vol avec une source de photoionisation douce (SPI-TOFMS). La photoionisation (PI) est une méthode d'ionisation efficace et douce pour l'analyse en ligne des substances volatiles de pyrolyse de la biomasse. D'autres expériences de pyrolyse rapide ont été effectuées au Canada dans « l'institut de carburants de sources alternatives » (ICFAR), dans un lit-fluidisé avec un système de condensation étagée, permettant de séparer la fraction riche en sucres d'autres produits légers (acide acétique, furanes, etc…). Les produits solubles dans le méthanol ont été analysés par chromatographie en phase gaz couplée au détecteur à ionisation de flamme et la spectrométrie de masses (GC-FID-MS). Les résultats de pyrolyse ne sont pas présentés dans ce rapport.

3. La fermentation des sucres dérivés de la (ligno)cellulose

Le mélange d'oligosaccharides obtenu par conversion thermochimique de la (ligno)cellulose est fermenté par des bactéries du type *Clostridium acetobutylicum* ATCC 824. Pour ce faire, il est nécessaire de préparer les microorganismes et les milieux de culture à l'avance. Un stock d'inoculum des bactéries dans un milieu RCM (Reinforced Clostridial Medium) est maintenu actif grâce à un entretien journalier des cultures à petite échelle, afin de pouvoir ensemencer les pré-cultures nécessaires pour les bioréacteurs ou fioles. La pré-culture est ensemencée 14 h avant d'inoculer le mélange à étudier. Pour les études de la cinétique microbienne et l'utilisation des sources carbonées, des prélèvements sont réalisés toutes les heures (ou demi-heures pendant la phase exponentielle). Les manipulations dédiées pour étudier le rendement final en solvants et acides organiques ont été effectuées pendant 72 heures avec des prélèvements toutes les 12 heures. A la fin de la fermentation, l'ensemble de la réaction (contenant et contenu) est inactivé par autoclave pendant 25 min. L'analyse des produits de fermentation est effectuée par HPLC-UV, l'utilisation des substrats par HPAEC-PAD et les sucres totaux par méthode phénol/acide sulfurique.

IV. Résultats

1. Conversion thermochimique de la (ligno)cellulose

L'ensemble « fractionnement et liquéfaction » est représenté dans la figure R2.

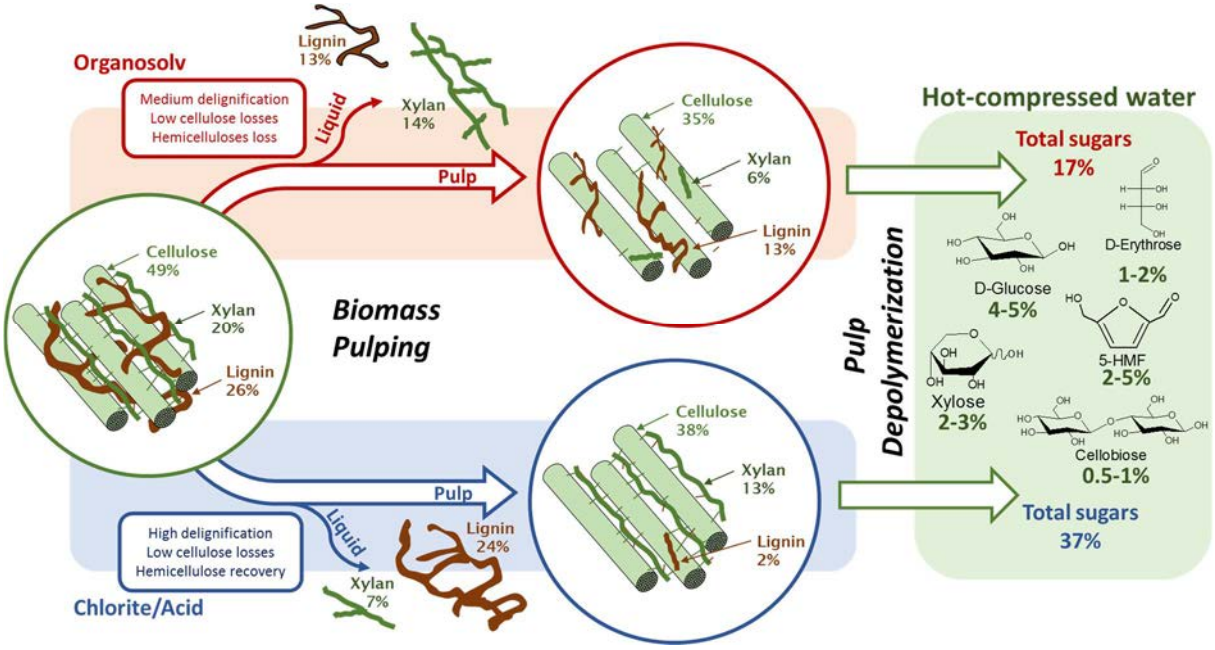

Figure R2. Schéma du fractionnement et liquéfaction de la biomasse lignocellulosique. Les rendements typiques obtenus sont indiqués pour les composants principaux.

Le bois de hêtre a été délignifié avec succès par les deux méthodes utilisées. La figure R3 présente les rendements massiques en cellulose, hémicellulose et lignine calculés par rapport à la masse initiale de hêtre anhydre. Ces méthodes de fractionnement ont permis d'extraire 74% (organosolv) et 92% (SC / AA) de la lignine initiale. La différence la plus importante entre les deux méthodes de fractionnement est la récupération des hémicelluloses dans la pâte quand la méthode SC / AA est utilisée. Contrairement à la méthode organosolv où presque toutes les hémicelluloses partent dans la phase liquide.

Dans un premier temps, la liquéfaction de la cellulose Avicel a été étudiée sur un intervalle de températures entre 180°C et 260°C. Les résultats obtenus ont permis de confirmer que les températures supérieures à 220°C favorisent la dégradation des sucres solubles en acide carboxyliques, aldéhydes et éventuellement des gaz permanents (dioxyde de carbone, monoxyde de carbone et hydrogène). Les mécanismes réactionnels principaux ont été décrits (en intégrant ceux de la littérature) et les réactions prédominantes à chaque température ont été identifiées (figure R4).

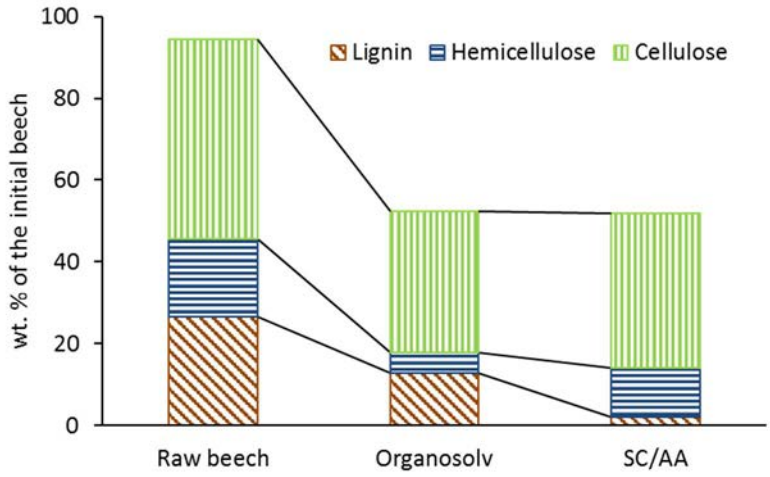

Figure R3. Rendements en cellulose, hémicellulose et lignine par rapport au hêtre initial.

La liquéfaction du hêtre brut et délignifié a été effectuée entre 180-220°C, sous conditions isothermes. Les produits principaux sont présentés dans la figure R5.

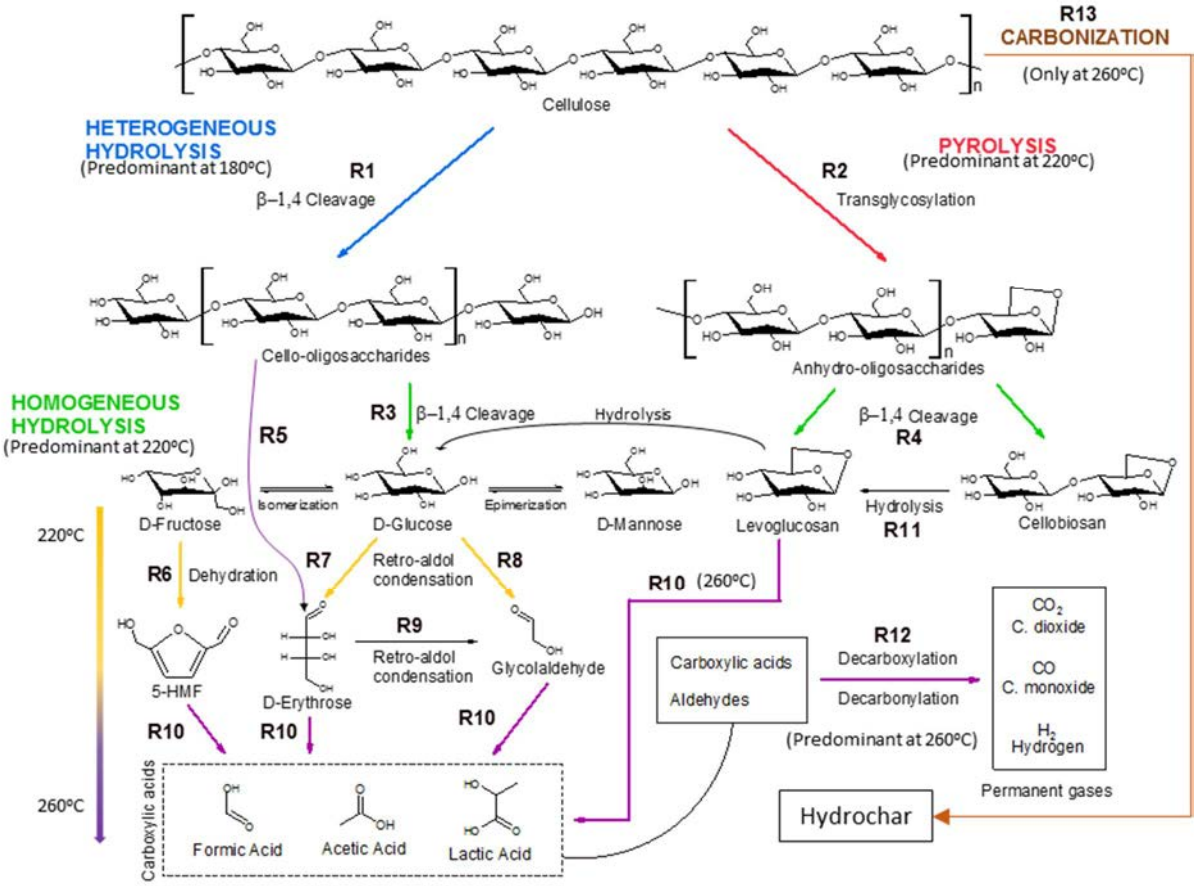

Figure R4. Schéma des mécanismes réactionnels principaux de la liquéfaction de la cellulose en milieu hydrothermal. Adapté de (Matsumura et al. 2006) et les contributions de ce travail. Les températures qui promouvoir chaque réaction (R) sont indiquées selon les conditions étudiés dans ce travail. R1, R3=(Sasaki et al. 1998), R2, R4=(Matsumura et al. 2006), R5=(Sasaki et al. 2002; Yu, Shafie, and Wu 2013), R6=(Kabyemela et al. 1999), R7, R8, R9=(Sasaki et al. 2002; Yu, Shafie, and Wu 2013), R10=(Watanabe et al. 2005; Yu and Wu 2011), R11=(Bilik 1972; Román-

Leshkov et al. 2010), R12=(Williams and Onwudili 2006) and R13=(Guo et al. 2015; Y. Liu et al. 2017).

La méthode SC/AA est la meilleure pour la production des sucres à partir d'une biomasse délignifiée. Le maximum de sucres fermentescibles (37 % m/m) a été produit quand la température de liquéfaction était 220°C pendant 2h. Cependant, une quantité importante (51.4 mg/g de biomasse) de 5-hydroxy-methyl-furfural (5-HMF), considéré comme un fort inhibiteur de la croissance microbienne, a été aussi générée lors de cette expérience.

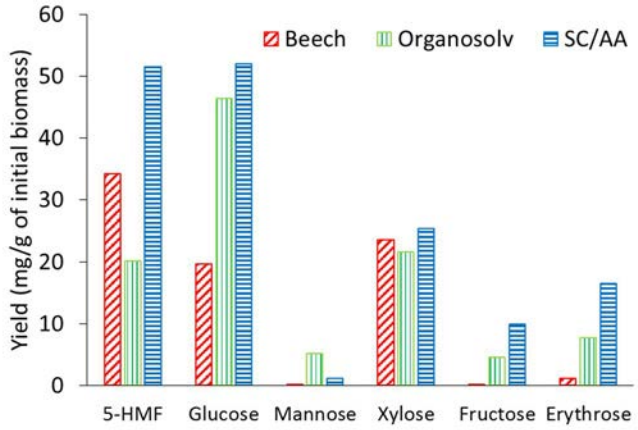

Figure R5. Rendement massique des composés principaux identifiés parmi les produits hydrosolubles issus de la liquéfaction de différentes biomasses pendant 2h à 220°C (conditions isothermes).

2. La fermentation des sucres dérivés de la (ligno)cellulose

L'étude des oligomères de glucose lors de la fermentation est indispensable pour comprendre le métabolisme du *C. acetobutylicum* pour les hydrolysats. Des fermentations à pH constant (5.5 – 6.3 - 7) ont été conduites dans un bioréacteur pour étudier l'effet du substrat et de la pré-culture sur la croissance cellulaire quand *C. acetobutylicum* est cultivé dans un mélange de glucose et de cellobiose (composés qui représentent les produits d'hydrolyse de la cellulose).

Concernant les cultures en mono-substrat, *C. acetobutylicum* a été capable d'utiliser le cellobiose seulement quand le pH était de 5.5. En plus, les cultures dans un mélange de glucose et cellobiose ont montré une croissance diauxique (croissance en deux phases) (figure R6). La maitrise de ce phénomène de croissance est cruciale pour la valorisation de la cellulose dans une bioraffinerie.

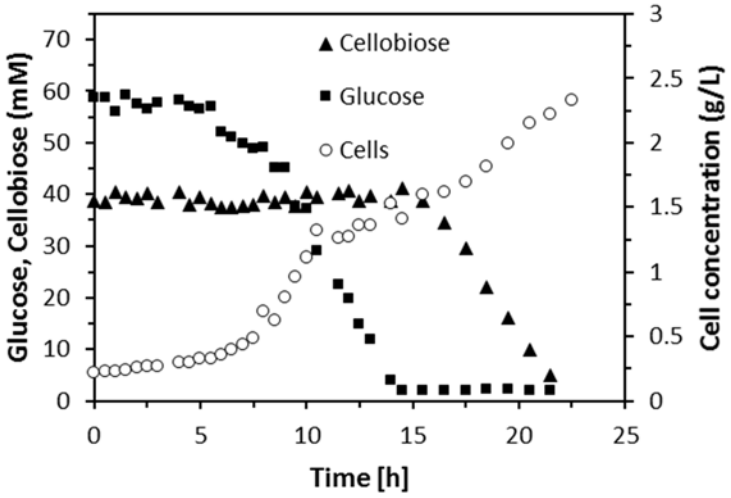

Figure R6. Courbes de croissance de *Clostridium acetobutylicum* avec un mélange glucose / cellobiose comme substrat.

L'étude des hydrolysats de la cellulose modèle (Avicel PH-101) a permis d'étudier l'utilisation séquentielle de différents carbohydrates produits par liquéfaction en milieu hydrothermal. Ces résultats présentent la première évidence d'utilisation d'oligomères de DP>2 (cellotriose, cellotetraose et cellopentaose) ce qui est montré dans la figure R7. La présence du 5-HMF n'a pas empêché l'utilisation des différentes sources carbonées. Ce composé a été consommé par *C. acetobutylicum* mais son métabolisme exact n'est pas encore connu. La capacité de *C. acetobutylicum* d'utiliser une large variété de substrats présents dans un mélange peut réduire significativement le coût de la saccharification par voie enzymatique qui est destinée aux fermentation à base de glucose.

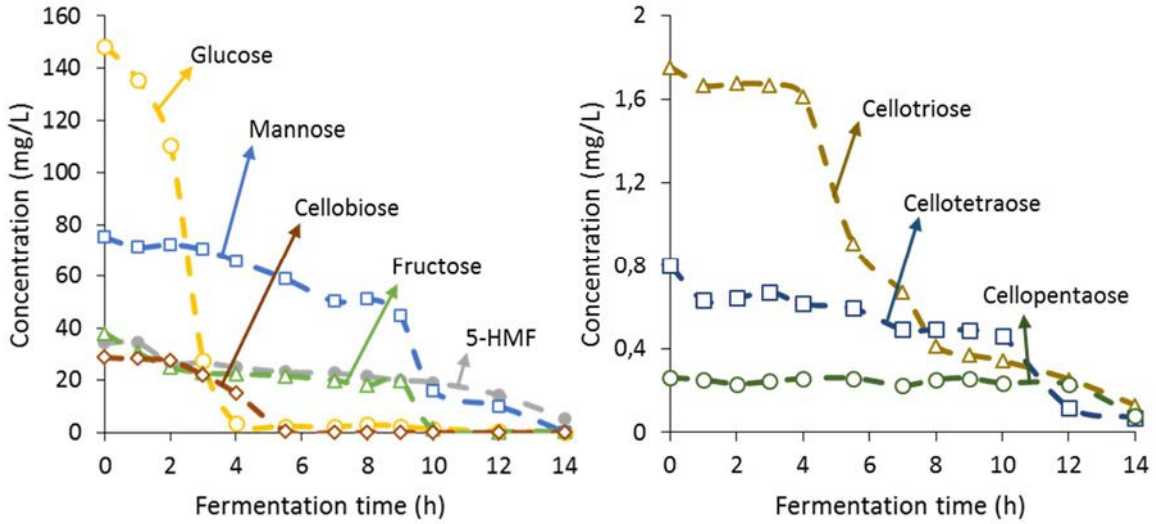

Figure R7. Utilisation d'un mélange de carbohydrates, par *C. acetobutylicum,* produit par liquéfaction en milieu hydrothermal de la cellulose Avicel pendant 2h à 180°C (conditions isothermes).

Des fermentations en fioles ont été effectuées pour les cultures avec des mélanges réels. Les rendements maximauax en butanol, 14.7% pour organosolv et 15.5% pour SC / AA (pourcentage par rapport à la quantité de sucres totaux au début de la fermentation), ont été trouvés quand la température de liquéfaction était 220°C et pour un temps de fermentation de 72h (figure R8).

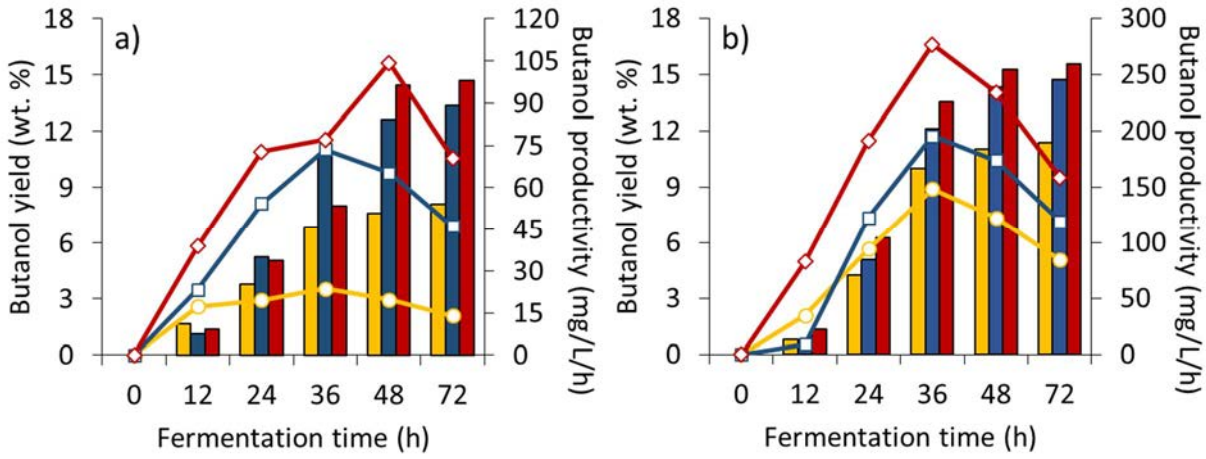

Figure R8. Rendements et productivités en butanol lors de la fermentation de mélanges de carbohydrates obtenus par liquéfaction pendant 2h à (jaune, ○) 180°C, (bleu, □) 200°C et (rouge, ◇) 220°C (conditions isothermes) de pâtes lignocellulosiques délignifiés par la méthode a) organosolv et b) SC / AA.

Dans les mêmes conditions, le rendement ABE (acétone-butanol-éthanol) a été de 19.2% pour la pâte organosolv et 20.7% pour la pâte SC / AA. D'autres métabolites valorisables ont été produits en même temps : l'hydrogène, l'acide butyrique et l'acide acétique.

Ces rendements sont comparables à ceux trouvés dans la littérature (Jang et al. 2012; K. Liu et al. 2015; Lee et al. 2016). Néanmoins, l'apport plus significative de ce travail est la méthodologie simplifiée qui peut augmenter significativement la viabilité économique de ce procédé de bioraffinerie : une saccharification très rapide de la biomasse par voie thermochimique couplée à une fermentation avec une souche capable de transformer tous les carbohydrates en produits d'intérêt industriel.

En plus, un modèle sous le logiciel Aspen Plus a été developé permettant de faire une analyse energétique de l'ensemble du procédé.

CELLULOSE VALORIZATION IN BIOREFINERY: SYNERGIES BETWEEN THERMOCHEMICAL AND BIOLOGICAL PROCESSES

Because fossil resources are exhaustible by definition, the carbon needed for energy and materials production could be obtained from lignocellulosic biomass. Fermentation processes are able to provide a wide variety of interesting products that can replace the crude oil based "building blocks". However, the abundance of lignocellulosic biomass in the environment contrasts with its very low bioavailability. Indeed, because of (i) its insoluble nature, (ii) its more or less crystalline structure and (iii) the nature of the bonds between the polymer fibers, cellulose is a carbon substrate difficult to valorize by biochemical / fermentation processes alone. Fast pyrolysis or liquefaction of cellulose are mainly studied to produce a bio-oil, which would be upgraded by catalytic hydrotreatment into fuels or building blocks. In the current state of the art, studies at the interface of these two fields involving a biochemical or microbiological conversion of these bio-oils are still rare. The aim of this thesis is the coupling of a thermochemical conversion process of cellulose, to depolymerize it, to a microbial transformation process to produce solvents, acids and gases (butanol, ethanol, acetone, acetic acid, butyric acid, lactic acid, hydrogen) that are of great interest for the fuel or green chemistry industry. To do this, beech wood was fractionated by organosolv and chlorite / acid (SC / AA) methods in order to recover a cellulose-rich pulp. Hydrothermal liquefaction and fast pyrolysis processes were used to obtain sugars that were transformed into building blocks by fermentation. Many analytical methods have been developed for the characterization of products from each step of the process. Finally, a model of the process using the commercial software Aspen Plus® was developed to establish mass and energy balances of the integrated process including: the fractionation of the wood, then the liquefaction of the cellulosic fraction and the fermentation of bio-oils.

Hydrothermal, Biorefinery, Biomass, Biofuels, Pyrolysis, Fermentation, Cellulose

VALORISATION DE LA CELLULOSE DANS UNE BIORAFFINERIE: SYNERGIES ENTRE LE PROCEDES THERMOCHIMIQUES ET BIOLOGIQUES

Parce que les ressources fossiles sont épuisables par définition, le carbone nécessaire à la production d'énergie et de matériaux pourrait provenir en grande partie de la biomasse lignocellulosique. Les procédés de fermentation sont capables de fournir une grande variété de produits d'intérêts capables de remplacer les synthons d'origine pétrolière. Cependant, en raison (i) de son caractère insoluble, (ii) de sa structure plus ou moins cristalline et (iii) de la nature des liaisons entre les maillons du polymère, la cellulose est un substrat carboné difficile à valoriser par voie biochimique / fermentaire seule. La pyrolyse rapide ou la liquéfaction de la cellulose sont principalement étudiées pour produire une bio-huile, qui serait valorisée par hydrotraitement catalytique en carburant ou en building blocks. Dans l'état de l'art actuel, les travaux à l'interface de ces deux domaines portant sur une conversion biochimique ou microbiologique de ces bio-huiles sont encore rares. L'objectif de cette thèse est de coupler un procédé de conversion thermochimique de la cellulose, pour la dépolymériser, à un procédé de transformation microbienne pour produire des solvants, des acides et des gaz (butanol, éthanol, acétone, acide acétique, acide butyrique, acide lactique, hydrogène) qui suscitent un fort intérêt dans l'industrie des carburants ou de la chimie verte. Pour ce faire, le bois de hêtre a été fractionné par les méthodes organosolv et chlorite / acide (SC / AA) afin de récupérer une pâte riche en cellulose. Des procédés de liquéfaction hydrothermale et de pyrolyse rapide ont été utilisés pour obtenir des sucres qui ont été finalement transformés par fermentation en synthons. De nombreuses méthodes analytiques ont été développées pour la caractérisation des produits issus de chaque étape du procédé. Enfin, un modèle du procédé utilisant le logiciel commercial Aspen Plus® a été développé pour établir les bilans de matière et énergie du procédé intégré : du fractionnement du bois, puis la liquéfaction de la fraction cellulosique et à la fermentation des bio-huiles.

Hydrothermal, Bioraffinerie, Biomasse, Biocarburants, Pyrolyse, Fermentation, Cellulose