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Abstract: The present work evaluates a two-step pretreatment process based on steam explosion and extrusion technologies for the optimal fractionation of lignocellulosic biomass. Two-step pretreatment of barley straw resulted in overall glucan, hemicellulose and lignin recovery yields of 84%, 91% and 87%, respectively. Precipitation of the collected lignin-rich liquid fraction yielded a solid residue with high lignin content, offering possibilities for subsequent applications. Moreover, hydrolysability tests showed almost complete saccharification of the pretreated solid residue, which when combined with the low concentration of the generated inhibitory compounds, is representative of a good pretreatment approach. *Scheffersomyces stipitis* was capable of fermenting all of the glucose and xylose from the non-diluted hemicellulose fraction, resulting in an ethanol concentration of 17.5 g/L with 0.34 g/g yields. Similarly, *Saccharomyces cerevisiae* produced about 4% (*v*/*v*) ethanol concentration with 0.40 g/g yields, during simultaneous saccharification and fermentation (SSF) of the two-step pretreated solid residue at 10% (*w*/*w*) consistency. These results increased the overall conversion yields from a one-step steam explosion pretreatment by 1.4-fold, showing the effectiveness of including an extrusion step to enhance overall biomass fractionation and carbohydrates conversion via microbial fermentation processes.

Keywords: lignocellulosic biomass; steam explosion; extrusion; *Scheffersomyces stipitis*; *Saccharomyces cerevisiae*; simultaneous saccharification and fermentation

1. Introduction

Uncertainties about future energy supplies and the current effects of global warming promoted by massive greenhouse gas emissions make it imperative to develop and implement competitive technologies for establishing a sustainable bio-based economy.

Lignocellulosic biomass is the major renewable organic matter in nature. It is composed of cellulose, hemicellulose and lignin polymers, bonded through non-covalent and covalent cross-linkages to form a complex and recalcitrant structure. Similar to current petroleum-based refineries, future biorefineries will efficiently convert the different components of lignocellulosic biomass into fuels, materials, high value-added chemicals, and other energy forms [1].

Biochemical conversion of lignocellulose includes pretreatment, enzymatic hydrolysis and fermentation steps. Pretreatment is needed to alter the structural characteristics of lignocellulose and increase the accessibility of cellulose and hemicellulose polymers to the hydrolytic enzymes, which

are responsible for breaking down these polysaccharides into fermentable sugars. From a biorefinery point of view, pretreatment processes must guarantee optimal and efficient biomass fractionation in order to maximize the potential value obtained from each component (cellulose, hemicellulose and lignin). Over the last four decades, different chemical, physical, physicochemical and biological methods have been developed for the pretreatment of lignocellulose [2,3]. Among pretreatment processes, hydrothermal-based technologies such as steam explosion or liquid hot water (with or without the addition of catalysts) have proven to be effective in deconstructing biomass structure. In the case of steam explosion, biomass accessibility is enhanced mainly by opening lignocellulosic fibers, solubilizing hemicellulosic sugars, and promoting partial solubilization and redistribution of lignin polymers [4]. This hydrothermal pretreatment is usually performed at elevated temperatures and pressures, with varying residence times. In general, temperatures ranging from 200 to 230 °C with short residence times (2–10 min) results in high cellulose saccharification yields (>70%; however, saccharification yields are highly dependent on biomass feedstock), but also in extensive hemicellulose degradation [4]. This side effect lowers the amount of sugars available for fermentation, and releases several biomass-derived products (aliphatic acids, furan derivatives and phenolic compounds), which inhibit hydrolytic enzymes and fermenting microorganisms [5,6]. In addition to hemicellulose degradation, the residual lignin present in the resulting pretreated solid material promotes the unspecific adsorption of hydrolytic enzymes, decreasing saccharification yields [7].

Besides hydrothermal methods, extrusion has been considered as another cost-effective pretreatment technology [2]. Extrusion represents a promising pretreatment method for industrial applications, since it has a highly versatile configuration process for the use of lignocellulosic feedstocks. This physical pretreatment provides effective mixing, rapid heat transfer, and high shear stress, which increases biomass accessibility by (1) promoting defibrillation and shortening of fibers; (2) increasing the surface area available to hydrolytic enzymes; and (3) reducing the crystallinity index and the degree of polymerization of cellulose [8,9]. Furthermore, chemical and or biological catalysts can be integrated in the process to boost saccharification processes. For instance, the addition of alkali during extrusion pretreatment has been shown to promote lignin solubilization and provoke a water-swollen effect, which leads to higher sugar yields in the subsequent saccharification step [8,9].

The combination of both hydrothermal and extrusion technologies can contribute to the balancing of biomass accessibility and biomass degradation, by using milder pretreatment conditions, while offering efficient biomass fractionation. In this context, the present work sequentially combines a mild acid-catalyzed steam explosion with an alkali-based extrusion process for optimal fractionation of lignocellulosic biomass. Using barley straw as a lignocellulosic source, the two-step pretreatment was designed to obtain (1) a liquid fraction containing mainly hemicellulosic sugars; (2) a lignin-rich liquid fraction; and (3) a solid fraction with a high cellulose content. To explore the full potential of the two-step pretreatment process in terms of subsequent applications, collected fractions were studied by analytical techniques and/or fermentation processes. First, the chemical compositions of collected fractions were analyzed to determine recovery yields. Second, the precipitated solid residue (PSR) from collected lignin-rich liquid fraction was analyzed by attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) to evaluate lignin purity. Finally, the corresponding water-insoluble solid fractions obtained from steam explosion (WIS) and extrusion (LE-WIS) were subjected to saccharification and fermentation processes, to evaluate their hydrolysability and fermentability in the context of bioethanol production.

2. Materials and Methods

2.1. Raw Material and Pretreatment Process

Barley straw, supplied by CEDER-CIEMAT (Soria, Spain), was used as lignocellulosic feedstock. It had the following composition in terms of percentage dry weight (DW): cellulose, 31.1 ± 0.8; hemicelluloses, 27.2 ± 0.4 (xylan, 22.3 ± 0.2; arabinan, 3.6 ± 0.1; galactan, 1.3 ± 0.1); Klason lignin,

18.8 ± 0.2; ashes, 3.9 ± 0.1; extractives, 10.5 ± 0.6; others components (including acid soluble lignin, acetyl groups, etc.), ~6%.

In order to collect hemicellulosic sugars, raw material was first pretreated by acid-catalyzed steam explosion. Prior to steam explosion, barley straw was milled in a laboratory cutting mill (Cutting Mill Type SM2000; Retsch GmbH, Haan, Germany) to obtain a chip size between 2 and 10 mm. Milled material was then impregnated with H_2SO_4 at an acid/biomass ratio of 10 mg/g, and pretreated in a 10 L steam explosion reactor (CIEMAT, Madrid, Spain) at mild conditions: 180 °C (~9 bar), 3.5 min. This condition was selected on the basis of preliminary studies showing a good balance between cellulose accessibility and hemicelluloses solubilization (data not shown). The recovered slurry was vacuum filtered to obtain a WIS fraction rich in cellulose and lignin, and a liquid fraction rich in hemicellulosic sugars and biomass-derived inhibitors. One portion of the WIS residue was stored for comparison purposes in the hydrolysability and fermentability tests.

Since the lignin polymer remains in the recovered solid fraction after steam explosion pretreatment, the corresponding WIS was subsequently subjected to an alkali-based extrusion process for lignin solubilization. Reactive extrusion was performed in a twin-screw extruder (Clextral Processing Platform Evolum® 25 A110, Clextral, Firminy, France) at 100 °C, 1 min of residence time (rotor speed: 150 rpm), with a biomass feeding rate of 2.5 kg/h, and at a final NaOH/biomass ratio of 80 mg/g (2 L/h of 10% (*w*/*v*) NaOH). Extrusion conditions and screw configuration were adapted from Duque et al. [10]. Similar to steam-pretreated slurry, extruded slurry was vacuum filtered to obtain a lignin-rich liquid fraction and a lignin-extracted solid residue (LE-WIS), which contained mainly cellulose and the remaining lignin polymers. The resulting lignin-rich liquid fraction was subsequently supplemented with H_2SO_4 (1N) to reach a final pH of 2, to produce a PSR fraction. The PSR was collected by centrifugation at 5000 g in a fixed-angle rotor for 10 min, washed once with distilled water, and lyophilized with a LyoQuest lyophilizer (Telstar, Terrassa, Spain).

Compositional analysis of raw material and collected fractions was determined as described in Section 2.6.1. Before usage, all collected liquid and solid fractions were stored at 4 °C.

2.2. Microorganisms and Growth Conditions

Scheffersomyces stipitis CBS 6054 (Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands) and *Saccharomyces cerevisiae* Ethanol Red (Fermentis, Marcq-en-Baroeul, France) were used as fermenting microorganisms in the present study. Active cultures for inoculation were obtained in 100-mL flasks containing 50 mL of growth medium: 30 g/L sugar (*S. cerevisiae* was grown on glucose, while xylose was used for growing *S. stipitis*), 5 g/L yeast extract, 2 g/L NH_4Cl, 1 g/L KH_2PO_4, and 0.3 g/L $MgSO_4 \cdot 7H_2O$. Flasks were incubated in an orbital shaker at 150 rpm and under controlled temperatures (35 °C for *S. cerevisiae* and 30 °C for *S. stipitis*) for 16 h (reagents for culture medium were purchased from Merck; Darmstadt, Germany). After incubation, cells were harvested by centrifugation at 5000 g in a fixed-angle rotor for 5 min, washed once with distilled water and diluted accordingly to obtain an inoculum concentration of 1 g/L cell dry weight (CDW).

2.3. Enzymes

Saccharification processes were carried out by using the commercial cocktails Celluclast + Novozyme 188 or Cellic CTec2 (Novozymes, Bagsvard, Denmark). Both Celluclast and Cellic CTec2 are mainly cellulase preparations. Due to its low β-glucosidase activity, Celluclast requires supplementation with Novozyme 188 (β-glucosidase) for the hydrolysis of cellobiose into glucose monomers. In contrast to Celluclast, Cellic CTec2 incorporates β-glucosidase activity, and does not therefore require supplementation with additional cocktails. Moreover, Cellic CTec2 also contains endoxylanase activity, which aids in hydrolyzing hemicellulosic sugars.

Overall cellulase activity, measured as filter paper units (FPU), was determined using filter paper (Whatman No. 1 filter paper strips), while β-glucosidase and xylanase activities were determined using cellobiose and birchwood xylan (filter paper, cellobiose and birchwood xylan were purchased

from Sigma-Aldrich Quimica SL; Madrid, Spain), respectively [11,12]. One unit of enzyme activity was defined as the amount of enzyme that transformed 1 μmol of substrate per minute.

2.4. Fermentation of the Hemicellulosic-Rich Liquid Fraction

The non-diluted liquid fraction obtained after filtration of steam-pretreated slurry was subjected to fermentation with *S. stipitis*, to evaluate its inhibitory capacity during revalorization of hemicellulosic sugars. Before inoculation, an enzymatic hydrolysis with Cellic CTec2 was carried out to hydrolyze both glucan and xylan oligomers. Enzymatic saccharification was performed in 100 mL shake flasks containing 50 mL of the corresponding liquid fraction. After adjusting the pH to 5, the liquid was supplemented with 2% (*v*/*v*) Cellic CTec2, and then incubated at 50 °C and 150 rpm for 24 h. Once oligomers were hydrolyzed, the pH was adjusted to 6, and nutrients (5 g/L yeast extract, 2 g/L NH_4Cl, 1 g/L KH_2PO_4, and 0.3 g/L $MgSO_4 \cdot 7H_2O$) and 1 g/L CDW of *S. stipitis* were added. Fermentation assays were performed at 30 °C and 150 rpm for 72 h. Samples were withdrawn periodically during fermentation for analytical purposes. Assays were performed in triplicate, and the corresponding average and standard deviation values were calculated to present the results.

2.5. Hydrolysability and Fermentability Studies of the Pretreated Solid Fractions

2.5.1. Enzymatic Hydrolysis

WIS and LE-WIS fractions were subjected to enzymatic hydrolysis to evaluate the pretreatment process in terms of hydrolysability potential. In this case, 2.5 g of the corresponding solid residue were first diluted in 100 mL shake flasks to a final substrate concentration of 5% (*w*/*v*). Saccharification was performed at pH 5, 50 °C and 150 rpm for 72 h, with an enzyme loading of 15 FPU/g DW substrate of Celluclast and 15 IU/g DW substrate of Novozyme 188. Assays were performed in triplicate, and the corresponding average and standard deviation values were calculated to present the results.

2.5.2. Simultaneous Saccharification and Fermentation

In addition to hydrolysability tests, collected WIS and LE-WIS residues were also subjected to SSF processes with *S. cerevisiae* to evaluate the fermentability potential of these residues. SSF processes were performed at 35 °C and pH 5 for 72 h in an orbital shaker (150 rpm). For this method, 5 g of the corresponding solid residue was first diluted to a final substrate concentration of 10% (*w*/*w*) and supplemented with 15 FPU/g DW substrate of Celluclast and 15 IU/g DW substrate of Novozyme 188, and 1 g/L CDW of *S. cerevisiae* Ethanol Red. Samples were withdrawn periodically during SSF for analytical purposes. Assays were performed in triplicate, and the corresponding average and standard deviation values were calculated to present the results.

2.6. Analytical Methods

2.6.1. Compositional Analysis of Biomass

Chemical composition of raw and pretreated material was determined using the Laboratory Analytical Procedures (LAP) for biomass analysis, provided by the National Renewable Energies Laboratory (NREL, Golden, CO, USA) [13]. Sugars and degradation compounds contained in the liquid fraction were also measured. For analysis of the oligomeric forms in the liquid fraction, a mild acid hydrolysis (4% (*w*/*w*) H_2SO_4, 120 °C and 30 min) was required to determine the concentration of all monomeric sugars. Monomeric sugars and degradation compounds were analyzed as described in Section 2.6.3.

2.6.2. ATR-FTIR Analysis of Solid Residues

Raw material, WIS, LE-WIS and PSR were analyzed by ATR–FTIR to determine chemical changes during pretreatment process. Dried biomass was analyzed in a FTIR spectrometer (Thermo Scientific

Nicolet 6700 spectrometer; Thermo Fisher Scientific Inc., Waltham, MA, USA), using an attenuated total reflectance (ATR) accessory and a deuterated triglycine sulfate detector. Spectra were collected at room temperature in the 4000–600 cm^{-1} range with a 1.928 cm^{-1} resolution and with an average of 64 scans.

2.6.3. Identification and Quantification of Metabolites

Ethanol was analyzed by gas chromatography (GC), while high-performance liquid chromatography (HPLC) was used to analyze sugars and biomass degradation compounds. In the case of ethanol, a 7890A GC System (Agilent, Waldbronn, Germany) equipped with an Agilent 7683B series injector, a flame ionization detector and a Carbowax 20 M column was used. The column oven was kept constant at 85 °C, while injector and detector temperatures were maintained at 175 °C. The carrier gas, helium, was set at a flow rate of 30 mL/min.

Sugars were analyzed by HPLC (Waters, Mildford, MA, USA) using a CarboSep CHO-682 carbohydrate analysis column (Transgenomic, San Jose, CA, USA). The operating temperature was 80 °C and the flow rate of the mobile phase (ultrapure water) was 0.5 mL/min. The identification of sugars was performed with a refractive index detector (Waters, Mildford, MA, USA).

Syringaldehyde, vanillin, ferulic acid, *p*-coumaric acid, furfural and 5-hydroxymethylfurfural (5-HMF) were analyzed and quantified by HPLC (Agilent, Waldbronn, Germany). The system was equipped with a Coregel 87H3 column (Transgenomic, San Jose, CA, USA). The operating temperature was 65 °C, and the mobile phase was 89% 5 mM H_2SO_4 and 11% acetonitrile, with a flow rate of 0.7 mL/min. All these compounds were identified by a 1050 photodiode-array detector (Agilent, Waldbronn, Germany). Finally, formic acid and acetic acid were also quantified by HPLC (Waters, Mildford, MA, USA). The system was equipped with a Bio-Rad Aminex HPX-87H column (Bio-Rad Labs, Hercules, CA, USA) and a 2414 refractive index detector (Waters, Mildford, MA, USA) for the separation and identification of acids, respectively. The operating temperature was 65 °C, and the flow rate of the mobile phase (5 mM H_2SO_4) was 0.6 mL/min.

3. Results and Discussion

3.1. Pretreatment of Barley Straw

Many pretreatment technologies have already been studied and developed to overcome the recalcitrant structure of lignocellulosic biomass. However, improvements are still necessary to maximize sugar recovery and establish a competitive lignocellulosic-based biorefinery process [2,3]. A two-step pretreatment process was designed for improving lignocellulosic biomass fractionation and facilitating its conversion into value-added compounds via fermentation processes (Figure 1).

Pretreatment consisted of a mild acid-catalyzed steam explosion, and an alkali-based extrusion process. First, steam explosion of acid impregnated barley straw resulted in a slurry with a total solid content of 20.4% (*w*/*w*) (12.7% and 7.7% insoluble and soluble solids, respectively). Steam explosion increased the cellulose and lignin content in the WIS fraction from 31.1% (*w*/*w*) and 18.8% (*w*/*w*), to 55.1% (*w*/*w*) and 32.1% (*w*/*w*), respectively (Table 1). This result is explained by an extensive hemicellulose solubilization, indicated by the low hemicellulose content in the pretreated WIS fraction (less than 10% (*w*/*w*)), and the high content of xylan and xylose in the recovered liquid fraction (Table 1). Biomass degradation compounds including acetic acid, furfural, 5-HMF and certain phenolic compounds (such as vanillin, syringaldehyde, *p*-coumaric acid and ferulic acid) were also identified in the liquid fraction of steam-exploded barley straw. Acetic acid is released by hydrolysis of the acetyl groups present in hemicelluloses. Formic acid derives from furfural and 5-HMF degradation, which results from the degradation of pentoses (mainly xylose) and hexoses respectively. Finally, phenols are released during partial solubilization and degradation of the lignin polymer [14,15].

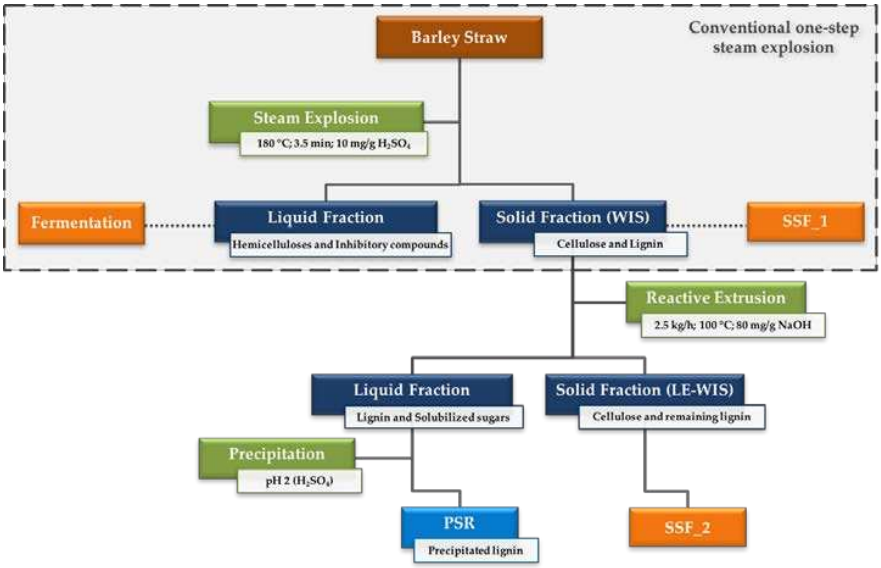

Figure 1. Process scheme depicting the two-step pretreatment process followed in the present study. SSF_1, simultaneous saccharification and fermentation (SSF) of the solid fraction obtained after steam explosion (WIS); SSF_2, SSF of the solid fraction obtained by the two-step pretreatment process (LE_WIS).

Table 1. Composition of steam-exploded barley straw.

WIS Fraction				
Component			**% (*w*/*w*)**	
Cellulose			55.1 ± 0.3	
Hemicellulose			8.8 ± 0.2	
Lignin			32.1 ± 1.9	
Ashes			2.5 ± 0.3	
Others			~1.5	
Liquid Fraction				
Sugar	**Monomeric Form % (*w*/*w*) [a]**	**Oligomeric Form % (*w*/*w*) [a]**	**Inhibitor**	**% (*w*/*w*) [a]**
Glucan	0.7 ± 0.1 (1.7)	2.8 ± 0.2 (7.6)	Acetic ac.	0.23 ± 0.04 (0.6)
Xylan	7.2 ± 0.4 (18.0)	13.9 ± 1.2 (31.9)	Formic ac.	n.d.
Arabinan	2.5 ± 0.3 (6.2)	1.1 ± 0.2 (2.9)	Furfural	0.17 ± 0.03 (0.4)
Galactan	0.7 ± 0.2 (1.8)	0.7 ± 0.1 (1.7)	5-HMF	0.04 ± 0.01 (0.1)
			Vanillin	<0.01 (12×10^{-3})
			Syringaldehyde	<0.01 (7×10^{-3})
			p-courmaric ac.	0.01 ± 0.00 (15×10^{-3})
			Ferulic ac.	0.01 ± 0.00 (21×10^{-3})

5-HMF, hydroxymethylfurfural; n.d., not determined; WIS, water insoluble solids; [a] Values expressed in g/L are listed in brackets.

Temperatures above 200 °C are needed during steam explosion pretreatment for enhancing biomass accessibility. Under these severe conditions, extensive biomass degradation—mainly hemicellulosic sugars—is also promoted, resulting in higher concentrations of inhibitory compounds. The use of lower pretreatment temperatures increases the recovery of hemicelluloses, and decreases the amount of lignocellulose-derived inhibitors in pretreated streams. However, at lower temperatures, longer pretreatment times (20–60 min) are needed to obtain similar saccharification yields in the subsequent enzymatic hydrolysis step, which increases pretreatment costs [4]. In order to reduce pretreatment time and lower the concentration of inhibitory compounds, an acid catalyst can be added to boost hemicellulose solubilization at temperatures below 200 °C. Thus, the pretreatment condition used in the present work for steam explosion was fairly sufficient for the solubilization of a major fraction of hemicellulosic sugars, reducing the hemicellulose content from 27.2% to 8.8% (*w*/*w*)

(Table 1). Furthermore, it is important to highlight the very low concentration of lignocellulose-derived inhibitors in the resulting liquid fraction, which can be indicative of a good pretreatment balance.

In the second stage of the pretreatment process, the recovered WIS fraction was subjected to an alkali-based extrusion process. The obtained extruded slurry contained 25% (*w*/*w*) insoluble solids out of 30% (*w*/*w*) total solids. After the extrusion process, the cellulose content of collected LE-WIS increased to 64.2% (*w*/*w*) (Table 2). Such an increase was promoted by lignin solubilization, even though similar lignin content was measured for both WIS and LE-WIS residues (Tables 1 and 2). The effectiveness of alkali-catalyzed extrusion processes to solubilize lignin has been previously observed. Duque et al. [10] reported a minimum NaOH/biomass ratio of 2.5–5% (*w*/*w*) to promote lignin solubilization in barley straw. Furthermore, these authors showed the highest lignin solubilization when using similar NaOH/biomass ratio and temperatures (7.5% (*w*/*w*) and 100 °C) to those used in the present study.

Another advantage of reactive extrusion with alkali is the possibility of lignin revalorization. Lignin represents an economic raw material for a wide range of applications. Although it has not yet been converted into high-value products at large scales, lignin has been utilized for the production of fertilizers, bioplastics or carbon fibers, among others products [16]. In this context, solubilized lignin was recovered by precipitation from the corresponding lignin-rich liquid fraction, resulting in a PSR fraction with about 85% and 3.5% (*w*/*w*) of lignin and sugar content, respectively (Table 2).

Table 2. Composition of extruded barley straw.

LE-WIS Fraction	
Component	% (*w*/*w*)
Cellulose	64.2 ± 2.0
Hemicellulose	6.8 ± 0.1
Lignin	29.3 ± 0.6
Ashes	2.1 ± 0.0
PSR Fraction	
Component	% (*w*/*w*)
Glucan	0.9 ± 0.1
Xylan	2.5 ± 0.2
Lignin	85.1 ± 1.5
Ashes	8.6 ± 0.6

LE-WIS, lignin-extracted water insoluble solids; PSR, precipitated solid residue.

In addition to determining the chemical composition of each collected fraction, the global mass balance for each component was estimated by comparing both raw and pretreated biomass yields. As listed in Table 3, high overall recovery yields were observed for glucan (84% (*w*/*w*)), hemicellulose (91% (*w*/*w*)) and lignin (87%, (*w*/*w*)), when considering all collected fractions. As well as the low concentration of biomass degradation compounds, these high recovery yields are representative of the well-balance pretreatment strategy, which offers high potential for the revalorization of lignocellulose.

Table 3. Mass balance during the two-step pretreatment process.

Component	**Steam Explosion**		**Extrusion**	
	Solid [a]	**Liquid**	**Solid** [b]	**Liquid**
Glucan	90	9	75	n.d.
Hemicellulose	17	82	9	n.d.
Lignin	87	n.d.	55	32 [c]

Values expressed as g/100 g DW of initial biomass; n.d., not determined; [a] Solid refers to WIS fraction; [b] Solid refers to LE-WIS fraction; [c] Value considering precipitated lignin in PSR fraction.

3.2. Characterization of Solid Residues by Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) Spectroscopy

Chemical changes promoted during pretreatment process were analyzed by ATR-FTIR on each solid residue. Figure 2 shows the absorbance in the mid-infrared region (2000–800 cm^{-1}) for all collected solid fractions. In the case of the non-pretreated barley straw (Figure 2a), typical peaks related to lignocellulosic biomass were observed [17]. The carbohydrate region (1370–890 cm^{-1}), including peaks characteristic of C–H deformation (900 cm^{-1}), C–O stretching (1105–1050 cm^{-1}), C–O–C vibration (1159 cm^{-1}) and C–H stretching (1375 cm^{-1}), showed the highest absorbance values. In addition, a lignin region (1595–1261 cm^{-1})—including signal for aromatic rings vibration (1595, 1510, 1421, 1329 and 1261 cm^{-1}) and C–H symmetric deformation (1498 cm^{-1})—and a peak related to ester groups in hemicelluloses (1731 cm^{-1}) could be also identified. This peak pattern of barley straw was modified during the two-step pretreatment process. First, the WIS fraction obtained after steam explosion pretreatment showed a significant reduction in the carbohydrate region, and at band 1731 cm^{-1} (Figure 2a). This reduction was supported by the extensive hemicellulose solubilization induced during the first stage of the pretreatment process (Table 1). In the case of extrusion pretreatment, an increase in the peak intensity of the carbohydrate region was noted when comparing WIS and LE-WIS fractions (Figure 2a). The higher absorbance in the carbohydrate region of LE-WIS can be explained by lignin solubilization, which increased the glucan/lignin ratio (Table 2).

A completely different absorbance profile was obtained with the collected PSR fraction (Figure 2b). This spectrum presented clearly defined peaks in the lignin region, which shows evidence of the high lignin content of this residue (Table 2). This result, combined with the high lignin content measured for the PSR fraction, offers possibilities for the subsequent revalorization of this residue from a biorefinery point of view. Nevertheless, further studies are needed to evaluate the actual potential of PSR utilization, since lignin polymers are usually altered during steam explosion pretreatment (e.g., cleavage of the β–O–4 ether bonds and other acid labile linkages) [4].

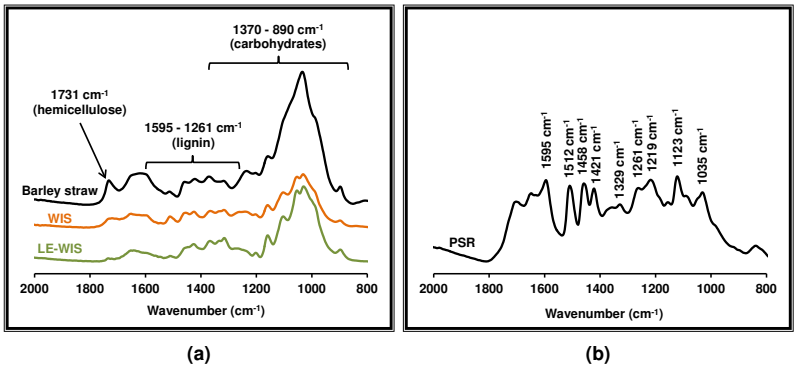

Figure 2. Infrared absorption spectra (cm^{-1}) of non-pretreated barley straw and pretreated collected fractions. (**a**) Solid fractions collected during the two-step pretreatment process: (black) non-pretreated barley straw, (orange) WIS fraction obtained after steam explosion, (green) LE-WIS fraction obtained after extrusion pretreatment; (**b**) PSR obtained by precipitation of the liquid fraction collected after extrusion pretreatment.

3.3. Saccharification of Pretreated Solid Residues

Saccharification is a key step during lignocellulosic biomass conversion as it highly influences overall production yields [18]. In this context, an efficient saccharification step is essential to obtain higher concentrations of fermentable sugars. After steam explosion pretreatment, 75% of potential sugars were enzymatically hydrolyzed from the collected WIS fraction (Figure 3a). This sugar yield was increased to about 100% by introducing the extrusion process, showing the effectiveness of this second stage for improving biomass accessibility.

Both steam explosion and extrusion are considered effective pretreatment technologies for enhancing biomass accessibility to the hydrolytic enzymes [2,3,8,9]. Moreover, these methods are highly versatile with regards to biomass feedstock and process configuration (such as the use of chemical catalysts). By combining steam explosion and extrusion processes, steam explosion can be performed at lower temperatures, decreasing the amount of released biomass degradation compounds without compromising biomass recovery and accessibility (Figure 3, Tables 1 and 3). Extrusion has been previously combined with other pretreatment technologies, with the aim of reaching high sugar yields and use milder process conditions (such as using lower temperatures and pressures, reducing the amounts of chemicals or solvents required during the process, decreasing enzyme loadings, etc.) [8,19,20]. For instance, Chen et al. [20] obtained an enzymatic hydrolysis yield of 80% (with about 84% xylan recovery), when subjecting rice straw to a combined extrusion and dilute acid pretreatment process. Similarly, Lee et al. [19] combined extrusion with hot-compressed water to pretreat Douglas fir, obtaining five-fold higher sugar yields.

In addition of increasing the concentration of fermentable sugars, higher saccharification yields also benefit the potential utilization of the remaining lignin polymer. Thus, the 55% (*w*/*w*) of the lignin that was left in the LE-WIS fraction could be recovered after an enzymatic hydrolysis step, increasing the overall lignin recovery yield from 32% (*w*/*w*) (in the PSR) to 87% (*w*/*w*) (Table 3).

When considering the initial sugar content, however, similar overall saccharification yields were observed for both WIS and LE-WIS fractions (Figure 3b). This result can be explained by the fact that some glucan and hemicellulose is co-solubilized with lignin during extrusion pretreatment, as indicated by the lower glucan and hemicellulose recovery yields for the LE-WIS fraction [10] (Table 3).

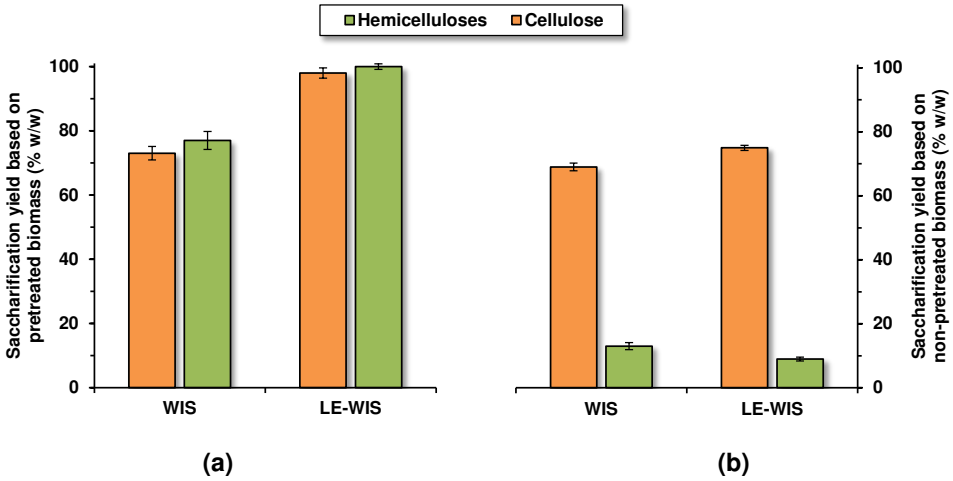

Figure 3. Saccharification yields obtained by enzymatic hydrolysis (72 h) of the WIS and LE-WIS fractions at 5% (*w*/*v*) substrate loadings. (**a**) cellulose and hemicellulose yields based on the composition of each pretreated fraction; (**b**) overall saccharification yields based on the initial composition of non-pretreated barley straw.

3.4. Conversion of Lignocellulosic Sugar by Microbial Fermentation Processes

From a biorefinery perspective, several biofuels and biochemicals (ethanol, methane, lactic acid, lipids, etc.) can be obtained via microbial fermentation of lignocellulosic sugars [21]. Among biofuels, lignocellulosic bioethanol is considered to be a promising alternative for the partial replacement of fossil fuels in the short to medium prospect. In this context, the two-step pretreatment process was evaluated in terms of ethanol production from pretreated sugar fractions: the hemicellulose-rich liquid fraction and the solid WIS and LE-WIS fractions. Results related to these assays are discussed in the following subsections.

3.4.1. Fermentation of the Hemicellulose-Rich Liquid Fraction

The presence of biomass degradation compounds in pretreated biomass is one of the main limitations for the fermentation of lignocellulosic sugars. These compounds have a negative impact on cell growth by inhibiting specific intracellular enzymes, causing an energy imbalance, and/or affecting the integrity of cell membranes [6,14,22,23]. After steam explosion pretreatment, the collected liquid fraction contained, in addition to solubilized hemicellulosic sugars, those compounds released from biomass degradation (Table 1). With the aim of evaluating the inhibitory capacity of this stream, the hemicellulose-rich liquid fraction was subjected to fermentation with *S. stipitis*. This yeast was chosen as a fermentative microorganism since it is capable of assimilating and converting xylose, the major component of this fermentation medium (Table 1). Most of the non-*Saccharomyces* yeast strains, including *S. sitipitis*, are known to be more sensitive to the inhibitory compounds released from biomass [24]. This means that lower concentrations of lignocellulose-derived compounds are needed to inhibit these fermentative microorganisms. To overcome microbial inhibition, different physical, chemical and biological detoxification processes have been developed to lower the concentration of degradation compounds [6,23,25]. Typical detoxification methods include filtration and washing, vacuum evaporation, and the use of resins and/or chemical/biological catalysts [26,27]. These processes, however, should be avoided since they usually require higher quantities of freshwater, the use of extra equipment, produce a loss of soluble sugars, and increase wastewater and overall process costs [25].

Biomass degradation promoted by steam explosion pretreatment can be reduced by using milder pretreatment conditions. As discussed above, the liquid fraction resulted from the first pretreatment stage (steam explosion) showed low concentrations of lignocellulose-derived compounds (Table 1). Nevertheless, the synergistic interaction between degradation compounds might cause the inhibition of the fermenting microorganisms, even at low concentrations [28,29], depending mainly on the inhibitory mixture and the inoculum size. In this case, the non-diluted liquid fraction caused no inhibition on *S. stipitis*, confirming the low inhibitory potential of this collected fraction. During the fermentation process, a maximum ethanol concentration of 17.5 g/L and a maximum ethanol volumetric productivity of 0.46 g/L·h were obtained, showing glucose and xylose depletion within 24 h and 72 h, respectively (Figure 4, Table 4). The observed ethanol concentration corresponds to a final ethanol yield of 0.34 g/g, which represents to about 70% of the theoretical ethanol that can be produced from the initial concentration of glucose and xylose.

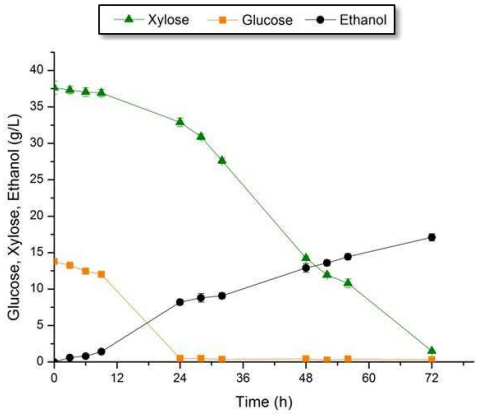

Figure 4. Fermentation of the non-diluted hemicellulose-rich liquid fraction (equivalent to about 13% WIS (*w*/*w*)). Time course of glucose and xylose consumption and ethanol production by the yeast *S. stipitis* CBS 6054. Prior to inoculation, the liquid fraction was enzymatically hydrolyzed with Cellic CTec2 at 50 °C for 24 h. Mean values and standard deviations were calculated from replicates to present the results. Note: glucose concentration is higher than that reported in Table 1 due to the presence of glucose in Cellic CTec2 preparation.

From a biorefinery point of view, the resulting ethanol concentration was below the minimum required for scaling up the process [30]. In this context, the low inhibitory capacity of the obtained hemicellulose-rich liquid fraction may offer possibilities for alternative microbial-based processes, such as the production of xylitol, lactic acid or microbial oils [31–33].

3.4.2. SSF of Pretreated Solid Fractions

Taking into account the good hydrolysability of LE-WIS (Figure 3a), this fraction was subjected to SSF processes to evaluate the fermentability potential of this pretreated material. The WIS fraction collected after steam explosion pretreatment was also subjected to SSF for comparison purposes. Due to its superior fermentation capacity of hexose sugars, the yeast *S. cerevisiae* was chosen as the fermentative microorganism for SSF processes. When using the WIS fraction as substrate, a maximum ethanol concentration of 19.6 g/L was obtained after 72 h of SSF process (Figure 5a, Table 4). This value was increased up to 31.7 g/L when using the LE-WIS fraction instead (Figure 5b, Table 4). With a 16% higher glucan content (Tables 1 and 2), higher ethanol concentrations during SSF of LE-WIS were expected. Nevertheless, the obtained ethanol concentrations respectively correspond to 0.29 g/g and 0.40 g/g overall yields, which were equivalent to 57% and 78% of the theoretical ethanol yield (Table 4). Both higher ethanol concentrations and yields were consequently observed for the LE-WIS fraction, being representative of the better hydrolysability of the two-step pretreated solid fraction. The differences in the glucan content, however, had an effect on the corresponding increase in ethanol concentration and yield. Thus, ethanol concentration increased by 60%, while overall ethanol yields increased by 1.4-fold.

In addition to ethanol concentration and yields, slightly higher maximum ethanol volumetric productivities were also observed during SSF of LE-WIS (0.96 g/L·h, compared to 0.83 g/L·h for WIS). In SSF processes, ethanol volumetric productivities are highly influenced by hydrolysis rates. Therefore, these small differences could be justified by the differences in the hydrolysability capacity of pretreated fractions.

The better fermentation parameters observed for LE-WIS fraction could be explained by the better hydrolysability of LE-WIS, as indicated by the higher glucose concentration within the first 12 h of SSF processes, and the higher overall yields (Figure 5, Table 4). However, although hydrolysability tests showed 75% and 98% saccharification yields for the WIS and LE-WIS fraction, respectively, only 57% and 78% ethanol yields were obtained –even though glucose concentration remained below 0.5 g/L after 72 h of SSF (Figure 5). This result hints at enzymatic hydrolysis as the main impeding factor for reaching higher conversion yields. Differences between saccharification yields during hydrolysability tests and SSF could be explained by the increase in substrate concentration (from 5% (*w*/*v*) to 10% (*w*/*w*)) and the lower temperature (35 °C instead of 50 °C) used during SSF processes. The increase in substrate loadings influences enzymatic hydrolysis by promoting (1) end-product inhibition of hydrolytic enzymes; (2) unproductive adsorption of proteins to the remaining lignin polymer; (3) protein deactivation or denaturalization and (4) the decline in the binding capacity of enzymes to cellulose [34,35]. For instance, Moreno et al. [36] reported a 35% decrease on the overall ethanol yields after increasing the substrate concentration from 10% to 20% (*w*/*w*) during SSF processes. Another factor that highly influences saccharification yields is SSF temperature. Enzymatic hydrolysis has an optimal temperature around 50 °C, while most fermenting yeasts work at 30–37 °C. In this context, the use of thermotolerant strains that can ferment at temperatures above 40 °C, may contribute to obtain increased overall conversion yields [37,38].

Energy balance is another important aspect for evaluating the economic feasibility of the process [39]. In this context, the present work provides the basic scenario to set optimal conditions for the future success of the process. Also, it is remarkable to mention that a final ethanol concentration of 4% (*v*/*v*) was obtained with the present two-stage pretreatment strategy. Notwithstanding, with the aim of increasing final ethanol concentration and overall yields, different experiments at higher substrate concentrations and using novel enzyme cocktails are now being performed.

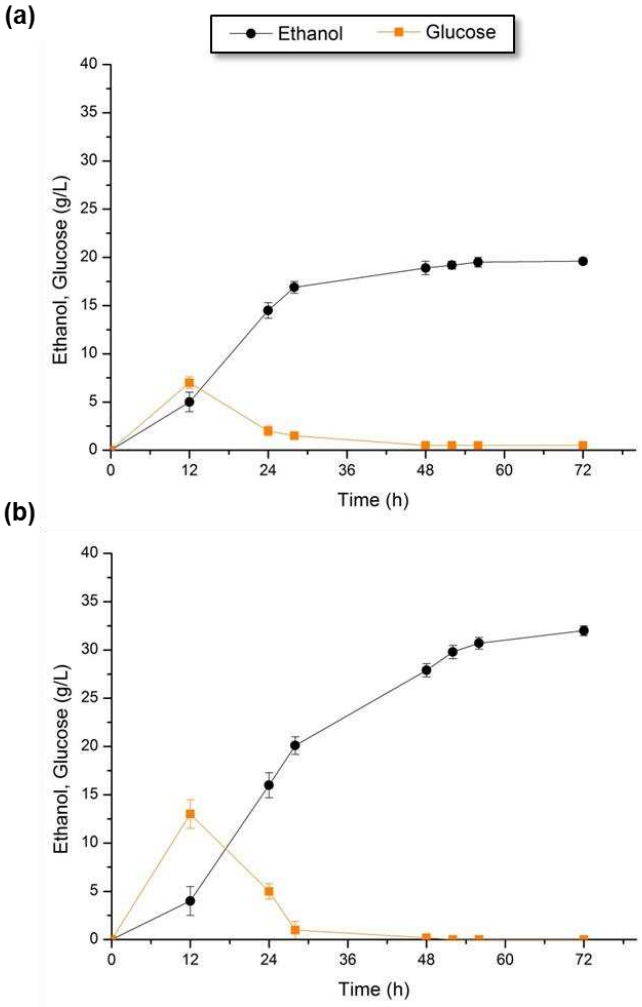

Figure 5. Simultaneous saccharification and fermentation (SSF) of (**a**) WIS and (**b**) LE-WIS at 10% (*w*/*w*) substrate loading. Time course of glucose and xylose consumption and ethanol production by the yeast *S. cerevisiae* Ethanol Red. Mean values and standard deviations were calculated from replicates to present the results.

Table 4. Summary of the fermentation parameters obtained for collected sugar fractions.

Substrate (*w*/*w*)	Yeast	$EtOH_{max}$ (g/L)	$Y_{E/S}$ (g/g)	$Y_{E/ET}$ (%)	Q_{Emax} (g/L·h)
Liquid fraction [a]	*S. stipitis*	17.5 ± 0.2	0.34 ± 0.01 [b]	66.7	0.46 ± 0.01
10% WIS	*S. cerevisiae*	19.6 ± 0.1	0.29 ± 0.00 [c]	56.9	0.83 ± 0.04
10% LE-WIS	*S. cerevisiae*	31.7 ± 0.3	0.40 ± 0.01 [c]	78.4	0.96 ± 0.09

[a] The liquid fraction used was equivalent to about 13% (*w*/*w*) WIS. $EtOH_{max}$, maximum ethanol concentration reached at 72 h; $Y_{E/G}$, ethanol yield based on [b] initial glucose and xylose concentration or [c] potential available glucose (considering the glucan content of substrate); $Y_{E/ET}$, percentage of the theoretical ethanol, assuming maximum ethanol yields of 0.51 g/g for both glucose and xylose; Q_{Emax}, maximum volumetric ethanol productivity, estimated within 12–24 h. Ethanol yield was calculated with the assumption that the liquid volume of the SSF system is constant [40].

4. Conclusions

By combining an acid-catalyzed steam explosion and an alkali-based extrusion process, lignocellulosic biomass (barley straw) can be fractionated with high overall recovery yields, producing (1) a solid residue with high lignin content, (2) a non-inhibitory liquid fraction containing hemicellulosic sugars and (3) a solid residue with high glucan content. From a sugar platform perspective, the majority of uses for sugar are via microbial fermentation. The present two-step pretreatment process has

demonstrated not only the possibility for maximizing lignin and sugar recovery, but also for enhancing the hydrolysability and fermentability of collected residues. Thus, this pretreatment favors the revalorization of each lignocellulosic component when considering a fermentation-based biorefinery.

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Author Contributions: All authors have participated in the design of the study. José Miguel Oliva, María José Negro and Mercedes Ballesteros conceived and designed the experiments; Ignacio Ballesteros and Paloma Manzanares performed pretreatment of barley straw; José Miguel Oliva and Miguel Ángel Chamorro performed fermentation experiments and analyzed the data; Felicia Sáez contributed with biomass analysis; Antonio D. Moreno wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

5-HMF	5-hydroxymethylfurfural
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared spectroscopy
CDW	Cell Dry Weight
DW	Dry Weight
$EtOH_{max}$	Maximum Ethanol concentration
FPU	Filter Paper Units
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
LE-WIS	Lignin-Extracted Water Insoluble Solid fraction
NREL-LAP	National Renewable Energies Laboratory-Laboratory Analytical Procedures
PSR	Precipitated Solid Residue
Q_E	Ethanol Volumetric Productivity
WIS	Water Insoluble Solid fraction
$Y_{E/ET}$	Ethanol Yield based on the maximum theoretical ethanol
$Y_{E/S}$	Ethanol Yield based on potential sugars

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