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PART 1
Second and Third Generation Biofuels: Towards Sustainability and Competitiveness
Deuxième et troisième génération de biocarburants : développement durable et compétitivité

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Upgrading the Hemicellulosic Fraction of Biomass into Biofuel

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Abstract — Upgrading the Hemicellulosic Fraction of Biomass into Biofuel — Hemicelluloses are polymers composed mainly of C5 sugars (pentosans). They constitute a significant part of lignocellulosic biomass (LCB), as they can be up to 30% of the total mass. The upgrading of the hemicellulosic components is thus a prerequisite for profitable biofuel production from LCB. When LCB undergoes acid pretreatment, the hemicellulose-derived fraction is mainly composed of monomeric pentoses (xylose, arabinose) and oligomeric pentoses both resulting from the thermo-chemical hydrolysis. The hemicellulosic fraction is not fermentable into ethanol by wild type strains of Saccharomyces cerevisiae. Over the past 20 years, several groups have worked to genetically modify this yeast in order to render it capable of fermenting pentose constituents. These efforts were met with varying degrees of success, especially in the case of industrial substrates. In this paper, we describe two other possible ways of using the hemicellulosic fraction, each of which may contribute to the economic viability of biofuel production from LCB. The first one is its use as a carbon substrate for the production of cellulases by Trichoderma reesei, since cellulases are needed for the enzymatic hydrolysis of cellulose. The second is the Acetone-Butanol-Ethanol (ABE) fermentation using anaerobic bacteria of the genus Clostridium. The produced ABE mixture has very interesting fuel properties and can be directly blended with gasoline.
ABBREVIATIONS

5-HMF 5-Hydroxymethylfurfural
ABE Acetone-Butanol-Ethanol
ATP Adenosine triphosphate
CCR Carbon Catabolite Repression
LCB Lignocellulosic biomass
MCIMB National Collection of Industrial, Food and Marine Bacteria, Aberdeen, UK
MFS Major Facilitator Superfamily
NAD Nicotinamide Adenine Dinucleotide (oxidized form)
NADH Nicotinamide Adenine Dinucleotide (reduced form)
PEP Phosphoenolpyruvate
PPP Pentose-Phosphate Pathway
PTS Phosphotransferase system
US DOE United States Department of Energy
XDH Xylose dehydrogenase
XI Xylose Isomerase

INTRODUCTION

Wood and agricultural by-products, such as wheat straw, are considered to be the most abundant bioresource. Since they are built by photosynthesis from solar energy and atmospheric CO₂, these woody materials (or lignocellulosic biomass, LCB) are considered to be renewable and virtually inexhaustible. Compared with biofuels produced from sucrose or starch, those derived from LCB have the advantage of not competing with edible biomass. The biofuels derived from non-edible biomass are commonly called "2G biofuels" signifying "2nd generation biofuels".

As part of the Futurol program, IFPEN has been working on a 2G biofuel process aiming at converting LCB, such as wheat straw or wood, into ethanol. A scheme of the overall process is shown in Figure 1.

In a first step, LCB (straw or wood) is prepared by removing stones and chopping the biomass. Then, LCB is pretreated with dilute sulphuric acid at high temperature, with or without steam-explosion, to break down the native woody structure. In optimised conditions, hemicellulose is hydrolysed to produce monomeric or oligomeric pentoses (C5 sugars) that are water-soluble and extractable with water. The residual cellulose is subsequently hydrolysed by cellulase enzymes to give a C6 sugar solution, which can be fermented into ethanol by Saccharomyces cerevisiae.

Upgrading the C5 sugars in such a process is not easy, since not all types of microorganisms utilize pentoses (Singh and Mishra, 1995). In addition, LCB treatment at high temperature under acidic conditions leads to the formation of a wide range of compounds, including acetic acid, furfural, 5-hydroxymethylfurfural (5-HMF) and phenolics. These degradation compounds are known to inhibit fermentation (Palmqvist and Hahn-Hägerdal, 2000). When the severity of the pretreatment is further increased, 5-HMF generates formic and levulinic acids that are even more toxic than furanics toward microbial activity.

In the Futurol process, a C5 sugar fraction derived from LCB is used for the preparation of the cellulases. The cellulase production requires a carbon source, such as glucose, and a cellulase inducer, such as lactose (Pourquie et al., 1988). The C5 sugar fraction derived from the pretreated biomass can partially substitute both the carbon source and the cellulase inducer (Warzywoda et al., 1992). At IFPEN, the upgrading of C5 sugars has also been studied through another approach consisting in the Acetone-Butanol-Ethanol (ABE) fermentation. The ABE fermentation is carried out anaerobically on sugars with Clostridium acetobutylicum or Clostridium beijerinckii strains, both of which are capable of utilizing pentoses. Similarly to ethanol, the mixture produced from ABE is a valuable biofuel that can be readily incorporated to gasoline or diesel fuel (Dürré, 2007).

In the present paper, the most relevant features of the problems surrounding the conversion of LCB derived C5 sugars into biofuels are described. The hemicellulose compositions are detailed, as well as the composition of the C5 sugar mixture resulting from the hemicellulose hydrolysis. The concerns of pentose uptake and catabolism by microorganisms are also addressed in terms of results obtained at IFPEN on productions of cellulase by T. reesei and ABE by C. beijerinckii.

1 HEMICELLULOSIC SUGARS FROM LIGNOCELLULOSIC BIOMASS

1.1 Composition of Hemicelluloses

LCB are composed of three main components: cellulose, hemicellulose and lignin. Cellulose, the most abundant constituent, is a linear polymer of D-glucose units linked by β-glycosidic bonds. In its native state, cellulose has an average polymerisation degree of about 10 000 to 20 000. Unlike cellulose, hemicellulose is a branched-chain heteropolymer of D-xylose, D-glucose, D-mannose, G-galactose, L-arabinose, 4-O-methyl-D-glucuronic acid
Figure 1
Basic scheme of the process studied at IFPEN for the conversion of lignocellulosic biomass into biofuels.
and occasionally another monomeric sugar. The close association of hemicellulose with both cellulose and lignin contributes to the cell rigidity and flexibility of plants. In contrast to cellulose, hemicellulose exhibits variability in both structure and sugar constituents. The hemicelluloses are usually composed of two to six sugar residues with a polymerization degree of approximately 50-400. The inner chain consists of polysaccharides linked to various sugar residues that can be the same or different from the sugars of the side chain. Most of the linkages are β-1,4-linkages except for galactose (β-1,3-linkages). The main types of hemicellulose structures are shown in Table 1 and Figure 2.

The common structures of hemicellulose contain xylose, glucose and arabinose. In hardwoods, the main type of polymer is a β-(1-4)-linked xylan with side chains of 4-O-methyl-D-glucuronic acid units, whereas in softwoods the most abundant polymer is a β-(1-4)-linked glucomannan. The mannose content is significant and distinguishes coniferous trees (high mannose content) from deciduous trees (low mannose content).

### Table 1: Basic structures of hemicelluloses (from Amidon and Liu, 2009)

<table>
<thead>
<tr>
<th>Hemicellulose type</th>
<th>General structure</th>
<th>Content in softwoods (%)</th>
<th>Content in hardwoods (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactoglucomannan (1:1:3)</td>
<td>G - M - M - M - G - M - M - M</td>
<td>5-8</td>
<td>0</td>
</tr>
<tr>
<td>(Galacto)glucomannan (0.1:1:4)</td>
<td></td>
<td>10-15</td>
<td>0</td>
</tr>
<tr>
<td>Glucomannan (1:2-1:1)</td>
<td>G - M - M - G - M</td>
<td>0</td>
<td>2-5</td>
</tr>
<tr>
<td>Arabinoglucuronoxylan</td>
<td>X X X X X X X</td>
<td>7-10</td>
<td>trace</td>
</tr>
<tr>
<td>Glucuronoxylan</td>
<td>X X X X X X X</td>
<td>trace</td>
<td>15-30</td>
</tr>
<tr>
<td>Extractives</td>
<td></td>
<td>5-8</td>
<td>2-4</td>
</tr>
<tr>
<td>Ash</td>
<td></td>
<td>0.2-0.5</td>
<td>0.2-0.8</td>
</tr>
<tr>
<td>Others</td>
<td>Terpenes, fatty acids, phenols, isoflavones, carbohydrates, salts, etc.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total hemicellulose</td>
<td></td>
<td>25-30</td>
<td>25-35</td>
</tr>
</tbody>
</table>

G: glucose, Ga: galactose; M: mannose; X: xylose; A: arabinose; Ac: acetyl; Gu: 4-O-methylglucuronic acid.

* Composition in monomers of each hemicellulose type is given in parentheses.
Table 2 shows the percentage of various monomeric sugars in several lignocellulosic feedstocks.

1.2 Extraction of Hemicellulosic Sugars

The optimized upgrading of LCB requires a treatment for partial or total fractionation. In the paper industry, the main goal is to obtain high quality, insoluble cellulose fibre. To this end, alkaline treatments are performed, since they solubilize and remove lignin and most of the hemicellulose. For biofuel production from LCB, alkaline pretreatments are not adapted, because they solubilize and remove a substantial fraction of the hemicellulose that could otherwise be used by microorganisms for the production of valuable compounds. Therefore, dilute-acid pretreatments ($\text{H}_2\text{SO}_4$) as described by Warzywoda et al. (1992), with or without steam-explosion, are preferable for making 2G biofuels.

The moderate acidic hydrolysis of lignocellulosic materials such wood or straw results in the release of lignin, which is partially hydrolysed in the process (Ropars et al., 1992). Similarly to lignin, hemicellulose is also partially hydrolysed resulting in hexoses (e.g. glucose), pentoses (e.g. xylene) and a variety of oligosaccharides. However, cellulose retains its polymeric structure and is later hydrolysed by cellulases.

### 2 ASSIMILATION OF PENTOSES BY MICROORGANISMS

Glucose is the most common carbon source for microorganisms. Since it constitutes the building block of starch; glucose is utilised by many species such as *Escherichia coli*, Saccharomyces cerevisiae and Corynebacterium glutamicum. In contrast, the hemicellulose-derived monomers such as xylene or arabinose are only utilized by a limited number of microorganisms. The uptake of C5 or C6 sugars by microorganisms involves various enzymatic transport systems.

2.1 Transport Systems for Sugar Uptake

The enzymes involved in the selective permeability of cell membranes to substrates are called “transporters”. Several types of transport systems have been identified in the microbial world (Madigan and Martinko, 2006; Jojima et al., 2010):

- the ATP-Binding Cassette (ABC) transport system is the most widely-distributed system in living organisms. In prokaryotes, more than 20 different types of ABC transport systems have been identified. The ABC system is composed of three types of components:
  - periplasmic-binding proteins;
  - membrane-spanning proteins;
  - ATP-hydrolysing proteins (kinases);
- in gram-negative bacteria, the periplasmic-binding proteins for sugar transport are located in the periplasm. These enzymes are characterized by high affinity to substrate. The membrane-spanning proteins carry the substrate through the cell membrane, while the ATP-hydrolysing proteins (kinases) supply energy for substrate transport;
- the Major Facilitator Superfamily (MFS) transporters build another ubiquitous class of transport system.
in living organisms. The MFS transporter family is composed of three subfamilies:

- uniporters that transport molecules unidirectionally;
- symporters that transport one molecule along with another one;
- antiporters that simultaneously transport one molecule in one direction and another molecule in the opposite direction;

- the Phosphoenolpyruvate (PEP) Carbohydrate Phosphotransferase system (PTS) has been reported for the uptake of glucose, mannose and fructose by bacteria. PTS is composed of five types of proteins. As with sugar transport, the first four proteins (Enzymes I, HPr, IIa, IIb) of the phosphotransferase system are alternatively phosphorylated and dephosphorylated in a cascade manner. As a result, the membrane-spanning protein (enzyme IIc) receives the phosphate group and phosphorylates the sugar molecule that crosses the membrane. In bacteria, glucose and mannose transports commonly occur through PTS.

Among the ABE producing bacteria, PTS has been identified in both *C. beijerinckii* NCIMB8052 (Mitchell et al., 1991) and *C. acetobutylicum* ATCC824 (Tangney and Mitchell, 2007). However, PTS is not usually involved in the uptake of pentoses. In *E. coli* for instance, two distinct transporters for xylose (XylFGH) and arabinose (AraFGH) have been identified, as well as a proton-linked arabinose (AraE) symporter and a proton-linked galactose symporter that can transport xylose (Henderson, 1990).

In yeasts, the major process of hexose transport is facilitated diffusion. The main transporters are transporter Hxt for glucose and transporter Gal2 for galactose. It is worth noting that even though wild type *S. cerevisiae* is unable to grow on xylose, xylose can be transported through cell membrane by both systems Gal2 and Hxt (Sedlak and Ho, 2004).

### 2.2 Pathways of Pentose Degradation in Bacteria and Yeasts

Inside the microbial cell, the C5 sugars enter degradation pathways that are specific to the bacteria or yeasts (or fungi) (Fig. 3).

In bacteria, D-xylose is first isomerised into D-xylulose by xylose isomerase. Then, xylulose is phosphorylated into xylulose 5-phosphate, which enters the Pentose Phosphate Pathway (PPP). In yeasts, because of the lack of xylose isomerase, two consecutive redox reactions lead to D-xylitol (Rizzí et al., 1989). Accordingly, xylose is first reduced into xylitol by a NAD(P)H-dependent reductase (XR). Then, xylitol is oxidized into xylulose by a NAD-dependant xylitol dehydrogenase (XDH). After phosphorylation, xylulose enters the PPP as previously indicated for bacteria.

L-arabinose uptake by bacteria involves an initial isomerisation into L-ribulose by L-arabinose isomerase (AraB), then a phosphorylation into L-ribulose-5-phosphate (by AraB). L-ribulose-5-phosphate is isomerised into D-xylulose-5-phosphate (AraD) that enters PPP. In yeasts, four consecutive redox reactions occur for the formation of D-xylulose-5-phosphate (Karhumaa et al., 2005).

The PPP forms two main intermediary metabolites that are essential to biosynthetic pathways. First, ribose-5-P is a major building block for nucleic acid formation. Second, erythrose-4-P is a precursor for the aromatic rings in three amino acids (phenylalanine, tyrosine and tryptophan). In addition, PPP is a major source of NADPH, a reduced coenzyme involved in many anabolic routes.

### 2.3 Catabolic Repression

Carbon Catabolite Repression (CCR) is the process by which microorganisms preferentially consume glucose when exposed to a mixture of several sugars. As long as the glucose in culture medium is not exhausted, the enzymes devoted to the utilization of the less favourable substrates are repressed. As a consequence, the growth curve displays a so-called diauxic pattern corresponding to the sequential substrate utilization. The microorganism grows on the best energy source first. After a lag period, growth resumes on a worse carbon source. The CCR is of major importance when using hemicellulosic monomers as the growth substrate for microorganisms, since xylose and arabinose coexist in hydrolysates together with glucose. For that reason, the sugar monomers resulting from hemicellulosic breakdown were utilised incompletely and produced limited amounts of solvent.

### 2.4 Contributions of Molecular Biology to Pentose Utilization

The contribution of molecular biology to improve pentose uptake has greatly benefited the ethanol-excreting capabilities of *S. cerevisiae* (Hahn-Hägerdal et al., 2007). This is briefly summarized below. Recently, it was found that insertion of the PPP genes into *S. cerevisiae* resulted in ethanol excretion in recombinant strains. However, these new bacterial capabilities were judged to
Figure 3
Compared degradation pathways of pentoses in bacteria and yeasts.
be insufficient (Kuyper et al., 2003) and several strategies were developed to increase ethanol excretion from pentoses by means of engineered strains.

Since xylitol was frequently detected in yeast cultures grown on xylose, it was hypothesized that the cofactor specificities with XR and XDR (Fig. 3) were the limiting steps in xylose catabolism (Brunenberg et al., 1984). The bacterial Xylose Isomerase (XI) was expressed in S. cerevisiae but unfortunately without any significant positive effect. Then, XR and XDR genes from Pichia stipitis were heterologously expressed but xylitol remained a significant by-product, probably because of the higher specificity of XDH for NADPH than for NADH (Sonderegger et al., 2004). Xylitol actually derived from xylose reduction due to a reductase was encoded by gene GRE3. Since xylitol is an inhibitor of Xylose Isomerase (XI), it was expected that GRE3 deletion might improve xylose utilization in S. cerevisiae recombinants. This was only partially successful, since GRE3 was proven to be a stress-induced protein whose deletion negatively affected growth.

In the PPP of S. cerevisiae, transaldolase (TAL1) and transketolase (THL1) were thought to have the limiting enzymatic activities (Jin et al., 2005). Overexpression of only TAL1 increased xylose uptake (Walfriðsson et al., 1995); the combined overexpression of TAL1, THL1, ribulose-5-phosphate epimerase (RPE1) and ribulose kinase (RKII) had a more pronounced effect (Johansson and Hahn-Hägerdal, 2002). As a result of the metabolic engineering work, the engineered strain RWB217 exhibited an anaerobic growth rate of 0.09 h⁻¹ on xylose. It produced ethanol from xylose due to reductase was encoded by gene GRE3. Since xylitol is an inhibitor of Xylose Isomerase (XI), it was expected that GRE3 deletion might improve xylose utilization in S. cerevisiae recombinants. This was only partially successful, since GRE3 was proven to be a stress-induced protein whose deletion negatively affected growth.

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Since the protonated acid is the only permeant form in the process, the rate of active anion exclusion is a crucial step. When large amounts of protonated carboxylic acid are present in medium, the microbial biomass must consume a lot of ATP in order to preserve the functionality of the cellular proton gradient. The energy normally devoted to biosyntheses is wasted; growth rate and yield are markedly affected.

### 3.1.2 Limitation of Acetic Acid Inhibition

Lignocellulosic hydrolysates, in particular the C5 sugar fraction, contain substantial amounts of acetic acid. This mainly comes from hemicellulose deacetylation during pretreatment. In the fermentation process, the usual method to cope with acetic acid inhibition consists of increasing the medium pH as high as possible in order to lower the concentration of the protonated acid form. In ABE fermentation, the initial culture pH can be brought up to 6.2-6.5 (i.e. 1.5 pH unit higher than the pKa value of acetic acid) to alleviate inhibition. After growth starts, supplementary acetic acid is excreted simultaneously with butyric acid. During the solventogenic phase, both carboxylic acids are taken up and utilized as co-substrates for solvent formation.

The sensitivity of the process to high acetic acid concentrations decreased when initial cell biomass concentration was increased, probably because high levels of cellular biomass could withstand proton influx thanks to sufficient active-export. A cell biomass productivity of 3.4 g/L/h with a yield of 0.41 g/g/h has been reported (Palmqvist et al., 1996) in a culture on a willow hydrolysate using an initial cell biomass of 6.0 g/L. Stenberg et al. (1998) reported an ethanol productivity of 3.0 g/L/h and a yield of 0.44 g/g/h on a spruce hydrolysate with pH adjustment to 4.8 and initial cell biomass concentration of 10 g/L. Schneider (1996) reported a selective removal of acetic acid from hardwood-spent sulphite liquor with a S. cerevisiae mutant that grew on acetic acid but not on D-xylene, D-glucose, D-mannose or D-fructose. T. reesei could be also used for removing acetic acid from hydrolysates (Larsson et al., 1999).

### 3.2 Furanic Compounds

Furfural and 5-HMF are formed by the thermal degradation of sugars during pretreatment. Furfural is the main product of thermal C5-sugars (xylose) degradation whereas 5-HMF is mainly produced from C6-sugars (glucose, mannose and galactose). Physical, chemical or biological methods have been applied to detoxify the hemicellulosic hydrolysates obtained by washing the pretreated LCB with water according to the process shown in Figure 1.

#### 3.2.1 Physicochemical Detoxification Methods

Furfural can be removed by evaporation and separation of volatile fraction from the solid residue (Palmqvist and Hahn-Hägerdal, 2000). It is also possible to use organic solvents, such as ethyl acetate or diethyl ether, to remove furfural. Nilvebrant et al. (2001) used a cation- and anion-exchange resin to remove both 5-HMF and furfural, in roughly equal proportions. However, many of these methods cannot be easily scaled up for an industrial process.

#### 3.2.2 Chemical Detoxification Methods

Alkali treatments are considered to be the most efficient detoxification methods. Treatment with calcium hydroxide (overliming) and ammonia has been considered to be better than treatment with KOH or NaOH but the reasons were not explained (Sanchez and Cardona, 2008). Amartey and Jeffries (1996) improved the fermentation of acid-hydrolysed hemicellulose by overliming. The total sugar utilisation increased from 18% to 82% because of partial removal of inhibitors such as 5-HMF. In the classical overliming procedures, lime is added to adjust pH to a high value, typically in the range of 9 to 11. Then, the hydrolysate is heated to a temperature of 50-60°C (Mohagheghi et al., 2006). The solution is held at this temperature for 30-40 min and then filtered to remove the precipitate. Finally, the hydrolysate is neutralized by sulphuric acid addition.

The overliming treatments may degrade sugars. To reduce losses, shifts to high pH must be kept as moderate as possible (Fein et al., 1984), since sugar loss increases with pH. The loss in xylose, the dominant sugar of hydrolysates, increases from 7% at pH 9 to 34% at pH 11. Millati et al. (2002) reported glucose losses greater than 70% when the overliming treatment was carried out for 170 h at 60 °C and pH 12 (Mohagheghi et al., 2006).

Treatment with a reducing agent such as sodium sulphite was also suggested to adjust the unfavourable redox potential of lignocellulosic hydrolysates (Palmqvist et al., 2000). Larsson et al. (1999) used this treatment on dilute acid hydrolysates of spruce. They observed a decrease in the furfural and 5-HMF concentrations.

#### 3.2.3 Biological Methods

Nichols et al. (2008) used the fungus Coniochaeta ligniaria (NRRL30616) to remove the inhibitors present in a...
corn stover hydrolysate, as confirmed by HPLC-UV-ms/MS analysis. The removal of furfural and 5-HMF by Coniochaeta ligniaria C8 was also reported by Lopez et al. (2004). Talebnia and Taherzadeh (2006) reported the effective 5-HMF detoxification of spruce hydrolysate by encapsuled S. cerevisiae.

3.3 Phenolic Compounds

Phenolic compounds are formed by a partial degradation of lignin. For example, vanillic acid and vanillin are formed from the degradation of the guaiacylpropane units of lignin. Phenolics have been detected in hydrolysates of spruce, willow, poplar and pine (Palmqvist and Hahn-Hägerdal, 2000). Syringaldehyde, syringic acid and 4-hydroxybenzoic acid are formed from the degradation of syringylpropane units, and have been detected in hardwood hydrolysates (Jönsson et al., 1998). These compounds (especially those with the lower molecular weights) exert an inhibitory effect on the fermentation of the hemicellulosic hydrolysates. The mechanism of inhibition is not clear, largely due to a lack of accurate analyses and because inhibition studies have been performed at far higher concentrations than are actually present in the hydrolysates (Palmqvist and Hahn-Hägerdal, 2000). The inhibitory compounds probably perturb the integrity of the cellular membrane (Heipieper et al., 1994).

3.3.1 Microbial Detoxification

Many microorganisms utilise phenolics, alleviating their inhibitory effect. T. reesei was reported to degrade inhibitors in a hemicellulose hydrolysate of willow (Palmqvist et al., 1997). The absorbance at 280 nm that is indicative of the presence of aromatic compounds decreased by 30% after the treatment by T. reesei (Palmqvist and Hahn-Hägerdal, 2000). Okuda et al. (2008) used the thermophilic bacterium Ureibacillus thermosphaercus to detoxify a hydrolysate of waste house wood and confirmed the degradation of phenolics using chromatographic analysis.

3.3.2 Enzymatic Treatment with Laccase

Phenolics can be removed by enzymatic treatments. Jönsson et al. (1998) increased the ethanol productivity of a hemicellulosic willow hydrolysate by treatment with peroxidase and laccase from Trametes versicolor. The detoxifying mechanism was suggested to be an oxidative polymerisation of the low molecular weight phenolics, because absorbance at 280 nm did not change after laccase treatment (Palmqvist and Hahn-Hägerdal, 2000). As a reminder, the phenolic monomers are much more inhibitory than polymers.

3.3.3 Other Treatments

Larsson et al. (1999) compared different detoxification methods using a dilute acid hydrolysate of spruce (overliming, laccase, anion and cation exchange at different pHs, etc.). The anion exchange at pH 10 was found to be the best method and led to the removal of more than 80% of the phenolics. This method also allowed for the removal of other inhibitors (levulinic acid, acetic acid, formic acid, 5-HMF and furfural) but was difficult to use at an industrial scale because of the product costs and the important losses of fermentable sugars.

4 CELLULASE PRODUCTION BY TRICHODERMA REESEI

4.1 Fermentation Profile

The production of the cellulolytic enzymes needed for glucose production is a main step of 2G bioethanol process. Commonly, cellulase excretion is carried out using T. reesei as a producer. The fungus is first grown with both sugar and nutrients in excess, in order to produce a large concentration of cellular biomass.

In the subsequent phase, a limiting rate of sugar is fed to the culture (Pourquié et al., 1988). Simultaneously, an inductive co-substrate, such as lactose, is also provided at a limiting rate. In fact cellulase production is very low in the presence of readily-metabolized sugars in excess, which is the case in batch culture. Therefore, soluble inducing carbon sources must be fed continuously, in a fed-batch protocol, so that their residual concentrations in the bioreactor will remain close to zero. This typical substrate feeding results in an initial batch culture in which the initial xylose or hemicellulosic hydrolysate are rapidly consumed. During the fed-batch phase, excretion of cellulases occurs, as a result of the low residual concentrations of both inductor and main carbon source.

At IFPEN, the feed rate of substrate has been optimized to maintain a constant level of biomass in the reactor during the whole phase of cellulase excretion (Pourquié et al., 1988). The protocol was successfully applied to a 30 m³ fermentation volume (Ballerini et al., 1994) and even larger volume reactors (data not shown).

4.2 Cellulase Production on Xylose as Major Carbon Source

The strategy of substrate feeding deeply impacts the production performances of the cellulase-producing cultures.
Cellular growth and protein excretion by *T. reesei* CL847 when only xylose was used as carbon source for cell growth and a mixture of xylose/lactose was used for cellulase excretion are shown in Figure 5. In the batch phase on xylose, the protein production was low because of the inhibitory effect of xylose excess on excretion. Then, the feed of lactose/xylose (from 50 h to 167 h) induced the cellulase excretion, at the beginning of the carbon source limitation, resulting in the sharp increase of protein measured in the reactor. When biomass growth stopped because of xylose exhaustion, protein excretion started and increased up to 34 g/L. The mean value of protein productivity ($r_p$) in this culture was 0.21 g.L$^{-1}$.h$^{-1}$ and yield of protein based on substrate (lactose and xylose) consumed ($Y_{P/S}$) was 0.35 g/g.

The specific growth rate ($\mu$) on xylose during the batch phase was 0.07 h$^{-1}$. When cultivated on glucose, the specific growth rate was 0.08 h$^{-1}$ (data not shown) with approximately the same yield of biomass produced on substrate consumed ($Y_{X/S}$ equal to 0.5 g/g). This is a relevant characteristic of *T. reesei*, that utilizes xylose very efficiently. Unfortunately, xylose does not induce cellulase excretion. Figure 5 shows a similar experiment (culture B) where the xylose/lactose feeding-solution was substituted by xylose. The final obtained protein concentration was significantly lower (less than 7.5 g/L).

The comparison of specific protein productivity ($q_p$) over time in cultures A and B is shown in Figure 6. The specific protein production rate ($q_p$, in mg.p.g$^{-1}$.h$^{-1}$) was one of the hardest characteristics to extract from the experimental data but one of the most meaningful. The mean $q_p$ value over the production phase was 20-25 mg.p.g$^{-1}$.h$^{-1}$ for culture A and 0-5 mg.p.g$^{-1}$.h$^{-1}$ for culture B. The xylose appears as poor substrate for cellulase production when it is utilized as the sole carbon source. However, supplementation of this sugar by lactose (Culture A) enhances significantly the production performances.

**4.3 Cellulase Production on Hemicellulosic Hydrolysates as Major Carbon Source**

Hemicellulosic hydrolysates could be used instead of xylose during the growth phase or during the cellulase production phase as a major carbon source.

**4.3.1 Use of the Hemicellulosic Hydrolysates in the Growth Phase**

As mentioned in Section 3.1, hemicellulosic hydrolysates contains substantial amounts of acetic acid that inhibit the growth of the microorganisms. In the cellulase
production by *T. reesei*, various fermentation strategies can be applied to reduce acetic acid inhibition. For example, culture pH, initial cell mass of inoculum or both can be increased. Figure 7 shows the positive effect of increasing the pH from 4.8 to 5.5. Growth of *T. reesei* started immediately after pH shift.

### 4.3.2 Use of the Hemicellulosic Hydrolysates in the Production Phase

The hemicellulosic hydrolysate corresponds to the water extracts of steam-exploded biomass where pentoses are predominant. Before being used for cellulase biosynthesis, the hemicellulosic hydrolysates were mixed with lactose in the feeding solution as described by Warzywoda *et al.* (1992). A representative kinetics is shown in Figure 8.

The cellulase productivity on hemicellulosic hydrolysates/lactose was close to that on xylose/lactose with 0.18 g.L⁻¹.h⁻¹ (*Tab. 3*). The partial substitution of the main carbon source (xylose) by hemicellulosic hydrolysate did not significantly affect cellulase production by *T. reesei*.

### TABLE 3

<table>
<thead>
<tr>
<th>Culture</th>
<th>Carbon source in excretion phase</th>
<th>Final protein concentration (g.L⁻¹)</th>
<th>Protein productivity (g.L⁻¹.h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Xylose</td>
<td>7.1</td>
<td>0.05</td>
</tr>
<tr>
<td>B</td>
<td>Xylose/lactose</td>
<td>34.4</td>
<td>0.21</td>
</tr>
<tr>
<td>C</td>
<td>C5 hydrolysate/lactose</td>
<td>30.4</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* The bovine serum albumin was used as standard for protein measurement.

### 5 SOLVENT PRODUCTION BY CLOSTRIDIUM BEIJERINCKII

The LCB hydrolysates from acidic hydrolysis of LCB can be converted into ABE by using the anaerobic bacterium *C. beijerinckii*. The main problems that arise with this type of substrate are the slower degradation rate of xylose compared to glucose and the occurrence of growth inhibitors (carboxylics, phenolics and furanics) in hydrolysates.

#### 5.1 Metabolic Pathway of ABE Formation

The ABE formation pathways from glucose and xylose are shown in Figure 9.

With either glucose, xylose or arabinose as the substrate, catabolism of the substrate leads to pyruvate as a key intermediate. Pyruvate is concomitantly decarboxylated, reduced and thio-esterified in the so-called “phosphoroclastic reaction” catalyzed by the pyruvate: ferredoxin oxidoreductase. Ferredoxin, the electron acceptor of the reaction, is reduced but, due to its low
redox potential, it can cede electrons to $H^+$ through hydrogenase. This reaction sequence constitutes the “hydrogen valve” that is essential for the clostridial cell-functioning. The regeneration of ferredoxin into an oxidized form can also be performed using NAD$^+$ or NADP$^+$ as alternative electron acceptors.

The phosphoroclastic reaction forms an acetyl-CoA molecule that is the precursor of all the main products of the ABE fermentation. Acetyl-CoA gives acetic acid and ethanol in one process and acetoacetyl-CoA by a separate self-condensation process. In turn, acetoacetyl-CoA is the precursor of the C3 compounds, i.e. acetone in the ABE fermentation or isopropanol in the IBE fermentation and the precursor of the C4 compounds, i.e. butyric acid and butanol. The detailed pathway and the regulation of solventogenesis have been excellently described in numerous reviews (Gheshlaghi et al., 2009; Paredes et al., 2008; Sullivan et al., 2008; Papoutsakis, 2008; Lee et al., 2008; Dürr, 2008; Girbal and Soucaille, 1998; Rogers, 1986; Jones and Woods, 1986; Ranjan and Moholkar, 2012).

### 5.2 Fermentation Profile

According to the current classification of microorganisms, the solventogenic Clostridia belong to four species: *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutyolicum* and *C. saccharobutylicum* (Keis et al., 2001). In batch cultures, bacterial growth starts at the same time as the acetic and butyric acids excretions. When not regulated, the pH of the culture begins to drop for 12 to 18 h (Spivey, 1978). An almost-equimolar $H_2/CO_2$ gas mixture evolves from the culture medium. The acidogenic phase corresponds to the active microbial growth.
although exponential growth is short-lasting (3-4 h). In a second fermentation phase (solventogenesis), solvents are produced from the carbon source. The acetic and butyric acids previously excreted are more or less extensively reassimilated, accounting for the observed pH increase. The evolved gas mixture is enriched in CO₂ at the expense of H₂. The fermentation ceases when the toxicity of the accumulated butanol reaches a threshold level.

5.3 ABE Production from Lignocellulosic Hydrolysates

The large-scale development of ABE production on whole lignocellulosic hydrolysates was performed by IFP (presently IFPEN) in the 1980s at the experimental biotechnology facility of Soustons in 50 m³ working-volume reactors (Ropars et al., 1992; Marchal et al., 1992). The goal of the French alternative-fuel program of the time was to mix ABE with methanol, a cheap automotive fuel, to avoid fuel demixing in car tanks. Nevertheless, ABE can be also considered for use in upgrading the hemicellulosic fraction. In this process, the hemicellulosic fraction (often called the C5 fraction) results from water extraction of pretreated biomass. In contrast to cellulose, hemicellulose from LCB is solubilised during acidic or steam-explosion pretreatments. The C5 fraction is mainly composed of monomers and soluble pentosans with short carbon chains. As indicated above, the C5 fraction also contains phenolics, furanics and carboxylic acids that stem from lignin or carbohydrate degradation. As with _S. cerevisiae_, the degradation compounds act as inhibitors to clostridial growth. For efficient culturing, LCB-based media needs to be treated. Overliming, as described by Mohagheghi et al. (2006) was found efficient and made possible clostridial growth.

At IFPEN, a steam-exploded C5 fraction from wheat straw was obtained through the European program Biosynergy. Per liter, it contained 46 g of xylose, 5 g of arabinose, 9 g of glucose and 1 g of mannose. Furanics (5-HMF at 0.65 g/L and furfural at 1.8 g/L) and acetic acid (at 6.6 g/L) were also detected. The total dry weight was 15%. The C5 fraction was not directly fermentable. It was overlimed at 50°C for one hour and the resulting solid phase was discarded. The overlimed C5 fraction supplemented with nutrient salts of Mutschlechner et al. (2000) was fermented using _C. beijerinckii_ NCIMB 8052. Figure 10 shows the kinetic profile obtained.

Table 4 indicates the mass balance in ABE at the end of the culture.

Compared to ABE fermentation performed on glucose, ABE fermentation on the C5 fraction was slightly less productive. The final ABE titre is only 17.5 g/L compared to 20-25 g/L on glucose substrate. In addition, the fermentation time was longer (3 days versus 2 days), restricting the solvent productivity.
6 DISCUSSION

The use of the hemicellulosic fraction resulting from the lignocellulosic pretreatment is a prerequisite for the profitability of biofuels production, since this fraction represents up to 30% of the total biomass. Therefore, the conversion of C5 sugars into ethanol was expected to enhance the yield of ethanol produced, however pentoses were found to not be directly fermentable by the wild type strains of *S. cerevisiae*. In this article, we describe two other possible ways of using the hemicellulosic fraction, in order to increase the economic viability of the 2G biofuel process.

The first way is to use pentose sugars as a carbon source for the production of cellulases required for cellulose hydrolysis. There are actually three major types of cellulosases: endocellulases, exocellulases (cellobiohydrodylases) and cellobiases (β-glycosidase). Other cellulases, such as xylanases, are essential for the hydrolysis of lignocellulolytic materials but only represent a limited fraction of the excreted enzyme mixture. The specific composition of the produced enzymatic cocktail is largely dependent on the nature of the carbon source. An inducing substrate is also necessarily required for the expression of the cellulolytic enzymes. When associated with the cellulase inducer (*i.e.* cellobiose or lactose) in the feed, the hemicellulosic hydrolysates were found to significantly stimulate the xylanase activity in the excreted enzyme mixture.

Since it displays evident cellulase-inducing properties, lactose appears to be the most appropriate substrate for cellulase production. However, the price of lactose amounts to approximately one third of the enzyme cost, which is already as high as 30% to 50% of the whole process. In addition, when using lactose as the carbon source, the process is dependent on an external, commercial source of carbon. Therefore, the use of a process-derived substrate, *i.e.* hydrolysed hemicelluloses, would be a substantial improvement that would contribute to the decrease of the total cellulose cost, which is currently the major bottleneck in the process development.

The second method for taking advantage of pentose sugars is to convert them into solvents (ABE) using *C. acetobutylicum* or *C. beijerinckii*. As with *T. reesei*, furfural and 5-HMF are toxic to the bacterial cell. However, they can easily be reduced into their corresponding alcohols by *Clostridia*, as soon as the metabolic activity of the cell biomass is sufficiently high. With glucose as the carbon source, the high flux of NAD(P)H resulting from substrate degradation quickly reduces furfural or 5,5-HMF into furfuryl and 5-hydroxymethylfurfuryl alcohols. However, when xylose is used as substrate, it is biodegraded more slowly than glucose and the corresponding NAD(P)H flux may be insufficient to relieve the C5 fraction from furanic aldehyde inhibitors.

In the ABE fermentation on glucose/xylose mixtures, *C. acetobutylicum* ATCC 824 displayed a diauxic growth pattern, which highlights the catabolic repression of xylose uptake by glucose (Ounine *et al.*, 1985). The glucose repression for xylose utilisation was removed by disrupting the pleiotropic regulator CcpA (Ren *et al.*, 2010) that is required for the induction of the gapA operon encoding glyceraldehyde-3-phosphate dehydrogenase (Ludwig *et al.*, 2002). The ccpA-disrupted mutant utilised xylose and glucose concurrently with no CCR effect. In some other *Clostridium* strains, CCR did not occur (Liu *et al.*, 2010).

In order to increase the activity of transaldolase in PPP of *C. acetobutylicum*, the gene *talA*, which encodes transaldolase from *Escherichia coli* K-12, was cloned and overexpressed in *C. acetobutylicum* ATCC 824 (Cu *et al.*, 2009). When compared with *C. acetobutylicum* ATCC 824 wild type, the transformant bearing the *E. coli* talA gene (824-TAL) showed improved abilities for both xylose utilisation and solvent excretion. In fermentations on mixtures of xylose and glucose, the rate of xylose consumption and final solvents titres of 824-TAL were higher than the corresponding 824-WT. However, the transformant still expressed glucose repression of xylose uptake. Further efforts to stimulate PPP by metabolic engineering would undoubtedly improve the C5 sugar conversion by *Clostridium* and alleviate the inhibitory effect of furanics. Therefore, a major challenge for optimal use of LCB substrates in ABE production resides in accelerating PPP functioning. To this end, the gene *talA*, which encodes transaldolase from *E. coli* could be cloned and overexpressed in *Clostridium*.

The pretreatment of LCB remains a crucial step in the process that has admittedly to be improved. LCB pretreatment has usually been operated at high temperature under acidic conditions leads to the formation of a wide range of compounds, including acetic acid, furfural, 5-HMF and phenolics that inhibit the cultures of *T. reesei* or *C. acetobutylicum*. A great number of methods have been proposed in the literature in order to remove or to reduce the inhibitory effect of these compounds but many of the proposed methods are difficult to extrapolate at industrial scale. For example, enzymatic treatment by laccase and cation/anion-exchange resins are commonly considered as being too expensive when process do not produce any valuable by-product together with biofuels. In fact, a low cost alternative method is the adaptation of microorganisms to inhibitors by repeated subculturing. Other fermentation strategies could be used like increasing culture pH or initial cell biomass.
LCB It has usually been operated at high temperature. LCB is admittedly a cheap raw material for the production of biofuels. However, the processing of biomass into biofuel through fermentation is still in the initial steps of development when compared to the processing of petroleum. The development of industrial products from LCB in the so-called “biorefineries” meets with two types of problems: the price of raw materials processing and the rather low performances of the fermentations. Accordingly, the economic success of using LCB as a fermentation substrate in industry will largely be dependent on microbial capabilities to utilise C5 sugars efficiently. In a biorefinery context, C5 sugars might also be a realistic source for the production of carboxylic acids (acetic, lactic, propionic and fumaric acids, etc.) 2,3-butanediol or ethanol, by using unconventional yeasts derived from *S. cerevisiae*.

7 EXPERIMENTAL PROCEDURES

**Cellulase Production**

*Trichoderma reesei* strain CL-847 (Durand et al., 1984) has been used throughout this work. Enzyme productions were performed in 3 L working volume fermentors, using fed-batch technology as previously described (Pourquie et al., 1988; Warzywoda et al., 1992). Standard analytical procedures were used to assay for excreted proteins (Lowry et al., 1951).

**ABE Production**

*C. beijerinckii* NCIMB 8052 was used. Overliming of the hemicellulosic hydrolysate, resulting from extraction by water was performed according to Mohagheghi et al. (2006). Cultures for ABE production were carried out at 34°C in a 6 L magnetically-stirred bioreactor with 4 L working volume and no pH regulation. Solvents and carboxylic acids were determined according to Collas et al., (2012).

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