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Development of Pretreatment Strategies for the Fractionation of Hazelnut Shells in the Scope of Biorefinery

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Abstract: Hazelnut shells are an important waste from the hazelnut processing industry that could be valorized in a multi-product biorefinery. Individual or combined pretreatments may be integrated in processes enabling the integral fractionation of biomass. In this study, fractionation methods based on alkaline, alkaline-organosolv, organosolv, or acid-catalyzed organosolv treatments were applied to raw or autohydrolyzed hazelnut shells. A comparative analysis of results confirmed that the highest lignin removal was achieved with the acid-catalyzed organosolv delignification, which also allowed limited cellulose losses. When this treatment was applied to raw hazelnut shells, 65.3% of the lignin was removed, valuable hemicellulose-derived products were obtained, and the cellulose content of the processed solids increased up to 54%. Autohydrolysis of hazelnut shells resulted in the partial solubilization of hemicelluloses (mainly in the form of soluble oligosaccharides). Consecutive stages of autohydrolysis and acid-catalyzed organosolv delignification resulted in 47.9% lignin removal, yielding solids of increased cellulose content (55.4%) and very low content of residual hemicelluloses. The suitability of selected delignified and autohydrolyzed-delignified hazelnut shells as substrates for enzymatic hydrolysis was assessed in additional experiments. The most susceptible substrates (from acid-catalyzed organosolv treatments) reached 74.2% cellulose conversion into glucose, with a concentration of 28.52 g glucose/L.

Keywords: biorefinery; hazelnut shells; hydrothermal pretreatment; delignification; enzymatic hydrolysis; fractionation; cellulose; hemicelluloses

1. Introduction

Lignocellulosic biomass (LB) is a key resource for the sustainable manufacture of bio-based chemicals and fuels. LB can be processed according to the biorefinery concept, which involves consecutive treatment stages to achieve an integral benefit of the feedstock, with minimal or no waste generation [1]. The success of biorefineries also depends on the right application of technology methodologies for obtaining multi-products [2]. The contribution of biorefineries to a future bio-economy inspires business opportunities based on product diversification while improving environmental performance [3].

In the field of biomass valorization, the biorefinery acts as a platform for chemicals and energy production through the inclusion of diverse conversion technologies [3,4].

The complexity of biomass utilization lies in both the polymeric nature of its main constituents (cellulose, hemicelluloses, and lignin) and their different chemical reactivities. In the last few decades, several fractionation methods have been developed, as summarized in recent literature reviews [2,5–8]. Galbe and Wallberg [6] classified the conventional methods according to their mode of action:

mechanical treatments (e.g., milling or grinding) reduce the particle size of biomass, increasing the surface area of the particles and improving the enzyme accessibility; dilute acid pretreatments (with H_2SO_4, H_3PO_4 or other strong acids), and hydrothermal pretreatments (with hot, compressed water or steam) promote the hydrolysis of hemicelluloses; whereas alkaline and organosolv processing enable the extraction of lignin.

Hydrothermal pretreatments (autohydrolysis, performed with hot, compressed water) causes the selective solubilization of hemicelluloses by depolymerization into soluble compounds of lower molecular weight. Soluble, low molecular weight polymers from hemicelluloses show potential for applications in the manufacture of barrier films or hydrogels [6]; oligosaccharides show prebiotic properties with potential in the pharmaceutical and food markets [9,10]; and monosaccharides can be transformed into value-added chemicals such as xylitol and furfural [11]. Additionally, autohydrolysis increases the biomass surface area and decreases the crystallinity of the cellulosic fraction remaining in the solid phase, facilitating its further hydrolysis.

Solutions of alkalis (such as NaOH, KOH, or ammonia) are suitable for removing lignin by saponification of intermolecular ester bonds, and for increasing the digestibility of cellulose [5]. Alternatively, delignification can be achieved by organosolv treatments with organic solvents (e.g., ethanol) or solvent-water mixtures, in the presence or absence of catalysts. From an environmental point of view, the separation of lignin from the organosolv media by precipitation with water facilitates the wastewater treatment. From an economic perspective, organosolv lignin is a valuable product with potential applications in diverse fields, including the manufacture of adhesives, fibers, films, biodegradable polymers [12], and natural antioxidants [13].

The solids from delignification treatments, with increased cellulose contents, may show increased susceptibility to enzymatic saccharification [1,14,15], or serve as substrates for manufacturing cellulose nanocrystals [16]. In the first case, glucose can be transformed in value-added compounds (e.g., bioethanol or lactic acid) [15,17]; whereas in the second one, cellulose nanocrystals are used in a number of fields, including biological applications.

"Conventional" pretreatments (or their combinations) are expected to play an important role in the future full-scale biorefineries [6]. Combined pretreatments may allow process designs enabling an improved fractionation and an efficient processing into a spectrum of multi-products [8]. For example, consecutive stages of autohydrolysis and delignification allow the separate recovery of hemicellulose-derived saccharides, lignin, and cellulose [18], enabling the integral fractionation of biomass, an aspect of crucial importance for a successful biorefinery approach [19].

Hazelnut (*Corylus avellana* L.) is a commercial crop, whose fruit is widely used in food industries [20]. Turkey is the main producer and exporter of hazelnut, followed by Italy, Azerbaijan, USA, China, Georgia, Iran, France, Chile, and Spain. According to the Food and Agriculture Organization (FAO), 863,888 tons of hazelnuts (with shell) were produced worldwide in 2018 [21]. Hazelnut shells (HS) are the most important byproduct of the hazelnut processing industry, representing more than half of the total nut weight. HS are a low–cost byproduct, usually burned, but could be valorized on the basis of their lignocellulosic nature. Recently, getting value from selected biochemicals in HS has become a motivation for research, due to the compelling economic benefits [3,22]. In terms of composition, HS are mainly made up of lignin: Perez-Armada et al. [23] reported 40.1 wt% of lignin, whereas Demirbas [24] and Surek and Buyukkileci [25] found around 46%, and Hoşgün and Bozan [20] near 50%. HS hemicelluloses are mainly made up of xylan, acetyl, and uronic moieties, which accounted jointly for 25–32.5% of the dry weight, whereas the reported cellulose contents are about 26–27% [10,23,26].

Based on the above ideas, this work deals with the development of HS fractionation methods suitable for a multi-product biorefinery. HS and solids resulting from HS autohydrolysis (denoted AS) were subjected to diverse delignification treatments to assess their efficiency. The soluble hemicellulose-derived products were identified, and the solids from selected delignification stages were assayed as substrates for enzymatic hydrolysis. In summary, this work provides an experimental assessment on biorefinery schemes enabling an integral valorization of HS.

2. Materials and Methods

2.1. Raw Material

Hazelnuts were purchased locally (Ourense, Spain). The shells (HS) were separated, milled to particle sizes within the range 0.250–1 mm, and subjected to analysis and chemical processing as described below.

2.2. Autohydrolysis of Hazelnut Shells

HS autohydrolysis was performed in a stirred 600 mL stainless steel reactor (Parr Instrument Company, Moline, IL, USA). HS were mixed with distilled water at a liquid to solid ratio (LSR) of 10 g/g of dry HS, heated up to 210 °C, and cooled immediately. Heating profiles previously reported for non-isothermal autohydrolysis shown the reaction time to reach the target temperature. Then, heating up from 60 °C to 210 °C lasted 16 min. These conditions were reported as optimal in literature [10,23]. The autohydrolyzed solids (AS) were separated by vacuum filtration, washed with distilled water, and air-dried before chemical characterization and solid yield determination. The autohydrolysis liquors (AL) were assayed for composition as described in Section 2.5.

2.3. Delignification Pretreatments

HS and AS were subjected to different delignification treatments. All experiments were performed at a LSR of 8 g/g, under the following conditions:

1. Alkaline delignification experiments of HS and AS were performed in an autoclave at 121 °C for 60 min using 2, 4, or 8 wt% NaOH solutions. The alkaline solutions and the operational conditions were selected according to the literature [20,27–29].
2. Alkaline-organosolv pretreatments of HS and AS were carried out in an autoclave at 121 °C or 135 °C for 60 min, in media with equal amounts of ethanol and alkaline solutions, containing 2–8 wt% NaOH with respect to the total solution.
3. Organosolv pretreatments were carried out in a stirred, 600 mL stainless steel reactor (Parr Instrument Company, Moline, IL, USA). HS and AS were treated with a mixture of ethanol/water (55/45 *w*/*w*) at 200 C for 60 or 120 min, according to the conditions reported in the literature [1,15].
4. Acid-catalyzed organosolv treatments of HS and AS were performed in the Parr reactor indicated above. The media contained a mixture of ethanol/aqueous H_2SO_4 (60/40 *w*/*w*), where the amount of H_2SO_4 corresponded 1 g H_2SO_4/100 g substrate. The reaction media were kept at 160–180 °C for 60–120 min.

Table 1 sumarizes the different delignification experiments performed in this work.

The solid and liquid phases from treatments were separated by vacuum filtration. The treated solids were washed first with solutions of the same composition as the ones used in the respective pretreatments, and then with distilled water. The solid phases from treatments were assayed for composition and solid yield. From the experimental data, the percentages of delignification, cellulose removal and hemicellulose removal were calculated as follows:

$$\%\ \text{Solid Yield (SY)} = 100 \times \frac{DS_{AD}}{DS_{BD}} \quad (1)$$

where DS_{AD} and DS_{BD} are the dry weights of the solid before and after delignification, respectively;

$$\%\ \text{delignification} = 100 \times \frac{TL_{BD} - TL_{AD} \times \frac{SY}{100}}{TL_{BD}} \quad (2)$$

where TL_{BD} and TL_{AD} are the percentages of total lignin present in the solids before and after delignification, respectively;

$$\% \text{ cellulose removal } = 100 \times \frac{C_{BD} - C_{AD} \times \frac{SY}{100}}{C_{BD}} \quad (3)$$

where C_{BD} and C_{AD} are the percentages of cellulose present in the solids before and after delignification, respectively;

$$\% \text{ hemicelluloses removal } = 100 \times \frac{H_{BD} - H_{AD} \times \frac{SY}{100}}{H_{BD}} \quad (4)$$

where H_{BD} and H_{AD} are the percentages of hemicelluloses (measured as the overall contributions of xylan, arabinan and acetyl groups) present in the solids before and after delignification, respectively.

The lignin present in the liquid phases from treatments performed under selected conditions was precipitated by adding HCl 5M to pH = 2, kept overnight at 4 °C, recovered by filtration, and dried in a vacuum oven at 40 °C. Aliquots of the liquid phases from treatments were analyzed for hemicellulose-derived compounds (oligosaccharides, monosaccharides, acetic acid, and furans) following the methods listed in Section 2.5.

Table 1. Set of experiments performed in this work for the delignification of HS and AS (LSR = 8 g/g).

Delignification Method	Experiment	Substrate and Operational Conditions
Alkaline (NaOH-water)	1	HS 121 °C 60 min, 2% NaOH
	2	AS 121 °C 60 min, 2% NaOH
	3	HS 121 °C 60 min, 4% NaOH
	4	AS 121 °C 60 min, 4% NaOH
	5	HS 121 °C 60 min, 8% NaOH
	6	AS 121 °C 60 min, 8% NaOH
Alkaline-organosolv (ethanol-aqueous NaOH)	7	HS 121 °C 60 min, 50/50 ethanol/aqueous NaOH, 2% total solution
	8	AS 121 °C 60 min, 50/50 ethanol/aqueous NaOH, 2% total solution
	9	HS 121 °C 60 min, 50/50 ethanol/aqueous NaOH, 4% total solution
	10	AS 121 °C 60 min, 50/50 ethanol/aqueous NaOH, 4% total solution
	11	HS 121 °C 60 min, 50/50 ethanol/aqueous NaOH, 8% total solution
	12	AS 121 °C 60 min, 50/50 ethanol/aqueous NaOH, 8% total solution
	13	HS 135 °C 60 min, 50/50 ethanol/ aqueous NaOH, 4% total solution
	14	AS 135 °C 60 min, 50/50 ethanol/aqueous NaOH, 4% total solution
Organosolv (ethanol-water)	15	HS 200 °C 60 min, 55/45 ethanol/water
	16	AS 200 °C 60 min, 55/45 ethanol/water
	17	AS 200 °C 120 min, 55/45 ethanol/water
Acid-catalyzed organosolv (ethanol/aqueous H_2SO_4)	18	HS 180 °C 60 min, 60/40 ethanol/aqueous H_2SO_4,
	19	AS 180 °C 60 min, 60/40 ethanol/aqueous H_2SO_4,
	20	AS 180 °C 120 min, 60/40 ethanol/aqueous H_2SO_4,
	21	HS 160 °C 120 min, 60/40 ethanol/aqueous H_2SO_4,
	22	AS 160 °C 120 min, 60/40 ethanol/aqueous H_2SO_4,

2.4. Enzymatic Hydrolysis

Solids resulting from consecutive stages of autohydrolysis (AS) and delignification under selected conditions were subjected to hydrolysis using the enzymatic complex Cellic CTec2 (Novozymes, Denmark). The enzymatic activity of Cellic CTec2 was 137 FPU (Filter Paper Units)/g of enzyme [30]. Hydrolysis assays were performed in Erlenmeyer flasks kept at 50 °C in an orbital incubator (150 rpm). The pH of media was adjusted to 4.8 by adding 50 mM citrate buffer. Experiments were performed for 0–96 h at LSR = 15 g/g using an enzyme to solid ratio (ESR) of 15 FPU/g substrate. All assays

were performed by triplicate. Aliquots of samples were withdrawn at selected times, centrifuged, filtered, diluted and assayed for glucose by HPLC as described in Section 2.5. Cellulose conversion into glucose, and xylan conversion into xylose were calculated as follows:

$$\%\ \text{cellulose conversion into glucose} = 100 \times \frac{[Glu]}{[Glu_{Pot}]} \quad (5)$$

where $[Glu]$ is the glucose concentration and $[Glu_{Pot}]$ is the potential glucose concentration (calculated assuming total conversion of the cellulose contained in the substrate);

$$\%\ \text{xylan conversion to xylose} = 100 \times \frac{[Xyl]}{[Xyl_{Pot}]} \quad (6)$$

where $[Xyl]$ is the xylose concentration and $[Xyl_{Pot}]$ is the potential xylose concentration (calculated assuming total conversion of the xylan contained in the substrate).

2.5. Analytical Procedures

The chemical composition of HS, AS, and solids from delignification were analyzed using the following TAPPI standard methods: moisture: T-264-cm-97, ash: T-211-om-02, structural carbohydrates, and Klason lignin: T-249-cm-85 [31–33]. The latter method is based on a two-step quantitative acid hydrolysis (QAH) performed with 72 and 4% H_2SO_4, respectively. The insoluble residue from QAH was oven-dried and weighed for Klason lignin determination. The liquid phase from QAH was assayed for glucose, xylose, arabinose, acetic acid, furfural, and hydroxymethylfurfural by HPLC, using a 1200 series instrument (Agilent Technologies, Santa Clara, CA, USA) fitted with a refractive index detector) and a 300 × 7.8 Aminex HPX-87H column (BioRad Life Science Group Hercules, CA, USA). The instrument detector was kept at 50 °C. The mobile phase was 0.003 N H_2SO_4 eluted at 0.6 mL·min^{-1}. The results allowed the determination of cellulose, xylan, arabinan, and acetyl groups present in the solid substrates. The acid-soluble lignin (ASL) was quantified spectrophotometrically at 205 nm. The total lignin was calculated as the sum of Klason lignin and ASL. All the analyses were performed in triplicate.

Aliquots from autohydrolysis and liquid phases from delignification assays were subjected to quantitative posthydrolysis (4% of H_2SO_4 at 121 °C for 20 min), and the increase in the concentrations of monosaccharides and acetic acid caused by posthydrolysis measured the amounts oligomers and their degree of substitution with acetyl groups. All the analyses were performed in triplicate.

3. Results and Discussion

3.1. Autohydrolysis and Composition of HS and AS

In previous studies reported by our research group, 210 °C was identified as the optimal autohydrolysis temperature for producing soluble hemicellulosic oligosaccharides [10]. Under the same conditions, soluble antioxidant compounds were extracted from the substrate [23]. Both oligosaccharides and antioxidant compounds find applications in a number of fields, including the food, cosmetic and pharmaceutical industries. Based on this information, HS autohydrolysis assays were performed at 210 °C, and the corresponding AS were selected for assessing the separation of cellulose from lignin.

Upon autohydrolysis, 35.4% of the dry HS mass was dissolved, yielding an aqueous phase with the following composition (in g/L): xylooligosaccharides, 16.15; glucooligosaccharides, 0.07; acetyl groups, 3.65; xylose, 1.02; arabinose, 0.31; acetic acid, 0.86; and total phenolic content, 1.60 g equivalent gallic acid/L. The average compositions of HS and AS are presented in Table 2. As expected, autohydrolysis causes the selective separation of hemicelluloses resulted in AS with increased percentages of cellulose (38.7%) and total lignin (50.4%).

Table 2. HS and AS composition, expressed as g of component/100 g of dry HS or AS, respectively. Data reported as average values ± standard deviations.

Component	HS (g/100 g of dry HS)	AS (g/100 g of AS)
Cellulose	24.2 ± 0.1	38.7 ± 0.2
Xylan	23.2 ± 0.1	7.5 ± 0.2
Arabinan	0.3 ± 0.0	0.0 ± 0.0
Acetyl groups	4.6 ± 0.1	1.6 ± 0.1
Klason lignin	38.5 ± 0.6	49.7 ± 0.7
Acid Soluble Lignin (ASL)	1.2 ± 0.1	0.7 ± 0.1
Other components	8.0	1.80

3.2. Delignification Treatments

Table 3 lists data concerning the HS and AS delignification treatments, including SY and the removal percentages of the structural components (lignin, cellulose, and hemicelluloses). For comparative purposes, Table 4 lists results reported for the delignification of hazelnut shells and other biomasses. Little information has been reported on the delignification of native or pretreated HS, with alkaline delignification being the most studied processing method [20,27,28].

Table 3. Solid yield and removal percentages of lignin, cellulose, and hemicelluloses achieved in the experiments 1–22 in Table 1. The removal percentages were calculated from their source (HS and AS, respectively).

Experiment	Solid Yield (%)	Lignin Removal (%)	Cellulose Removal (%)	Hemicellulose Removal (%)
1	91.7	7.2	10.0	26.6
2	81.8	12.7	22.5	74.7
3	88.0	15.7	9.0	38.8
4	78.1	20.7	22.3	76.7
5	76.1	19.2	7.4	47.8
6	76.9	24.3	20.3	79.2
7	86.8	11.3	11.5	27.5
8	82.3	17.2	20.0	70.6
9	82.4	18.0	10.9	42.5
10	88.0	22.4	13.8	62.2
11	82.7	18.9	10.3	52.4
12	85.5	29.1	24.9	69.1
13	73.5	21.2	11.0	49.2
14	75.8	26.6	16.2	70.1
15	53.6	53.3	0.0	67.5
16	76.8	35.2	2.6	42.0
17	75.8	32.8	5.2	51.0
18	46.2	65.3	0.0	76.0
19	64.9	47.9	7.1	87.7
20	57.9	50.5	17.3	93.2
21	61.4	47.9	0.0	56.2
22	73.2	37.2	5.6	62.1

Table 4. Literature reported on the delignification of diverse types of biomass using operational conditions related to the ones used in this work.

Raw Material	Reagents	T (°C)	Time	LSR	% Deligni-fication	Pre-Processing	Reference
Hazelnut shell	NaOH 4% (*w*/*v*)	121	60 min	10/1 (*v*/*w*)	32	Steam explosion, 5 min, 198–200 °C	[27]
	NaOH 4% (*w*/*v*)	121	90 min	10/1 (*v*/*w*)	42.5	Steam explosion, 5 min, 198–200 °C	[27]
	H_2O_2 4% (*w*/*v*)	121	30 min	10/1 (*v*/*w*)	36	Steam explosion, 5 min, 198–200 °C	[27]
	$NaBH_4$ 4% (*w*/*v*)	121	60 min	10/1 (*v*/*w*)	48	Steam explosion, 5 min, 198–200 °C	[27]
	NaOH 5% (*w*/*v*)	121	60 min	10/1 (*v*/*w*)	19.7	-	[28]
	NaOH 2.25%	120	60 min	10/1 (*v*/*w*)	60	-	[20]
	NaOH 2.25%	200	60 min	10/1 (*v*/*w*)	73.28	-	[20]
Almond shell	Ethanol 70/30 (*v*/*v*)	200	90 min	6/1	10.8	Autohydrolysis, 180 °C, 30 min, LSR 8/1	[34]
	NaOH 7.5 wt.%	121	90 min	6/1	18.4	Autohydrolysis. 180 °C, 30 min, LSR 8/1	[34]
Rice husks	Ethanol 54/46, NaOH 8% (*w*/*w* on solid)	160	60 min	10/1	90.1	Acid, 0.3% H_2SO_4 (*w*/*v*), 152 °C, 33 min	[35]
	Ethanol 54/46, NaOH 8% (*w*/*w* on solid)	160	100 min	10/1	91.47	Acid, 0.3% H_2SO_4 (*w*/*v*), 152 °C, 33 min	[36]
Hazelnut tree prunings	NaOH 2%	121	60 min	10/1	30.7	-	[37]
	NaOH 2%	121	60 min	10/1	51.2	Hydrothermal, 190 °C, 45 min, LSR 10/1 (*v*/*w*)	[37]
Olive tree pruning	Ethanol 70/30 (*v*/*v*)	200	90 min	6/1	31.2	Autohydrolysis, 180 °C, 30 min, LSR 8/1	[34]
	NaOH 7.5 wt.%	121	90 min	6/1	14.6	Autohydrolysis, 180 °C, 30 min, LSR 8/1	[34]
Sugarcane bagasse	Ethanol 50/50 (*v*/*v*), NaOH 1.5% on dry fiber (*w*/*w*)	175	60 min	5/1 (*v*/*w*)	44.3	-	[38]
	Ethanol 30/70 (*v*/*v*), NaOH 3% on dry fiber (*w*/*w*)	195	60 min	7/1 (*w*/*w*)	17.1	Acid, 0.2 M H_2SO_4, LSR 5/1 (*w*/*w*), 40 min, 120 °C	[39]
Miscanthus biomass	Ethanol 80/20 (*v*/*v*), H_2SO_4 1% (*w*/*w*, based on solid)	170	60 min	8/1	84	-	[40]
	Ethanol 80/20 (*v*/*v*), H_2SO_4 1% (*w*/*w*, on solid)	170	60 min	8/1	88.5	Autohydrolysis. LSR 9/1. 150 °C, 8h	[40]
Eucalyptus globulus	Ethanol 60:40 (*w*/*w*)	180–200	60 min	8/1 (*w*/*w*)	81	Autohydrolysis. LSR 8/1 (*w*/*w*), Severity) 3.65–3.94	[1]
Eucalyptus nitens bark	Ethanol 52-65%	192–200	60–86 min	8/1 (*w*/*w*)	49–52	-	[15]
Wheat straw	Ethanol 60/40 (*w*/*w*)	200	60 min	10/1 (*v*/*w*)	67	-	[41]
	Ethanol 60/40 (*w*/*w*)	200	60 min	10/1 (*v*/*w*)	64.3	Acid, LSR 7.5/1 (*v*/*w*), 160 °C, 30 min	[41]

Alkaline treatments of HS performed with 2–8% NaOH solutions (experiments 1, 3, and 5) did not exceed 19.2% delignification. These results are in agreement with the data reported by Uzuner et al. [28], who assessed the effects of NaOH concentration on delignification, and achieved a limited delignification degree (20%). The data were justified on the basis of the high recalcitrance of HS lignin to depolymerization. Hoşgün and Bozan [20] reported 60% of lignin removal operating at 120 °C for 60 min in media containing 2.25% NaOH, conditions that allowed almost complete cellulose recovery.

In this study, as a general trend, the solid dissolution and delignification effects reached in experiments performed with AS (exp. 2, 4, and 6) increased with the NaOH concentration more than they did in assays using HS (exp. 1, 3, and 5). The highest lignin removal on the delignification stage (24.3%) was reached when AS was treated with 8% NaOH at 121 °C for 60 min (experiment 6). These results are in accordance with literature using steam-exploded substrates, taking into account both delignification and autohydrolysis (31.6% of lignin removal for the experiment 4) but are below for experiments containing NaOH during longer times or $NaBH_4$ (42.5 and 48% lignin removal, respectively) [27]. The alkaline delignification of hazelnut tree prunings (before and after hydrothermal processing) was also assessed in media containing 2% NaOH, with a substantially improved delignification when the pretreated biomass was used as a substrate [37].

In the set of experiments performed with aqueous alkaline solutions (exp 1–6), AS led to higher hemicellulose removal than the ones carried out with HS. For example, 79.2% hemicellulose removal was achieved when AS was treated with 8% of NaOH at 121 °C for 60 min (exp. 6), conditions under which more than 20% of cellulose was removed (revealing poor selectivity).

The comparison between experiments performed with aqueous alkaline solutions and alkaline-organosolv mixtures (7–12) showed that the presence of ethanol resulted in limited additional delignification (11.3–26.6% of lignin removal). Related results were observed for the solubilization of cellulose and hemicelluloses. The experiments performed at higher temperatures (exp. 13 and 14) led to improved delignification degrees. In related studies, alkaline-organosolv methods were successfully applied to rice husks [35,36]. Native and pretreated sugarcane bagasse were also delignified by alkaline-organosolv delignification, resulting in a significantly higher lignin removal in the native samples [38,39].

Fernández-Rodríguez et al. [34] compared organosolv and alkaline delignification treatments of pre-processed biomass, looking at the manufacture of lignin isolates suitable for valorization alkaline methods led to the best results for almond shell delignification, whereas the opposite behavior was observed for olive tree pruning. In our study, as a general trend, organosolv treatments (exp. 15–17) and acid-catalyzed organosolv assays (exp. 18–22) showed that the amount of cellulose remaining in the solid phase was hardly altered. In assays using HS as a substrate, the delignification increased with respect to the ones performed with AS. This conclusion can be confirmed by comparing the results obtained in assays free from the catalyst (for example, 53.3% lignin removal in exp. 15 in comparison with 35.2% in experiment 16), and also by data analysis of experiments performed in H_2SO_4 containing media (65.3% delignification in exp. 18 in comparison with 47.9% in exp. 19).

Coupling stages of autohydrolysis and organosolv delignification has been reported as a suitable strategy for the complete fractionation of biomass [1,15,37], providing an alternative to conventional, single-stage delignification. In a related study, Huijgen et al. [41] compared organosolv and prehydrolysis-organosolv treatments of wheat straw, concluding that the prehydrolysis before organosolv resulted in decreased lignin recovery yields, a fact ascribed to the formation of "pseudo-lignin" and to lignin recondensation during prehydrolysis. El Hage et al. [42] found that the severity of the autohydrolysis modifies the lignin structure, affecting the subsequent organosolv delignification. Obama et al. [40] reported lignin alteration upon autohydrolysis, with the participation of repolymerization reactions (C-C linkages) that negatively affect the further delignification.

In this study, enhanced removal of hemicelluloses from HS and AS was observed in experiments performed in acid-catalyzed organosolv assays. In runs with AS, high degrees of hemicellulose removal (87.7% in exp. 19, performed at 180 °C for 60 min; or 93.2% in exp. 20, which lasted 120 min) were

achieved in acid-catalyzed organosolv treatments. These results are significantly higher than the ones observed for HS (76% hemicellulose removal in exp. 18). However, it can be noted that the AS hemicellulose content (9.1%) was significantly lower than the one of HS (28.1%). An opposite pattern was observed in experiments performed in the absence ofan acid catalyst, in which HS reached higher hemicellulose removal.

According to the above ideas, SY (which is affected by the contents of lignin and hemicelluloses) were lower for acid-catalyzed experiments. In experiment 18, the limited SY (46.2%) corresponded to 65.3% lignin removal and 76% hemicellulose removal from HS. Using AS as a substrate under the same conditions (exp. 19), the SY (64.9%) corresponded to 47.9% and 87.7% removal of lignin and hemicelluloses, respectively.

A comparative analysis of results confirmed that the conditions of experiments 18 and 19 (dealing with HS and AS, respectively) were the best ones identified in this study. The liquid phase from HS in exp. 18 contained the following hemicellulose-derived compounds: xylooligosaccharides, 10.01 g/L; arabinooligosaccharides, 0.28 g/L; glucooligosaccharides, 0.07 g/L; acetyl groups, 2.25 g/L; xylose, 10.74 g/L; glucose, 0.07 g/L; acetic acid, 2.29 g/L; furfural 1.38 g/L. In comparison, the composition of the liquid resulting from the acid-catalyzed delignification of AS (exp. 19) contained the following hemicellulose-derived compounds: glucooligosaccharides, 2.34 g/L; xylooligosaccharides, 1.25 g/L; acetyl groups, 0.28 g/L; xylose, 4.20 g/L; glucose, 1.98 g/L; acetic acid, 1.06 g/L; furfural 2.06 g/L; hydroxymethylfurfural, 0.24 g/L.

3.3. Enzymatic Hydrolysis

The results included in this section provide a quantitative assessment of the susceptibility of selected delignified and autohydrolyzed-delignified solids towards enzymatic hydrolysis. The composition of the solids is listed in Table 5. The experimental plan included acid-catalyzed organosolv treatments (assays 18–19), and experiments in ethanol/NaOH media (runs 13–14).

Table 5. Substrates and composition of solids employed as substrates for enzymatic hydrolysis. Data expressed as average values ± standard deviations.

Substrate for Enzymatic Hydrolysis	Delignified HS (from exp. 13)	Delignified AS (from exp. 14)	Delignified HS (from exp. 18)	Delignified AS (from exp. 19)
Solid composition (g/100 g of delignified solid)				
Cellulose	29.3 ± 0.5	42.8 ± 0.7	54.0 ± 1.2	55.4 ± 1.9
Xylan	19.3 ± 0.8	3.6 ± 0.1	11.9 ± 0.9	1.7 ± 0.3
Arabinan	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Acetyl groups	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.2	0.1 ± 0.1
Klason lignin	41.5 ± 3.5	48.3 ± 2.5	29.0 ± 0.7	40.1 ± 1.2
Acid soluble lignin (ASL)	1.1 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.0
Other	8.7	4.7	2.5	2.3

Both AS and the solids obtained under the conditions listed in Table 5 presented different behavior as hydrolysis substrates. Figure 1a shows the cellulose conversions into glucose achieved at selected reaction times. The highest cellulose conversion (74.2%, corresponding to a volumetric glucose concentration of 28.52 g/L) was reached when the solids from exp. 18 (HS subjected to acid organosolv delignification) were hydrolyzed for 96 h. In comparison, the solid from exp. 19 (AS delignified under the same conditions) was poorly hydrolyzed (conversion below 15%, or 5.62 g glucose/L). HS delignified with aqueous NaOH/ethanol under the conditions of exp. 13 showed higher hydrolysis conversion (52.5%) than the one achieved using AS delignified under the same conditions (exp. 14). Despite the low conversions reached in the experiments performed with autohydrolyzed or autohydrolyzed-delignified solids (obtained in exp. 14 and 19), the highest enzymatic susceptibility was observed for the substrate from exp. 14, carried out in aqueous NaOH/ethanol media. In comparative terms, the susceptibility of the diverse substrates to hydrolysis varied as follows: AS < acid-catalyzed-organosolv AS (exp. 19) <

aqueous NaOH-organosolv AS (exp. 14) < aqueous NaOH-organosolv HS (exp. 13) < acid-catalysed organosolv HS (exp. 18).

Literature data reports that the lignin content affects the enzymatic hydrolysis, since cellulases may bound to lignin irreversibly [40]. In our study, the solid from exp. 18 (which showed the highest conversion into glucose) presented the highest cellulose/lignin ratio (1.86). In comparison, the solid from exp. 19 (cellulose/lignin ratio, 1.36) reached considerably lower conversion into glucose than the solids from exp. 13 and 14 (which presented cellulose/lignin ratios of 0.68 and 0.87, respectively). Çöpür et al. [27] reported the behavior of different pretreatment techniques on regard to their efficiencies for the subsequent enzymatic hydrolysis of glucan into glucose from hazelnut husks. The highest glucan to lignin ratio of pretreated solids was used as criteria for the subsequent saccharification. Then, they reported a glucan to lignin ratio of 0.99 when HS were treated with combined steam explosion-NaOH delignification, and a high conversion of glucan into glucose (74.7%) in enzymatic hydrolysis performed at LSR 20. Hoşgün and Bozan [20] compared the enzymatic susceptibility of HS treated by different methods (acid, alkali, and steam) and found that the maximum glucose recovery (58.7%) corresponded to samples pretreated with NaOH. Similar results were reported with alkali delignified HS when solids were enzymatically hydrolyzed at higher LSR and ESR than were used in this work [28].

Hallac et al. [43] reported that the relationship between the lignin content of pretreated *Buddleja davidii* samples and their enzymatic susceptibility was not proportional. Huijgen et al. [41] compared the digestibility of delignified and prehydrolyzed-delignified wheat straw, and concluded that prehydrolysis prior to organosolv improved the enzymatic cellulose digestibility, despite the low lignin removal in delignification. Yang and Pan [44] discussed some aspects of the complexity of lignin effects on the enzymatic hydrolysis: a) high lignin content does not necessarily predict poor enzymatic digestibility; b) not all lignins have the same inhibitory effect on enzymatic hydrolysis of cellulose, since lignin from different sources had varying inhibitory effects, greatly related to lignin structures and properties; c) not all the lignin in the same substrate has the same inhibitory effect. On the basis of these observations, they studied the inhibitory effect of lignin with varied physicochemical properties from different biomass sources on the enzymatic hydrolysis of cellulose, and concluded that the lignin inhibition to enzymatic hydrolysis of cellulose was related to the hydrophobicity or the phenolic hydroxyl groups of lignin.

The data in Figure 1b show that the residual xylan in the hydrolysis substrates was extensively hydrolyzed into xylose. For example, in exp. 18, 80% of xylan was converted into xylose, which reached 7.03 g/L. Sun et al. [30] reported that some accessory enzymes in the enzymatic hydrolysis of natural lignocellulosic substrates favored an efficient conversion, and underlined the role of the high specific xylanase activity of the complex Cellic CTec2.

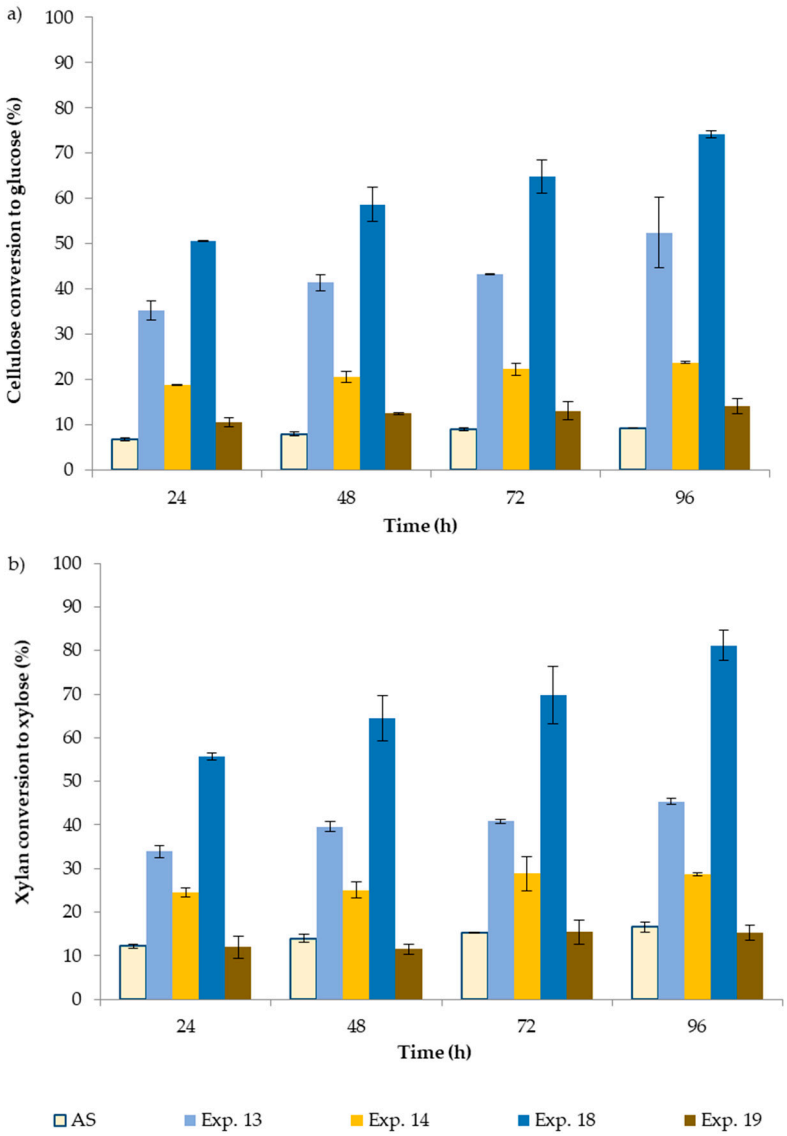

Figure 1. Effect of the enzymatic hydrolysis in the conversion of: (**a**) cellulose to glucose; (**b**) xylan to xylose, of spent solids from HS and AS processed under selected conditions of alkaline-organosolv, acid-catalyzed organosolv, and AS.

4. Conclusions

This study deals with the integral fractionation of HS, based on selected delignification methods (in alkaline, alkaline-organosolv, organosolv or acid-catalyzed organosolv media). Both HS and autohydrolyzed HS (AS) were used as substrates for delignification.

HS treated in acid-catalyzed organosolv media reached the highest delignification degree (65.3%), enabling the production of valuable hemicellulose-derived products (about half of them in the form of oligosaccharides), with limited cellulose losses from the solid phase. Alternatively, autohydrolysis led to the partial solubilization of hemicelluloses (mainly as oligosaccharides), and the subsequent acid-catalyzed organosolv of AS resulted in the simultaneous solubilization of the remaining hemicelluloses (87.7%) and lignin (47.9%).

The enzymatic hydrolysis of solids from delignification or autohydrolysis-delignification treatments confirmed that acid-catalyzed organosolv of HS provided the best substrate for enzymatic hydrolysis (74.2% cellulose conversion into glucose, with a volumetric concentration of 28.52 g glucose/L).

Then, the strategy approaches reported in this work can be considered as "conventional" but promising alternatives for the integral fractionation of HS in the scope of biorefinery.

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