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1 **Hydrolysis and fermentation steps of a pretreated sawmill mixed feedstock for bioethanol**
2 **production in a wood biorefinery**

3

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10 **Abstract**

11 The aim was to demonstrate the feasibility of second-generation bioethanol production using
12 for the very first time a sawmill mixed feedstock comprising four softwood species,
13 representative of biomass resource in Auvergne-Rhône-Alpes region (France). The feedstock
14 was subjected to a microwave-assisted water/ethanol Organosolv pretreatment. The
15 investigation focused on the enzymatic hydrolysis of this pretreated sawmill feedstock (PSF)
16 using Cellic[®] Ctec2 as the enzyme, followed by fermentation of the resulting sugar solution
17 using *Saccharomyces cerevisiae* strain. The cellulose-rich PSF with 71% w/w cellulose
18 content presented a high saccharification yield (up to 80%), which made it perfect for
19 subsequent fermentation; this yield was predicted vs. time up to 5.2% w/v PSF loading using
20 a mathematical model fitted only on data at 1.5%. Finally, high PSF loading (7.5%) and
21 scaleup were shown to impair the saccharification yield, but alcoholic fermentation could still
22 be carried out up to 80% of the theoretical glucose-to-ethanol conversion yield.

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1 **Keywords:** Organosolv pretreatment, mixed sawdust, cellulose hydrolysis, fermentation,
2 lignocellulosic bioethanol

3 **1. Introduction**

4 Biofuels, in particular second-generation biofuels, have gained interest from academic
5 research, government, and large companies nowadays. Lignocellulosic biofuel production is
6 indeed widely accepted in the society as it is perceived as non-competitive with agri-food
7 (Bryngemark, 2019). Also, these biofuels represent a renewable option to replace the
8 depleting oil supply and can help mitigate the climate change impacts resulting from fossil
9 fuels. For low-cost biofuel production, lignocellulosic biomass is a potential candidate
10 feedstock because its price is estimated as being the lowest compared to starch which is
11 presently used to produce bioethanol (Chovau et al., 2013).

12 Auvergne Rhône-Alpes is one of the largest forest regions in France with a forest density
13 estimated at $439 \cdot 10^6 \text{ m}^3$. As a consequence, the local wood processing industries generate a
14 significant amount of sawdust or other wastes that are not valorized otherwise than being
15 burnt for heat in this region. An alternative way of valorization of this waste is the production
16 of second-generation bioethanol in order to develop a reliable and sustainable regional energy
17 model that fits well with the energy transition policy and the fight against climate change.

18 This could have the advantage of promoting the valorization of local wood waste with a view
19 to setting up a forest biorefinery. Beyond the regional impact, large-scale second-generation
20 biofuel facilities could also contribute to enhance the security of energy supply and, more
21 generally, to strengthen the world economy (Sarks et al., 2014).

22 To increase lignocellulose digestibility, numerous lignocellulose pretreatment methods have
23 been matured. Among all the thermomechanical and chemical processes, Organosolv
24 pretreatment can be considered to be an eco-friendly and attractive method to remove lignin

1 from lignocellulose materials using pure or diluted organic solvents in order to purify
2 cellulose (Akgul and Kirci, 2009; Zhao et al., 2009). Thus, Organosolv pretreatments are very
3 efficient techniques for the production of second-generation bioethanol because they can
4 provide both cellulose and lignin with high purity, and minimize at the same time the
5 production of fermentation inhibitors (Mupondwa et al., 2017).

6 As a subsequent step to pretreatment, hydrolysis transforms the cellulose and the remaining
7 hemicelluloses into fermentable sugars. The enzymatic hydrolysis step has been recognized as
8 a major techno-economic bottleneck in the whole wood-to-ethanol bioconversion process. For
9 example, the enzymatic hydrolysis and fermentation of starch-based substrates (e.g. corn) are
10 generally performed at a substrate loading greater than 20% solids, leading to a final ethanol
11 content of the order of 10% (w/v) (Zhang et al., 2009). Conversely, the standard enzymatic
12 hydrolysis of lignocellulosic biomass is generally carried out at a substrate proportion of less
13 than 5% solids content. This results in a concentration of sugar lower than 4% (w/v) in the
14 hydrolysate and, subsequently, an ethanol concentration less than 2% (w/v).

15 Elevating the substrate loading during the enzymatic hydrolysis should result in an increase in
16 the concentration of sugar and a higher final ethanol concentration after fermentation. This
17 strategy will allow cutting the costs of the bioconversion process, *e.g.* by reducing the
18 operational and capital expenditures of hydrolysis and fermentation, and by saving energy in
19 the distillation/evaporation and other downstream processes. When proceeding to a techno-
20 economic assessment on enzymatic hydrolysis, Stenberg et al. (2000) and Wingren et al.
21 (2003) reported that an augmentation in the proportion of the substrate from 5% to 8% (w/v)
22 could diminish the total production cost by almost 20%. A further increase in the substrate
23 proportion could then result in even greater savings. Nevertheless, their investigation
24 conducted at that time failed to achieve effective hydrolysis at a substrate proportion greater
25 than 10% using simultaneous saccharification and fermentation approaches (SSF), or separate

1 hydrolysis and fermentation (SHF). The authors had thus identified several impediments to
2 high substrate hydrolysis, among which the cases were: (i) a high concentration of fibrous
3 materials reduces the mass transfer rate; (ii) a high level of inhibiting substances is produced;
4 (iii) a high sugar concentration leads to severe inhibition effects of the finished products.
5 Similarly, Qiu et al. (2017) concluded that increasing initial solid concentrations above 2%
6 w/w reduced significantly the conversion rate.
7 To circumvent these issues, process optimization through mathematical modeling is the key.
8 Thus, various models have been proposed to describe enzymatic hydrolysis. The simplest
9 approach considers conventional or modified Michaelian kinetics with competitive, non-
10 competitive or uncompetitive inhibition, whereas the most complex modeling strategy may
11 take cellulose polymerization degree or particle size and morphology through a population
12 balance into account (Zhang et al., 2014; Lebaz et al., 2015). Alternative mechanistic models
13 sometimes account for chemical or physical phenomena which may affect the kinetics of
14 enzymatic hydrolysis of cellulose, such as concurrent reactions (Tsai et al., 2014), adsorption
15 equilibria without or with inactivation of adsorbed cellulase (Kadam et al., 2004; Wang and
16 Feng, 2010; Tervasmäki et al., 2017), fractal kinetics for heterogeneous reactions (Ye and
17 Berson, 2011), or mixing conditions through mass transfer rate (Kinnarinen et al., 2012;
18 Wojtusik et al., 2016). It must be pointed out that the complete mechanism of enzymatic
19 hydrolysis is still unknown, and could also depend on the type and purity of the substrate and
20 of the enzymes.

21 In this work, the goal was, therefore, to hydrolyse and ferment an industrial and readily
22 available sawmill feedstock previously pretreated through an “Organosolv pretreatment”
23 based on an original microwave heating (Alio et al., 2019). This process presents the
24 simultaneous advantage to produce high quality lignin that can be used in the closed-loop
25 biorefinery concept with potentially high added-value coproducts. So, this study focussed first

1 on glucose concentration and cellulose-to-glucose conversion yield as a function of the
2 operating parameters in a batch enzymatic hydrolysis process; from these data, a model aimed
3 to predict the glucose concentration, and the cellulose-to-glucose conversion yield could be
4 developed. Then, the feasibility of bioethanol production by fermentation using the obtained
5 enzymatic hydrolysate was studied using the yeast *Saccharomyces cerevisiae*. Finally, the
6 scale-up from flasks to batch bioreactors of the hydrolysis and fermentation steps for the
7 production of second-generation bioethanol was investigated.

8 **2. Materials and methods**

9 **2.1. Feedstocks and reagents**

10 Organosolv pretreated samples of mixed sawdust from four softwood species were used: fir,
11 spruce (*Picea abies*), Scots pine (*Pinus sylvestris*), and Douglas fir, respectively. The sawdust
12 mixture was provided by a local sawmill company (Les Scieries du Centre Marcel Esbelin et
13 Cie), located in Cournon d'Auvergne (France), and was extracted from a monthly feedstock.
14 The initial composition of the sawdust mixture was determined to be 44.3% \pm 0.5% glucan
15 (dry wt.), 25.6% \pm 0.2% hemicellulose (dry wt.), 26% \pm 3% Klason lignin (dry wt.), ash
16 content 0.3% \pm 0.2% (dry wt.), and 3.2% \pm 0.2% extractives (dry wt.) (Alio et al., 2019).

17 The pretreated mixed sawdust was subjected to a microwave-assisted Organosolv
18 pretreatment in which four parameters were studied to optimize the fractionation of the wood,
19 including cellulose recovery yield and cellulose purity, lignin recovery yield, and the absence
20 of formation of inhibitors; these parameters were, namely: (i) the concentration of sulfuric
21 acid (H₂SO₄) as a catalyst; (ii) the ethanol/water ratio in the extraction solvent; (iii) the
22 treatment temperature; (iv) the process time. The optimal conditions determined
23 experimentally on sawdust could be summarized as follows: an ethanol/water ratio of 60:40
24 with 0.25% H₂SO₄ for one-hour extraction at 175°C. These conditions made it possible to

1 remove 50% of the lignin while preserving $82\% \pm 3\%$ of the initial cellulose with a purity of
2 $71\% \pm 3\%$ w/w. The composition of the pretreated sawdust mixture was then: 70.6% w/w
3 cellulose, 9.8% w/w hemicellulose, and 19.6% w/w lignin, as determined by strong acid
4 hydrolysis (Alio et al., 2019).

5 **2.2. Enzymes and other chemicals**

6 The enzymatic hydrolysis was carried out using an enzyme preparation: Cellic[®] Ctec 2
7 (Novozymes, Denmark). The activity on filter paper fibers (FPU) was determined as
8 described by Ghose (1987), and the protein content as described by Bradford (1976). Thus,
9 the obtained activity was found to be 189 FPU/mL and the protein content as 77.0 ± 0.3
10 mg/mL. The chemicals, such as acetate buffer solution (pH 4.8), streptomycin antibiotics and
11 other chemicals (reagent grade) were purchased from Sigma-Aldrich (France).

12 **2.3. Enzymatic hydrolysis**

13 All enzymatic hydrolysis runs were performed according to the NREL standard procedure
14 (Selig, 2008). All experiments were carried out using the pretreated sawdust mixture obtained
15 through the best pretreatment previously described. Depending on the assays, pretreated
16 sawdust mixture solids could be oven-dried (110°C for 24h) or not. Enzymatic hydrolysis of
17 pretreated mixture was performed in 150 mL Erlenmeyer flasks under batch conditions at
18 50°C in a shaker water bath (Julabo SW22, France) with a mixing speed of 180 rpm according
19 to Mukasekuru et al. (2018). Hydrolysis was carried out in a 50 mM acetate buffer solution at
20 pH 4.8 using the cocktail of enzyme mentioned above and streptomycin antibiotics were
21 added to prevent contamination. Different biomass loading concentrations were tested: 1.5 %,
22 3.75 %, 4.5%, and 5.2 % w/v. These were selected because the homogeneity of the liquid-
23 solid dispersion could be maintained, despite the increase in viscosity of this dispersion in this
24 range, which was not the case at higher solid substrate loadings. The carbohydrate profile in

1 the hydrolysate was determined using HPLC, as described below in **section 2.6**. The
2 enzymatic hydrolysis yield was calculated using the following equation:

$$3 \text{ Cellulose – to – glucose conversion yield (\%)} = 100 \times \frac{C_{glucose}(\frac{g}{L}) \times V_{liquid}(L) \times 0.90}{m_{solids}(g) \times x_{cellulose}} \quad (1)$$

4 where $C_{glucose}$ is the concentration of glucose determined by HPLC (g/L), V_{liquid} represents
5 the volume of the liquid (L), 0.90 is the correction factor for the conversion of cellulose-to-
6 glucose, $x_{cellulose}$ is the mass fraction of cellulose (g cellulose/g solid) and m_{solids} is the mass
7 of dry solids (g).

8 **2.4. Fermentation**

9 For the fermentation step, the yeast *Saccharomyces cerevisiae* ATCC 7754 was used. The
10 strain was supplied as a lyophilizate, taken in a sterile water and spread onto YM agar-agar
11 (Yeast Medium, Difco 0712-01-8), before incubated 24 hours at 28 °C. The strain was then
12 stored at 4°C and subcultured on a Petri dish 24 hours, before being used in the culture vessels
13 (Bahry et al., 2017). Then, the fermentation itself was carried out in 125 mL Erlenmeyer
14 flasks and the hydrolysate obtained after the enzymatic step was used as a culture medium.
15 First, the culture medium (denoted as “unenriched hydrolysate” or UE.H) was considered as
16 the control assay in order to assess the intrinsic potential of the hydrolysate to undergo growth
17 and fermentation. The second culture medium (denoted as “enriched hydrolysate” or E.H)
18 was supplemented with additional elements defined by Kristiansen (1994). The biomass
19 growth was followed by spectrophotometry at 550 nm (Biomate 3S, UV/vis
20 spectrophotometer, Thermo Scientific, France), and the dry matter was determined by
21 gravimetry.

1 ***2.5. Enzymatic hydrolysis and fermentation scale-up***

2 For the scale-up experiments, enzymatic hydrolysis was conducted as described in **section 2.3**
3 using a biomass concentration of 7.5% w/v in a 1000 mL glass bottle with a working volume
4 of 800 mL; this higher substrate loading was allowed by the improved mixing conditions, so
5 as to maintain spatially homogenous reaction rate and concentrations for sampling purpose.
6 Then, the fermentation step was carried out in a 500 mL bioreactor (Infors HT, Multitron 2)
7 with a working volume of 400 mL. The hydrolysate obtained after the enzymatic hydrolysis
8 was completed as described in **section 2.4**. Only (E.H) was used as the culture medium.

9 ***2.6. Sugar, ethanol and inhibitors quantification***

10 In order to monitor the evolution of the carbohydrate concentrations during the enzymatic
11 hydrolysis and during fermentation, samples of 1 mL were taken at specific time intervals. In
12 the case of enzymatic hydrolysis, enzymes were first deactivated in all samples at 100 °C for
13 10 min. Prior to analysis, each sample was deproteinized to prevent clogging of
14 chromatography column. For this purpose, 125 µL of barium hydroxide solution (Ba(OH)₂;
15 0.3 M) and 125 µL of zinc sulfate solution (ZnSO₄, 7H₂O; 5% w/v) were added to the
16 samples. After centrifugation (Thermo scientific, France) for 5 minutes at 10,000 g, the
17 supernatant was filtered using a 0.2 µm cellulose acetate filter (Chromafil, Germany). The
18 concentrations of glucose, cellobiose, xylose, ethanol, and by-products (formic acid, levulenic
19 acid, and acetic acid) were measured using a high-performance liquid chromatography
20 (HPLC) device (1260 Infinity Quaternary LC system, Agilent Technology, USA). This was
21 equipped with two ionic exclusion columns in series (Rezex ROA 300×7.8 mm, Phenomenex,
22 USA). The mobile phase was a solution of 5 mM sulfuric acid at 0.7 mL/min flowrate.
23 Products detection was done using a refractometer (HP 1100 series, Agilent Technologies,
24 USA).

1 **2.7. Data analysis and number of samples replicate**

2 All the results reported for batch hydrolysis and fermentation are the mean values of at least
3 two replicates based on two batches of repeated experiments under the same conditions. Three
4 samples were taken for each time point. The mean and standard deviation were calculated by
5 Excel[®] Sheets version 1902 (Microsoft[®] Office package).

6 **2.8. Enzymatic hydrolysis kinetic modeling**

7 As described in **section 1**, various models had been proposed in the literature to describe this
8 process. A rapid analysis of preliminary data had shown that the rate of glucose production
9 could not be fitted by a Lineweaver-Burk plot, which suggested a more complex mechanism
10 than classical or modified Michaelian kinetics as a function of substrate content. Similarly,
11 glucose production rate increased with substrate loading, so that the effect of enzyme mass
12 transfer resistance never seemed to become predominant, despite the increase in dispersion
13 viscosity. Actually, the initial reaction rate appeared to be nearly proportional to the initial
14 substrate loading, while reaction rate decreased over time when substrate consumption
15 proceeded, indicating a limitation by enzyme availability. Among the possible mechanistic
16 models able to simulate the enzymatic hydrolysis of the pretreated sawdust mixture with the
17 enzymatic cocktail, an approach similar to that proposed by Kadam et al. (2004), considering
18 enzyme adsorption, was adopted. Enzymatic hydrolysis of lignocellulosic materials is a
19 heterogeneous reaction where the adsorption of enzymes is one of the key steps in the overall
20 reaction mechanism. Thus, hydrolysis can be seen as a succession of two rate-limited reaction
21 steps schematized in the following equation: a heterogeneous reaction involving
22 endoglucanases (EG) and cellobiohydrolases (CBH) to produce cellobiose, and then a
23 homogeneous reaction where cellobiose is transformed into glucose by β -glucosidases (BGL),
24 as follows:

1 Cellulose (EG / CBH) $\xrightarrow{r_1}$ Cellobiose (BGL) $\xrightarrow{r_2}$ Glucose

2 The transformation rate of the cellulose r_1 by the pair EG/CBH considering the (competitive)
3 inhibition effect by the products (cellobiose and glucose) is given by:

$$4 \quad r_1 = \frac{k_{1r} \times E_{1b} \times R_S \times S}{1 + \frac{C_C}{K_{1IC}} + \frac{C_G}{K_{1IG}}} \quad (2)$$

5 Then, the rate of transformation of cellobiose into glucose r_2 by BGL is described by a
6 Michaelian kinetics:

$$7 \quad r_2 = \frac{k_{2r} \times E_2 \times C_C}{K_{2M} \left(1 + \frac{C_G}{K_{2IG}} \right) + C_C} \quad (3)$$

8 where S , C_C , C_G , E_{1b} and E_2 represent the respective concentrations of substrate, cellobiose and
9 glucose and of the EG/BGL and BGL enzyme species (g.kg^{-1}), the subscript b corresponding
10 to bound enzymes. In these equations, K_{iIG} , K_{1IC} represent the inhibition constants of glucose
11 in reaction i and of cellobiose (g.kg^{-1}), respectively; K_{2M} designates the enzyme Michaelis
12 constant in r_2 (g.kg^{-1}), and finally, k_{1r} and k_{2r} are the respective kinetic constant of r_1 and r_2 ,
13 and R_S the substrate reactivity. Adsorption was modelled using a Langmuir isotherm, which
14 assumes that equilibrium is rapidly reached and can be described by

$$15 \quad E_{1b} = \frac{E_{1max} \times K_{1ad} \times E_1 \times S}{1 + K_{1ad} \times E_1} \quad (4)$$

16 where the amount of free enzyme E_1 is related to the amount of bound enzyme E_{1b} by the
17 enzyme conservation law and two additional parameters: K_{1ad} the dissociation constant for the
18 enzyme/substrate complex, and E_{1max} the maximum mass of enzyme that can adsorb onto
19 cellulose, both expressed in g enzyme/g cellulose. Equation (4) also expresses the relationship
20 between E_{1b} and substrate concentration S .

21 From this model, a set of differential equations vs. time was derived, which was solved using a
22 first-order Euler forward algorithm. The equations were finally coded and solved using the
23 MATLAB programming environment. Langmuir adsorption parameters were derived from

1 Kadam et al. (2004), while the kinetic parameters were deduced by fitting the model with
2 experimental data.

3 **3. Results and discussion**

4 **3.1. Enzymatic hydrolysis**

5 *3.1.1. Effect of size reduction on the dried substrate*

6 Prior to the preliminary assays of enzymatic hydrolysis, pretreated sawdust mixture was oven-
7 dried (110°C for 24h) for dosage purpose (Alio et al., 2019). Then, this was subjected to
8 enzymatic hydrolysis, conducted at 1.5% (w/v) substrate loading, with an enzyme content of
9 50 FPU/g of dry pretreated sawdust mixture for two different particle sizes. A first fraction of
10 the solids was crushed using a domestic blender to reach an average particles size passing
11 through 0.5 mm sieve (IG), while the other one was kept intact with an average size larger
12 than 0.5 mm (SG). Thus, the hydrolysis of SG resulted in a glucose concentration about $1.6 \pm$
13 0.3 g/L, while the hydrolysis of IG produced approximately 3.1 ± 0.1 g/L glucose in the final
14 hydrolysate. When the cellulose-to-glucose conversion yield was calculated (**Fig. 1** Erreur !
15 Source du renvoi introuvable.), it was found that only $12.4\% \pm 0.7\%$ of the cellulose present
16 in the SG pretreated sawdust mixture was converted to glucose within 8 days of incubation.
17 Reducing the substrate particle size to a smaller granulometry (IG) doubled cellulose-to-
18 glucose conversion yield, which reached approximately $24\% \pm 1\%$. Other studies had already
19 investigated the effect of particle size on the rate of enzymatic hydrolysis of cellulose (Dasari
20 and Berson, 2007; Fu et al., 2018; Sangseethong et al., 1998; Yeh et al., 2010). According to
21 Sangseethong et al. (1998), reducing particle size from 0.10 to 0.02 mm at 0.1% (w/v)
22 substrate proportion almost doubled the saccharification rate of cellulose (Avicel).
23 Nevertheless, with the same size reduction, only 50% increase in the saccharification rate was
24 observed at 2% (w/v) substrate concentration according to Yeh et al. (2010). Finally, Zeng et

1 al. (2007) also reported that reduction of size from 425–710 to 53–75 μm raised glucose
2 production rate from 0.1 to 0.18 g/L/h. In agreement with the literature, experimental results
3 obtained in this study highlight that a reduction in particle size significantly enhanced the
4 hydrolysis rate of cellulose. However, the grinding process may be costly because of its
5 important power requirements that counterbalance its beneficial effects on cellulose-to-
6 glucose conversion yield (Fu et al., 2018; Shastri et al., 2014). Shastri et al. (2014) determined
7 the evolution of the cost of *Miscanthus* and switchgrass size reduction as a function of output
8 particle size using a hammer mill for the following particle sizes: 1, 2, 4, 6, 8, 12.7, 16, 25.4
9 mm. According to this study (**Table 1**), the costs were dramatically increased when particle
10 size was reduced below 2 mm. However, for both the *Miscanthus* and the switchgrass, the
11 optimal particle size ranged between 4 and 6 mm, and the corresponding total cost (grinding,
12 storage and transportation) were about \$55 and \$61 per ton for *Miscanthus* and switchgrass,
13 respectively.

14 3.1.2. Effect of substrate humidity

15 **Fig. 1** also displays the evolution of the cellulose-to-glucose conversion yield as a function of
16 time for two different substrate humidity values: oven-dried pretreated substrate, and wet
17 pretreated substrate. Both runs were performed on 0.5–1 mm pretreated substrate particule
18 size to cut the cost of the grinding process, at 1.5% (w/v) substrate concentration and an
19 enzyme loading of 50 FPU/g of pretreated substrate. Regarding these results for both cases, it
20 can be noticed that the enzymatic hydrolysis was strongly affected by the humidity level. So,
21 a low conversion rate was observed with the dried pretreated substrate with a maximum
22 conversion yield reached after 8 days of $12.4 \pm 0.5\%$, while a value of $56\% \pm 2\%$ was reached
23 for the wet pretreated substrate in the same period. There is no doubt that this difference was
24 due to the low digestibility of the dried substrate, linked itself to a structure modification of

1 the substrate as a direct impact of the drying process. According to Kang et al. (2018), the
2 drying step of NaOH pretreated *Eucalyptus* samples caused a decrease in the volume and the
3 surface area of the porosity of wood of about 45% of the initial value. So, the pore volume
4 and surface area of dried biomass substrates should not provide sufficient access to the
5 enzymes for adsorption onto the cell surfaces, thus significantly slowing down the enzymatic
6 hydrolysis of the cellulose. Moreover, these authors also explained the structure modification
7 as a result of a significant pore collapse in the cellulose fibers during the oven-drying
8 procedure, based on the low adsorption capacity of dried pretreated *Eucalyptus* samples.
9 Bhagia et al. (2018) also reported that drying possibly caused pore collapse and thereby
10 impaired cellulose accessibility. In their investigations, Huang et al. (2010) advocated,
11 however, that the changes in the chemical composition might also be an alternative reason, as
12 the removal of lignin and hemicellulose had also a significant effect on the surface area and
13 the pore size distribution. Another possible reason in the present study could be linked to the
14 Organosolv pretreatment that did not significantly delignify the raw sawdust material, as a
15 significant fraction of the lignin remained in the resulting pretreated substrate ($39\% \pm 1\%$
16 w/w of the raw feedstocks lignin content, which represents $20\% \pm 2\%$ w/w of the pretreated
17 substrate). High lignin content is, indeed, known to hinder enzymatic hydrolysis through the
18 nonproductive binding of cellulase enzymes. Likewise, as a non-cellulose component,
19 hemicellulose is generally considered as a physical hindrance in enzymatic hydrolysis of
20 cellulose, and prevents the access of cellulase from cellulose surface (Qiu et al., 2017).
21 Finally, it emerges from experimental data that there is no need to dry the pretreated substrate,
22 which saves energy and enhances hydrolysis rate at the same time, and that hydrolysis must
23 be carried out rapidly after pretreatment to prevent natural drying.

24

1 3.1.3. Effect of enzyme loading on the wet substrate

2 The wet pretreated sawdust mixture was hydrolyzed at both 50 and 70 FPU/mL enzyme
3 content under the same conditions as applied before, *i.e.* 1.5% substrate loading and particle
4 size between 0.5–1 mm. As shown in **Fig.1**Erreur ! Source du renvoi introuvable., it is
5 apparent that increasing enzyme loading leads to an increase in sugar concentration and
6 cellulose-to-glucose conversion rate. Thus, the pretreated sawdust with the higher enzyme
7 loading demonstrated a higher hydrolysability which reached nearly 68% of the available
8 cellulose. Hydrolysis using 70 FPU/g enzyme loading also yielded a greater glucose
9 concentration that reached 7.8 ± 0.2 g/L in the hydrolysate after 10 days, while 5.7 ± 0.5 g/L
10 and only about 50% conversion yield were obtained at 50 FPU/g. Thus, 50 FPU/g substrate
11 induced a 28% reduction in enzyme loading in comparison to the maximum value, which
12 reduced the final glucose amount by about 27% (from 8 to 6 g/L), *i.e.* approximately the same
13 value. The same conclusions were reported by Zhang et al. (2009) when conducting an
14 enzymatic hydrolysis on pretreated hardwood substrates with three different enzyme charges
15 (3, 10 and 20 FPU/g). Moreover, in another study by Bhagia et al. (2018), 1% glucan loading
16 (Avicel, 97% cellulose and 3% xylan) was enzymatically hydrolyzed using Accelerase® 1500
17 at two different enzymes loadings, 5 and 30 mg of enzyme/100 ml of solvent, designated as
18 low and high cellulase loadings, respectively; as a result, these authors observed that the
19 kinetic curves displayed a cellulose conversion yield of 60% for up to 17 days of enzymatic
20 hydrolysis at low enzyme loading, while the conversion yield reached 97% for up to 9 days at
21 high enzyme loading. Similar results were also reported in other studies conducted in quite
22 identical experimental conditions of the present work (Stenberg et al., 2000; Zhang et al.,
23 2009). As a conclusion, enzymatic hydrolysis remains strongly dependent on enzyme content,
24 even though 1.5% (w/v) substrate loading used is the lowest value used in this work.

1 3.1.4. Effect of wet substrate loading

2 Erreur ! Source du renvoi introuvable. depicts the evolutions of glucose concentration and
3 hydrolysis yield as a function of time for four different wet pretreated substrate loadings (1.5%,
4 3.75%, 4.5%, and 5.2% w/v) with a particle size between 0.5 and 1 mm, at 50 FPU/g to.
5 Basically, it can be noted that, in general, the higher the amount of hydrolyzed substrate, the
6 higher the amount of glucose produced, as expected. However, a surprising result was that the
7 conversion yield first increased rapidly when the substrate loading was increased from 1.5% to
8 3.75%, and then became nearly constant, independent from substrate loading (**Fig. 2**). This
9 means that the hydrolysis rate was nearly proportional to the initial substrate loading, even
10 though it decreased over time when substrate consumption proceeded. Thus, a cellulose-to-
11 glucose conversion yield about $77 \pm 3\%$ could be reached when wet substrate loading was
12 between 3.75% and 5.2% (w/v). As the conversion yield was not significantly different for
13 3.75%, 4.5%, and 5.2% (w/v) substrate proportion, the consequence is that the glucose
14 concentration increased nearly proportionally to the substrate content up to 32 ± 1 g/L at 5.2%
15 (w/v) substrate loading, corresponding to a conversion yield of $80\% \pm 2\%$ after 12 days of
16 hydrolysis.

17 Similar outcomes on the evolution of glucose concentration had been already reported by
18 several studies (Zhang et al., 2009; Qiu et al., 2017). For example, Qiu et al. (2017)
19 investigated the effect of substrate loading during enzymatic hydrolysis using a pretreated
20 wheat straw by phosphoric acid and hydrogen peroxide. Four substrate loadings (2, 10, 15,
21 and 20%) were tested, and it was pointed out that the highest substrate consistency led to the
22 highest glucose concentration in the final hydrolysate. However, the cellulose-to-glucose
23 conversion yield decreased slightly when the substrate loading increased.

24 Finally, contrary to expectations which predicted an increase in glucose concentration coupled
25 to a decrease in yield when substrate content was increased, it was found that the lowest

1 substrate loading investigated, 1.5% (w/v), lead simultaneously to the lowest conversion yield
2 ($57\% \pm 3\%$) and the lowest concentration of glucose (6.7 ± 0.3 g/L), whereas the best results
3 were achieved for the highest proportion of substrate (5.2% w/v) in terms of yield and glucose
4 content. Thus, by increasing pretreated substrate loading, higher concentrations of
5 fermentable sugars are available and then, higher ethanol concentration can be achieved. Due
6 to mixing issues resulting from the high viscosity of the cellulose suspension, experiments in
7 150 mL Erlenmeyer flasks had to be limited to 5.2% (w/v) substrate loading, but a higher
8 concentration will be tested in the scale-up assays in **section 3.4**, as better mixing condition
9 could be achieved.

10 **3.2. Fermentation tests in flasks**

11 To produce bioethanol, the upstream enzymatic hydrolysis process must provide, as much as
12 possible, a hydrolysate highly concentrated in glucose, accompanied by minor quantities of
13 other hydrolyzed components (hemicellulose, inhibitors, etc.). The hydrolysates obtained
14 from the enzymatic hydrolysis of 3.75% (w/v) substrate loading present a satisfactory glucose
15 concentration to critically investigate the capacity of the yeast to develop fermentation (24.7
16 g/L glucose). As already stated, (UE.H) corresponds to the medium without supplementation,
17 whereas the culture medium is supplemented by the minerals and vitamins nutrients as
18 described by Kristiansen (1994) in (E.H), both being prepared with an identical concentration
19 of pure glucose.

20 **Fig. 3** illustrates the evolution of glucose, ethanol and dry cell weight concentrations for the
21 two hydrolysates tested (UE.H and E.H). Regarding the results, the sugars undergo ethanol
22 fermentation in both cases, but the difference lies in the rates of glucose and of ethanol
23 production, and consequently, in the concentration values compared at the same time.

24 In the Kristiansen's medium, all the sugars were consumed after 9 hours and the produced
25 ethanol reached approximately 9.5 ± 0.4 g/L (data not shown). In the case of the (E.)H assay,

1 the maximum concentration of ethanol was found to be 10.1 ± 0.7 g/L after 24 hrs., which
2 constituted 72% of the theoretical glucose-to-ethanol conversion yield (0.51 g ethanol per
3 gram of glucose). In parallel, the maximum concentration of ethanol was only 3.1 ± 0.2 g/L at
4 the same time for the (UE.H) assay. The latter concentration represented only $22 \pm 1\%$ of the
5 theoretical glucose-to-ethanol conversion yield. This demonstrates the need to enrich the
6 hydrolysates with components necessary for the growth of yeast, such as the source of
7 phosphorus (KH_2PO_4), and nitrogen ($(\text{NH}_4)_2\text{SO}_4$), as well as various salts and vitamins
8 (Kristiansen, 1994). **Table 2** summarizes the kinetic parameters calculated from the
9 fermentation inputs and outputs. Finally, the fermentation of the glucosidic hydrolysate by a
10 strain of *Saccharomyces cerevisiae* led us to reach an alcoholic fermentation yield close to
11 80% of the theoretical yield, which seems consistent with the absence of inhibiting
12 compounds produced during the pretreatment and hydrolysis steps.

13 ***3.3. Scale-up of the enzymatic hydrolysis and fermentation processes***

14 The enzymatic hydrolysis scale-up was carried out in a 800 mL working volume under the
15 same conditions applied to the other pretreated substrate loadings, except that better mixing
16 conditions allowed to increase solid loading up to 7.5 % (w/v) pretreated sawdust mixture
17 substrate. This value is closer to the solids loading range proposed by Stenberg et al. (2000)
18 and Wingren et al. (2003). In these assays, the final glucose concentration was measured at
19 about 34.5 g/L, which represents a cellulose-to-glucose conversion yield of $59 \pm 2\%$ for up to
20 12 days (data not shown). This value is lower than in 150 mL flasks, which can probably be
21 explained by the high viscosity of the 7.5% (w/v) substrate suspension. The fermentation step
22 was then performed in a 500 mL bioreactor using the obtained hydrolysate enriched with
23 vitamins and minerals. The fermentation assays were carried out for 24 hrs.; the produced
24 ethanol and the decrease in glucose contents were monitored during the fermentation.

1 According to **Fig. 4**, approximately all the glucose was consumed after only 8 hours. The
2 produced ethanol in fermentation broth reached nearly 16 ± 2 g/L at the same time, which
3 represents almost 80% of the theoretical glucose-to-ethanol conversion yield.

4 According to the information provided by **Table 2**, comparison of the control-based
5 fermentation and the enriched hydrolysate fermented in bioreactor demonstrates the interest to
6 use a bioreactor, in terms of yields and rates. Moreover, the high substrate proportion during
7 enzymatic hydrolysis not only reduces the capital cost for installation of hydrolysis vessel, but
8 it also more significantly provides a concentrated glucose stream for subsequent fermentation
9 which will lead to important savings in the distillation cost (Zhang et al., 2009).

10 **3.4. Model for enzymatic hydrolysis**

11 From the previous sections, it emerges that enzymatic hydrolysis is the bottleneck of the
12 process. Thus, modelling must focus on this step. Using the adsorption-reaction model
13 developed in **section 2.8** and inspired by Kadam et al. (2004) to fit enzymatic experimental
14 data, adjusted model parameters were derived from the data monitored for the enzymatic
15 hydrolysis performed at 3.75% (w/v) substrate loading in 150 mL Erlenmeyer flasks, as
16 described in **section 3.2**. The estimated value for maximum glucose concentration was found
17 to be 19.3 g/L versus 21.4 ± 0.2 g/L derived experimentally for 3.75% (w/v) substrate. **Fig.**
18 **5** displays the comparison between calculated and experimental results after using the
19 adjusted parameters in the model ($k_{1r} = 3.61$ g/mg.h, and $k_{2r} = 11.5$ g/mg.h), not only for 3.75%
20 substrate loading, but also for 4.5% and 5.2% (w/v). The plot of glucose concentration as a
21 function of time shows that the model correctly fits glucose production for the three
22 concentrations until day 8, whereas glucose production is underestimated for longer times,
23 especially when loading is increased. However, the model was not able to predict correctly
24 the scale-up in a 1000 mL bottle: it overestimated about 38.2 g/L glucose production the
25 experimental value at day 12, while it underestimated experimental values at lower loading in

1 **Fig. 5.** This result agrees with the decrease in conversion yield and with the effect of
2 suspension viscosity at high substrate loading that slows down mass transfer phenomena, as
3 Kadam's model assumes a Langmuir equilibrium model for enzyme adsorption, accounting
4 only for the kinetics of chemical steps. This highlights that more complex models are
5 probably required and that the respective influences of mixing conditions and dispersion
6 rheology must be studied for scale-up purpose.

7 **4. Conclusion**

8 The feasibility of lignocellulosic bioethanol production using a sawmill mixed feedstock of
9 softwood species was assessed. Enzymatic hydrolysis, applied to an undried microwave-
10 assisted water/ethanol Organosolv pretreated substrate (71% w/w cellulose) with particle size
11 higher than 0.5 mm, reached 80% saccharification yield with up to 5.2% (w/v) substrate
12 loading. Higher loading decreased this yield, but higher ethanol concentration could be
13 achieved from subsequent fermentation using *Saccharomyces cerevisiae*, e.g. 16 g/L when
14 substrate loading was 7.5% (w/v). As an outcome, this process which tends to a closed-loop
15 biorefinery, is promising for regions where only mixed softwood feedstock is available.

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18 Development Fund (FEDER/ERDF) to promote the valorization of local feedstocks (wood-
19 wastes) on a regional scale.

20

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21

1 **Figure captions**

2 **Fig. 1:** Evolution of cellulose-to-glucose conversion vs. time at 1.5% (w/v) initial substrate
3 content: effects of drying (wet vs. dry substrate), particle size (>0.5 mm vs. <0.5 mm) and
4 enzyme loadings (50 vs. 70 FPU/g).

5 **Fig. 2:** Analysis of enzymatic hydrolysis of pretreated sawdust at four different substrate
6 loadings (1.5%, 3.75%, 4.5%, and 5.2% w/v), monitoring the glucose concentration and the
7 cellulose-to-glucose conversion yield.

8 **Fig. 3:** Evolution of ethanol, biomass and glucose concentrations in the submerged
9 fermentation broth in flasks (a): enriched hydrolysate (E.H), and (b): unenriched hydrolysate
10 (UE.H).

11 **Fig. 4:** Evolution of glucose, ethanol and yeast cells concentrations during fermentation scale-
12 up in the bioreactor.

13 **Fig. 5:** Kinetic model validation based on enzymatic hydrolysis conducted in Erlenmeyer flasks
14 for 3.75, 4.5, and 5.2% (w/v) pretreated substrate loading: (a) the glucose concentration; (b) the
15 cellulose-to-glucose conversion yield.

16

17

1 **Table captions**

2 **Table 1:** Evolution of size reduction costs of *Miscanthus* and switchgrass as a function of the
3 output particle size using a hammer mill (data extracted from Shastri et al., 2014).

4 **Table 2:** Comparison of kinetic data obtained from the different fermentation trials.

5