Synergetic effect of hydrothermal and deep eutectic solvents (DES) pretreatments on Robinia wood fractionation for the manufacture of bioethanol and cellulose nanocrystals

Alba Pérez-Pérez [a,b], Pablo G. Del-Río [a,b,c,*], Álvaro Lobato-Rodríguez [a,b], Gil Garrote [a,b], Beatriz Gullón [a,b]

[a] *Universidade de Vigo, Departamento de Enxeñaría Química, Facultade de Ciencias, 32004 Ourense, Spain*
[b] *Instituto de Agroecoloxía e Alimentación (IAA). Universidade de Vigo – Campus Auga, 32004 Ourense, Spain*
[c] *Stokes Laboratories, School of Engineering, Bernal Institute, University of Limerick, Limerick V94 T9PX, Ireland*

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ABSTRACT

This study dealt with the use of environmentally friendly processes based on microwave-assisted autohydrolysis (MAA) and deep eutectic solvents (DES) for the selective fractionation of *Robinia pseudoacacia* wood (RW) within a biorefinery approach. MAA enabled the recovery of 76% of hemicelluloses in the form of oligomers. Afterwards, different conditions were assessed for the optimal delignification of RW with the DES choline chloride combined with lactic acid reaching delignification ratios up to 86%. Two different methods were accomplished to valorize the cellulosic-rich solid fraction after delignification: (i) bioethanol via enzymatic-fermentative pathway (attaining 53.3 g ethanol/L, about 83% of ethanol yield), and (ii) cellulose nanocrystals (length of 27–550 nm, width of 2–12 nm). Hence, this study presents a novel multiproduct biorefinery to selectively separate the main components of RW and valorize its cellulosic fraction using eco-friendly procedures.

1. Introduction

The ever-increasing human demand for fuels and chemicals is implying the depletion of non-renewable reserves and the increasing of environmental issues such as greenhouse gas emissions, climate change and global warming (del Río et al., 2022a; Kim et al., 2020). This urgent need leads to the search for renewable and sustainable sources, being the most abundant and promising the lignocellulosic biomass (LB) (W. W. Li et al., 2021; C. Li et al., 2021).

Regarding the different LB, *Robinia pseudoacacia* (otherwise called black locust) represents a fast-growing tree, native to the SE of the United States that has been naturalized in Europe since the 17th century (Martín-Sampedro et al., 2019; Yang et al., 2020). The black locust has a high biomass production (14 t·ha^{-1}·yr^{-1}) (Sixto et al., 2015; Straker et al., 2015), and has a good behavior in stressful situations such as lack of water, extreme temperatures or severe conditions of salinity, acidity, and alkalinity (Kamperidou et al., 2021; Straker et al., 2015). Nevertheless, *Robinia pseudoacacia* may be considered an environmental problem due to its easy spread and the negative impact on native vegetation (Straker et al., 2015), being deemed as one of the 40 most

invasive woody angiosperms in the world due to its high invasiveness (Vítková et al., 2017). Therefore, a strategy based on the employment of this plant species as a source to produce commercial commodities (Jiménez-López et al., 2020) may provide both environmental relief and economic profitability.

To fully exploit the possibilities of LB and to selectively separate its main components (cellulose, hemicellulose and lignin), the selection of specific pretreatments is key, looking for economic, efficient, and sustainable procedures (Padilla-Rascón et al., 2022). Accordingly, microwave-assisted autohydrolysis meets these requirements, using only water, avoiding the use of toxic and corrosive reagents, and being highly selective with the solubilization of hemicelluloses. In addition, the use of microwaves provides uniform heating of the samples, accelerating the hydrolysis reactions and shortening the reaction times, with the consequent energy efficiency (Aguilar-Reynosa et al., 2017; del Río et al., 2022b; Kuittinen et al., 2016).

On the other hand, the integral valorization of LB would require the lignin solubilization to efficiently employ the remaining cellulose. Several methods (organosolv, alkali…) have been studied for biomass delignification; however, deep eutectic solvents (DES) have been

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introduced as emerging green solvents around lignin extraction due to being non-toxic, safe, easy to synthesize, can be tailored, and are recyclable and economic. These mixtures comprising a hydrogen bond donor (HBD) and acceptor (HBA) can break ester and hydrogen bonds between lignin and carbohydrates, hence their high selectivity (del Río et al., 2022a; Draszewski et al., 2021; Mankar et al., 2022). Pretreatments of lignocellulosic biomass using intensification technologies such as microwaves and smart solvents such as DES have already been postulated by other authors in the literature (Jose et al., 2023) and the combination of pretreatments based on the solubilization of hemicelluloses and lignin has a high potential to be used in a multiproduct biorefinery due to their selectivity.

Obtaining separate streams of the three main fractions of lignocellulosic biomass allows the conversion of each of them into different products of interest, such as platform chemicals (methanol, ethanol, glycerol or propanol), food additives (lactic acid, ethyl lactate, succinic acid or lysine), biomaterials and biopolymers (polysaccharides-based hydrogels or films), or bioenergy (bioethanol, biobutanol, biomethane or syngas) (Cheng et al., 2020; Sriariyanun and Kitsubthawee, 2020). For example, hemicelluloses can be used to obtain oligosaccharides (prebiotic activity) and lignin is a precursor of bioproducts of wide applicability (antioxidants). On the other hand, cellulose is often hydrolyzed to glucose to obtain bioethanol or other compounds, like cellulose nanocrystals, which are very interesting in the medical or food sector (García et al., 2016; Morales et al., 2020). This valorization of *Robinia pseudoacacia* wood following a biorefinery concept would fit within the concept of circular economy and zero waste production proposed in the literature for other similar biomasses (Dávila et al., 2019).

This work aims the study of the combined effect of microwave-assisted autohydrolysis followed by DES-delignification for the selective separation of components from *Robinia pseudoacacia* wood. Hence, different conditions for the optimal separation of hemicelluloses and lignin were evaluated, obtaining an aqueous phase rich in hemicellulosic oligomers, a black bleach from DES extraction containing lignin and a solid phase enriched in cellulose. The cellulose fraction was also further evaluated for the formation of cellulose nanocrystals (CNC) and to produce bioethanol via biological pathway. To our knowledge, there are no studies on delignification processes with DES of this raw material and its use to produce cellulose nanocrystals and bioethanol has not been investigated either.

2. Materials and methods

2.1. Reagents employed

Acetic acid (CH_3COOH, glacial, ACS reagent, Reag. USP, $\geq$99.8%), Ethanol (C_2H_5OH, Ph. Eur., 96.0% v/v), Glycerol 30°Bé (CH_2OH-$CHOHCH_2OH$, USP-Fu-Ph.Franc.-BP-DAB), L (+)-Lactic acid ($CH_3CHOHCOOH$, BP-DAB-FU-Ph.Eur.-Ph. Franc., 90.0%), Sodium hydroxide pellets (NaOH, for analysis ACS-ISO, 97.0%), Sulfuric acid (H_2SO_4, for agroalimentary analysis, 72%) and Sulfuric acid (H_2SO_4, for microanalysis, 98%) were purchased from Carlo Erba Reagents. Cellic CTec2 cellulase was supplied by Novozymes. The Choline chloride ($HOCH_2CH_2N(CH_3)_3Cl$, 98 + %) was acquired from Alfa Aesar. *Saccharomyces cerevisiae* Ethanol Red ® was obtained from Fermentis, S. I. Lesaffre, France. Sodium Chlorite solution ($NaClO_2$, for synthesis, 25% w/w) was purchased from PanReac AppliChem (ITW Reagents).

2.2. Feedstock and analytics

Robinia pseudoacacia wood (RW) used in this study was provided by a local business (Aserradero de Madera de J. González Groba, S.L., O Porriño, Pontevedra, NW Spain). RW was stripped of its bark and dried until constant moisture. Subsequently, the wood was milled using a woodchipper and a laboratory-cutting mill to reduce the particle size to

below 1 mm, necessary for microwave treatment. Finally, it was stored in a dry and dark place at room temperature. The chemical composition was determined as explained in Table 1.

2.3. Microwave-assisted autohydrolysis (MAA) of Robinia pseudoacacia wood

A mixture of RW and water, at a solid loading (SL) of 6 g of wood/100 g of total weight, were introduced in G30 reaction vials and processed in a Monowave 450 microwave (Anton Paar, GmbH, Austria) equipped with an air compressor to cool the sample after the pretreatment. This equipment allows the selection of certain variables, such as the treatment temperature and the time it takes to reach that temperature. Accordingly, the power consumed is adjusted internally by the equipment to comply with the established parameters. After 5 min of continuous heating to reach the target temperature (230 °C), the sample was maintained for 0–10 min at continuous agitation (900 rpm) based on previous studies (Pérez-Pérez et al., 2023). The solid and liquid fractions were separated by filtration, and the solid was washed with water until neutral pH. The chemical composition of both fractions was determined as explained in Table 1.

The severity (S_0) was selected as parameter to measure the intensity of the pretreatment, using the following equation:

$$S_0 = \log R_0 = \log(R_{0HEATING} + R_{0ISOTHERMAL} + R_{0COOLING}) = \quad (1)$$

$$= \log\left(\int_0^{t_H} \exp\cdot\left(\frac{T(t) - T_{REF}}{\omega}\right)\cdot dt + \exp\cdot\left(\frac{T_{ISOT} - T_{REF}}{\omega}\right)\cdot t_{ISOT} \right.$$

$$\left. + \int_{t_H+t_{ISOT}}^{t_H+t_{ISOT}+t_C} \exp\cdot\left(\frac{T'(t) - T_{REF}}{\omega}\right)\cdot dt \right)$$

where R_0 represents the severity factor (min), t_H is the time (min) required to achieve the target temperature T_{ISOT} (°C), which is maintained for a time t_{ISOT} (min), the cooling period time is t_C (min), T(t) and T'(t) are the temperature profiles in the heating and cooling stages, and T_{REF} (reference temperature) and ω (activation energy parameter) present values established by the bibliography (100 °C and 14.7 °C, respectively).

2.4. DES-delignification of autohydrolyzed Robinia pseudoacacia wood

Deep eutectic solvents (DES), specifically choline chloride (ChCl) mixed with lactic acid (LA, molar ratios 2:1, 1:1, 1:3), glycerol (Gly, molar ratio 1:1) or oxalic acid (OxA, molar ratio 1:1), were prepared at 80 °C under magnetic stirring until obtaining a transparent and uniform liquid.

For the delignification procedure, dry autohydrolyzed RW and the selected DES were blended, at a SL of 6 g of wood/100 g of total weight, in a pyrex bottle with screw cap and placed in an autoclave at desired temperature and time. Afterwards, the solid and liquid phases were separated by filtration, and 50% v/v ethanol (50 °C) and a 0.1% solution of NaOH (Zhou et al., 2021) (60 °C) were used to wash the solid, prior to washing with water until neutral pH. The chemical composition of the solid was determined as explained in Table 1.

2.5. Enzymatic susceptibility of the processed Robinia pseudoacacia wood

Enzymatic hydrolysis of processed RW after (i) autohydrolysis, and (ii) DES-delignification were evaluated using the enzymatic cocktail Cellic CTec 2 (kindly provided by Novozymes, Denmark). The cellulosic activity was determined as explained in Table 1 and resulted in 116 FPU/mL.

The processed solids were subjected to enzymatic susceptibility evaluation in an orbital incubator at 50 °C, pH of 5 (adjusted with citrate buffer), SL= 5 g of RW/100 g total weight and enzyme to substrate ratio (ESR) of 20 FPU/g. Samples were withdrawn and centrifuged at

Table 1

Analytical methods and equipment employed in this study.

Analysis	Sample	Equipment	Reference
Moisture	Raw, autohydrolyzed, and delignified Robinia wood	Oven Memmert UNB500	(Sluiter et al., 2008a)
Ashes	Raw Robinia wood	Muffle furnace Select-Horn-TFT	(Sluiter et al., 2008b)
Extractives	Raw Robinia wood	Soxhlet extractors	(Sluiter et al., 2008d)
Quantitative acid hydrolysis (Polysaccharides determination)	Raw, autohydrolyzed, and delignified Robinia wood	HPLC-RI (Agilent, CA, USA). Rezex ROA-Organic Acid H+ (8%) column	(del Río et al., 2022b; Sluiter et al., 2008c)
Acid posthydrolysis (Oligosaccharides determination)	Liquors after microwave-assisted autohydrolysis	HPLC-RI (Agilent, CA, USA). Rezex ROA-Organic Acid H+ (8%) column	(del Río et al., 2022b)
Direct HPLC analysis (Monosaccharides determination)	Liquors after microwave-assisted autohydrolysis, saccharification samples, fermentation samples	HPLC-RI (Agilent, CA, USA). Rezex ROA-Organic Acid H+ (8%) column	(del Río et al., 2022b)
Non-volatile content and volatile content	Liquors after microwave-assisted autohydrolysis	Oven Memmert UNB500	(del Río et al., 2022b)
Cellulase activity	Cellic CTec2	Spectrophotometer ONDA UV-20	(Adney, B and Baker, 2008)
Surface morphology and size of the nanocrystals	Nanocrystals of Robinia wood	Veeco's Multimode 8 Nanoscope, peak force tapping mode (Tip Roc<10 nm, Silicon Tip on Nitride Lever, K=0.4 N/m, Frequency= 70 KHz).	(Morales et al., 2020)

12000 rpm (14000 x g) for 10 min, using the supernatant for glucose quantitation via HPLC (see Table 1).

The performance of enzymatic hydrolysis was measured as concentration (g/L) and as glucan to glucose conversion (GGC, %), which is determined as hereunder:

$$GGC = \frac{G_t - G_{t=0}}{\frac{Gn}{100} \cdot \frac{180}{162} \cdot \frac{\rho}{\frac{100}{C} - \frac{KL}{100}}} \cdot 100\% \qquad (2)$$

where G_t denotes the glucose content (g/L) at a time t and $G_{t=0}$ denotes the glucose concentration (g/L) at the beginning of the assay. Gn denotes the glucan content per 100 g of pretreated RW, 180/162 is the stoichiometric factor for glucan hydration regarding hydrolysis, ρ describes the density of the medium (commonly using 1005 g/L), SL represents the solid loading of the assay, measured in g of solid per 100 g of total weight, and KL represents the g of Klason lignin per 100 g of pretreated biomass.

2.6. Pre-saccharification and simultaneous saccharification and fermentation (PSSF) of the processed Robinia pseudoacacia wood

Saccharomyces cerevisiae Ethanol Red ® strain was incubated in sterilized liquid medium containing glucose, peptone, and yeast extract (concentrations of 20, 20 and 10 g/L, respectively) at 30 °C and 200 rpm for 15 h. Afterwards, the medium was centrifuged for 10 min at 2000 rpm (1050 x g), obtaining a pellet which was resuspended with 0.9% NaCl to get 200 g of fresh yeast/L (corresponding to 8 g of fresh yeast/L within the fermentation media, around 1.50 g of dry yeast/L within the fermentation media).

Processed RW (after autohydrolysis and DES-delignification) was sterilized with water at 121 °C for 15 min. The presaccharification stage was set at 48 h employing the hereunder conditions: SL of 11 or 14 g of solid/100 g of total weight, ESR of 20 FPU/g, at 50 °C and 150 rpm. After that 48 h, the previously said amount of inoculum was introduced in the medium to start the fermentation stage, the temperature and agitation were modified to 35 °C and 120 rpm, respectively. Samples were withdrawn and centrifuged at 12000 rpm (14000 x g) for 10 min, using the supernatant for glucose and ethanol quantitation via HPLC (see Table 1).

The ethanol yield was calculated as:

$$\text{Ethanol yield} = \frac{\text{EtOH}_t}{\frac{Gn}{100} \cdot \frac{180}{162} \cdot \frac{92}{180} \cdot \frac{\rho}{\frac{100}{SL} - \frac{KL}{100}}} \cdot 100\% \qquad (3)$$

where EtOHt describes the ethanol concentration (g/L) reached at time t, and the denominator represents the potential ethanol concentration where Gn is the glucan content per 100 g of pretreated biomass, 180/162 denotes the stoichiometric factor for glucan hydration regarding hydrolysis, whereas 92/180 denotes the stoichiometric factor for glucose to ethanol conversion, ρ designates the density of the medium (commonly using 1005 g/L), SL designates the solid loading of the assay, measured in g of solid per 100 g of total weight, and KL represents the g of Klason lignin per 100 g of pretreated biomass.

2.7. Bleaching of DES-delignified solid and cellulose nanocrystals (CNC) formation

RW after delignification procedure was subjected to bleaching as described by Morales et al. (2021), using acetic acid, sodium hypochlorite and distilled water at 75 °C with constant agitation for 2 h. The resulting mixture was vacuum-filtered, and the solid fraction was washed with water until neutral pH and dried for 24 h at 50 °C.

The resulting solid after bleaching was subjected to acid hydrolysis as described by Barbosa et al. (2016) using 50% H_2SO_4 at SL of 6 g/100 g for 1 h at 60 °C in an ultrasonic bath. Once the hydrolysis time was completed, the reaction was stopped with distilled water, and the solid and liquid were separated by filtration with vacuum, washing the solid phase (that corresponded to cellulose nanocrystals, CNC) until neutral pH. Eventually, the CNC were resuspended in water and sonicated to keep the suspension stable. For the topographic analysis by Atomic Force Microscopy (AFM) of the CNC, the previous suspension was stirred at 1600 rpm for 1 min, 30 µL of solution were deposited on the mica substrate (exfoliated several times before deposition), previously adhered to the AFM support. Once deposited, the sample was left to dry on a hot plate for a few minutes (30–40 °C). The characteristics of the equipment used are given in Table 1. In addition, a 11.9 µm scanner was employed to obtain the pictures. Images were made in diverse regions with various fields of view/magnifications, in different positions and significantly separated from each other. Fig. 1 shows a general scheme of the processing of *Robinia pseudoacacia* wood for the separation and valorization of its components.

3. Results and discussion

3.1. Effect of microwave-assisted autohydrolysis of Robinia wood

Robinia pseudoacacia wood, employed in this study, was chemically characterized prior to the microwave processing, obtaining the following composition (expressed in g/100 g of dry biomass): glucan 34.5 ± 0.3, Klason lignin 23.1 ± 0.4, hemicelluloses 16.9 ± 0.2 (including xylan 13.8 ± 0.2, arabinan 0.21 ± 0.05, acetyl groups 2.88
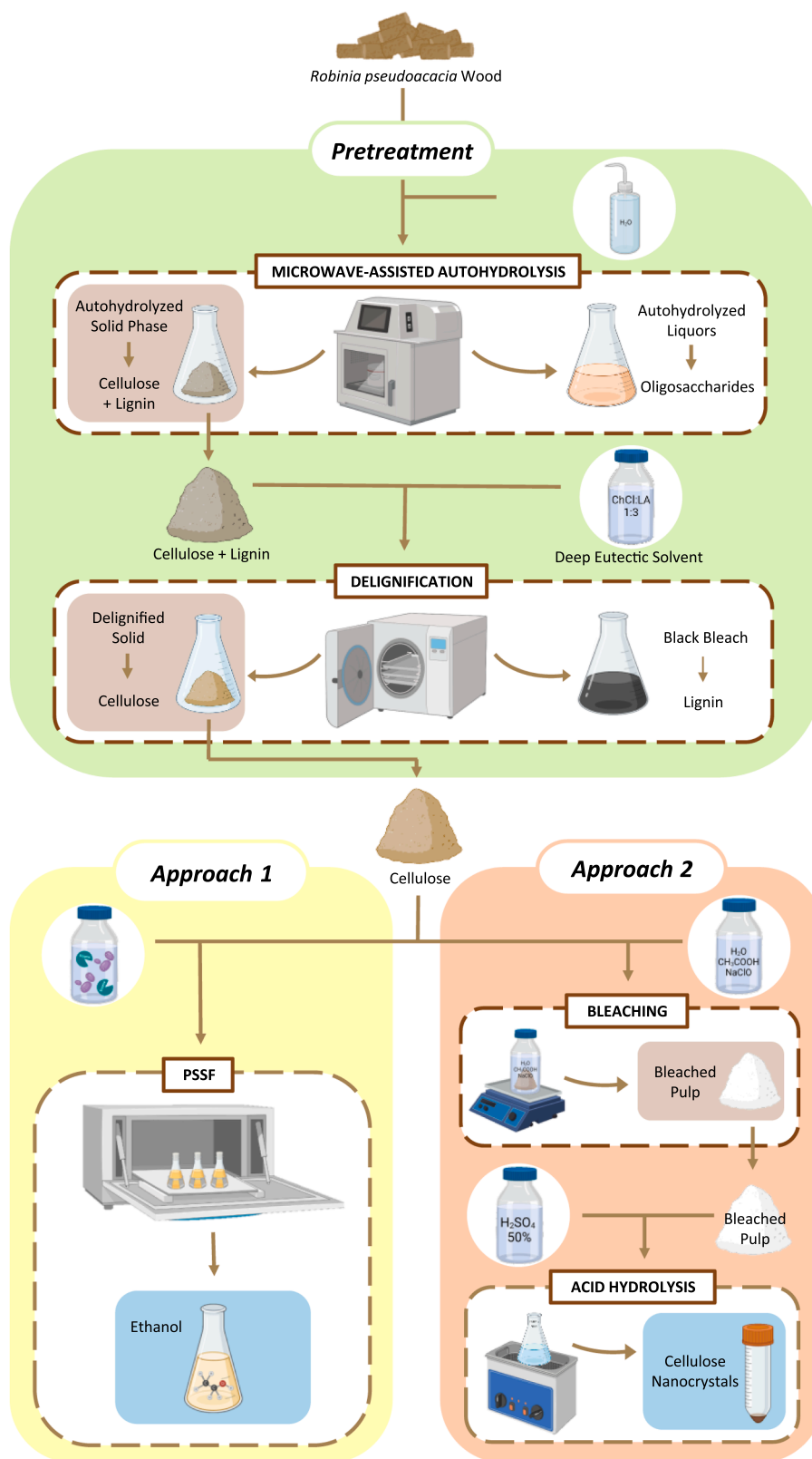
Fig. 1. Schematic representation of the processes tackled in this study for the integral valorization of *Robinia wood*. Created with BioRender.com.

± 0.04), ethanol extractives 13.81 ± 0.44, ashes 0.74 ± 0.01 and uronic acids 8.05 ± 0.20 (expressed as galacturonic acid equivalents). These results are similar to those obtained by other authors regarding the chemical composition of Robinia wood (Jiménez-López et al., 2020; Yang et al., 2020).

To assess the capacity of MAA to remove hemicelluloses and, consequently, obtain solids with higher enzymatic susceptibility, RW was subjected to that treatment employing a maximum temperature of 230 °C, at residence times of 0–10 min (severities of 3.62–4.85). The chemical characterization of both liquid and solid phases is displayed in

Table 2

Solid and liquid phases' composition of *Robinia wood* after MAA at 230 °C (the standard deviation is expressed in triplicate determinations).

T_{MAX} (°C)	230								
Reaction time (min)	0	0.25	0.5	0.75	1	2	5	7	10
S_0 (dimensionless)	3.62	3.79	3.87	3.96	4.04	4.25	4.57	4.71	4.85
Material balance data (g/100 g raw material, o. d. b.)									
Solid yield (SY)	72.4	72.0	70.9	69.9	69.9	67.6	66.2	66.3	66.9
Non-volatile compounds (NVC)	22.5	23.8	23.8	23.9	23.7	23.3	16.4	14.0	13.1
Volatile compounds (VC)	5.07	4.20	5.34	6.19	6.37	9.08	17.3	19.7	20.0
Solid phase composition (g/100 g autohydrolyzed Robinia, o. d. b.)									
Glucan (Gn)	49.9 ± 0.8	50.5 ± 0.3	51.9 ± 1.5	51.2 ± 0.9	54.0 ± 1.2	52.0 ± 2.7	51.5 ± 0.7	50.2 ± 1.3	49.2 ± 1.2
Xylan (Xn)	6.77 ± 0.09	5.63 ± 0.05	4.94 ± 0.04	4.35 ± 0.09	4.01 ± 0.13	2.83 ± 0.06	1.19 ± 0.07	0.79 ± 0.05	0.56 ± 0.08
Arabinan (Arn)	0.10 ± 0.03	0.14 ± 0.05	0.10 ± 0.02	0.09 ± 0.01	0.09 ± 0.03	0.06 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.02
Acetyl groups (AcG)	1.57 ± 0.10	1.39 ± 0.13	1.19 ± 0.15	1.46 ± 0.09	1.45 ± 0.02	1.09 ± 0.17	0.35 ± 0.05	0.12 ± 0.04	0.17 ± 0.05
Klason lignin (KL)	35.7 ± 1.4	37.9 ± 0.9	36.9 ± 1.5	38.3 ± 0.9	37.9 ± 0.5	38.0 ± 2.7	42.1 ± 0.7	43.3 ± 1.4	43.8 ± 1.7
Liquid phase composition (g/L)									
Oligomers	9.00 ± 0.02	9.67 ± 0.04	9.42 ± 0.07	9.47 ± 0.04	9.48 ± 0.04	8.70 ± 0.10	3.08 ± 0.05	1.63 ± 0.02	0.85 ± 0.02
Monomers	0.68	0.82	0.90	1.01	1.13	1.46	2.34	1.82	1.43
Acetic acid and furans	0.42	0.60	0.81	0.92	1.09	1.44	3.76	4.41	4.99

Oligomers: sum of glucooligomers, xylooligomers, ararbinooligomers, and acetyl groups linked to oligomers; monomers: sum of glucose, xylose, and arabinose; furans: sum of hydroxymethylfurfural and furfural

Table 2.

Firstly, the solid yield (SY, a quantifiable measure of the effect of the treatment on the raw material) decreased in a narrow range between 72.4 and 67.6 g of autohydrolyzed RW/100 g of raw material, which implied that higher severities led to higher solubilization ratios. Regarding the non-volatile compounds (NVC), the values ranged 22.5–23.9 g of NVC/100 g of raw material for severities 3.62–4.25; however, its value decreased at higher severities up to 13.1 g/100 g of raw material. Conversely, volatile compounds (VC) increased with the severity from 4.20 to 20.0 g VC/100 g of raw material, due to the formation of volatile degradation compounds, such as acetic acid or furfural. Similar behaviors in these parameters (SY, NVC, VC) has been observed by other authors, such as by Domínguez et al. (2020) after non-isothermal autohydrolysis of Paulownia wood or when that same raw material was subjected to microwave-assisted hydrothermal treatment, as shown in the work by del Río et al. (2021).

Concerning the solid phase, the hemicelluloses were the most affected fraction, with solubilization raising from 64% to 97% as residence time was increased. On the other hand, the glucan content varied in a narrow range with an average value of 51.2 g glucan/100 g of autohydrolyzed RW (around 100% recovery of the initial glucan). The lignin fluctuated between 35.7 and 43.8 g lignin/100 g of autohydrolyzed RW, reaching recoveries higher than 100% that may be explained owing to the formation of pseudo-lignin and lignin degradation products (del Río et al., 2021, 2020; Sannigrahi et al., 2011). These results were higher than those observed by Vargas et al. (2015), who achieved 90% glucan and lignin recovery and a maximum hemicellulose removal of 86% after an autohydrolysis treatment under non-isothermal conditions of barley straw.

On the topic of the liquid phase, the main components were oligomers (expressed as sum of glucooligomers, xylooligomers, arabinooligomers and acetyl groups linked to oligomers). The maximum oligomer content was 9.67 g/L (of which 97% were hemicellulosic oligomers) reached at 230 °C for 0.25 min ($S_0 = 3.79$) and being mainly represented by xylooligomers (80% of the total of oligomers). At longer residence times, the oligomers triggered the formation of monomers (maximum of 2.34 g/L at $S_0 = 4.57$), and afterwards, degradation products such as furans (specifically furfural and hydroxymethylfurfural). Del Río et al. (2022b) and Dávila et al. (2021) also reported this trend when they carried out the microwave hydrothermal treatment of Paulownia wood and vine shoots, respectively.

3.2. Delignification of autohydrolyzed Robinia wood using deep eutectic solvents (DES)

Considering the abovementioned, conditions of MAA at 230 °C for 0.25 min were selected in order to recover high-added value hemi-cellulosic oligosaccharides, and the remaining spent solid was subjected to DES-delignification.

Although different choline chloride-DES have been studied, the combination with lactic acid (ChCl:LA, 1:3), glycerol (ChCl:Gly, 1:1) or oxalic acid (ChCl:OxA, 1:1) have reported interesting results for the solubilization of lignin (C. W. Li et al., 2021; C. Li et al., 2021). In this
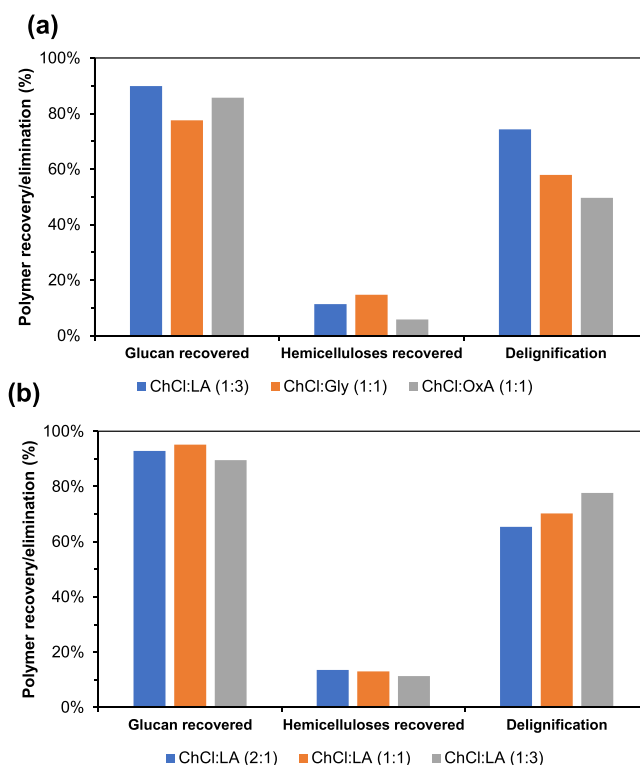
Fig. 2. Glucan and hemicelluloses recovery, and lignin removal after DES-delignification of autohydrolyzed RW (230 °C, 0.25 min): (a) evaluation of choline chloride:lactic acid (ChCl:LA (1:3)), choline chloride:glycerol (ChCl:gly (1:1)), choline chloride:oxalic acid (ChCl:OxA (1:1)) at 120 °C for 45 min, and (b) evaluation of choline chloride:lactic acid (ChCl:LA) at different molar ratios (2:1; 1:1 and, 1:3) at 120 °C for 60 min. Standard deviation was lower than 5%.

sense, preliminary evaluation of these DES is shown in Fig. 2a, displaying the glucan and hemicelluloses recovery, and the delignification ratio regarding initial composition of RW. The delignification conditions employed were a temperature of 120 °C, and a time of 45 min (based on previous experiments, data not shown). The glucan recovery varied in a narrow range (78–90% of initial glucan), similarly to what occurred with hemicelluloses (6–15% of the raw material hemicelluloses). If compared with the autohydrolyzed solid composition, this delignification procedure slightly affected the solubilization of glucan (74–85% of glucan is recovered regarding the autohydrolyzed solid), but contrariwise, between 52% and 80% of residual hemicelluloses was removed. This phenomenon is convenient since the elimination of excess hemicelluloses favors the development of subsequent enzymatic hydrolysis. On the other hand, the lignin was largely influenced, reaching delignification percentages between 50% and 74%. These results were in contrast with those obtained by C. W. Li et al. (2021); C. Li et al. (2021), who pretreated sugarcane bagasse at 100 °C for 4 h, achieving a lower lignin solubilization of 47.8% when using ChCl:OxA (1:1).

In this context, the combination ChCl:LA enabled a higher solubilization of lignin (74%), maintaining the cellulose practically unaltered, so this DES was selected for further experiments. Fig. 2b depicts the glucan and hemicelluloses recovery and the delignification of pretreated RW at 120 °C for 60 min with ChCl:LA at different molar ratios. As previously described, the cellulosic polysaccharide was barely affected by the processes, displaying a recovery of 90–95% of the initial glucan present in the raw material. As for hemicelluloses, only 11–14% of those present in the initial RW were recovered in the solid. This fact can be seen as positive, not only due to the great selectivity of this DES towards the non-solubilization of glucan, but also in order to obtain a lignin with high purity and low carbohydrate content (pseudo-lignin) (Alvarez-Vasco et al., 2016; Wang et al., 2020) due to the low hemicelluloses content in the solid after autohydrolysis. Finally, the effect on lignin was extensive, with solubilizations up to 78% when using a molar ratio of 1:3 (ChCl:LA). Other authors, such as Tan et al. (2019), have reported that the molar ratio had a meaningful impact on the lignin solubilization. In this sense, augmenting the amount of the hydrogen bond donor (HBD), for instance ChCl:LA to 1:3, 1:5, 1:10, or 1:15, may increase the extraction of lignin. Their work stated that using ChCl:LA at molar ratio of 1:15 instead of 1:1 would improve the solubilization of lignin from 33.5% to 61% (Tan et al., 2019). Similar behavior was observed by Li et al. (2017) when varying the molar ratio of ChCl:LA from 1:2–1:10 in the pretreatment of willow at 120 °C for 6 h, augmenting the delignification from 52% to 67% (Li et al., 2017).

In this sense, ChCl:LA at a molar ratio of 1:3 was selected since these conditions enabled a higher delignification ratio (78%) while maintaining a glucan recovery over 90%. The variation of the solid residue composition at different temperatures (110–130 °C) and reaction times (30–60 min) was studied and can be seen in Table 3. Firstly, the hemicellulosic content was partially affected by the pretreatments, with slightly content reduction at harsher conditions. Regarding the glucan content, it accounted for 74.3–81.5 g glucan/100 g of pretreated RW, which corresponded with an average glucan recovery of 92%, regarding

glucan in the initial RW. In relation to the lignin fraction, contents between 17.0 and 8.6 g of lignin/100 g of pretreated RW were achieved, corresponding to delignification ratios of 68–86% regarding initial lignin content. Similar delignification ratios were achieved by Alvarez-Vasco et al. (2016) who reached up to 72.1% of delignification after subjecting poplar wood to delignification with ChCl:LA at 120 °C for 3 h (Alvarez-Vasco et al., 2016).

Although the highest delignification was performed during the harsher conditions (130 °C, 60 min), it must be highlighted that at these conditions, the cellulose recovery was lessened to 87%, and that the increase in delignification was provided due to an increase in temperature and reaction time, with the consequent increment of energy consumption. Besides that, the increment of 15 min in the reaction time (if comparing 130 °C for 30 and 45 min) only increased a 2% the delignification ratio (from 76% to 78%), which does not support the waste of energy. Considering all these, intermediate conditions such as 120 °C 45 min and 130 °C 30 min, reached interesting delignification ratios (74% and 76%, respectively) with similar cellulose recovery (93–94%). For that reason, the energy spent by the autoclave, for the previously stated conditions, was quantified using a power-meter (Zaeel). Thus, 120 °C for 45 min was set as preferable condition since it enabled to consume almost 10% less energy that the other condition, reaching similar results of delignification and cellulose recovery.

3.3. Enzymatic susceptibility of Robinia wood solids either after microwave-assisted autohydrolysis (MAA) or after MAA followed by DES-delignification

The evaluation of the enzymatic saccharification of the pretreated biomass is key for the manufacture of bioethanol. In this sense, RW solids after MAA and after sequential MAA and DES-delignification treatments were enzymatically assessed. Fig. 3 displays the glucan to glucose conversion course of such experiments.

As previously stated by other authors, in autohydrolysis treatments, the increase of severity (i.e., larger reaction times), enable an easier access to cellulose, prompting higher glucan to glucose conversions (GGC) (Jesus et al., 2017; Pontes et al., 2018). In this sense, RW pretreated at 230 °C for 10 min achieved a maximum conversion of 71%, whereas when the residence time was reduced to 0.25 min, a conversion of 26% at 96 h was reached. Intermediate residence times in the pretreatment, between 0.25 and 10 min, led to GGC between 26% and 71% at 96 h of saccharification.

Only taking into account the enzymatic hydrolysis assay, the harsher conditions in autohydrolysis treatment seemed to be an interesting option for bioethanol production. Nonetheless, those pretreatment conditions would only enable to obtain a solid with high enzymatic susceptibility, but with a liquid phase with low hemicellulosic oligomers (2% regarding raw material) and monomers (8% regarding raw material) and high content in furfural (24% regarding raw material). On the other hand, the pretreatment at 230 °C for 0.25 min presented scarce enzymatic digestibility but with high concentration of oligomers (9.67 g/L), characterized by a high content of xylooligomers (80% of the

Table 3

Solid phase composition of *Robinia wood* pretreated by MAA (230 °C, 0.25 min) followed by DES-delignification with ChCl:LA (1:3) at different temperatures and reaction times (the standard deviation is expressed in triplicate determinations).

| Temperature (°C)	110	120	120	120	130	130	130
Reaction time (min)	60	30	45	60	30	45	60
Material balance data (g/100 g autohydrolyzed RW, o. d. b.)							
Solid yield (SY)	58.8	60.7	57.1	55.2	55.2	54.6	50.9
Solid phase composition (g/100 g delignified RW, o. d. b.)							
Glucan (Gn)	74.3 ± 0.0	76.0 ± 0.3	78.6 ± 0.6	77.7 ± 1.0	80.7 ± 0.7	79.7 ± 0.4	81.5 ± 0.2
Xylan (Xn)	4.14 ± 0.03	4.11 ± 0.09	4.12 ± 0.07	3.93 ± 0.06	4.27 ± 0.63	3.75 ± 0.07	3.54 ± 0.04
Arabinan (Arn)	0.31 ± 0.04	0.77 ± 0.03	0.75 ± 0.04	0.23 ± 0.03	0.78 ± 0.05	0.61 ± 0.41	0.37 ± 0.05
Acetyl groups (AcG)	0.95 ± 0.25	0.85 ± 0.16	0.70 ± 0.21	1.04 ± 0.25	0.63 ± 0.17	0.50 ± 0.05	1.21 ± 0.09
Klason lignin (KL)	14.9 ± 0.5	17.0 ± 0.1	15.0 ± 0.5	12.2 ± 0.9	14.0 ± 1.1	13.1 ± 0.4	8.60 ± 0.11
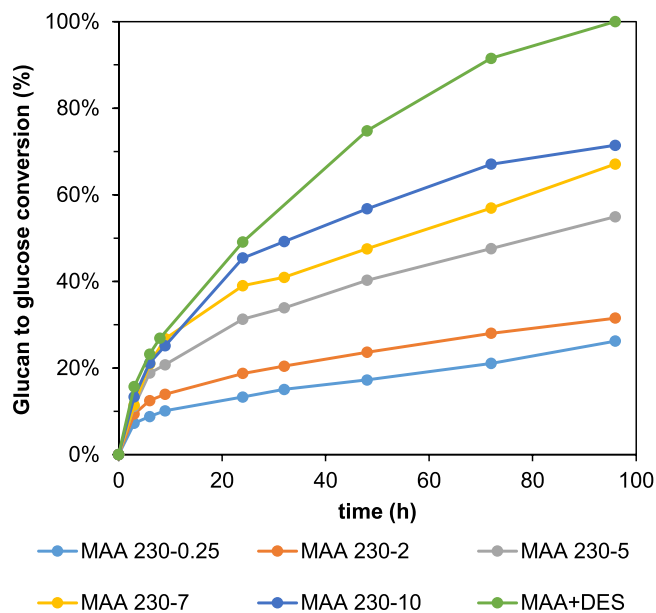
Fig. 3. Glucan to glucose conversion course during enzymatic saccharification of autohydrolyzed RW at 230 °C for 0.25–10 min and of RW subjected to autohydrolysis (230 °C, 0.25 min) followed by delignification (choline chloride: lactic acid (1:3), 120 °C for 45 min).

total oligomers). In this sense, 230 °C for 0.25 min may be the most interesting conditions as a first step, since these conditions resulted in the maximum solubilization of hemicelluloses in the form of oligomers, leaving a solid phase enriched in cellulose and lignin that could be subjected to other pretreatment, such as delignification, to improve its enzymatic susceptibility (Gullón et al., 2017). Moreover, since it is a shorter time (0.25 min vs 10 min), the energy consumption will be lower (18.12 vs 28.13 MJ/kg), making it a more efficient and economical treatment, as stated in previous works by the authors (Pérez-Pérez et al., 2023).

Based on the above, the selected solid phase after microwave-assisted autohydrolysis (230 °C 0.25 min) was subjected to a delignification treatment with DES (120 °C 45 min) to remove the lignin that have remained embedded in the solid phase. The saccharification assay of RW after the sequential process showed an improvement in enzyme accessibility to the cellulose in the processed solid with respect to the only autohydrolyzed RW. On the one hand, the autohydrolyzed RW under the most severe conditions reached less than 75% of GGC at 96 h, whereas for autohydrolyzed and delignified solid, quantitative glucose conversion was reached at the same saccharification time. Moreover, 48 h were enough to reach 75% of glucose conversion for the double-pretreated solid. In this sense, it is demonstrated the suitability of DES-delignification to remove the lignin with the aim of increasing the enzymatic susceptibility of the processed solid. Other authors have also observed a strong positive relationship between lignin elimination and the efficacy of enzymatic saccharification. This phenomenon is because lignin constitutes a physical barrier (lignin-carbohydrate complexes) that prevents contact between cellulases and the cellulosic fraction. In addition, residual lignin causes the non-productive adsorption of the cellulolytic enzyme, which limits proper enzymatic digestibility (Liang et al., 2021; Xie et al., 2021; Zhou et al., 2023).

In this context, del Río et al. (2020) studied that the combination of two pretreatments (autohydrolysis and delignification) on Paulownia wood led to solids with high enzymatic susceptibility, while at the same time allowed the recovery of other fractions of interest. Likewise, a work by Chen et al. (2017) demonstrated that the combination of autohydrolysis and subsequent delignification favored the enzymatic hydrolysis of bamboo (*Phyllostachys pubescens*) due to an increase in the

contact area between the enzyme and the cellulose. Vargas et al. (2016) concluded that a combined pretreatment strategy on barley straw resulted in solids with high enzymatic digestibility, suitable for bioethanol production. Hence, a delignification pretreatment step would be key in order to obtain high enzymatically digestible cellulose after autohydrolysis without the loss of hemicellulosic oligosaccharides.

3.4. Pre-saccharification and simultaneous saccharification and fermentation (PSSF) of Robinia wood after microwave-assisted autohydrolysis (MAA) followed by DES-delignification

Finally, PSSF was assayed with the RW after MAA followed by DES-delignification at SL of 11 and 14 g of biomass/100 g of total weight, choosing 48 h as presaccharification time, prior to the addition of inoculum. The glucose and ethanol time courses are displayed in Fig. 4.

The production of glucose during the first 48 h reached 88.7 g/L at a SL= 11 g/100 g, corresponding to a GGC= 91%. Similar glucose conversion was achieved when employing SL= 14 g/100 g, reaching 116.7 g of glucose/L at 48 h. At that moment, the inoculum was added, and 6 h afterwards, maximum ethanol titers of 40.3 and 53.3 g of ethanol/L were achieved for SL= 11 g/100 g and SL= 14 g/100 g, respectively. These values corresponded to ethanol yields of 81–83%, implying the obtainment of around 0.46 g of ethanol/g of glucose.

Other authors have also studied the production of bioethanol after treating biomass with DES, but most of them employed agricultural or agriforestal waste as biomass. One example is the work from Xian et al. (2022), who reported that wheat straw after DES treatment with triethylbenzyl ammonium chloride mixed with glycerol and acetic acid (TEBAC:GL:ACH, 1:2:0.05) at 120 °C for 1 h, may achieve up to 81.4% of ethanol conversion in SHF (separate hydrolysis and fermentation) at SL= 16%. Similar ethanol yield (81.5%) was achieved by Isci et al. (2020) using wheat straw pretreated with choline chloride and formic acid. On the other hand, miscanthus pretreated with ChCl:Gly at 100 °C for 6 h enabled to reach up to 72% of ethanol yield (Hassan and Mutelet, 2022).

3.5. Cellulose nanocrystals' characterization by Atomic Force Microscopy (AFM)

As a second way of valorizing the cellulosic fraction after DES-delignification of RW, the resulting spent solid was bleached and
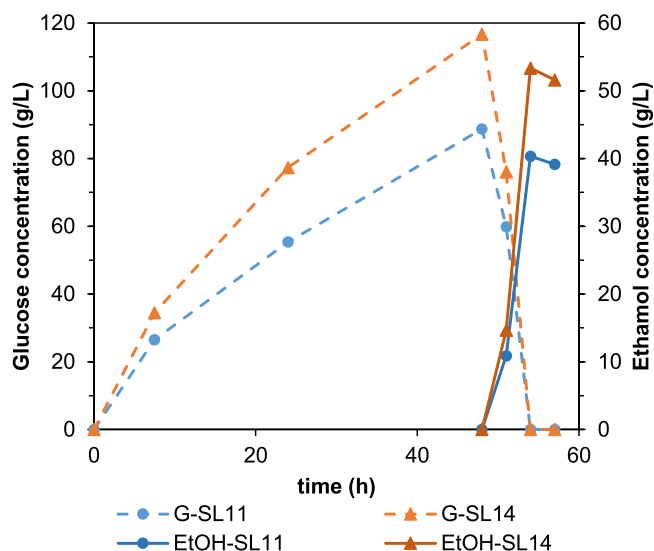

Fig. 4. Glucose (G) and ethanol (EtOH) course in the pre-saccharification and simultaneous saccharification and fermentation (PSSF) of pretreated RW (autohydrolysis and DES-delignification) at solid loading (SL) of 11 and 14 g/100 g.

subjected to acid hydrolysis, as explained in the materials and methods section. The cellulose nanocrystals (CNC) obtained were resuspended and sonicated, and the sample was employed in AFM as observed in Fig. 5.

The image shows elongated structures with a typical rod-shape morphology, with assorted dimensions: length varying from 27 to 550 nm, width (diameter) between 2 and 12 nm, and height of 0.762 (probably corresponding to an individual nanocrystal) to 15 nm (probably corresponding to a stacking and aggregation of multiple nanocrystals). The integration of the crystals is probably due to the establishment of hydrogen bonds and Van der Walls forces between them, as a consequence of their small size and their high specific surface area (Xiao et al., 2019). On the other hand, the observed unevenness in the dimensions of these nanostructures could be explained by the randomness in the degradation of cellulose during acid hydrolysis (Hemmati et al., 2018).

Kang et al. (2018) produced CNC with a length (120–400 nm) and diameter (3–10 nm) in the range of those obtained in this work, whereas Mujtaba et al. (2017) produced nanocrystals from flax fibers with an average length of 305 nm, similar to that found in this work (288.5 nm). Nonetheless, the average diameter obtained in this work is much smaller than that reported by Mujtaba et al. (2017) of 50 nm (flax fibers). In addition, Morales et al. (2020) also reported greater values of diameter of 70 nm and 61 nm for almond shells pretreated via organosolv and alkaline delignification. The differences observed in CNC dimensions can be associated with variations in treatment conditions. The size of the crystals may decrease when the hydrolysis time is increased or when ultrasound technologies are employed, due to the breaking of glycosidic bonds. In addition, other factors such as temperature and acid concentration also influence the size of these structures (Hemmati et al., 2018).

The dimensions of CNC are closely related to their properties and their applications. In this context, the average aspect ratio (length/ diameter) is an important factor. In this work, the aspect ratio observed was 41.21, a value considerably higher than that obtained by other authors using other raw materials, such as walnut shells (10.77) (Morales et al., 2021) or *Linum usitatissimum* (6.06) (Mujtaba et al., 2017). In general, those CNC with high aspect ratios (above 10) have a greater potential for application as a reinforcing agent, for example, in the production of edible films from biopolymers such as chitosan (Azeredo

et al., 2010; Xiao et al., 2019).

3.6. Mass balance of the multiproduct biorefinery

For obtaining an appropriate understanding of the complete process carried out in this study, a mass balance, exhibited in Fig. 6, was performed. In this figure, 100 kg of dry RW would be exposed to sequential extraction of hemicelluloses and lignin by microwave-assisted autohydrolyisis and delignification with DES to obtain a cellulose-rich solid valorize in two strategies: (i) ethanol obtainment and (ii) cellulose nanocrystals production.

During the first pretreatment (MAA), a liquor rich in oligomers (13.1 kg) and small amounts of monomers (1.15 kg) and furans+acetic acid (0.80 kg) was obtained, maintaining a solid enriched in cellulose and lignin. This value of oligomers is in accordance with that obtained after optimized autohydrolysis process of Paulownia wood by autohydrolysis (S_0 =4.08), reaching 13.94 kg (Domínguez et al., 2020). Subsequently, after the delignification by means of DES, a liquor enriched in lignin (16.9 kg) was obtained, which is higher than that obtained by del Río et al. (2020) after organosolv pretreatment (200 °C, 3 h, 50% ethanol) on autohydrolyzed Paulownia wood with 12.11 kg, and by Romaní et al. (2016) using autohydrolysis followed by lime pretreatment of oat straw with 7.54 kg.

Finally, the cellulose-rich solid was subjected to two different strategies, specifically CNC production and bioethanol manufacture via enzymatic-fermentative pathway. For the latter option, 13.7 kg of ethanol were obtained. This value is in accordance with that obtained after sequential autohydrolysis and lime delignification of oat straw with 15.91 kg (Romaní et al., 2016) and is higher that tat obtained by Morales et al. (2018) after alkali delignification of chestnut shells (7.5 kg).

Considering the above mentioned, the strategy carried out in this study enables an efficient separation of LB components by emerging technologies such as MAA and DES-delignification, opening the path to novel multiproduct biorefineries.

4. Conclusion

Robinia wood was subjected to sequential microwave-assisted autohydrolysis and DES-delignification as pretreatments. The first step enabled to recover 76% of hemicelluloses in oligomeric form, while the second permitted up to 86% of delignification. The spent solid was employed to produce bioethanol, reaching high ethanol yield of 83% (53.3 g ethanol/L), and was also transformed in cellulose nanocrystals. This work proposed a novel and green biorefinery scheme to selectively separate Robinia wood components and valorize the glucan fraction, enabling new chances for the sustainable valorization of the lignocellulosic biomass.

CRediT authorship contribution statement

Alba Pérez-Pérez: Formal analysis, Investigation, Writing – original draft, Visualization, Writing – review & editing. **Pablo G. del Río**: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Visualization, Writing – review & editing. **Álvaro Lobato-Rodríguez**: Investigation, Visualization, Writing – review & editing. **Gil Garrote**: Funding acquisition, Supervision, Validation, Writing – review & editing. **Beatriz Gullón**: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Writing – original draft, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence
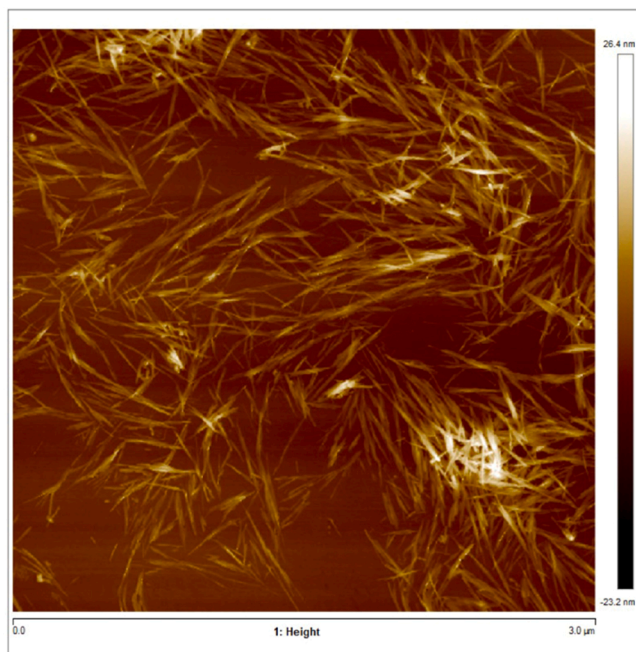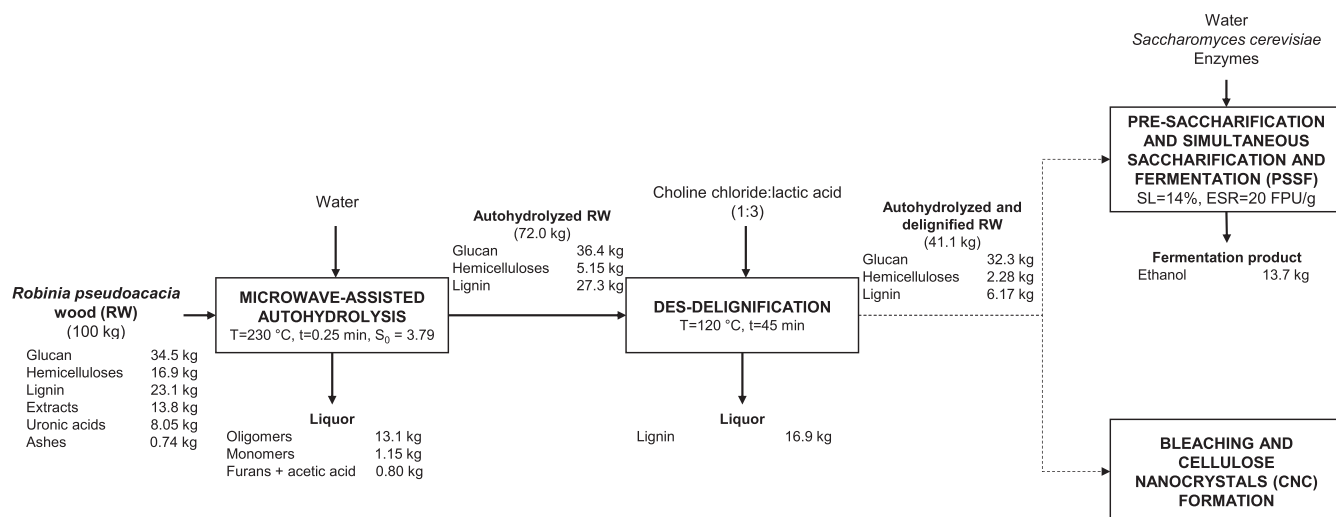

Fig. 5. 2D AFM images of RW nanocrystals.

Fig. 6. Mass balance of the RW biorefinery process.

the work reported in this paper.

Data Availability

Data will be made available on request.

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