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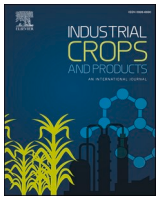

Co-valorization of discarded wood pinchips and sludge from the pulp and paper industry for production of advanced biofuels

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ABSTRACT

Several lignocellulosic wastes are generated in the pulp and paper industry (PPI), such as small wood chips (pinchips) and paper sludge, presenting a high cellulose content suitable to be converted into biofuels or bio-products in a forest biorefinery scheme. In this work, two schemes of biorefinery were proposed for their valorization, processing small eucalyptus wood pinchips in two different strategies: (i) autohydrolysis at 230ºC, and (ii) autohydrolysis at 195ºC followed by organosolv process (47.7% ethanol-water, 198ºC for 60 min). More than 95% of cellulose was recovered in both schemes. In the combined process, 76% of delignification was achieved and 78% of xylan was solubilized as xylooligosaccharides. To reduce operational cost of lignocellulosic biomass-to-ethanol fermentation, the mixture of the treated eucalyptus pinchips from two processes with sludge was also proposed to increase the initial glucan content and to supply a rich source of nitrogen (present in the sludge). For that, two experimental designs were carried out for ethanol production by simultaneous saccharification and fermentation (SSF) process. Ethanol from SSF assays using sludge as co-substrate at 0.6 g of sludge/g of treated wood pinchips and 16 FPU/g of pretreated solids allowed to obtain 59 g/L (90% of conversion) and 46 g/L (96% of conversion) when blended with the wood from autohydrolysis and with the wood from autohydrolysis followed by organosolv, respectively. Overall, this study shows an alternative process valorization of biomasses derived from PPI for production of advanced biofuels and bio-products (such as xylooligosaccharides and lignin) contributing to achieving a circular economy.

1. Introduction

Developing bio-based resources, processes, and products that are environmentally, economically, and socially sustainable is key to the emerging circular bioeconomy as a global approach to lessen or remove fossil fuels to decrease global warming and the emission of greenhouse gases (GHG). In a circular economy, wastes, co-products, and residues from manufacturing industries are re-used and recycled in the most efficient way possible (Sette et al., 2020; Zambare and Christopher, 2020). In this context, the pulp and paper industry (PPI) is making important efforts to transition towards a Forest Biorefinery, reducing the production of useless wastes to obtain cleaner and greener products (Rathour et al., 2023). Nowadays, this industry manufactures more than 500 million tons of paper worldwide every year (FAOSTAT, 2022), consequently generating solid wastes (that account for over 10% of the total paper production) such as sludge, lime or fly and boiler ash, among others, and consuming large amounts of water (between 20–90 m^3 per ton of product), causing extensive environmental issues (Namdarimonfared et al., 2023; Zambare and Christopher, 2020; Suhr et al., 2015) due to the poor management of these wastes including landfilling, incineration and burning (de Azevedo et al., 2019; Dey et al., 2021).

The pulp and paper sludge (PPS) is usually generated from the primary and secondary treatment of wastewater where the water streams after wood treatment (commonly Kraft technology) are performed, implying a significant loss of valuable bio-energy resources, and questions about energy security, environmental safety, and methane emissions, leading to an increased risk of GHG emissions (Glińska et al., 2019). Among the generated sludge, primary sludge is obtained after mechanical treatments of wastewater and it is usually composed of fines fibers, fillers, metallic components, sand and coatings (Mendes et al.,

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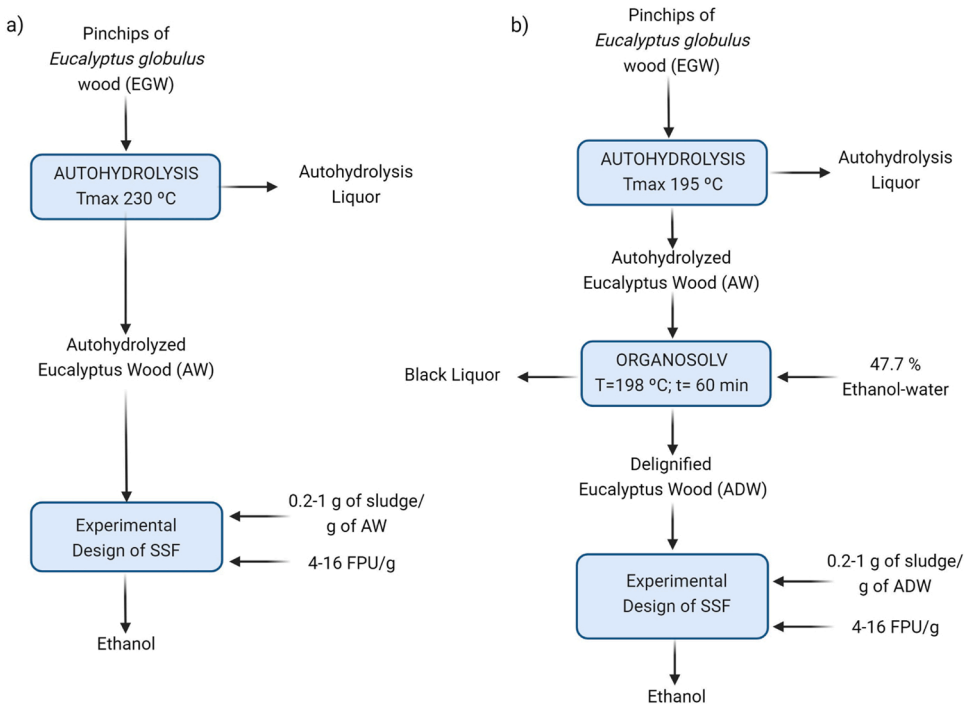

Fig. 1. Scheme of biomass processing for valorization of by-products derived from pulp and paper mill using a) autohydrolysis treatment at 230ºC of *Eucalyptus globulus* wood (EGW) and b) autohydrolysis at 195ºC followed by organosolv treatment (198ºC, 60 min) 0 f *Eucalyptus globulus* wood (EGW).

2014). On the other hand, secondary sludge (known also as activated sludge or biosludge) is produced by microorganism action to remove and decompose organic materials and its chemical composition compromises lignin, protein, hemicellulose, cellulose and lipids (Jokela et al., 1997). Usually, primary and secondary sludge can be combined, being denoted as mixed sludge. Therefore, PPS is considered one of the most promising resources, in this industry, to generate biofuels such as bioethanol since it is composed of small fibers with up to 40% of carbohydrates, especially cellulose and hemicelluloses, and microbial biomass (Dey et al., 2021; Romaní et al., 2008), including other advantages such as: (i) high enzymatic digestibility (ii) high protein content, which can be used as nutrients in fermentation; (iii) negative cost; and (iv) environmental benefit due to the reduction of waste volume (Romaní et al., 2008). Hence, the use of PPS (mixed sludge) as a source of carbohydrates and nitrogen for a more economically attractive microorganism growth supplementation is encouraged (Pereira et al., 2010), and was already optimized to produce lactic acid via homolactic fermentation with no addition of commercial supplementation (Romaní et al., 2008).

However, the glucan content of PPS is usually not enough to reach a desirable ethanol concentration (higher than 4% w/w, necessary to reduce distillation costs). For instance, ethanol titers can attain around 4 or 9.7 g/L (Alkasrawi et al., 2021; Zambare and Christopher, 2020) using low solid loadings. On the other hand, 42 g/L can be obtained at high solid loadings with ethanol yield of 0.53 g ethanol/g glucose and xylose (Dey et al., 2021). For that reason, the integration of this waste with another residue from PPI in a multi-waste valorization approach could be an interesting strategy to enhance ethanol fermentation, since the initial carbon source is increased (Cunha et al., 2018). In this case, within the paper and pulp processing, there are small wood chips (pinchips) that may be discarded due to their size and that would increase the cellulosic content in the fermentation media. In order to enhance the enzymatic digestibility of these pinchips, the use of a pretreatment is encouraged. One example would be autohydrolysis, which consists of mixing the biomass with water in a pressurized reactor at high temperatures which provokes the self-ionization of water and consequently the solubilization of hemicelluloses and the improvement of the cellulosic digestibility (Díaz et al., 2021; Rahmati et al., 2023). On the other hand, the use of organosolv treatment as a second step of wood biorefinery has been employed to solubilize the lignin fraction after autohydrolysis, facilitating an easy recovery of the three main components (cellulose, hemicelluloses derived compounds and lignin) of the wood in separated streams (del Río et al., 2020; Dornelles et al., 2021). In this sense, the combination/mixture of discarded wood pinchips and PPS may provide a novel and underexplored source of carbohydrate and nitrogen for a cleaner advanced production of bioethanol, transforming several residues into valuable products. Literature collects some examples of mixture of residues such as wheat meal or flour, cheese whey, potato tubers and starch or mixed vegetable wastes for ethanol production (Cunha et al., 2018; Erdei et al., 2010; González-Guerra et al., 2023; Jin et al., 2016; Nakamura et al., 2012). Nevertheless, most of these studies are focused on the employment of first generation biomasses (Oke et al., 2016), being the use of lignocellulosic feedstock still a challenge (Nguyen et al., 2017).

The main goal of this study relies on the assessment of by-products derived from PPI (namely, wood pinchips and mixed sludge) as suitable and renewable resources for the production of bioethanol and other high-value compounds. For that, fractionation processes based on autohydrolysis and autohydrolysis followed by organosolv were proposed as two schemes of biorefinery (Rochón et al., 2022). Moreover, cellulosic ethanol production was evaluated by simultaneous saccharification and fermentation (SSF) strategy using two experimental designs in order to evaluate operational conditions such as enzyme loading (4–16 FPU/g of substrate) and mixture of pretreated eucalyptus wood with mixed sludge as an alternative source of carbon and nitrogen at ratio of 0.2–1 g of sludge/g of pretreated biomass. Overall, this work searches for sustainable ways of valorization of industrial bio-based wastes using sustainable processes.

2. Materials and methods

2.1. Raw materials and chemical composition

The raw materials used in this work were the pinchips of *Eucalyptus*

globulus wood that are rejected due to small size and paper sludge (PS) obtained by the combination of primary and secondary sludge from wastewater treatments generated in ENCE pulp industry (Galicia, NW Spain). Eucalyptus pinchips wood were milled to a particle size smaller than 8 mm (denoted EGW), dried at room temperature and kept in a homogeneous batch in a dry and dark location. On the other hand, the initial moisture content of sludge (more than 75%) was decreased by air drying at 60ºC to less than 10% in order to prevent deterioration.

These two feedstocks were subjected to the following procedures for their chemical composition determination: for moisture (Sluiter et al., 2008a), ashes (Sluiter et al., 2008b), extractives (Sluiter et al., 2008d) and carbohydrate and Klason lignin determination via quantitative acid hydrolysis (Sluiter et al., 2008c). The liquid phase obtained after such hydrolysis was injected in an Agilent HPLC (series 1200) for monosaccharides (glucose, xylose, arabinose) and acetic acid determination using a BioRad Aminex HPX-87-H column, 3 mM H_2SO_4 as mobile phase at 0.6 mL/min. The spent solid obtained after the hydrolysis was quantified as Klason lignin.

Paper sludge were also analyzed for the nitrogen and metals content. A Thermo Finnigan Flash EATM 1112 analyzer (130 and 100 mL/min of He and O_2 and an oven temperature of 50ºC) was used to determine elemental nitrogen content. The metal content was performed employing an atomic Absorption Spectrometer 220 Fast Sequential. In selected experiments, samples were digested in an MLS-1200 Microwave Labstation mega with 5 mL HNO_3 65%, 1 mL H_2O_2 and 0.5 mL HF 40%. The determinations were carried out in three replicates.

2.2. Autohydrolysis treatment

A 3.75-L Parr reactor (Parr Instruments Company Moline, IL) was used for autohydrolysis pretreatment, where EGW and water were blended at a liquid-to-solid-ratio (LSR) of 8 g of distilled water/g of EGW on dry basis (Fig. 1). The reactor used four-bladed turbine impellers, the exterior fabric mantle provided heating, and an internal loop circulated tap water for cooling. The stirring was maintained at 150 rpm during the whole process, and the maximum temperatures (Tmax) reached were 195 and 230ºC under non-isothermal conditions (Romaní et al., 2010). Severity of these treatments can be expressed by log of $R_0 = S_0$ (del Río et al., 2020):

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

$$= \log\left(\int_0^{t_H} \exp \bullet \left(\frac{T(t) - T_{REF}}{\omega}\right) \bullet dt + \int_{t_H}^{t_H+t_C} \exp \bullet \left(\frac{T'(t) - T_{REF}}{\omega}\right) \bullet dt \right)$$

where R_0 denotes the severity factor, T(t) and T′(t) represent the temperature profiles in the heating and cooling steps, t_H and t_C (min) represent the time needed to reach the target temperature and the time used in the cooling period, ω denotes the empiric parameter related to activation energy and T_{REF} denotes the temperature of reference (generally values: ω = 14.75ºC and T_{REF} = 100ºC).

The two conditions of autohydrolysis treatment were selected for oligosaccharide recovery in the liquid phase and to improve the enzymatic saccharification of cellulose, respectively. Vacuum filtration was employed to separate the liquid and solid fractions. Water was used to wash the solid fraction (autohydrolyzed wood, AW) until neutral pH, the solid yield was gravimetrically determined, and the chemical composition was assayed as described previously (see Section 2.1). The liquor was used for monosaccharides, acetic acid and furans determination (direct injection in HPLC after filtration via 0.45 μm membranes) and oligosaccharides determination (after acid posthydrolysis 4% H_2SO_4, 20 min, 121ºC).

2.3. Organosolv treatment

Eucalyptus wood resulting from autohydrolysis treatment at 195ºC was submitted to organosolv delignification with 47.7% w/w ethanol at LSR= 8 g/g at 198ºC for 60 min (Fig. 1). Afterwards, the autohydrolyzed and delignified wood (ADW) was washed with warm 47.7% w/w ethanol, and water until neutral pH, the solid yield was assessed by weight and the chemical composition was assayed as described previously (see Section 2.1). The experimental conditions were set grounded on prior studies (Romaní et al., 2010, 2011).

2.4. Inoculum preparation

Saccharomyces cerevisiae CECT-1170 from Spanish Collection of Type Cultures (Valencia. Spain) was used in this study. A liquid medium made of glucose, peptone yeast and malt extract (concentrations of 10, 5, 3 and 3 g/L, respectively) was used to grow the cells at 32ºC during 24 h. After that, biomass cells were recovered by centrifugation and used to start the experiments of simultaneous saccharification and fermentation, described below.

2.5. Simultaneous saccharification and fermentation (SSF) of discarded wood pinchips and sludge

2.5.1. SSF of sludge and autohydrolyzed eucalyptus wood

Preliminary four assays of SSF using sludge or autohydrolyzed EGW at 230ºC as two different sources of carbon were performed at 35ºC in an orbital shaker using enzyme loading of 15 FPU/g and liquid to pretreated solid ratio (LSR) of 10 g/g. The enzymes employed in SSF experiments were kindly supplied by Novozymes (Madrid, Spain, reaching a cellulase activity (Adney and Baker, 2008) of 70.1 FPU/mL for Celluclast 1.5 L cellulases from *Trichoderma reesei*, and 630 IU/mL (Paquot and Thonart, 1982) for Novozyme β-glucosidase from *Aspergillus niger*. The β-glucosidase to cellulose ratio was set at 10 IU/FPU. SSF using sludge was not supplemented with nutrients. On the other hand, three SSF experiments using autohydrolyzed EGW (AW) were performed: (i) without nutritional supplementation, (ii) with addition of only yeast extract (10 g/L) and (iii) using peptone and yeast extract (20 and 10 g/L, respectively). Samples were withdrawn a desired times and analyzed by HPLC for glucose and ethanol quantification.

2.6. SSF of sludge mixed with eucalyptus biomasses: experimental design

SSF assays were also carried out at 35ºC in an orbital shaker with temperature control. The substrate employed, either AW or ADW, was blended with the cellulosic sludge and sterilized at 121ºC for 15 min. The LSR (considering the sum of the weight of sludge and AW or ADW, for each assay) was set at 6 g/g (or 14.3% of solid loading) in the SSF experiments. Enzymes and *Saccharomyces cerevisiae* inoculum (to reach a concentration of 1.85 g of dry cells/L) were introduced in the mixture. The other operational conditions and independent variables (sludge to autohydrolyzed Eucalyptus-AW or delignified Eucalyptus-ADW ratio, enzyme to solid ratio (ESR) used in this work varied as described by the experimental design, see below. The SSF was performed up to 96 h, withdrawing samples at set times. These samples were centrifuged, filtered through 0.45 μm membrane and injected into HPLC for monosaccharides, acetic acid, and ethanol quantitation.

In order to optimize ethanol production from two pretreated solids (AW and ADW) obtained within the biorefinery schemes proposed (Fig. 1), two full factorial design were carried out. For that, the independent variables evaluated were sludge load or x_1 (ranged between 0.2–1 g of sludge/g of substrate) and enzyme to substrate ratio or x_2 (varying between 4–16 FPU/g of pretreated solid). Dependent variables obtained from SSF (Ethanol concentration or y_1 and y_3 and Ethanol yield or y_2 and y_4) were correlated with the independent variables following the expression:

Table 1
Chemical composition of eucalyptus wood pinchip (EGW) and paper sludge (PS) (based on mean value of three replicates ± standard deviation).

Component	*Eucalyptus globulus* wood pinchip (EGW) (g per 100 g of raw material, oven dry basis)	Paper Sludge (PS) (g per 100 g of raw material, oven dry basis)
Cellulose (as glucan)	44.39 ± 0.44	46.35 ± 0.85
Xylan	17.49 ± 0.65	9.20 ± 0.38
Arabinan	1.08 ± 0.05	-
Acetyl groups	3.27 ± 0.23	0.53 ± 0.08
Klason lignin	27.67 ± 0.37	11.60 ± 1.15
Ethanol extractives	2.40 ± 0.15	-
Ash	0.23 ± 0.03	6.40 ± 0.83
Protein	1.25 ± 0.03	20.13 ± 0.48

$$y_j = b_{oj} + \sum_{i=1}^{2} b_{ij}x_i + \sum_{i=1}^{2} \ldots \sum_{k\geq i}^{2} b_{ikj}x_i x_k \quad (2)$$

where y_j (j = 1 to 4) was the ethanol concentration or ethanol yield; x_i or x_k (i or k: 1 to 2, k ≥ i) are the independent and normalized variables (namely, sludge load and enzyme to substrate ratio). On the other hand, $b_{0j}\ldots b_{ikj}$ were regression coefficients that were calculated from data experimentally collected by multiple regression using the least-squares method. These results were fitted to the proposed model (Eq. 1) using commercial software (Microsoft Excel, Microsoft Office 365 ProPlus).

3. Results and discussion

3.1. Raw materials composition and biomass processing

3.1.1. Sludge and Eucalyptus wood characterization

In order to determine the potential of the by-products evaluated in this work to obtain ethanol, EGW and PS were chemically analyzed, and the results obtained are listed in Table 1. As shown, a glucan content higher than 40% was reported for both biomasses, and eucalyptus pinchips present a higher content in hemicelluloses (xylan, arabinan and acetyl groups) than that obtained for sludge, but similar to that found in other hardwoods such as paulownia (del Río et al., 2020) or acacia (Chung et al., 2021). The composition of eucalyptus wood used in this work shows a typical composition of eucalyptus species (such as *E. botryides, E, grandis,* and *E. nitens*) as can be compared with those reported by other authors (Penín et al., 2020). For instance, (Cebreiros et al., 2020) characterized eucalyptus sawdust, achieving a glucan, lignin, and hemicelluloses content of 43.2%, 27.5% and 19.7%, respectively, which is very similar to that obtained in this work. Other eucalyptus species (such as *E. benthamii*) may present slight differences such as higher lignin content (35.9%) of a bit less glucan content (40.2%) (Castro et al., 2014).

Opposed to eucalyptus wood pinchips, sludge presents a low content of lignin. This may be due to the origin of this waste, since it is generated in a wastewater treatment of a Kraft pulp mill, so it is formed by paper fines and fillers (composed mainly of cellulose). Generally, the composition of sludge can vary, depending on several factors, such as raw material from which it comes, the process of pulp and paper manufacturing, and the chemical products used (Monte et al., 2009). In fact, the content of cellulose is especially high compared with the reported by other authors, containing only 21% of carbohydrates (solid obtained from the wastewater treatment of paper recycling effluents) (Gomes et al., 2016), while other authors reported values up to 55.6% for paper mill sludges and 40.3% for sewage sludge (Chen et al., 2023). In addition, the ash content is lower than that reported by Chen et al. (2023) (48.4%), whereas the hemicelluloses content is in the range of other paper mill sludges (Tawfik et al., 2023). Donkor et al. (2021) also investigated the composition of different fractions of primary paper sludge, obtaining a similar polymeric composition for corrugated recycling mill, but higher ash content (25.9%).

Table 2
Composition of minerals and nitrogen of paper sludge and commercial media of growth for yeast (peptone, yeast extract, malt extract).

	N (g/kg)	K (g/kg)	Fe (mg/kg)	Mn (mg/kg)	Mg (mg/kg)
Paper sludge	32.22	1.38	3268.81	35.72	1051.37
Peptone	158.7	6.30	35.72	<7.0	75.97
Yeast extract	109.7	57.45	1051.34	<7.0	284.87
Malt extract	15.3	3.81	1.38	<7.0	375.45

Table 3
Chemical composition of solid and liquid phases based in three replicates (mean value ± standard deviation) obtained from autohydrolysis treatment at 230ºC, and autohydrolysis treatment at 195ºC combined with organosolv delignification (198ºC, 60 min).

Component	Autohydrolysis treatment ($AW_{195ºC}$)	($AW_{230ºC}$)	Delignification process using AW at 195ºC ($AW_{195ºC}$)
Conditions	195ºC	230ºC	198ºC, 60 min
Solid yield (g pretreated solid/100 g of raw material)	75.50	69.90	68.00
(a) Solid phase composition after treatments (g/100 g of initial EGW)			
Cellulose	44.31 ± 0.93	44.11 ± 1.63	42.36 ± 1.24
Xylan	3.90 ± 0.58	0.30 ± 0.03	1.70 ± 0.33
Arabinan	-	-	
Acetyl groups	0.64 ± 0.67	0.64 ± 0.89	0.11 ± 0.03
Klason lignin	24.76 ± 1.83	25.97 ± 1.97	6.73 ± 0.89
(b) Autohydrolysis liquor composition (g/100 g of initial EGW)			
GOS	0.57	1.15	-
XOS	10.02	0.83	-
ArOS	0.17		-
Acetyl groups	1.68	0.06	-
Glucose	0.11	1.49	-
Xylose	1.74	4.60	-
Arabinose	0.41	0.01	-
Acetic acid	0.14	3.80	-
Hydroxymethylfurfural	0.03	0.53	-
Furfural	0.23	2.86	-

On the other hand, after biological treatment (secondary treatment), bacterial cells remain in the sludge generated. In this sense, sludge was also analyzed for its content in nitrogen and metal elements as shown in Table 2, being remarkably elevated protein content compared with the one present in eucalyptus wood (Table 1) and with other sludge derived from papermill which typically present a content of 1.5–8.3% (Norgren et al., 2023). Additionally, the presence of magnesium and iron is lower than that found in sewage (Jankowski et al., 2023) and paper sludges (Chen et al., 2023), respectively.

3.1.2. Eucalyptus wood pinchips processing: autohydrolysis and organosolv

EGW pinchips were processed to break down their recalcitrant structure following the biorefinery concept. For that, two schemes of a biorefinery based on autohydrolysis combined or not with organosolv treatment were proposed as selective pretreatments to recover hemicelluloses, cellulose, and lignin in separate streams, following previous optimization work of EGW (Romaní et al., 2011, 2012, 2013). The scheme of these experimental works is shown in Fig. 1 and chemical composition of several phases obtained from the biomass processing are collected in Table 3. On the other hand, autohydrolysis at 230ºC (Romaní et al., 2012) was selected for maximum cellulose to glucose

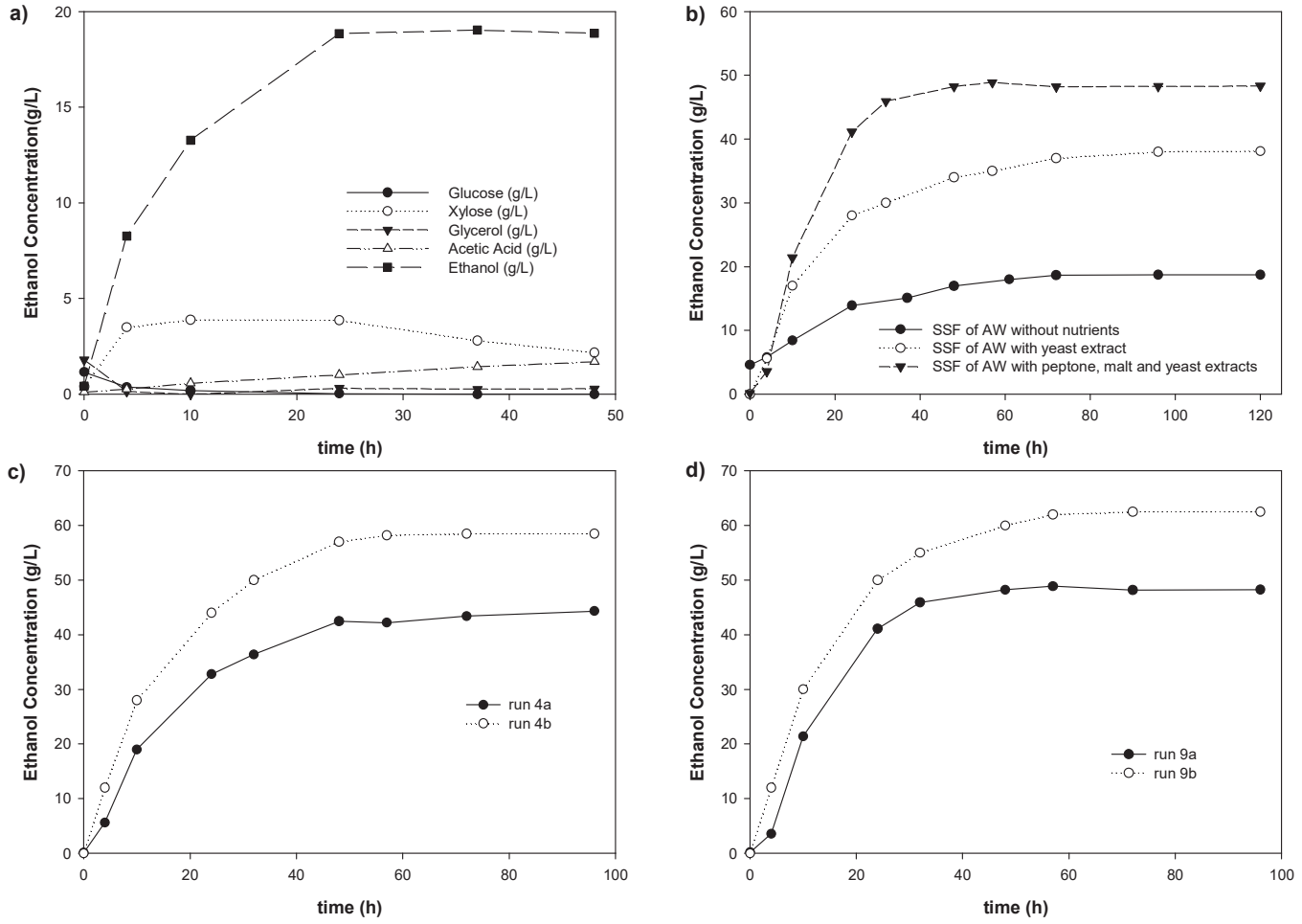

Fig. 2. Simultaneous saccharification of fermentation (SSF) of a) sludge paper without nutritional supplementation; b) autohydrolyzed eucalyptus wood at 230ºC (i) without nutritional supplementation, (ii) with yeast extract and peptone and (iii) with yeast extract; c) pretreated AW and ADW under conditions defined in Table 4 (run 4a and 4b), d) pretreated AW and ADW under conditions defined in Table 4 (run 9a and 9b).

conversion in the enzymatic hydrolysis, and other hand, the autohydrolysis at 195ºC was chosen for the maximum recovery of xylan as xylooligosaccharides (Romaní et al., 2011), taking into account their interest as new functional foods due to prebiotic activity (del Río et al., 2022).

It is well known that autohydrolysis treatment allows a high solubilization of hemicelluloses. Under the severest condition of autohydrolysis (Tmax 230ºC or S_0 of 4.67 under non-isothermal treatment), shown in Fig. 1a, 68% of xylan (the main component of hemicelluloses) was solubilized and recovered in the liquid phase as hemicellulosic derived compounds such as xylose, furfural, and xylooligosaccharides (36.0, 25.5 and 6.51 g per 100 g of xylan in raw material, respectively). In this sense, this severe condition implied a low recovery of xylan as xylooligosaccharides (XOS) of 0.83 g/100 g of initial EGW, and an elevated concentration of the other hemicellulosic derived compounds, namely xylose and furfural, in the autohydrolysis liquor, reaching values of 4.60 and 2.86 g/100 g of initial EGW, respectively. Otherwise, at lower severity conditions of autohydrolysis (S_0 3.64 or Tmax 195ºC) shown in Fig. 1b, 78% of xylan was solubilized, especially in the form of XOS achieving 10.02 g/100 g of initial EGW, while xylose and furfural appeared in lower amounts (1.74 and 0.23 g/100 g of initial EGW). Similar results for hemicelluloses solubilization to highly recover xylooligosaccharides were found for eucalyptus pinchips treated at S_0 = 3.66 (170 °C for 40 min) (Bariani et al., 2022). A similar behavior was observed when processing paulownia wood at temperatures from 202 to 230 °C. In this case, a softer temperature (205 °C) enabled the recovery of up to 13.08 g of XOS/100 g of initial wood (77% of xylan recovery), while employing higher temperatures triggered its hydrolysis into xylose and the formation of degradation products such as furfural (del Río et al., 2020). A comparable performance was achieved when pretreating barley straw between severities of 3.64–4.23 (Vargas et al., 2016).

The autohydrolysis treatment enabled a glucan recovery higher than 99% for both conditions of autohydrolysis and an increase of glucan content with respect to the raw material (44.39%) up to 58.69 g/100 g of $AW_{195ºC}$ and 63.10 g/100 g $AW_{230ºC}$ respectively, which could rise the ethanol concentration when used as substrate in a SSF process. On the other hand, the processing based on autohydrolysis followed by organosolv treatment yielded a solid phase composed mainly of cellulose (82.51 g of cellulose/100 g of ADW). Additionally, higher than 95% of cellulose was recovered for both schemes of biorefinery. As expected, the combined process using autohydrolysis and organosolv (Fig. 1b) enabled a delignification percentage of 75.68% through the solubilization of high-quality lignin in the black liquor (Portela-Grandío et al., 2021). These results of delignification and cellulose recovery can be compared with literature using other hardwoods such as the wood of Paulownia, in which 64% of lignin removal and 94% of glucan recovery were reported after a similar biorefinery scheme (del Río et al., 2020). Similarly, Cebreiros et al. (2020) processed eucalyptus wood by ethanol organosolv reaching up to 61% of delignification with 95% cellulose recovery in the resulting spent solid at conditions of 180 °C for 15 min using 75% ethanol.

Table 4
Experimental conditions of full factorial design using eucalyptus wood after autohydrolyzed (AW) or after autohydrolysis followed by delignification (ADW) as substrates, and main results obtained from simultaneous saccharification and fermentation (SSF).

Run Substrate: Autohydrolyzed Wood (AW)	Run Substrate: Autohydrolyzed Delignified Wood (ADW)	Independent variables Sludge to biomass Ratio (g/g)	Enzyme to Substrate Ratio (ESR, FPU/g)	AW EC (g/L) or y_1	AW EY (%) or y_2	ADW EC (g/L) or y_3	ADW EY (%) or y_4
1a	1b	0.2 (−1)	4 (−1)	17.6	34.5	30.7	42.5
2a	2b	0.6 (0)	4 (−1)	29.8	62.3	36.8	56.6
3a	3b	1 (1)	4 (−1)	35.9	78.2	38.6	63.6
4a	4b	0.2 (−1)	10 (0)	36.4	71.2	55.6	77.1
5a	5b	0.6 (0)	10 (0)	45.7	95.6	56.3	86.6
6a	6b	0.6 (0)	10 (0)	44.8	93.6	55.8	85.9
7a	7b	0.6 (0)	10 (0)	44.4	92.7	55.5	85.4
8a	8b	1 (1)	10 (0)	42.9	93.5	51.4	84.7
9a	9b	0.2 (−1)	16 (1)	48.2	90.5	58.8	81.5
10a	10b	0.6 (0)	16 (1)	46.3	96.8	58.8	90.5
11a	11b	1 (1)	16 (1)	44.4	96.7	56.1	92.5

*EC: Ethanol Concentration; EY: Ethanol Yield

3.2. Evaluation of sludge potential as a source of ethanol and as a low-cost nutrient

Despite the potential source of sugars that are present in paper sludge, a high amount of this waste is usually considered as a discard, which must be properly managed to avoid environmental problems. In order to search for other ways to valorize it, some authors have evaluated the suitability of this raw material for the production of advanced biofuels such as bioethanol (Gomes et al., 2016), or lactic acid (Marques et al., 2008). The chemical composition of this waste is very heterogeneous showing differences in the glucan content, which implies a wide range of ethanol concentrations reported from this raw material. For instance, 41.7 g/L of ethanol with a yield of 48.9% was obtained from SSF of primary sludge, supplemented with nutrients, namely peptone, malt extract, and yeast extract (Mendes et al., 2016). On the other hand, sludge pretreated to reduce ash content was converted into 27 g/L of ethanol (39% of yield) (Mendes et al., 2014).

Before the study of mixing of PS with EGW, the sludge obtained from Kraft pulp mill was assayed as only carbon source for ethanol production using 9.1% of solids and enzyme-substrate ratio (ESR) of 15 FPU/g of pretreated solid. Results from this SSF assay are shown in Fig. 2a, reaching an ethanol concentration of 18.8 g/L, corresponding with an ethanol yield of 72% within 48 h after the inoculation. As can be observed in Fig. 2a, the fermentation was successfully performed without additional supplementation of yeast extract and peptone, being the amount of protein present in this raw material enough (Table 1). Despite being a suitable source for biofuel production, ethanol concentration attained from these residues is far from reaching > 4% w/w, necessary to reduce distillation costs (Ko et al., 2016). Similarly, Gomes et al. (2016) also investigated the production of ethanol from recycled paper sludge (5% solid loading, ESR of 20 FPU/g of pretreated solid) reaching around 92% of conversion after 54 h of hydrolysis followed by fermentation.

On the other hand, Fig. 2b displays the results obtained from the SSF experiment using as substrate eucalyptus treated from autohydrolysis (without nutritional supplementation) at the same conditions of solid and enzyme loading as the SSF experiment from Fig. 2a. Maximum ethanol concentration was obtained at 72 h, attaining 18.73 g/L of ethanol and a low ethanol yield of 51.53% without the addition of commercial nutrients. In view of these results, the fermentation using autohydrolyzed eucalyptus ($AW_{230°C}$) obtained lower cellulose to ethanol conversion than those obtained with nutritional supplementation (Fig. 2b), in which 90% of conversion was achieved.

Considering all this, the mixture of both biomasses (sludge paper and pretreated eucalyptus) could be an interesting strategy for ethanol production (with the ultimate goal of obtaining > 4% w/w), firstly to increase final ethanol titers (since the sludge is a more susceptible substrate) and secondly to improve fermentation yield from pretreated eucalyptus, since sludge could provide a rich source of nitrogen (required for the synthesis of amino acids). In fact, the use of different agro-industrial by-products (such as corn steep liquor, raw yeast, and cheese whey) as low-cost supplements in lignocellulose-to-ethanol fermentations to increase ethanol productivity has been already evaluated by several authors (Jørgensen, 2009; Kelbert et al., 2015; Pereira et al., 2010). Moreover, trace elements (such as Fe, Zn, Cr and Se) are also necessary as co-factors for some metabolic pathways (Sun et al., 2022). For comparative reasons, sludge paper and commercial nutrients used for yeast growth and ethanol fermentation were analyzed for nitrogen and mineral salt content, as shown in Table 2. Even though the content of nitrogen in sludge is lower than commercial nutrients, this residue provides an elevated amount of Mn and Fe. These trace elements play an essential factor in yeast growth and the complete fermentation of glucose to ethanol (Ko et al., 2016). These are essential co-factors for redox process need for physiological functions such as cellular respiration, transport of O_2, amino acid synthesis (Martínez-Pastor et al., 2017).

3.3. Experimental design and response surface methodology assessment

Attending to previous results from SSF of sludge, the use of this residue not only as a carbon source but also as nutritional supplementation in the SSF of pretreated eucalyptus biomasses was evaluated through two experimental designs. For that, sludge was mixed at different proportions with both pretreated eucalyptus wood obtained from the two biorefinery schemes proposed (AW and ADW) as shown in Fig. 1, and the enzyme to substrate ratio was also evaluated.

Table 4 shows experimental conditions and the main results obtained from saccharification and fermentation experiments, namely ethanol concentration and corresponding ethanol yield at 48 h. For experiments carried out with sludge and AW (runs 1a-11a), ethanol concentration varied in the array of 17.6–48.2 g/L. In a wide range of conditions, the ethanol concentration obtained was higher than 40 g/L, even using medium enzyme loadings (10 FPU/g). Interestingly, SSF assays using the highest sludge proportion (1 g/g) and adding the lowest enzyme load (4 FPU/g) achieved 35.9 g/L of ethanol with an ethanol yield of 78% (experiment 3a). Nevertheless, the reduction of sludge percentage (experiments 1a) considerably limited the ethanol production (17 g/L with an ethanol yield < 35%). The highest cellulose to ethanol conversion obtained by the blend of AW with sludge was achieved with 16 FPU/g and a proportion of sludge/biomass of 0.6 g/g. These results indicate the strong relationship between the amount of enzyme necessary and the origin of cellulose (cellulose fibers in the sludge from wastewater treatment or treated wood).

For the other set of SSF experiments regarding ADW (runs 1b-11b), ethanol concentration from delignified biomass was in the range of

Table 5
Regression coefficients and statistical parameters that measure correlation and signification of the model for SSF study.

Regression coefficients	y_1	y_2	y_3	y_4
b_{0j}	44.16	92.48	55.71	85.64
b_{1j}	3.48[a]	12.03[a]	0.16	6.62[a]
b_{2j}	9.29[a]	18.17[a]	11.28[a]	16.97[a]
b_{12j}	-5.53[a]	-9.38[a]	-2.66[b]	-2.53[c]
b_{11j}	-3.32[b]	-7.91[a]	-2.01	-4.26[b]
b_{22j}	-4.92[a]	-10.71[a]	-7.75[a]	-11.61[a]
R^2	0.984	0.989	0.984	0.990
F exp	62.67	93.81	60.57	96.20
Significance level (%)	> 99	> 99	> 99	> 99

[a] Significant coefficients at the 99% confidence level.
[b] Significant coefficients at the 95% confidence level.
[c] Significant coefficients at the 90% confidence level.

30.7–58.8 g/L, corresponding to ethanol yield of 42.5% and 81.5%, respectively. If comparing with AW, the lowest enzyme load (4 FPU/g) (runs 1b to 3b) enabled the obtainment of higher ethanol concentrations. This fact can be due to lignin removal by organosolv treatment, improving kinetics parameters of enzymatic saccharification, such as the reaction time need to obtain 50% of maximum glucose conversion as stated by other authors (del Río et al., 2020). In view of these results, delignified biomass containing higher and more accessible cellulose yields a superior concentration of ethanol.

Moreover, Fig. 2c and d show the fermentation profiles of some selected SSF assays within the experimental designs (Table 4). Experiments using the lowest proportion of sludge and an enzyme loading of 10 FPU/g (run 4a and b) were displayed in Fig. 2c. On the other hand, Fig. 2d compares the fermentation profiles from AW and ADW mixed with sludge under conditions of run 9a and 9b, respectively. As expected, ethanol concentration was higher for delignified biomass, since ADW presents a superior glucan content compared with glucan remaining in AW (83% and 63%, respectively). Control, using AW supplemented with commercial yeast extract, showed the same behavior in the ethanol fermentation than run 9a (mixture of AW and sludge), confirming its suitability as a carbon and nitrogen source.

In order to attain a better understanding of the results obtained from SSF assays, experimental results (dependent variables, y_1 and y_3 or ethanol concentration from AW and AWD, respectively and y_2 and y_4 or ethanol yield from AW and AWD, respectively) were fitted to

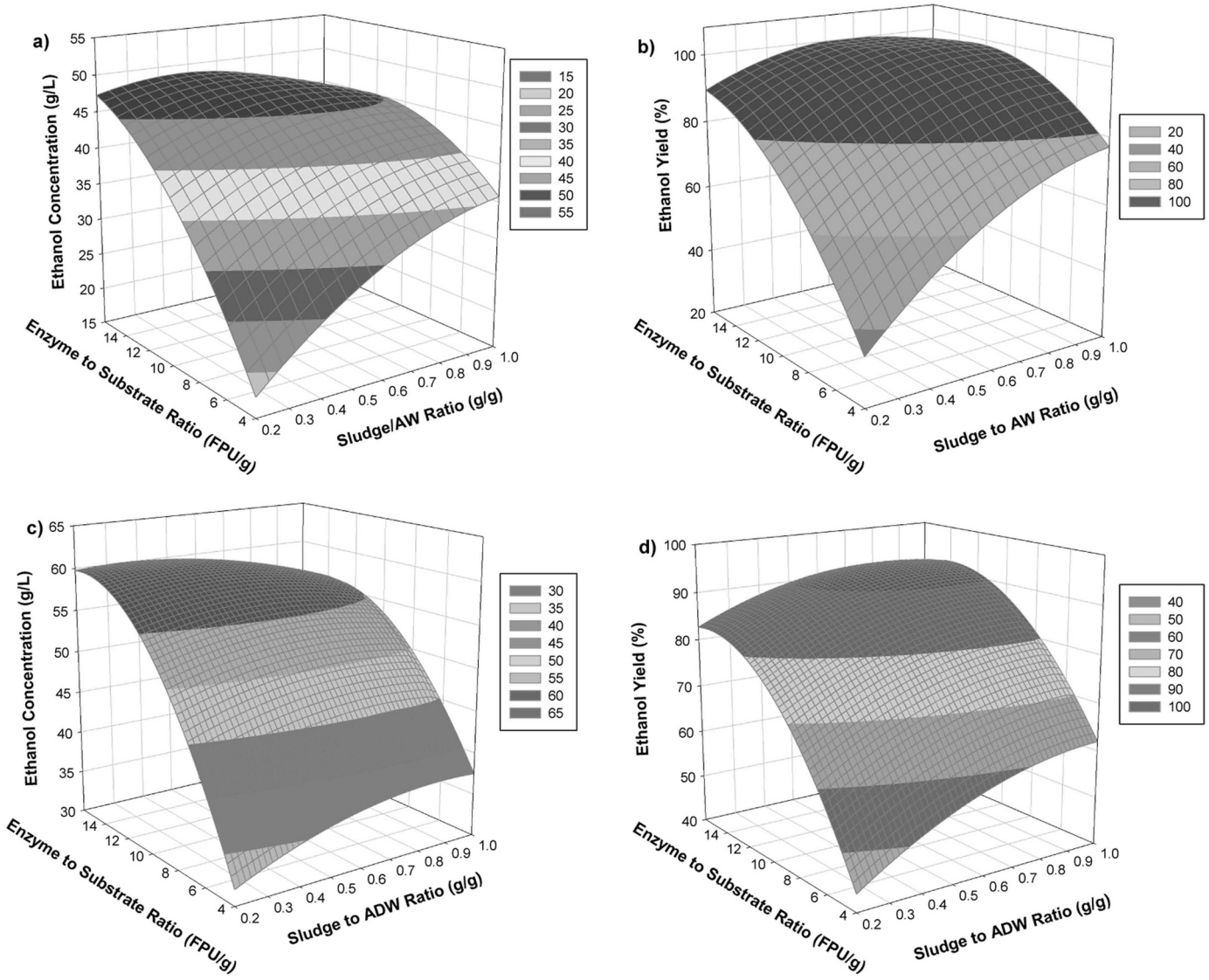

Fig. 3. Response surface varying sludge to pretreated wood ratio (g/g) and enzyme to substrate ratio (FPU/g) for: a) ethanol concentration at 48 h using autohydrolyzed wood (AW); b) ethanol yield at 48 h using as autohydrolyzed wood (AW); c) ethanol concentration using delignified wood (ADW); and d) ethanol yield at 48 h using delignified wood. The variables on sludge to ADW ratio (g/g) and enzyme to substrate ratio.

independent variables (sludge to biomass ratio and enzyme to substrate ratio) through empirical model shown in second order polynomial equation (Eq. 1). The resulting coefficients of regression and the statistical significance (Fischer's F parameter) are shown in Table 5. The results of regression showed a good fitting (R^2 varying in the range of 0.984–0.990) to the models proposed.

The response surface methodology (RSM) enables a better and visual interpretation of results obtained from the experimental design, also allowing the prediction of ethanol conversion within the conditions evaluated. Taking into account the different glucan content of both biomasses, the variable ethanol conversion at 48 h was selected for comparison of SSF results. Predicted values of ethanol conversion from AW and ADW (or y_2 and y_4) as function of sludge/biomass ratio and enzyme to substrate ratio were represented in Fig. 3. Comparatively, ethanol conversion was > 80% under the conditions for ESR > 8 FPU/g using as co-substrate the delignified eucalyptus wood, and higher than 80% using ESR > 12 FPU/g for autohydrolyzed wood. Influence of sludge proportion is more prominent when is mixed with autohydrolyzed biomass in comparison with the mixture with delignified wood. As seen, with 0.5 g/g of sludge/AW (corresponding to 50% of both substrates) ethanol yield achieved the 90% for ESR of 11 FPU/g. On the other hand, 90% of ethanol yield could be attained using 0.5 g/g of sludge/ADW with lower enzyme (9.5 FPU/g).

Comparatively, Donkor et al. (2021) studied the use of virgin pulp paper sludge to produce bioethanol using fed-batch simultaneous saccharification and fermentation (in 6 batches) but including additional supplementation. After 96 h of reaction, up to 49.6 g ethanol/L were reached using cellulose load of 20 FPU/g and final solid loading of 18%. In addition, Dey et al. (2021) used steam and NaOH pretreated primary paper sludge to produce bioethanol from glucose and xylose using additional supplementation, reaching 42.34 g ethanol/L (yield of 0.53 g/g) after 96 h of reaction.

4. Conclusions

In this work, two experimental designs were proposed to enhance ethanol production by means of the mixture of by-products generated in the pulp and paper industry, revealing a positive effect of including sludge carbon and nutrients source. Ethanol concentration using this mixture was increased 2.6 fold-higher in using AW. Ethanol yields around 90% were obtained using ESR of 10 FPU/g and sludge to AW ratio of 0.2–1 g/g. On the other hand, biorefinery scheme including sequential steps of autohydrolysis followed by organosolv delignification allowed the recovery of hemicellulosic derived compounds as xylooligosaccharides, solubilized lignin in the black liquor and cellulosic ethanol in three separated streams.

CRediT authorship contribution statement

Rubira Alexandre: Writing – original draft, Formal analysis. **Pérez María José:** Validation, Supervision. **del Río Pablo G.:** Writing – review & editing, Writing – original draft, Validation, Methodology, Conceptualization. **Romani Aloia:** Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Garrote Gil:** Writing – review & editing, Validation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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