



Eucalyptus lignin production coupled with Cellic® CTec3 HS enzymatic hydrolysis to monosaccharides

Antonio Caporusso¹ · Federico Liuzzi¹ · Vito Valerio¹ · Egidio Viola¹ · Isabella De Bari¹ · Nicola Di Fidio²

Received: 12 October 2025 / Revised: 6 March 2026 / Accepted: 10 March 2026
© The Author(s) 2026

Abstract

The aim of this work was to design, develop, and optimise a two-step process for extracting lignin from *Eucalyptus globulus* residues and subsequently converting the remaining holocellulose into monosaccharides, primarily glucose and xylose. Firstly, the solubility of high-quality lignin was optimised using a new hydro-organo-thermal method involving a diluted imidazole solution in water instead of pure imidazole. Subsequently, enzymatic hydrolysis of the polysaccharides-rich pretreated solid was optimised to give glucose and xylose by using the commercial enzymatic mixture Cellic® CTec3 HS (Novozymes, Bagsværd, Denmark) supplemented with endo-1,4- β -xylanase from *Trichoderma viride* (Merck, Darmstadt, Germany). The extraction process was thoroughly optimised by acting on several parameters, such as the imidazole concentration, temperature, reaction time, and solid-to-liquid ratio. Under the optimised reaction conditions (imidazole 35 wt%, biomass loading 10 wt%, 120 °C, 2 h, 400 rpm) over the entire process, about 70 wt% of lignin removal was achieved, together with glucose and xylose yields of 99 and 92 mol%, respectively, reaching a high total sugar concentration of 91 g/L in the hydrolysate.

Key points

- A new hydro-organo-thermal treatment of lignocellulosic biomass has been proposed.
- Glucose and xylose yields were 99 and 92 mol% in 48 h at low enzyme dosage.
- A total reducing sugar concentration of 91 g/L was achieved in the hydrolysate.

Keywords Biorefinery · *Eucalyptus globulus* · Lignin recovery · Sugars · Enzymatic hydrolysis

Introduction

In 2020, the European Union developed and approved the “EU Green Deal” plan aimed at achieving the carbon neutrality of the European Union by 2050 and decisively promoting a circular bioeconomy that is environmentally

friendly and more equitable. In this context, there is a need to develop innovative, low-impact solutions and technologies to replace fossil fuels with renewable sources in the production of sustainable fuels, chemicals, and materials (Asghar et al. 2022; Caporusso et al. 2025b).

Pentose and hexose sugars, such as xylose and glucose, are among the most important and common platform molecules in biorefinery processes (Pandey and Sharma 2024). These sugars are generated through the hydrolysis reaction of biomass polysaccharides, which can occur via acid (organic or inorganic) or enzymatic pathways (Di Fidio et al. 2024). Through different processes, such as pretreatment, fractionation, and hydrolysis, biomass-derived monosaccharide mixtures can be converted into a broad range of high-value chemicals via biological or chemical methods (Di Fidio et al. 2024; Fulignati et al. 2024; Pandey and Sharma 2024). The selection of technologies, transformation processes, and the final products of a biorefinery are strongly influenced by the chemical composition of the raw material, global market demands, and the technological readiness level of a given

Antonio Caporusso and Federico Liuzzi contributed equally to the manuscript.

✉ Nicola Di Fidio
nicola.difidio@uniba.it

¹ Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Laboratory for Processes and Technologies for Biorefineries and Green Chemistry, ENEA C.R. Trisaia S.S., 106 Jonica, 75026 Rotondella, MT, Italy

² Department of Biosciences, Biotechnologies and Environment, University of Bari “Aldo Moro”, Via E. Orabona, 4, 70125 Bari, Italy

process. In biorefineries using lignocellulosic biomass as the starting feedstock, pretreatment is applied to decrease biomass recalcitrance (Caporusso et al. 2025a). This results in the production of polysaccharide- and lignin-rich fractions. Due to the stringent conditions required for the conversion of lignocellulosic biomass, the lignin tends to undergo condensation reactions, forming a resistant chemical structure that is not suitable for further conversion reactions (D'Orsi et al. 2023). Consequently, this lignin is typically treated as waste and burned in waste-to-energy processes to generate energy, heat, and/or steam. However, in recent years, a new strategy called “lignin-first” has emerged, in which lignin valorisation is considered alongside carbohydrate valorisation in the biorefinery scheme (Luo et al. 2023; Paone et al. 2020).

In this work, an innovative lignin-first biorefinery scheme has been designed, developed, and optimised for the exploitation of *Eucalyptus globulus* mixed residues. *Eucalyptus globulus* is a rapidly growing tree and is now one of the key hardwood sources for global pulp and paper manufacturing, a process that generates significant amounts of residues, such as branches, bark, and leaves. All these residues can be repurposed to produce second-generation monosaccharides (López et al. 2020). The chosen lignocellulosic biomass was initially pretreated by a hydro-organo-thermal approach based, for the first time, on the use of an aqueous solution of imidazole, obtaining a solid residue rich in polysaccharides and a liquid fraction enriched in soluble lignin. The lignin was then recovered by precipitation in an acidic environment, followed by filtration, and its characterisation was initiated for future valorisation studies. The solid residue was subjected to enzymatic hydrolysis using the innovative commercial mixture Cellic® CTec3 HS (Novozymes, Bagsværd, Denmark) supplemented with the commercial endo-1,4- β -xylanase from *Trichoderma viride* (Merck, Darmstadt, Germany) in order to improve the hydrolytic efficiency of the hemicellulose fraction (D'Orsi et al. 2023).

Imidazole offers several advantages, including low toxicity, low surface tension, high thermal stability, high water solubility, and significant nucleophilicity and reactivity that make it suitable for solvothermal processes (Savian et al. 2024). Furthermore, it is more cost-effective than ionic liquids and deep eutectic solvents and does not require special corrosion protection equipment (Valladares-Diestra et al. 2023).

To date, only a few studies in the scientific literature have investigated the use of pure imidazole for biomass pretreatment. Morais et al. (2016) and Savian et al. (2024) were the first authors to employ pure imidazole in a wheat straw pretreatment process at temperatures ranging from 110 to 170 °C for durations between 1 and 4 h. They demonstrated that, under the optimised reaction conditions (170 °C, 2 h, biomass loading of 9 weight (wt)%), lignin removal reached 80 wt% (calculated with respect to the lignin content in the untreated biomass), yielding a solid residue enriched in polysaccharides.

This residue, when subjected to enzymatic hydrolysis using a mixture of the two commercial enzymes Celluclast® 1.5L and β -glucosidase Novozym 188, achieved a glucose yield of approximately 99 mol%.

Valladares-Diestra et al. (2023) used pure imidazole for the pretreatment of four different biomass types, namely oil palm empty fruit bunches, sugarcane bagasse, soybean hulls, and cocoa pod husks, containing a different lignin content. Under the optimised reaction conditions for each biomass (180 °C, 1 h for oil palm empty fruit bunches; 160 °C, 1 h for sugarcane bagasse; 120 °C, 1 h for soybean hulls; 180 °C, 2 h for cocoa pod husks), lignin removal was around 70 wt%. The polysaccharide-enriched solid residue subsequently yielded glucose in the range of 80–90 mol% via enzymatic hydrolysis using the Cellic® CTec2 enzyme mixture.

Moreover, according to the literature, infrared spectroscopy analysis revealed that the solid residue obtained after imidazole treatment contained a higher amount of type-II cellulose than the raw biomass. This crystalline phase of cellulose is more susceptible to enzymatic hydrolysis and can be obtained through basic biomass treatment (Morais et al. 2016; Nagarajan et al. 2017; Valladares-Diestra et al. 2023).

Despite its efficiency, using pure imidazole has some drawbacks. These include a high melting point (90 °C), a density higher than water (1.23 g/cm³ under standard conditions) and, above all, a higher cost than traditional organic solvents used in organosolv technology. The high boiling point of 256 °C makes it difficult to recover pure solvent at an industrial scale, as this increases the energy and cost of distillation and downstream processing. Furthermore, the combination of severe reaction conditions (140–170 °C for 2–4 h) with pure imidazole has a significant impact on the preservation of the lignin structure, as reported by Morais et al. (2016) and Pereira et al. (2021a, b). These studies found that depolymerisation of the lignin occurs.

In the present study, diluted imidazole was tested for the first time to reduce imidazole consumption and favour pure lignin precipitation after solubilisation. In particular, the use of different diluted aqueous imidazole solutions (25, 35 wt%) in combination with different reaction times (1, 2, and 3 h) and temperatures (60, 90, and 120 °C) was studied and optimised. This choice aimed to improve the process from both an environmental and economic point of view, in line with the principles of Green Chemistry and Circular Bioeconomy (Kururl et al. 2025).

Material and methodology

Biomass and reagents

Eucalyptus globulus mixed residues were kindly provided by The Navigator Company (Cacia, Portugal). Lignocellulosic

biomass was dried overnight at 105 °C in a muffle, ground up to reach a particle size of approximately 0.5 cm, and then stored in a desiccator.

Water for high-performance liquid chromatography (HPLC), imidazole ($\geq 99\%$), tetrahydrofuran ($\geq 99\%$), sulfuric acid (98%), glucose (99.5%), xylose (95%), arabinose (95%), mannose ($\geq 99\%$), galactose ($\geq 99\%$), glacial acetic acid (99.7%), acetyl bromide (99%), poly(styrene sulphate) sodium salt standards (M_w range 891–258,000 g/mol) and endo-1,4- β -xylanase from *T. viride* (200 International Unit (IU)/mg protein) were purchased by Merck (Darmstadt, Germany). The enzyme mixture Cellic® CTec3 HS was kindly provided by Novozymes (Bagsværd, Denmark) and characterised by an enzymatic activity of 195 Filter Paper Unit (FPU)/g of solution. All reagents were used as received.

Determination of the chemical composition of biomass streams

The chemical composition of the *E. globulus* mixed residues and all solid streams generated throughout the proposed biorefinery process was determined through the standard National Renewable Energy Laboratory (NREL) procedures (Sluiter et al. 2004a,b, 2008a,b). Each sample was analysed in triplicate, and its standard deviation was calculated.

Imidazole-based hydro-organo-thermal treatment for lignin solubilisation

The hydro-organo-thermal treatment of *E. globulus* residues with diluted imidazole aqueous solutions was carried out in a 300 mL Parr® reactor (Autoclave Buchi Limbo-li®, Buchiglas, Zurich, Switzerland), introducing 100 g of each solution and 10 g of dry biomass. The relative quantity of imidazole and water was set based on the desired imidazole dilution according to the experimental setup (25 and 35 wt%). The mixture was stirred at 400 rpm. The effect of the temperature and reaction time was investigated by testing 60, 90, and 120 °C and 1, 2, and 3 h, respectively. At the end of the reaction, the reactor was cooled by an external airflow, depressurised, and opened. The resulting slurry was filtered through a sintered glass filter under vacuum. The collected solid was washed with deionised water until neutrality, dried overnight in a muffle at 105 °C, and weighed to determine the amount of residue obtained (Sluiter et al. 2008a,b). The precipitation of the lignin contained in the filtered liquid phase was carried out by adding a proper amount of 98 wt% H₂SO₄ to reach the final pH of 2. The solution was left overnight at 4 °C, and then the precipitated lignin was separated by centrifugation at 5000 rpm for 5 min. The solid was washed with acetone (solid-to-liquid ratio of 1:10 w/v) to remove any remaining imidazole and then with deionised water. Finally, lignin was dried in an oven at 45 °C under vacuum until reaching a constant weight and then stored in a desiccator. Lignin solubilisation was calculated as follows:

$$\text{Lignin solubilisation (wt\%)} = [(\text{lignin in the raw biomass (g)} - \text{lignin in the pretreated solid (g)}) / \text{lignin in the raw biomass (g)}] \times 100 \quad (1)$$

$$\text{Glucan removal (wt\%)} = [(\text{glucan in the raw biomass (g)} - \text{glucan in the pretreated solid (g)}) / \text{glucan in the raw biomass (g)}] \times 100 \quad (2)$$

$$\text{Xylan removal (wt\%)} = [(\text{xylan in the raw biomass (g)} - \text{xylan in the pretreated solid (g)}) / \text{xylan in the raw biomass (g)}] \times 100 \quad (3)$$

Enzymatic hydrolysis

The enzymatic hydrolysis reactions were carried out in a 500 mL stirred bioreactor (Braun Biotech International, Melsungen, Germany) equipped with a helical impeller using 100 mL of the 0.05 M citrate buffer and a proper amount of pretreated polysaccharide-rich dry solid residue, enzyme mixture Cellic® CTec3 HS, and endo-1,4- β -xylanase, according to the experimental plan. The speed of the stirrer was set to 200 rpm. The effect of the biomass

loading (10, 15, 20 wt%) and the enzyme dosage (15, 25, 35 FPU of Cellic® CTec3 HS/g glucan supplemented with 10 international units (IU) of endo-1,4- β -xylanase/g xylan) was investigated. The kinetics of each reaction were studied until reaching the plateau phase according to the Michaelis-Menten model. At the end of the reaction (after 48 h), the suspension was cooled at 4 °C to stop the reaction and filtered through a sintered glass filter. An aliquot of the liquid fraction was filtered through a 0.22 μ m syringe filter and injected into high-performance ionic chromatography

(HPIC) for the quantification of sugars. The solid residue was placed in a muffle at 105 °C overnight to gravimetrically determine the dry matter (Sluiter et al. 2008a,b).

Glucose and xylose yields were calculated by the following equations:

$$\text{Glucose yield (mol\%)} = [\text{glucose in the hydrolysate (mol)}/\text{glucan in the pretreated solid (mol)}] 100 \quad (4)$$

$$\text{Xylose yield (mol\%)} = [\text{xylose in the hydrolysate (mol)}/\text{xylan in the pretreated solid (mol)}] 100 \quad (5)$$

Chemical analyses

High-performance ionic chromatography (HPIC) analysis

Monosaccharides were quantified by HPIC Dionex ICS-2500 System (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Nucleogel Ion 300 OA (Macherey-Nagel, Düren, Germany) operating at 40 °C with 2.5 mM H₂SO₄ solution as mobile phase (flow equal to 0.4 mL/min). The detector was a Shodex RI-101 Refractive Index (PerkinElmer Italia Spa, Milano, Italy). Acetic acid was quantified by an HP1100 system equipped with a Dionex AS1 column (Thermo Fisher Scientific, Waltham, MA, USA) operating at 30 °C with Milli-Q water/acetonitrile as mobile phase (flow of 0.7 mL/min) and a diode array detector. Standards and reaction samples were analysed three times, and the average experimental error was ≤ 4%.

Elemental analysis

Vario MICRO Cube organic elemental analyser (Elementar, Langensfeld, Germany) was used for the elemental analysis (C, H, N, S) of lignocellulosic samples. The oxygen content was calculated through the Eq. 6:

$$\text{O (wt\%)} = 100 \text{ (wt\%)} - (\text{C (wt\%)} + \text{H (wt\%)} + \text{N (wt\%)} + \text{S (wt\%)} + \text{ash (wt\%)}) \quad (6)$$

The higher heating value (HHV) was calculated using Eq. 7 proposed by Channiwala and Parikh (2002), as follows:

$$\begin{aligned} \text{HHV (MJ/kg)} = & 0.3491 \cdot \text{C (wt\%)} + 1.1783 \cdot \text{H (wt\%)} \\ & + 0.1005 \cdot \text{S (wt\%)} - 0.1034 \cdot \text{O (wt\%)} \\ & - 0.0151 \cdot \text{N (wt\%)} - 0.0211 \cdot \text{ash (wt\%)} \end{aligned} \quad (7)$$

Infrared (IR) spectroscopy

A PerkinElmer Spectrum GX spectrometer (PerkinElmer Italia Spa, Milano, Italy) equipped with a MIRacle ATR

accessory was used for the acquisition of attenuated total reflection (ATR) Fourier-transform (FT) IR spectra. The spectra were acquired in the range of 450–4000 cm^{−1} using a resolution of 8 cm^{−1} and 64 scans.

Thermogravimetric analysis (TGA)

4 mg of lignin sample was used for TGA to determine its thermal stability, moisture content, and volatile substances. TGA was performed using a Perkin Elmer TGA 7 apparatus (PerkinElmer Italia Spa, Milan, Italy). The tests were carried out with variable heating rates set as follows: 5.5 °C/min from 25 to 260 °C; then 6 °C/min up to 380 °C; 7.5 °C/min up to 510 °C; 7.5 °C/min up to 630 °C; 17 °C/min up to 900 °C. The N₂ gas flow was 20 mL/min and was kept constant at the inlet.

Gel permeation chromatography (GPC)

Prior to GPC analysis, the lignin obtained was acetylated to increase its solubility in the solvent (tetrahydrofuran, THF) used for elution in the chromatographic equipment. For the acetobromination reaction, 50 mg of lignin was suspended in 10 mL of glacial acetic acid/acetyl bromide (9:1 v/v) for 2 h. The solvent was then completely removed under vacuum, and the residue was dissolved in THF (10 mL) and filtered through a 0.45 µm syringe filter before injection. The lignin solution was analysed on an Agilent HP 1100 series LC system (Agilent Technologies, Santa Clara, CA, USA) equipped with GPC columns SUPELCO TSKgel-G4000HHR, TSKgel-G3000HHR and TSKgel-G2500HHR to determine the average molecular weight by coupling an Agilent 1260 ELSD detector, setting the evaporator temperature at 42 °C, the nebuliser temperature at 30 °C and using a nitrogen flow rate of 1.19 mL/min. Standard calibration was performed using poly(styrene sulphonate) sodium salt standards (*M_w* range 891–258,000 g/mol).

Results

Hydro-organo-thermal treatment of *E. globulus* to solubilise lignin

The chemical composition of raw *E. globulus* mixed residues (based on a dry matter) was as follows: (wt%): glucan 41.5 ± 1.8 , xylan 15.9 ± 0.9 , arabinan 0.4 ± 0.1 , galactan 0.6 ± 0.1 , mannan 0.9 ± 0.1 , acetyl groups 4.0 ± 0.2 , acid-insoluble lignin

24.7 ± 1.3 , acid-soluble lignin 2.9 ± 0.3 , ash 2.1 ± 0.1 , extractives (in absolute ethanol) 1.7 ± 0.1 , protein 2.3 ± 0.1 , other 3.0, substantially overlapping the data in the literature (Viola et al. 2021). The high polysaccharide content makes this residual biomass a valuable raw material for the development of biorefinery schemes based on the production of monosaccharides as platform chemicals (Pandey and Sharma 2024).

According to the literature, pure imidazole can solubilise lignin, yielding a solid residue enriched in cellulose and hemicellulose, which can then be hydrolysed into

Table 1 Experimental matrix. The biomass loading was fixed at 9 wt%

Entry	Imidazole concentration (wt%)	T (°C)	Time (h)	Lignin removal (wt%)	Glucan removal (wt%)	Xylan removal (wt%)
1	25	60	1	9.9	5.6	7.4
2	25	60	2	14.3	8.7	11.2
3	25	60	3	21.5	12.4	15.3
4	25	90	1	12.2	14.9	16.6
5	25	90	2	21.6	17.8	19.5
6	25	90	3	41.2	21.9	25.4
7	25	120	1	18.9	18.1	20.5
8	25	120	2	25.2	19.7	22.6
9	25	120	3	55.8	23.6	27.7
10	35	60	1	18.2	7.5	9.9
11	35	60	2	22.5	9.8	13.5
12	35	60	3	26.4	14.7	16.3
13	35	90	1	25.3	13.1	14.5
14	35	90	2	30.9	17.4	21.1
15	35	90	3	45.3	22.3	26.9
16	35	120	1	25.9	19.3	24.1
17	35	120	2	66.4	22.1	29.8
18	35	120	3	74.7	31.2	38.1

Table 2 Comparison of the chemical composition of *E. globulus* delignified with dilute imidazole with that of other biomasses pretreated with pure imidazole reported in the literature

Biomass	Reaction conditions	Glucan (wt%)	Xylan (wt%)	Lignin (wt%)	Reference
<i>E. globulus</i> residues ^a	120 °C, 2 h	55.9	19.4	16.1	This work
Wheat straw ^{b,c}	170 °C, 2 h	62.4	24.9	3.5	(Morais et al. 2016)
Wheat straw ^{b,d}	170 °C, 2 h	19.3	52.0	5.6	(Morais et al. 2016)
Wheat straw ^b	160 °C, 4 h	54.6	23.8	4.1	(Pereira et al. 2021b)
<i>Eucalyptus</i> residues ^b	160 °C, 4 h	54.3	24.4	16.3	(Pereira et al. 2021b)
<i>Cupressus lusitanica</i> ^{b,c,e}	145 °C, 3 h	~ 40	~ 20	~ 25	(Pereira et al. 2021a)
<i>Cupressus lusitanica</i> ^{b,d,e}	145 °C, 3 h	~ 18	~ 22	~ 35	(Pereira et al. 2021a)
Soybean hulls ^b	120 °C, 1 h	57.8	16.6	2.6	(Sayury Nishida et al. 2021)
Sugarcane bagasse ^b	160 °C, 1 h	64.8	21.6	11.7	(Valladares-Diestra et al. 2022)
Oil palm empty fruit bunches ^b	180 °C, 1 h	55.3	23.0	7.7	(Zevallos Torres et al. 2023)
Cocoa pod husks ^b	180 °C, 3 h	43.3	10.0	29.8	(Valladares-Diestra et al. 2023)

^aDiluted aqueous imidazole solution (35 wt%); ^bpure imidazole (100 wt%); ^crecovered cellulose-rich solid residue; ^drecovered hemicellulose-rich solid residue; ^edata extrapolated from a figure reported in the work

Table 3 Experimental matrix. Reaction conditions: 50 °C, 48 h, pH 4.8 (citrate buffer), 200 rpm, 10 IU of endo-1,4- β -xylanase/g xylan

Entry	Biomass loading (wt%)	Cellic® CTec3 HS dosage (FPU/g glucan)	Glucose yield (mol%)	Xylose yield (mol%)	Total sugars concentration (g/L) ^a
19	10	15	87.4 $\pm$ 3.6	80.3 $\pm$ 2.8	79.9
20	10	25	98.5 $\pm$ 1.5	92.0 $\pm$ 2.9	90.5
21	10	35	99.2 $\pm$ 1.2	95.2 $\pm$ 2.3	91.7
22	15	15	60.3 $\pm$ 3.6	60.1 $\pm$ 3.1	89.5
23	15	25	70.5 $\pm$ 3.1	69.2 $\pm$ 3.3	104.1
24	15	35	79.0 $\pm$ 3.5	77.2 $\pm$ 2.1	116.5
25	20	15	40.3 $\pm$ 3.6	31.1 $\pm$ 3.9	79.7
26	20	25	46.7 $\pm$ 3.7	39.7 $\pm$ 3.3	94.2
27	20	35	55.0 $\pm$ 3.5	47.9 $\pm$ 3.4	111.6
28 ^b	10	25	20.8 $\pm$ 1.4	15.6 $\pm$ 0.9	13.8

^aSum of glucose and xylose concentration; ^bcontrol test with raw *E. globulus*

monomeric sugars through enzymatic hydrolysis (Morais et al. 2016; Valladares-Diestra et al. 2023). In the present work, the pretreatment of biomass using aqueous solutions of imidazole at 25 and 35 wt% was investigated at 120, 90, and 60 °C for 1, 2, and 3 h with a biomass loading of 9 wt%. The adopted experimental plan was reported in Table 1 with the main results obtained for each reaction in terms of lignin solubilisation and glucan and xylan removal.

The chemical composition of the delignified solid residue (57.8 wt%) recovered under the optimal reaction conditions (imidazole 35 wt%, $T = 120$ °C, time = 2 h) was constituted of 55.9 wt% glucan, 19.4 wt% xylan, 16.1 wt% lignin, 3.5 wt% acetyl groups, 1.9 wt% ash, 2.3 wt% proteins, and 0.9 wt% other (Supplemental Table S1). The main components were reported in Table 2 and compared with the chemical composition of the pretreated polysaccharide-rich solids reported in the literature studies based on the use of pure imidazole.

Enzymatic saccharification of the pretreated solid

The effect of the biomass loading (10, 15, and 20 wt%) and enzyme dosage (15, 25, and 35 FPU/g glucan) was also investigated to find the optimal reaction conditions for maximising sugar yield and concentration. Table 3 shows the experimental plan implemented, with the reaction conditions tested and the glucose and xylose yield and total sugar concentration obtained after 48 h as response variables. Figure 1 shows the kinetics of glucose and xylose yields under the different reaction conditions.

Recovery of soluble lignin and its physical-chemical characterisation

The soluble lignin obtained under the optimised reaction conditions of the hydro-organo-thermal treatment (imidazole

35 wt%, $T = 120$ °C, time = 2 h) was precipitated by acidification of the solution (pH = 2) and centrifugation, washed to remove imidazole and sulphates, dried, and characterised as described below. The solid lignin was about 95 wt% of the removed lignin, confirming the efficiency of the proposed approach. To confirm the purity and quality of the isolated lignin, its chemical composition was determined using standard NREL methods. It was composed of (wt%): 0.3 $\pm$ 0.1 glucan, 0.2 $\pm$ 0.1 xylan, 86.5 $\pm$ 1.1 acid-insoluble lignin, 6.7 $\pm$ 0.9 acid-soluble lignin, 2.2 $\pm$ 0.2 ash, 1.5 $\pm$ 0.1 proteins, 2.6 others.

ATR-FTIR spectroscopy

Figure 2 shows the ATR-FTIR spectrum of the lignin recovered from dilute imidazole-based delignification.

The peak observed at 3390 cm^{-1} is due to the stretching of the aliphatic and aromatic hydroxyl groups typical of lignin. The signals at 2926, 2842, and 1460 cm^{-1} are characteristic of the stretching of the C-H bond in the $-\text{CH}_3$ - and $-\text{CH}_2$ -groups. Moreover, the sample shows characteristic peaks of the aromatic skeleton, such as 1600 cm^{-1} (stretching of the C=C bond of the aromatic ring), 1514 and 1424 cm^{-1} (vibrations of the aromatic skeleton associated with the in-plane deformation of the C-H bond) (Tavares et al. 2022). The characteristic bands of syringyl units fall at 1324 and 1114 cm^{-1} , and those characteristic of guaiacylic units at 1514 and 1214 cm^{-1} . In addition, the signal at 1706 cm^{-1} is characteristic of the stretching of the C=O group present in unconjugated ketones, carbonyls, and esters, and the peak at 1032 cm^{-1} is associated with the stretching of the C–O–C bond of the pyranose ring of cellulose (Romaní et al. 2016). The relatively low intensity of this peak is consistent with the negligible amount of glucan in the lignin composition.

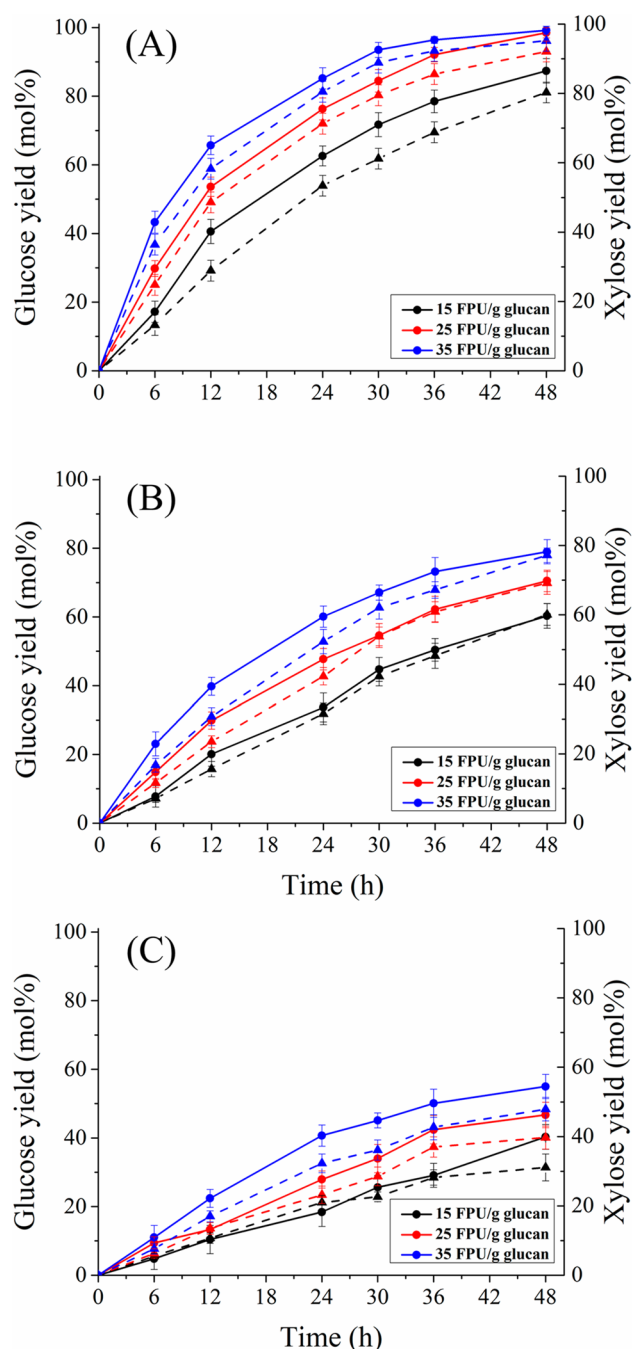


Fig. 1 Kinetics of glucose (solid line) and xylose (dashed line) yields during Cellic.[®] CTec3 HS-catalysed hydrolysis of pretreated *E. globulus* residues at biomass loadings of 10 wt% (A), 15 wt% (B), and 20 wt% (C)

Thermogravimetric analysis

Figure 3 shows TGA and derivative thermogravimetry (DTG) curves of pure lignin recovered under the optimised reaction conditions.

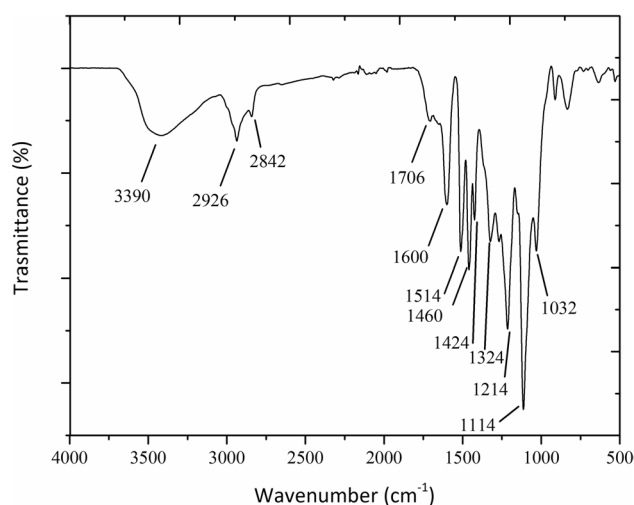


Fig. 2 ATR-FTIR spectrum of the isolated lignin

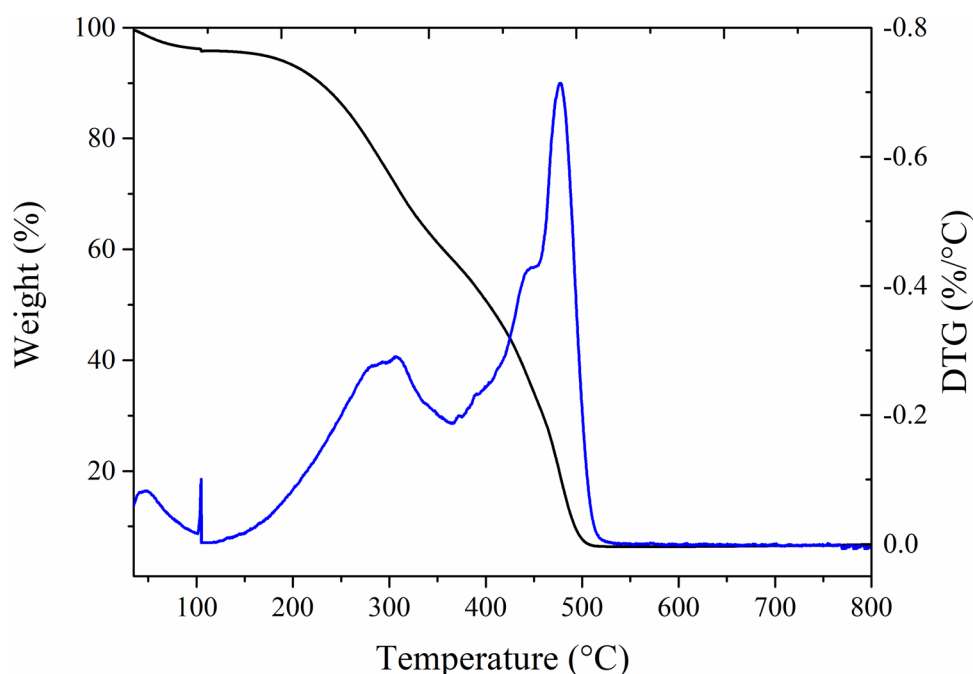
Lignin showed three main peaks in the DTG curve. The first was in the range of 30–130 °C with a mass loss of 4.3% and is related to the dehydration phenomenon. The second degradation peak (corresponding to a mass loss of around 31%) was in the range of 130–330 °C with a $T_{\max}$ of around 300 °C. The last one (corresponding to a mass loss of around 59%) was comprised between 330 and 540 °C with a $T_{\max}$ equal to 480 °C and a peak shoulder at around 440 °C. The peaks present after the first dehydration peak, from lower to higher temperatures, are related to the cleavage of the β -O-4 bond, the C–C bond, and the β - β bond respectively, and to condensation and polymerisation reactions (El Moustaqim et al. 2018). The resolution and intensity of the peaks corresponding to β -O-4 bond cleavage depend on the relative abundance of these bonds in the lignin. Lignins rich in these bonds show a prominent peak in the 200–300 °C region, as shown in Fig. 3 for the lignin isolated in the present work. Furthermore, the peaks in the 300–500 °C region (Fig. 3) correspond to the cleavage of C–C and β - β bonds, respectively, confirming the typical structure of pure lignin.

In addition, the sample showed a low ash residue at 800 °C (4.6 wt%), which is consistent with the ash content (2.2 wt%) determined by the standard NREL method and in agreement with the values of typical organosolv lignins (Başakçılardan Kabakcı and Tanış 2021).

Elemental analysis

Pure lignin from *E. globulus* showed the following elemental profile (wt% on dry mass): 63.81 ± 0.16 carbon, 6.17 ± 0.08 hydrogen, 0.24 ± 0.06 nitrogen, 0.15 ± 0.03 sulphur, and 27.43 ± 0.33 oxygen (calculated according to Eq. 6 in the Materials and methods section, considering the ash content of 2.2 wt%). The HHV of this material was calculated using Eq. 7 and was 26.7 MJ/kg.

Fig. 3 Black line: weight loss curve during the TGA of isolated lignin. Blue line: the calculated DTG curve of the same sample



Molecular weight determination

Supplemental Fig. S1 shows the GPC chromatogram obtained experimentally. The number average molecular weight (M_n) was 1003.3 g/mol, while the weight average molecular weight (M_w) was 2448.65 g/mol. The polydispersity index, calculated as the ratio between M_w and M_n , was 2.4, corresponding to a limited broad distribution of the lignin chains.

Discussion

In this work, an innovative hydro-organo-thermal treatment was tested in order to optimise the complete valorisation of *E. globulus* residues by using aqueous imidazole solutions under relatively mild process conditions in terms of temperature and solvent consumption. The main goal of this approach, which is greener than those based on pure imidazole, was to obtain pure, soluble lignin and a solid residue enriched in valuable polysaccharides, characterised by a deconstructed lignocellulosic matrix. The latter is expected to undergo enzymatic hydrolysis more easily than the raw biomass to produce glucose and xylose.

Lignin production

The main objective of this experimental matrix was to find the appropriate reaction conditions to maximise lignin solubilisation while minimising the solubilisation of the main polysaccharides of the biomass, namely cellulose and

hemicellulose. Based on the experimental results reported in Table 1, lignin solubilisation increased as a function of increasing the imidazole concentration, temperature, and reaction time. Polysaccharide solubilisation was mainly favoured by the increase in temperature and imidazole concentration. In particular, when comparing entries 1–3, 4–6, 7–9, 10–12, 13–15, and 16–18, lignin solubilisation tended to increase slightly from 1 to 2 h, whereas it increased significantly at 3 h. A similar trend was observed for glucan and xylan removal, although the difference in values was less pronounced than for delignification. From the comparison of entries 1, 4, and 7 (1 h) or 2, 5, and 8 (2 h) or 3, 6, and 9 (3 h), based on the use of the imidazole solution of 25 wt%, the effect of the temperature on the lignin solubilisation was more evident for a process duration of 3 h.

A similar trend was observed for the 35 wt% imidazole solution by comparing entries 10, 13 and 16 (1 h), 11, 14, and 17 (2 h) or 12, 15, and 18 (3 h). Finally, the effect of imidazole concentration on lignin and polysaccharide solubilisation was shown by comparing entries 1–3 with 10–12 (60 °C), 4–6 with 13–15 (90 °C), and 7–9 with 16–18 (120 °C). Increasing the imidazole concentration from 25 to 35 wt% had little effect on the solubilisation of biopolymers at 60 and 90 °C, but significantly increased lignin removal up to 75 wt% at 120 °C. Differently, a limited increase in glucan (from 24 to 31 wt%, entries 9 and 18) and xylan (from 28 to 38 wt%, entries 9 and 18) removal was observed. The recovery of the solid residues remaining after the hydro-organic extractions listed in Table 1, alongside their complete chemical composition, can be found in Supplemental Table S1.

Previous literature studies based on the use of pure imidazole thermal treatment show that this organic base can modify the structure of cellulose, resulting in a transition from type I cellulose to type II cellulose, which is less crystalline and more porous and therefore more susceptible to solubilisation (Morais et al. 2016; Valladares-Diestra et al. 2023). The proportion of cellulose that undergoes this transition is likely to increase with the concentration of imidazole used.

Under the reaction conditions reported in entry 18 (Table 1), the maximum lignin solubilisation of 75 wt% was achieved together with undesired significant removal of glucan and xylan, which should preferably remain in the solid residue to be exploited in the following process step. For this reason, the best compromise between lignin solubilisation and polysaccharide removal was represented by entry 17 (imidazole 35 wt%, $T = 120\text{ }^{\circ}\text{C}$, time = 2 h), where a high lignin removal of 66 wt% was achieved with a limited glucan and xylan solubilisation. The selection of this compromise condition was aligned with the techno-economic and environmental analysis of sugar production from lignocellulosic biomass reported in the literature (Baral et al. 2021). According to this analysis, in the pretreatment step, lignin removal should be higher than 65 wt%, glucan removal lower than 15 wt%, and xylan removal lower than 35 wt%.

The obtained results offer concrete perspectives to increase the sustainability of the delignification process at mild conditions and contribute to the advancement of technical knowledge and scientific understanding in the field of lignocellulosic biomass utilisation, as a high degree of delignification and limited polysaccharide removal were achieved under milder and more sustainable reaction conditions compared to literature studies based on the use of pure imidazole. There are only a few studies in the scientific literature where the pretreatment of biomass was based on the use of imidazole, and in all cases, imidazole was used as a pure solvent rather than in solution, as in the present investigation (Morais et al. 2016; Pereira et al. 2021a; Sayury Nishida et al. 2021; Valladares-Diestra et al. 2023). In the previous studies, the reactions were carried out in the temperature range of 110–180 °C, with a reaction time in the range of 1–4 h and a biomass loading of 9–10 wt%. In all cases, at the end of the reaction, the reactor was cooled to 90 °C (to keep the imidazole liquid) or room temperature, deionised water was added, the suspension was stirred for 1 h, and then filtered to recover the solid biomass. In contrast to that, in the present investigation, aqueous solutions of imidazole were used to reduce the consumption of organic solvents and, consequently, the environmental impact of the proposed process. In fact, the use of a 35 wt% imidazole solution resulted in significant reagent savings, which also implies an improved economic viability of the process. Furthermore, the use of dilute aqueous imidazole solution allowed the temperature to be reduced to even below the melting point of imidazole

(e.g. up to 60 °C in this work), thus implementing an energy-saving approach.

Morais et al. (2016) used pure imidazole in a wheat straw pretreatment process at temperatures ranging from 110 to 170 °C for durations between 1 and 4 h. They demonstrated that, under the optimised reaction conditions (170 °C, 2 h, biomass loading of 9 wt%), lignin removal reached 80 wt%, yielding a solid residue enriched in polysaccharides. The slightly higher degree of delignification achieved by Morais and co-workers compared to the present work is due to the use of a 50 °C higher temperature, together with the use of 100 wt% imidazole. The use of higher temperatures resulted in higher energy consumption and, therefore, higher process costs. Similarly, Valladares-Diestra et al. (2023) used pure imidazole for the pretreatment of four different biomass types, namely oil palm empty fruit bunches, sugarcane bagasse, soybean hulls, and cocoa pod husks, containing different lignin contents. Under the optimised reaction conditions for each biomass (180 °C, 1 h for oil palm empty fruit bunches; 160 °C, 1 h for sugarcane bagasse; 120 °C, 1 h for soybean hulls; 180 °C, 2 h for cocoa pod husks), lignin removal was around 70 wt%, in line with the value achieved in the present investigation.

The pretreatment with pure imidazole was carried out on several biomasses with very different initial chemical compositions, demonstrating the significant versatility and efficacy of this organic solvent. This is a fundamental characteristic in the perspective of scaling up a biorefinery process that could be fed with biomasses of different natures over time, ensuring the same performance of the proposed conversion processes.

As reported in Table 2, the glucan, xylan, and lignin contents of the solid residue obtained from the new hydro-organo-thermal process were in agreement with those reported in the literature for the polysaccharide-rich solids obtained from the pure imidazole-based pretreatment. The glucan and xylan contents achieved in the present work were slightly lower than the maximum value of their corresponding interval. In contrast, the lignin content was right in the middle of the range, confirming that the solid residue was obtained, which has a suitable composition for further chemo and/or biocatalytic valorisation, reducing both organic solvent consumption by two-thirds and process energy costs due to the lower temperature used (120 °C instead of the typical 160–180 °C).

The chemical composition of isolated lignin confirmed its very high purity (93.2 wt% of the solid), paving the way for various valuable applications of this aromatic biopolymer. This chemical composition is consistent with those reported in the literature for typical organosolv lignins (Başakçılardan Kabakçı and Tanış 2021).

Regarding the quality of the obtained lignin, the low nitrogen content due to the presence of proteins (1.5 wt%)

confirmed the absence of a significant amount of imidazole in the isolated lignin, confirming the effectiveness of the centrifugation and washing method used to selectively collect the solubilised lignin. The low sulphur content also confirmed the absence of a significant amount of sulphates resulting from the use of H_2SO_4 to reach pH 2 to promote lignin precipitation after the imidazole-based hydro-organothermal treatment of the raw biomass. The elemental profile obtained is consistent with that reported in the literature for major commercial lignin types, including Kraft lignin, Indulin® AT, Soda Protobind™ 1000, and Alcell™ organosolv lignin (Constant et al. 2016).

The typical HHV values of biomasses, biomass residues, and lignins suitable for direct combustion for energy production range from 20 to 35 MJ/kg. Therefore, the obtained HHV for pure lignin confirms its potential use for direct energy applications (Di Fidio et al. 2024). Alternatively, the lignin recovered in the proposed biorefinery scheme could be used as a raw material for the production of surfactants and hydrogels (Sethupathy et al. 2022), or it could be depolymerised to aromatic compounds by enzymatic (Reshmy et al. 2022) or by chemical (Lu et al. 2020) catalysis. The polydispersity index, M_n , and M_w values were consistent with those reported in the literature for commercial lignins (Constant et al. 2016).

Enzymatic hydrolysis to fermentable monosaccharides

In order to assess the effectiveness of the entire process, the degree of delignification needs to be integrated by the evaluation of the enzymatic hydrolysability of the biomass, which is also fundamental. Within this scope, the suitability of the hydro-organothermal treatment was evaluated in terms of monosaccharide (glucose and xylose) yields in the subsequent enzymatic hydrolysis tests. Factors influencing enzymatic hydrolysis include: i) the porosity of the biomass; ii) the type of crystalline phase of the cellulose; iii) the degree of polymerisation of the cellulose and hemicellulose; iv) the crystallinity of the latter; v) the amount of lignin present (Zhao et al. 2012).

The Cellic® CTec3 HS enzyme blend was used for the hydrolysis tests. This enzyme mixture represents one of the most recent and advanced enzyme formulations used industrially, as it has high enzyme activity and a relative enzyme composition optimised for the hydrolysis of lignocellulosic biomass (Sun et al. 2015). However, this enzyme mixture is characterised by a lower hydrolytic activity towards hemicellulose than Cellic® CTec2, because Cellic® CTec3 HS contains only xylan 1,4- β -xylosidase, which catalyses the hydrolysis reaction of (14)- β -D-xylans by removing successive D-xylose residues from the non-reducing termini (Sun et al. 2015). On this basis and considering the non-negligible

amount of xylan in the pretreated biomass (~20 wt%), endo-1,4- β -xylanase from *T. viride* was added to the enzyme mixture to improve the hydrolytic activity of the biocatalyst.

Relatively low enzyme dosages were tested to reduce the process costs of the proposed biorefinery model, as the cost of enzymes typically represents up to around 20% of the total cost of a biorefinery process (Gomes et al. 2020). Furthermore, with a view to a possible industrial development of the designed multi-stage process, an increase of the biomass loading in the hydrolysis reaction to 15 or 20 wt% (high gravity approach) was investigated in order to obtain hydrolysates even richer in monosaccharides. The pH was measured at the beginning and end of all tests and remained constant. This observation, together with the ATR-FTIR spectra and elemental analysis described above, showed that there was no imidazole in the solid residue after pretreatment, so washing with water at the end of each pretreatment was effective.

As shown in Table 3 and Fig. 1, increasing the enzyme dosage favoured the glucose and xylose yields for each biomass loading. Increasing the biomass loading significantly decreased the sugar yields but increased the total sugar concentration in the hydrolysate up to the maximum value of 116.5 g/L obtained by working with a biomass loading of 15 wt% and a Cellic® CTec3 HS dosage of 35 FPU/g glucan (entry 24, Table 3). However, under these reaction conditions, the glucose yield was 79.0 mol%, and the xylose yield was 77.2 mol%. These values were significantly lower than those obtained in entries 20 and 21 (Table 3), where almost complete conversion of the polysaccharides was achieved. In order to reduce the side streams of the proposed biorefinery process and to maximise the exploitation of the selected renewable resource, the best results were obtained in entry 20 (biomass loading = 10 wt%, Cellic® CTec3 HS = 25 FPU/g glucan) as a compromise between glucose and xylose yields, total sugar concentration, and enzyme consumption.

In addition, in order to demonstrate the importance of the pretreatment for obtaining high yields in the subsequent saccharification process, a control test was carried out by subjecting raw *E. globulus* to enzymatic hydrolysis under the optimised reaction conditions (entry 28, Table 3). As expected, the glucose and xylose yields were extremely low, confirming the fundamental role of the innovative hydro-organothermal treatment in the efficiency of the subsequent saccharification process.

As reported in Table 2, only a dozen studies have investigated the pretreatment of various biomasses with pure imidazole, but no studies have yet investigated the effect of imidazole-based hydro-organothermal treatment on the efficiency of the subsequent saccharification process of lignocellulosic biomass.

In the two studies of Pereira et al. (2021a,b), after the pretreatment with pure imidazole of wheat straw and

Eucalyptus residues at 160 °C for 4 h and *Cupressus lusitanica* at 145 °C for 3 h, the polysaccharides-rich solid residues were subjected to the enzymatic hydrolysis in the presence of the commercial enzyme mixture Cellic® CTec2, i.e. the earlier version than the mixture used in this work. Under the adopted reaction conditions (biomass loading of only 2 wt%, enzyme dosage of 60 FPU/g glucan, 72 h), the glucose yields were 93, 38, and 31 mol%, while the xylose yields were 77, 40, and 19 mol% for wheat straw, *Eucalyptus*, and *C. lusitanica*, respectively. With the sole exception of the glucose yield obtained for wheat straw, these sugar yields are significantly lower than those obtained in this work, which were also based on a fivefold higher biomass loading (10 wt% instead of 2 wt%) and 42% the enzyme dosage (25 FPU/g glucan instead of 60 FPU/g glucan).

In the study of Valladares-Diestra et al. (2023), sugarcane bagasse, soybean hulls, cocoa pod husks, and oil palm empty fruit bunches were subjected to imidazole pretreatment and subsequent enzymatic hydrolysis with Cellic® CTec2 enzyme. Also in this case, a very low biomass loading of 2 wt% was used in the presence of 20 FPU enzyme/g glucan for the saccharification process. The glucose yields obtained were 97, 100, 87, and 92 mol%, respectively, values comparable to those obtained in the present work. However, these high yields were obtained with a very low biomass loading, which is not suitable for a possible process scale-up, since the hydrolysates obtained have a low sugar concentration, which is not optimal for downstream operations and for subsequent catalytic upgrading processes, such as fermentation.

Morais et al. (2016) carried out the delignification of wheat straw at 170 °C, 2 h, and a biomass loading of 9 wt%. The pretreated solid residue was subjected to enzymatic hydrolysis for 72 h using a mixture of the two commercial enzymes Celluclast® 1.5L and β -glucosidase Novozym 188 at a biomass loading of 10 wt%, obtaining glucose and xylose yields of 99 and 81 mol%, respectively, corresponding to a total sugar concentration of 84.7 g/L. These results are similar to those obtained in the present study, in which, however, there was the additional advantage of reducing the amount of imidazole used in the pretreatment stage.

Regarding the efficiency of the enzyme mixture Cellic® CTec3 HS used in the present study, there are only a few studies in the literature using this new commercial product, and the combination of Cellic® CTec3 HS with the enzyme endo-1,4- β -xylanase from *T. viride* was proposed for the first time in the present work. In previous literature studies, different biomasses have been subjected to different pretreatment processes. In particular, Kim et al. (2019) enzymatically hydrolysed empty fruit bunches that had undergone steam-explosion pretreatment (190 °C, 15 min), using a solid loading between 20 and 30 wt% and an enzyme dosage between 20 and 60 FPU/g glucan, achieving a glucose yield of 99 mol% under the optimised conditions (20 wt%, 60

FPU/g glucan). However, in the hydrothermally pretreated residue, more than 65 wt% of the hemicellulose present in the starting biomass was lost, preventing its utilisation. In contrast, in this work, the proposed hydro-organo-thermal treatment resulted in the solubilisation of only 30 wt% of hemicellulose, improving the exploitation of the starting material.

Fockink et al. (2017) enzymatically hydrolysed the steam-exploded sugarcane bagasse (195 °C, 7.5 min) by varying the dosage between 7 and 40 FPU/g glucan and the biomass loading between 10 and 20 wt%. Under the optimal reaction conditions (38.6 FPU/g glucan, biomass loading of 10 wt%, 72 h), a glucose yield of 99 mol% was obtained, corresponding to a total sugars concentration of 120 g/L. This value and that obtained in the present work are much higher than those obtained by Gomes et al. (2020), who obtained a total sugars concentration of 28 g/L using the same enzyme mixture on sugarcane bagasse (8.5 FPU/g glucan, 2 wt% substrate loading) pretreated with an aqueous citric acid solution (citric acid concentration of 6 wt%) at a substrate loading of 9 wt%, 100 °C and a pretreatment time of about 1.5 h.

Mass balance flow diagram of the proposed biorefinery model

Figure 4 shows the mass balance flow diagram of the proposed biorefinery scheme.

Starting from 1 kg of *E. globulus* mixed residues, the solid residue recovered after the dilute imidazole-based hydro-organo-thermal treatment (120 °C, 2 h, biomass loading 9 wt%, 400 rpm) accounted for 57.8 wt% of the starting raw biomass. This solid residue was mainly composed of valuable polysaccharides (glucan and xylan), accounting for 75.3 wt% of dry matter, with a lower lignin content of 16 wt%. The liquid fraction contained 0.183 kg of lignin, corresponding to around 66 wt% of the lignin present in the raw biomass. After the precipitation of lignin in an acidic environment and its recovery and washing, the solid residue was 0.174 kg (95.1 wt% of the soluble lignin), containing 93.2 wt% of lignin and a negligible amount of other components, such as glucan, xylan, proteins, and ash.

The sugar-rich solid residue was used as a substrate for the subsequent saccharification step in the presence of the commercial enzyme mixture Cellic® CTec3 HS supplemented with endo-1,4- β -xylanase from *T. viride*. Under the optimal reaction conditions (biomass loading = 10 wt%, Cellic® CTec3 HS = 25 FPU/g glucan + 10 IU of endo-1,4- β -xylanase/g xylan, 48 h, 200 rpm, pH 4.8), there was almost complete conversion of glucan and xylan to glucose and xylose, yielding a hydrolysate with 0.353 kg glucose and 0.117 kg xylose per 5.2 L of solution (68.0 g/L glucose and 22.5 g/L xylose).

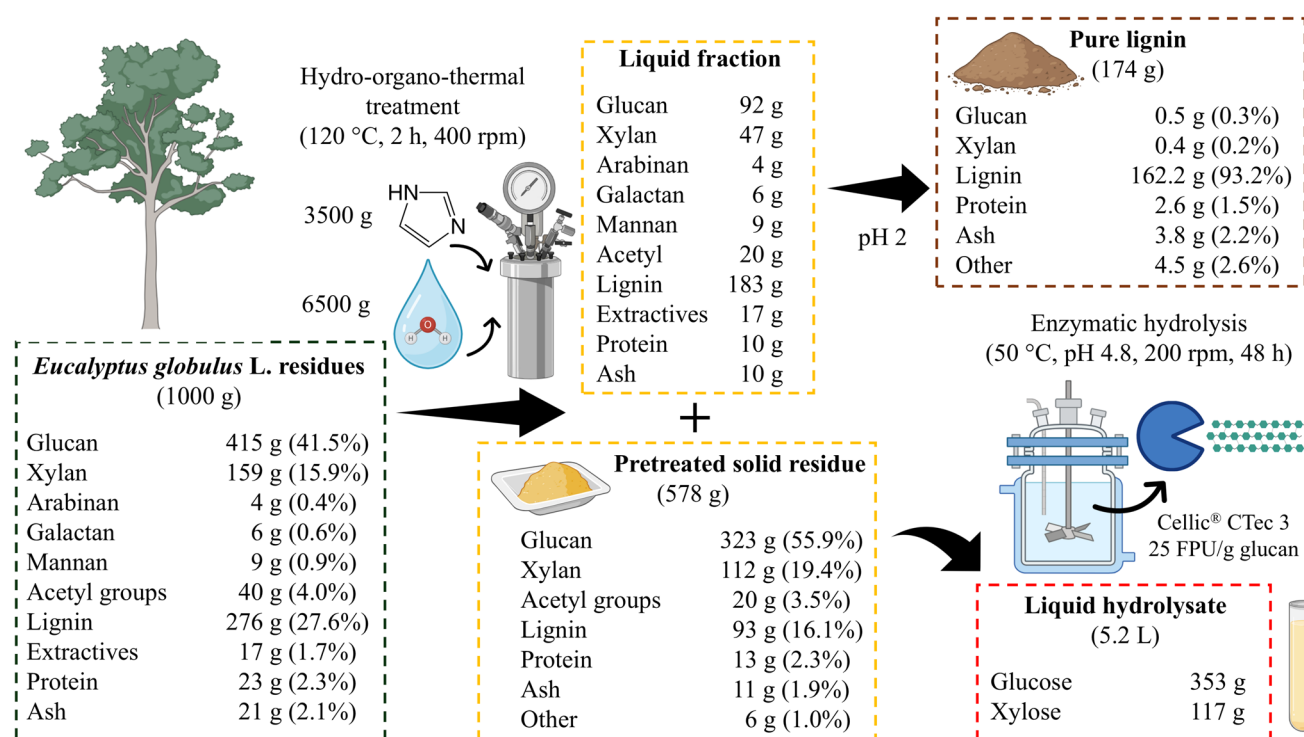


Fig. 4 A mass balance flow diagram of the cascade biorefinery model was developed

Based on this mass balance, 174 g of pure lignin, 353 g of glucose, and 117 g of xylose were obtained from 1.0 kg of dry lignocellulosic residual biomass, giving a total of 0.64 kg. These results agreed with the mass balance flow diagrams reported in the literature for the biorefinery models based on the use of pure imidazole in the first step and the enzymatic hydrolysis in the second step (Sayury Nishida et al. 2021; Valladares-Diestra et al. 2022; Zevallos Torres et al. 2023).

The complete valorisation of the starting *E. globulus* residues and the low-impact reaction conditions adopted in each step significantly improved the sustainability of the cascade process from the perspective of Green Chemistry principles and the Bioeconomy.

Sustainability metrics and solvent efficiency

To make a realistic, quantitative assessment of the process's sustainability, the E-factor was adopted as a preliminary metric. It is defined as the ratio of the mass of waste generated to the mass of desired products (Sheldon 2017). For this analysis, a conservative approach was adopted, including washing solvents (water and acetone) and the acidic precipitation step in the waste inventory. Imidazole was excluded from the waste stream as it is assumed to be quantitatively recovered due to its high water solubility, negligible volatility, and chemical stability. Imidazole can be recovered

using established separation strategies, such as pH adjustment followed by solvent extraction, vacuum evaporation, or membrane-based concentration (Morais et al. 2016). As imidazole is neither irreversibly consumed during pretreatment nor significantly degraded under the applied conditions, its recovery and reuse are feasible within a closed-loop solvent management system. In line with standard E-factor methodology, recovered solvents are not considered part of the waste stream.

Laboratory-scale lignocellulosic fractionation processes typically exhibit relatively high E-factors due to extensive washing and solvent handling steps that have not yet been optimised through solvent recovery or counter-current operation (Sheldon 2007; Clark et al. 2009).

Based on an experimental mass balance of 1000 g of *E. globulus* residues (Fig. 4), the total amount of desired products is 174 g of pure lignin and 470 g of monosaccharides (353 g of glucose and 117 g of xylose), making a total of 644 g of valorised products.

When both lignin and monosaccharides were considered valorised products within an integrated biorefinery approach, the total amount of desired products was 644 g (174 g of lignin and 470 g of sugars). The waste mass in this case included residual solids (approximately 200 g), the acetone used for lignin purification (1600 g), the sulfuric acid used for precipitation (approximately 200 g), and the concentrated aqueous effluent generated during washing

(approximately 5500 g). Assuming this scenario, the total waste amounted to around 7500 g, resulting in an estimated E-factor of 11.6.

This value highlights the improved material efficiency achieved when both lignin and carbohydrate fractions are fully valorised, rather than treating lignin as a single product. If the aqueous medium used during enzymatic hydrolysis is also included in the calculation, the waste mass increases further. With an estimated 5000 g of process water associated with hydrolysis at the reported sugar concentration of 91 g/L, the total waste rises to around 12,500 g, giving a corresponding E-factor of 19.4. This increase reflects the impact of aqueous process streams on material efficiency, particularly at a laboratory scale where solvent and water consumption have not yet been optimised through recovery or process intensification strategies.

Therefore, the range of 12–20 reported in the present study represents a conservative estimate that reflects laboratory practice while avoiding the overestimation that would be associated with counting all process water as unrecoverable waste. These results suggest that aqueous streams are the primary contributors to the material footprint at this stage of development and indicate that implementing water recycling strategies could reduce the effective E-factor further under industrial conditions.

In addition to the E-factor, the efficiency of the solvent was assessed using the active solvent intensity (ASI) metric, which is defined as the mass of active solvent used per unit mass of extracted lignin. From a Green Chemistry perspective, using diluted imidazole in water substantially improved solvent utilisation efficiency. When normalised to the amount of lignin removed, the ASI was reduced by around 65% compared to reports in the literature based on pure imidazole systems, while maintaining a similar degree of delignification (~70 wt%) (Nishida et al. 2021; Valladares-Diestra et al. 2023). Notably, introducing water as a low-impact co-solvent did not affect extraction performance but enabled a significant reduction in organic component consumption.

Finally, the operating conditions employed (120 °C, atmospheric pressure, an aqueous medium, and a low promoter concentration) were considered mild, as they are considerably less severe than those typically required for conventional acidic or alkaline pretreatments.

Based on these factors, the sustainability of the proposed process should be considered in a comparative and process-oriented sense, reflecting the following: (i) high carbon efficiency; (ii) improved solvent efficiency, as evidenced by the reduced ASI; and (iii) full utilisation of both the extracted lignin and the carbohydrate-rich solid, resulting in minimal solid waste generation and relatively low E-factor.

Conclusions

A new hydro-organo-thermal treatment based on the lignin-first approach has been optimised to close the cycle of complete valorisation of *E. globulus* mixed residues (branches, bark, and leaves), producing pure lignin and sugar-rich syrup. The raw lignocellulosic biomass was subjected to a delignification process based on the use of dilute aqueous imidazole solution (35 wt%) under mild reaction conditions (120 °C, 2 h, 9 wt% biomass loading) to selectively solubilise lignin and obtain a polysaccharide-rich solid material. The effect of imidazole concentration and reaction time on the removal of lignin, glucan, and xylan was investigated. Under the optimised reaction conditions, lignin solubilisation was 66 wt% with respect to the content in the raw biomass, and the solid residue accounted for about 58 wt%. This solid residue was composed of 56 wt% glucan, 19 wt% xylan, and 16.1 wt% lignin, confirming the enrichment of valuable sugars. It was then subjected to enzymatic hydrolysis in the presence of a new enzyme mixture based on the use of Cellic® CTec3 HS supplemented with endo-1,4- β -xylanase from *T. viride*. The effect of biomass loading (10, 15, and 25 wt%) and enzyme dosage (15, 25, and 35 FPU/g glucan) on glucose and xylose yields was investigated. Under the optimised reaction conditions (10 wt% biomass loading, 25 FPU/g glucan, 48 h), glucose and xylose yields were 98.5 and 92 mol%, respectively. A hydrolysate with about 90 g/L total reducing sugars was obtained, which represents a promising syrup for further catalytic upgrading to value-added compounds via chemical and/or biological routes. Finally, the complete and tailored fractionation and conversion of the different components of this non-edible biomass represents a step forward in the perspective of developing green and sustainable biorefinery processes.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00253-026-13793-2>.

Acknowledgements N.D.F. would like to thank the colleagues at the ENEA Trisaia Research Centre for the hospitality in their laboratories and Novozymes for providing the Cellic® CTec3 HS enzyme.

Author contributions A.C.: Methodology, Formal analysis, Validation, Writing - Review & Editing. F.L.: Validation, Formal analysis, Writing - Review & Editing. V.V.: Validation, Formal analysis. E.V.: Formal analysis, Supervision, Writing - Review & Editing. I.D.B.: Methodology, Supervision, Funding acquisition, Writing - Review & Editing. N.D.F.: Conceptualisation, Methodology, Formal analysis, Writing - Original Draft.

Funding Open access funding provided by Università degli Studi di Bari Aldo Moro within the CRUI-CARE Agreement.

Data availability All data supporting the findings of this study are available within the paper and its Supplementary Information.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Asghar A, Sairash S, Hussain N, Baqar Z, Sumrin A, Bilal M (2022) Current challenges of biomass refinery and prospects of emerging technologies for sustainable bioproducts and bioeconomy. *Biofuels Bioprod Biorefin* 16:1478–1494. <https://doi.org/10.1002/bbb.2403>
- Baral P, Munagala M, Shastri Y, Kumar V, Agrawal D (2021) Cost reduction approaches for fermentable sugar production from sugarcane bagasse and its impact on techno-economics and the environment. *Cellulose* 28:6305–6322. <https://doi.org/10.1007/s10570-021-03940-5>
- Başakçılardan Kabakcı S, Tanış MH (2021) Pretreatment of lignocellulosic biomass at atmospheric conditions by using different organosolv liquors: a comparison of lignins. *Biomass Convers Biorefin* 11:2869–2880. <https://doi.org/10.1007/s13399-020-00677-2>
- Caporusso A, Casiello M, De Leo V, Perna A, Catucci L, D'Accolti L, Capece A, De Bari I (2025a) Low impact methods for microbial lipids production for third generation biofuels and biobased polyurethanes. *J Environ Chem Eng* 13:117375. <https://doi.org/10.1016/j.jece.2025.117375>
- Caporusso A, Radice M, Biundo A, Gorgoglione R, Agrimi G, Pisano I (2025b) Waste cooking oils as a sustainable feedstock for biobased application: a systematic review. *J Biotechnol* 400:48–65. <https://doi.org/10.1016/j.jbiotec.2025.02.003>
- Channiwala SA, Parikh PP (2002) A unified correlation for estimating HHV of solid, liquid and gaseous fuels. *Fuel* 81:1051–1063. [https://doi.org/10.1016/S0016-2361\(01\)00131-4](https://doi.org/10.1016/S0016-2361(01)00131-4)
- Clark JH, Deswarte FEI, Farmer TJ (2009) The integration of green chemistry into future biorefineries. *Biofuels Bioprod Biorefin* 3:72–90. <https://doi.org/10.1002/bbb.119>
- Constant S, Wienk HLJ, Frissen AE, De Peinder P, Boelens R, Van Es DS, Grisel RJH, Weckhuysen BM, Huijgen WJJ, Gosselink RJA, Bruijninx PCA (2016) New insights into the structure and composition of technical lignins: a comparative characterisation study. *Green Chem* 18:2651–2665. <https://doi.org/10.1039/c5gc03043a>
- D'Orsi R, Di Fidio N, Antonetti C, Raspolli Galletti AM, Operamolla A (2023) Isolation of pure lignin and highly digestible cellulose from defatted and steam-exploded *Cynara cardunculus*. *ACS Sustainable Chemistry & Engineering* 11:1875–1887. <https://doi.org/10.1021/acssuschemeng.2c06356>
- Di Fidio N, Carmassi L, Kasmiarti G, Fulignati S, Licursi D, Raspolli Galletti AM, Antonetti C (2024) Chemical and enzymatic hydrolysis of waste wheat bran to sugars and their simultaneous biocatalytic conversion to valuable carotenoids and lipids. *Catal Today* 442:114941. <https://doi.org/10.1016/J.CATTOD.2024.114941>
- El Moustaqim M, El Kaihal A, El Marouani M, Men-La-Yakhaf S, Taibi M, Sebbahi S, El Hajjaji S, Kifani-Sahban F (2018) Thermal and thermomechanical analyses of lignin. *Sustain Chem Pharm* 9:63–68. <https://doi.org/10.1016/j.scp.2018.06.002>
- Fockink DH, Urio MB, Sánchez JH, Ramos LP (2017) Enzymatic hydrolysis of steam-treated sugarcane bagasse: effect of enzyme loading and substrate total solids on its fractal kinetic modeling and rheological properties. *Energy Fuel* 31:6211–6220. <https://doi.org/10.1021/acs.energyfuels.7b00818>
- Fulignati S, Antonetti C, Raspolli Galletti AM, Bonaldi L, Ribechini E, Mattonai M, Licursi D, Di Fidio N (2024) In-depth investigation of the reaction parameters tuning the ethyl levulinate synthesis from fructose and inulin. *ChemCatChem* 16:e202400381. <https://doi.org/10.1002/cctc.202400381>
- Gomes MG, Gurgel LVA, Baffi MA, Pasquini D (2020) Pretreatment of sugarcane bagasse using citric acid and its use in enzymatic hydrolysis. *Renew Energy* 157:332–341. <https://doi.org/10.1016/j.renene.2020.05.002>
- Kim JK, Yang J, Park SY, Yu JH, Kim KH (2019) Cellulase recycling in high-solids enzymatic hydrolysis of pretreated empty fruit bunches. *Biotechnol Biofuels* 12:138. <https://doi.org/10.1186/s13068-019-1476-x>
- Kurul F, Doruk B, Topkaya SN (2025) Principles of green chemistry: building a sustainable future. *Discov Chem* 2:68. <https://doi.org/10.1007/s44371-025-00152-9>
- López M, Santos V, Del Río JC, Rencoret J, Parajó JC (2020) One-pot processing of *Eucalyptus globulus* wood under microwave heating: simultaneous delignification and polysaccharide conversion into platform chemicals. *ACS Sustain Chem Eng* 8:10115–10124. <https://doi.org/10.1021/acssuschemeng.0c02023>
- Lu X, Zhu X, Guo H, Que H, Wang D, Liang D, He T, Hu C, Xu C, Gu X (2020) Efficient depolymerization of alkaline lignin to phenolic compounds at low temperatures with formic acid over inexpensive Fe-Zn/Al₂O₃ catalyst. *Energy Fuels* 34:7121–7130. <https://doi.org/10.1021/acs.energyfuels.0c00742>
- Luo Z, Qian Q, Sun H, Wei Q, Zhou J, Wang K (2023) Lignin-first biorefinery for converting lignocellulosic biomass into fuels and chemicals. *Energies* 16:125. <https://doi.org/10.3390/en16010125>
- Morais ARC, Pinto JV, Nunes D, Roseiro LB, Oliveira MC, Fortunato E, Bogel-Lukasik R (2016) Imidazole: prospect solvent for lignocellulosic biomass fractionation and delignification. *ACS Sustain Chem Eng* 4:1643–1652. <https://doi.org/10.1021/acssuschemeng.5b01600>
- Nagarajan S, Skillen NC, Irvine JTS, Lawton LA, Robertson PKJ (2017) Cellulose II as bioethanol feedstock and its advantages over native cellulose. *Renew Sustain Energy Rev* 77:182–192. <https://doi.org/10.1016/j.rser.2017.03.118>
- Nishida VS, Woiciechowski AL, Valladares-Diestra KK, Torres LAZ, de Souza Vannberghe LP, Zandona Filho A, Soccol CR (2021) Bioethanol and succinic acid co-production from imidazole-pretreated soybean hulls. *Ind Crops Prod* 172:114060. <https://doi.org/10.1016/j.indcrop.2021.114060>
- Pandey A, Sharma YC (2024) Advancements in biomass valorization in integrated biorefinery systems. *Biofuels Bioprod Biorefin* 18:2078–2090. <https://doi.org/10.1002/bbb.2670>
- Paone E, Tabanelli T, Mauriello F (2020) The rise of lignin biorefinery. *Curr Opin Green Sustain Chem* 24:1–6. <https://doi.org/10.1016/j.cogsc.2019.11.004>
- Pereira PMA, Bernardo JR, Oliveira MC, Roseiro LB, Łukasik RM (2021a) Depolymerization of lignin from extracted solid waste of *Cupressus lusitanica* mill. biomass using imidazole.

- Waste Biomass Valoriz 12:1341–1355. <https://doi.org/10.1007/s12649-020-01087-1>
- Pereira PMA, Bernardo JR, Roseiro LB, Gírio F, Łukasik RM (2021b) Imidazole processing of wheat straw and *eucalyptus* residues—comparison of pre-treatment conditions and their influence on enzymatic hydrolysis. *Molecules* 26:7591. <https://doi.org/10.3390/molecules26247591>
- Reshmy R, Athiyaman Balakumaran P, Divakar K, Philip E, Madhavan A, Pugazhendhi A, Sirohi R, Binod P, Kumar Awasthi M, Sindhu R (2022) Microbial valorization of lignin: prospects and challenges. *Bioresour Technol* 344:126240. <https://doi.org/10.1016/j.biortech.2021.126240>
- Romání A, Ruiz HA, Teixeira JA, Domingues L (2016) Valorization of *Eucalyptus* wood by glycerol-organosolv pretreatment within the biorefinery concept: an integrated and intensified approach. *Renew Energy* 95:1–9. <https://doi.org/10.1016/j.renene.2016.03.106>
- Savian CM, Scapini T, Martínez-Burgos WJ, Zevallos Torres LA, Zandoná Filho A, Ramos LP, Woiciechowski AL, Soccol CR (2024) Imidazole as a new strategy in the lignocellulosic pretreatment: an application in corn sub-products with a focus on bioenergy production. *Biomass Bioenergy* 183:107075. <https://doi.org/10.1016/j.biombioe.2024.107075>
- Sayury Nishida V, Lorenci Woiciechowski A, Valladares-Diestra KK, Zevallos Torres LA, de Porto Souza Vannberghe L, Zandoná Filho A, Soccol CR (2021) Bioethanol and succinic acid co-production from imidazole-pretreated soybean hulls. *Ind Crops Prod* 172:114060. <https://doi.org/10.1016/j.indcrop.2021.114060>
- Sethupathy S, Murillo Morales G, Gao L, Wang H, Yang B, Jiang J, Sun J, Zhu D (2022) Lignin valorization: status, challenges and opportunities. *Bioresour Technol* 347:126696. <https://doi.org/10.1016/j.biortech.2022.126696>
- Sheldon RA (2007) The e factor: fifteen years on. *Green Chem* 9:1273–1283. <https://doi.org/10.1039/B713736M>
- Sheldon RA (2017) The E factor 25 years on: the rise of green chemistry and sustainability. *Green Chem* 19:18–43. <https://doi.org/10.1039/C6GC02157C>
- Sluiter A, Hames B, Ruiz RO, Scarlata C, Sluiter J, Templeton D (2004a) Determination of ash in biomass: laboratory analytical procedure (LAP). Technical Report. National Renewable Energy Laboratory NREL/TP-510-42622 1–5. TP-510-42622
- Sluiter A, Ruiz RO, Scarlata C, Sluiter J, Templeton D (2004b) Determination of extractives in biomass: laboratory analytical procedure (LAP). Technical Report. National Renewable Energy Laboratory NREL/TP-510-42619 1–9. 2008
- Sluiter A, Hames B, Ruiz R, Slui CS, Templeton D, Crocker D (2008a) Determination of structural carbohydrates and lignin in biomass: laboratory analytical procedure (LAP). Technical Report. National Renewable Energy Laboratory NREL/TP-510-42618 1–15.
- Sluiter A, Hames B, Hyman D, Payne C, Ruiz R, Scarlata C, Sluiter J, Templeton D, Wolfe J (2008b) Determination of total solids in biomass and total dissolved solids in liquid process samples: laboratory analytical procedure (LAP). Technical Report. National Renewable Energy Laboratory NREL/TP-510-42621 9:1–6.
- Sun FF, Hong J, Hu J, Saddler JN, Fang X, Zhang Z, Shen S (2015) Accessory enzymes influence cellulase hydrolysis of the model substrate and the realistic lignocellulosic biomass. *Enzyme Microb Technol* 79:42–48. <https://doi.org/10.1016/j.enzmictec.2015.06.020>
- Tavares D, Cavali M, Tanobe VOA, Torres LAZ, Rozendo AS, Zandoná Filho A, Soccol CR, Woiciechowski AL (2022) Lignin from residual sawdust of *Eucalyptus* spp.—isolation, characterization, and evaluation of the antioxidant properties. *Biomass* 2:195–208. <https://doi.org/10.3390/biomass2030013>
- Valladares-Diestra KK, de Porto Souza Vannberghe L, Nishida VS, Zevallos Torres LA, Zandoná Filho A, Soccol CR (2022) Integrated sugarcane biorefinery for first- and second-generation bioethanol production using imidazole pretreatment. *J Clean Prod* 381:135179. <https://doi.org/10.1016/j.jclepro.2022.135179>
- Valladares-Diestra KK, de Porto Souza Vannberghe L, Nishida VS, Soccol CR (2023) The potential of imidazole as a new solvent in the pretreatment of agro-industrial lignocellulosic biomass. *Bioresour Technol* 372:128666. <https://doi.org/10.1016/j.BIORTECH.2023.128666>
- Viola E, Zimbardi F, Morgana M, Cerone N, Valerio V, Romanelli A (2021) Optimized organosolv pretreatment of biomass residues using 2-methyltetrahydrofuran and *n*-butanol. *Processes* 9:2051. <https://doi.org/10.3390/pr9112051>
- Zevallos Torres LA, Lorenci Woiciechowski A, Nishida VS, Valladares-Diestra KK, de Porto Souza Vannberghe L, Zandoná Filho A, Soccol CR (2023) Imidazole pretreatment of oil palm empty fruit bunches for ethanol and succinic acid co-production by *Saccharomyces cerevisiae* and *Pichia stipitis*. *Bioenergy Res* 16:990–1000. <https://doi.org/10.1007/s12155-022-10512-3>
- Zhao X, Zhang L, Liu D (2012) Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. *Biofuels Bioprod Biorefin* 6:465–482. <https://doi.org/10.1002/bbb.1331>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.