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# Does Acid Addition Improve Liquid Hot Water Pretreatment of Lignocellulosic Biomass towards Biohydrogen and Biogas Production?

George Dimitrellos <sup>1</sup>, Gerasimos Lyberatos <sup>1,2</sup>  and Georgia Antonopoulou <sup>1,\*</sup> 

<sup>1</sup> Institute of Chemical Engineering Sciences, Stadiou, Platani, GR 26504 Patras, Greece; gdimitrellos@hotmail.com (G.D.); lyberatos@chemeng.ntua.gr (G.L.)

<sup>2</sup> School of Chemical Engineering, National Technical University of Athens, GR 15780 Athens, Greece

\* Correspondence: geogant@chemeng.upatras.gr; Tel.: +30-26-1096-5318

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**Abstract:** The effect of liquid hot water (LHW) pretreatment with or without acid addition (A-LHW) on the production of hydrogen—through dark fermentation (DF)—and methane—through anaerobic digestion (AD)—using three different lignocellulosic biomass types (sunflower straw (SS), grass lawn (GL), and poplar sawdust (PS)) was investigated. Both pretreatment methods led to hemicellulose degradation, but A-LHW resulted in the release of more potential inhibitors (furans and acids) than the LHW pretreatment. Biological hydrogen production (BHP) of the cellulose-rich solid fractions obtained after LHW and A-LHW pretreatment was enhanced compared to the untreated substrates. Due to the release of inhibitory compounds, LHW pretreatment led to higher biochemical methane potential (BMP) than A-LHW pretreatment when both separated fractions (liquid and solid) obtained after pretreatments were used for AD. The recovered energy in the form of methane with LHW pretreatment was 8.4, 12.5, and 7.5 MJ/kg total solids (TS) for SS, GL, and PS, respectively.

**Keywords:** liquid hot water pretreatment; acid; hydrothermal; hydrogen; methane; anaerobic digestion; lignocellulosic biomass

## 1. Introduction

Recently, much research effort has been conducted for the transition from a fossil-fuel-based economy to a bio-economy, in which renewable resources of biological origin are used to sustainably produce food, energy, and new materials. Adoption of a biobased economy model is expected to decrease the world's dependence on natural resources and to contribute to the protection of the climate and environment through the reduction of greenhouse gas emissions [1]. Lignocellulosic biomass is considered among the most promising sources for the production of alternatives to petroleum-based fuels—the biofuels, which address the aforementioned challenges for a sustainable bio-economy model [2]. Agricultural residues, which are rich in lignocellulose, have a significant unutilized energy potential and are a renewable and readily available type of biomass [3].

Anaerobic digestion (AD) for methane production and dark fermentation (DF) for hydrogen generation have been extensively studied for the exploitation of a broad variety of lignocellulosic materials [4–7]. It is reported that only 20–30% of the biomass (mainly the extractives and hemicellulose) could be converted into methane, without any treatment, during the AD process [8]. Moreover, very low hydrogen yields have been reported for raw, untreated lignocellulosic substrates [2]. This is mainly due to the complex structure of the biomass, which limits the accessibility of microorganisms or enzymes, thus reducing its biodegradability and resulting in lower methane/hydrogen yields compared to the

theoretical ones [3,9]. Thus, in order to reduce the structural and compositional barriers, an appropriate pretreatment method should be applied, leading thus to improved biofuel yields [9].

Liquid hot water (LHW) pretreatment, often called hydrothermal pretreatment, has gained much attention for the production of hydrogen, methane, and ethanol from lignocellulosic feedstocks [10–12], since it is reported to be more efficient in terms of energy than thermo-chemical pretreatment methods [11]. During LHW pretreatment, lignocellulose is treated with liquid water at a temperature range of 90–260 °C [12], an initial pressure of 0–60 bar, and a retention time in the range of 2–40 min [3]. The effectiveness of this pretreatment method depends on the imposed operational conditions as well as on the type of pretreated biomass (softwood, hardwood, perennial crops) [13], owing to the different properties of water (such as ionic strength, pH, and viscosity) with the change of conditions. Water at high temperatures acts as an acid, reducing the pH to slightly acidic levels, thus causing a hydration of the cellulose, a significant solubilization of hemicellulose, and, depending on the substrate, partial or no degradation of lignin [11].

In order to achieve more severe conditions during LHW pretreatment by further decreasing the pH, addition of some catalysts, such as dilute acids, has been reported for enhancing the bioethanol production process [14]. More acidic conditions might lead to higher hemicellulose removal efficiency and increase cellulose digestibility [15]. However, inhibitory by-products or toxic compounds might be generated at the same time.

As there is a lack of literature regarding the effect of inhibitory compounds and the impact of acid LHW (A-LHW) pretreatment on hydrogen or methane production from lignocellulosics, the aim of the present study was to assess A-LHW (210 °C for 15 min and 1% w/v H<sub>2</sub>SO<sub>4</sub>) as a pretreatment method for enhancing hydrogen through DF and methane production through AD using three different lignocellulosic biomasses: sunflower straw (SS), grass lawn (GL), and poplar sawdust (PS). Direct comparison with the respective LHW pretreatment (210 °C, for 15 min) was performed. Compositional and structural changes after both pretreatment methods were determined, while potential inhibitors after pretreatment were quantified. To our knowledge, it is the first comparative study to simultaneously investigate A-LHW and LHW pretreatments and to evaluate the possible process schemes for hydrogen and methane production from different types of lignocellulosic biomass.

## 2. Materials and Methods

### 2.1. Biomass Used

PS and GL were collected in Attica, Greece, while SS was collected from Central Macedonia in Greece. All feedstocks were initially air-dried, ground with a domestic grinder (Izzy X3, E560T3, Titanium, Crete, Greece), milled with a laboratory grinder (A11 basic, IKA, Staufen, Germany), and sieved to a powder with a size smaller than 0.7 mm. Before being used in the experiments, all biomasses were air-dried.

### 2.2. LHW and A-LHW Pretreatment

For both pretreatment methods, the solid loading, expressed as total solids (TS), was 5% w/v, (5 g of TS in 100 mL water or dilute acidic water solution). Microwave digestion equipment (speedwave MWS-2, Berghof, Germany) with an infrared (IR) sensor for temperature monitoring was used for the LHW and A-LHW pretreatments. All biomasses were pretreated at 210 °C for 15 min (LHW) and at the same conditions, but with addition of 0.1% w/v of H<sub>2</sub>SO<sub>4</sub> (2 g H<sub>2</sub>SO<sub>4</sub>/100 g TS) (A-LHW). After pretreatment, the whole pretreated biomasses were separated through vacuum filtration (0.7 µm pore size), and two fractions (a liquid and a solid one) were obtained. A detailed physicochemical and structural characterization was performed on both fractions. The rich in cellulose solid fractions were used for the production of (a) hydrogen, through biological hydrogen potential tests (BHP), and (b) methane, through biochemical methane potential tests (BMP), while the liquid ones were used for the production of methane in BMP tests. The pretreatments were performed in duplicate.

### 2.3. BMP and BHP Experimental Assays

BMP experiments at 35 °C were carried out in both separated fractions (liquid and solid fraction) obtained after LHW and A-LHW pretreatment based on the modified method of Owen and Chynoweth [16] and as described in Antonopoulou et al. [17]. Briefly, in all experiments, 20% *v/v* of anaerobic sludge was used as inoculum in serum bottles of 160 mL, with a working volume of 100 mL. Regarding experiments with the solid fractions obtained after LHW and A-LHW pretreatments, a final loading of 2 g TS/L was used, while in the experiments with the liquid ones, the final chemical oxygen demand (COD) concentration was 2 g/L. Control (using cellulose and glucose) as well as blank experiments were also performed in order to check the methanogenic activity of the inoculum used. The biogas produced was collected via a syringe.

BHP tests at 35 °C were performed in the rich in cellulose solid fractions of the pretreated biomasses using commercial cellulose, at a loading of 40 FPU/g TS (Celluclast 1.5 L) and glycosidase (Novozyme 188) at a ratio of 3:1 (*v/v*) with a simultaneous saccharification and fermentation (SSF) concept, as described in Antonopoulou [2]. Briefly, 10% *v/v* of anaerobic sludge initially boiled at 100 °C for 15 min was used as inoculum in batch serum bottles of 160 mL, with a working volume of 50 mL. LHW and A-LHW pretreated solids at a final loading of 10 g TS/L were used along with a basal nutrient medium, which was properly diluted [2]. Control and blank experiments were also performed, and the initial pH was set to 6 in all vials. The gas produced was collected via a syringe. At the end of DF, the contents of the vials were centrifuged, filtered (0.7 µm filters), and then analyzed for volatile fatty acid (VFA) concentration.

### 2.4. Analytical Methods

Extractives and lignocellulose (cellulose, hemicellulose, and lignin) before and after the pretreatments were analyzed as described in Antonopoulou et al. [6,15]. Characterization of the liquid fractions obtained after the pretreatments (total soluble sugar content, glucose, xylose, arabinose, and cellobiose, as well as 5-hydroxymethylfurfural (5-HMF), furfural formic, and acetic acid) was performed as in Antonopoulou et al. [6,15].

COD, TS, volatile solids (VS), and Total Kjeldahl Nitrogen (TKN) were measured according to standard methods [18]. The protein content was calculated by multiplying TKN by 6.25 [19], which is a mean value for foods and feeds, and is also commonly used for lignocellulose.

The percentages of hydrogen and methane in the produced biogas of the BMP or BHP tests, as well as VFA (acetic, propionic, iso-butyric, butyric, iso-valeric, valeric, and caproic acid) concentrations at the end of BHP tests, were quantified via air chromatography as described in Antonopoulou et al. [6] and Alexandropoulou et al. [5,19]. The filter paper activity (FPU) of the commercial cellulase (Celluclast 1.5 L) was measured as presented in Antonopoulou et al. [6], while scanning electron microscopy (SEM) images and attenuated total reflection (ATR) spectra were obtained as in Antonopoulou et al. [15].

### 2.5. Calculations

The severity factor ( $R_o$ ) can be used for describing the pretreatment intensity, which, in the case of thermal processes, was first proposed by Overend et al. [20], taking into account the pretreatment conditions (temperature and retention time):

$$\log R_o = \log \left\{ t \exp \left( \frac{T - T_{ref}}{14.75} \right) \right\} \quad (1)$$

where  $R_o$  is a dimensionless severity factor,  $t$  and  $T$  are the reaction time (min) and temperature (°C), respectively,  $T_{ref}$  is the reference temperature (usually 100 °C), and 14.75 is an empirical parameter depending on the gas constant ( $R$ ) and the activation energy [15].

For taking into account the influence of pH on the pretreatment severity during A-LHW pretreatments, a combined severity factor ( $R'_0$ ), comprising the acidic pH value of the pretreated biomass slurry obtained after pretreatment, can be used [21]:

$$\log R'_0 = \log R_0 - pH \quad (2)$$

The BMP or BHP yields, which are defined as the cumulative methane or hydrogen production during AD or DF experiments and are expressed as mL CH<sub>4</sub> or H<sub>2</sub>/g VS<sub>pretreated</sub>, were calculated as:

$$\text{BMP or BHP yield} \left( \frac{\text{mL}}{\text{g VS}_{\text{pretreated}}} \right) = \frac{mL_t - mL_{\text{blank}}}{\text{g VS}_{\text{pretreated}}} \quad (3)$$

where  $mL_t$  is the volume of methane or hydrogen produced from the substrate at a given time, while  $mL_{\text{blank}}$  is the respective value of the blank experiment. In order to compare BMP or BHP with the respective untreated substrates, expressed as mL CH<sub>4</sub> or H<sub>2</sub>/g VS<sub>initial</sub>, the methane/hydrogen yields were calculated as:

$$\text{yield} \left( \frac{\text{mL}}{\text{g VS}_{\text{initial}}} \right) = \text{yield} \left( \frac{\text{mL}}{\text{g VS}_{\text{pretreated}}} \right) \times \text{Material Recovery} \left( \frac{\text{g VS}_{\text{pretreated}}}{\text{g VS}_{\text{initial}}} \right) \quad (4)$$

For calculating the methane yield of the hydrolysates in terms of mL CH<sub>4</sub>/g VS<sub>initial</sub>, Equation (5) was used:

$$\text{CH}_4 \text{ yield} \left( \frac{\text{mL}}{\text{g VS}_{\text{initial}}} \right) = \text{CH}_4 \text{ yield} \left( \frac{\text{mL}}{\text{mL}_{\text{hydrolysate}}} \right) \times \left( \frac{\text{mL}_{\text{hydrolysate}}}{\text{g VS}_{\text{initial}}} \right) \quad (5)$$

### 3. Results and Discussion

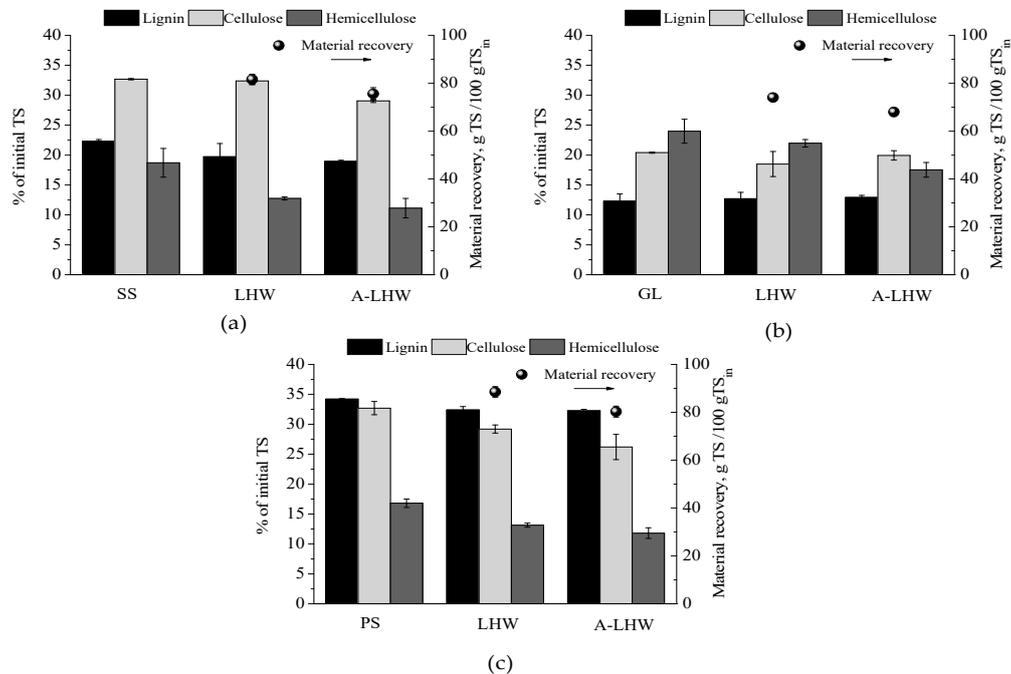
#### 3.1. Impact of Pretreatments on Chemical Composition of Biomasses

The lignocellulosic composition and the material recovery of three types of biomass before and after hydrothermal pretreatments and in the presence or absence of acid are presented in Figure 1. For the three substrates, the extractive and protein contents were (% of initial TS):  $8.1 \pm 0.1$  and  $1.7 \pm 0.2$  for SS,  $25.6 \pm 3.1$  and  $10.5 \pm 0.5$  for GL, and  $5.0 \pm 0.6$  and  $1.0 \pm 0.1$  for PS, respectively. For raw substrates, the sum of lignocellulose accounted for 73% (SS), 56.7% (GL), and 85.7% (PS) of the initial TS, suggesting the low content of soluble compounds or extractives in the case of PS.

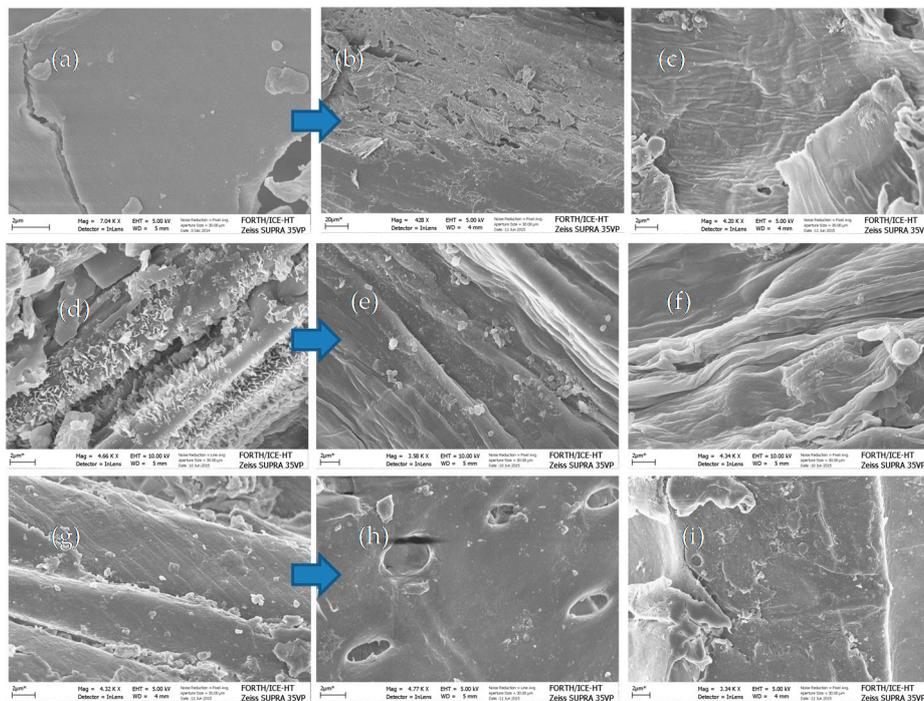
A-LHW was more severe for all substrates ( $\log R'_0 = 1.6$  for SS, 1.2 for GL, and 1.5 for PS) compared to the LHW pretreatment ( $\log R_0 = 4.4$  for all types of biomass). A-LHW pretreatment resulted in lower solid material recovery and higher reduction of the hemicellulose fraction due to solubilization ( $75.6 \pm 2.6\%$  and  $40.5\%$  for SS,  $68.0 \pm 1.8\%$  and  $27.0\%$  for GL, and  $80.3 \pm 2.3\%$  and  $29.8\%$  for PS) compared to the respective values for LHW pretreatment ( $81.6 \pm 2.2\%$  and  $31.8\%$  for SS,  $74.1 \pm 1.7\%$  and  $8.4\%$  for GL, and  $88.5 \pm 2.2\%$  and  $21.8\%$ , for PS). Based on Ahmad et al. [22], higher severity in hydrothermal pretreatment resulted in relatively higher hemicellulose solubilization, lower material recovery, and significant structural changes in cellulose and lignin. Based on Figure 1, both pretreatment methods tested were not so effective in removing lignin, something which is also confirmed by other studies [3]. It is reported that only alkali pretreatment is effective in lignin breakdown by causing cleavage of lignin–carbohydrate linkages [17,23]. A slight reduction in cellulose was observed only for PS, which was 5.0% for LHW and 5.6% for A-LHW.

In Figure 2, representative SEM images of raw (Figure 2a) SS, (Figure 2d) GL, and (Figure 2g) PS, as well as LHW (Figure 2b,e,h) and A-LHW (Figure 2c,f,i) pretreated samples, are presented. It can be seen that the morphology of all substrates is quite different after LHW and A-LHW pretreatment, since each method affected each substrate differently. For instance, raw SS and raw PS were characterized by a compact, connected, and rigid structure that was somewhat separated and exposed after pretreatments. A less compact structure was also observed after LHW pretreatment on other substrates, such as

safflower straw [24], Miscanthus [25], or wheat straw [26]. Regarding GL, the surface after LHW and A-LHW pretreatment (Figure 2e,f) was smoother compared to that of the raw substrate (Figure 2d). The smoother surface was also evident in previous studies in which HCl and NaOH as well as aqueous-ammonia-soaking pretreatment were applied in the same substrate [17,27].

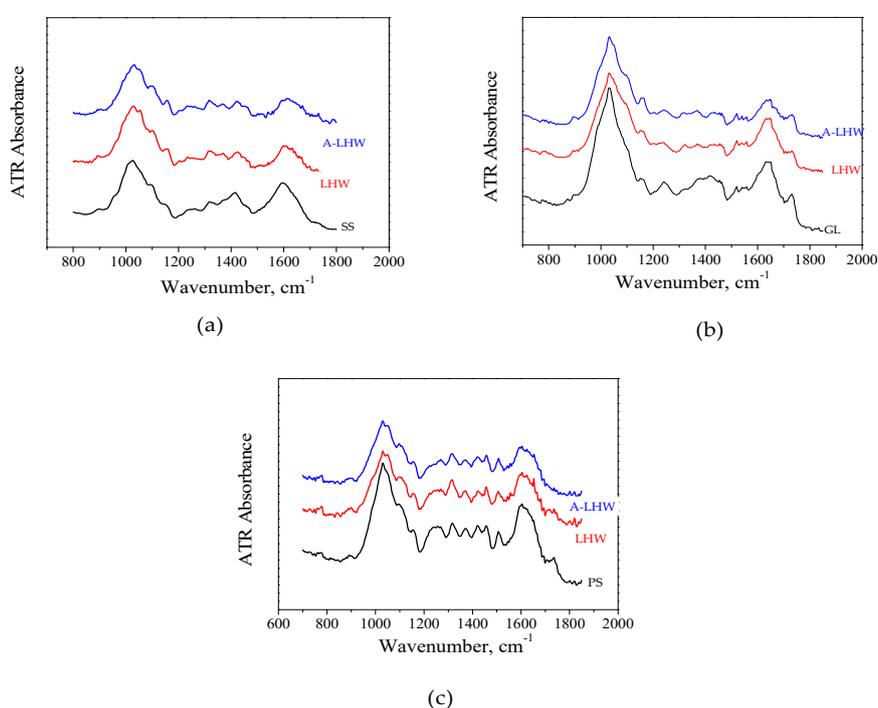


**Figure 1.** Effect of liquid hot water (LHW) and acid LHW (A-LHW) pretreatment on solid material recovery (right axis) and lignocellulosic content (left axis) of (a) sunflower straw (SS), (b) grass lawn (GL), and (c) poplar sawdust (PS).



**Figure 2.** Scanning electron microscopy (SEM) images of (a) raw sunflower straw (SS), (d) raw grass lawn (GL), and (g) raw poplar sawdust (PS), as well as of the liquid hot water (LHW) pretreated (b) SS, (e) GL, and (h) PS and acid-LHW (A-LHW) pretreated (c) SS, (f) GL, and (i) PS.

In Figure 3, the ATR spectra of raw and pretreated biomasses in a range from 600 to 1800  $\text{cm}^{-1}$  are presented. It is obvious that the spectrum of raw PS (Figure 3c) exhibited comparable absorption bands with the corresponding LHW and A-LHW pretreated substrates, while the spectra of A-LHW pretreated SS (Figure 3a) and GL (Figure 3b) had different profiles compared to the corresponding untreated biomasses. In the case of pretreated samples, the band at 894  $\text{cm}^{-1}$ —attributed to the deformation of the C-H bond or stretching of C-O-C at  $\beta$ -1, 4 glycosidic linkages, which corresponds to the cellulose amorphous fraction [17]—is intense, implying the decrease of the crystalline part of cellulose in favor of the amorphous part due to the LHW and A-LHW pretreatment. Despite the fact that the cellulose seems not to be affected due to pretreatments (Figure 1), IR spectra reveal that its form has changed from crystalline to amorphous, thanks to which cellulose might be more accessible to the enzymes during the next biochemical steps. Only in the spectrum of A-LHW pretreated SS was the band not so intense compared to the other substrates, implying that this pretreatment method did not influence the cellulose crystallinity of all the substrates to the same degree.



**Figure 3.** Attenuated total reflection (ATR) spectra of untreated, liquid hot water (LHW) pretreated, and acid LHW (A-LHW) pretreated (a) sunflower straw (SS), (b) grass lawn (GL), and (c) poplar sawdust (PS).

The C=O bond due to stretching of hemicellulose [28] at 1730  $\text{cm}^{-1}$  is less intense in the LHW and A-LHW pretreated samples due to hemicellulose reduction, as also confirmed by the values of Figure 1. Finally, in the spectra of pretreated samples, the signal at 1043  $\text{cm}^{-1}$  caused by C-O, C-C, and C-OH stretching vibrations, which are related to lignocellulose [29], corresponded to different peaks of different heights, which was not obvious in the spectra of all raw, untreated biomasses.

The total soluble sugar content (TSSC) (measured as glucose equivalent) and the monosaccharide concentration (glucose, xylose, and arabinose)—expressed as % of initial TS (or g/100 g TS)—contained in the hydrolysates obtained after pretreatment are presented in Table 1. Cellobiose was not detected. It is obvious that the TSSC was higher in the case of pretreated GL, confirming the lower solid material recovery (Figure 1) due to solubilization that was observed. For GL, the sum of xylose, arabinose, and glucose, which come from hemicellulose degradation, accounted for 85.4% of the TSSC measured in the case of LHW pretreatment and 80.5% for the A-LHW pretreatment, indicating that some other monosaccharides or oligosaccharides were probably produced.

**Table 1.** Total soluble sugar and monosaccharide concentrations (expressed as % of initial total solids (TS)) contained in the hydrolysates obtained after pretreatments of all types of biomass.

		Total Soluble Sugars	Glucose	Xylose	Arabinose
SS	LHW	3.11 ± 0.19	0.52 ± 0.02	0.46 ± 0.00	0.87 ± 0.01
	A-LHW	3.45 ± 0.38	0.56 ± 0.05	0.87 ± 0.03	1.22 ± 0.01
GL	LHW	7.34 ± 0.14	1.18 ± 0.08	2.63 ± 0.06	2.46 ± 0.05
	A-LHW	8.83 ± 0.63	1.25 ± 0.05	2.87 ± 0.03	2.99 ± 0.01
PS	LHW	2.00 ± 0.16	0.15 ± 0.02	0.16 ± 0.06	0.26 ± 0.05
	A-LHW	2.07 ± 0.29	0.15 ± 0.03	0.16 ± 0.02	0.28 ± 0.05

In general, in acid pretreatments (low pH values) at high temperatures, degradation of sugars such as pentoses and hexoses might lead to the production of compounds, such as furfural and 5-HMF, which are characterized as inhibitors to the microorganisms involved in the next biochemical process steps [30,31]. Further degradation of 5-HMF at severe pretreatment conditions might result in the formation of levulinic or formic acids, while acetic acid is another common product released due to hemicellulose bond cleavage.

In Table 2, the concentration of furanic compounds and that of the produced acids are presented. It is obvious that during both pretreatment methods, furans, acetic, and formic acids were produced at various concentrations for each substrate. Pretreatment of GL led to a higher production of inhibitors due to the higher solubilization (lower material recovery). The presence of acid in A-LHW pretreatment enhanced their production due to the low pH value and the higher severity, which is also confirmed by other studies [9]. For A-LHW pretreated SS, production of 3.76 g/100 g TS acids and 0.34 g/100 g TS furans occurred, while in the case of GL and PS, these values were 1.02 and 1.07 g/100 g TS and 0.9 and 0.87 g/100 g TS, respectively. Regarding furans, a lower concentration of 5-HMF in comparison to furfural was observed for both pretreatments in all types of biomass, which was also reported for other hydrothermally pretreated feedstocks [32], due to the limited hexose degradation coming from cellulose. In general, the release of inhibitory compounds during pretreatment and their concentration widely depend on the nature of the lignocellulosic biomass, the pretreatment severity (related to the reaction temperature and time), and the reactor vessel used [33]. A high concentration of furfural (1.2 g/L) was produced when sugarcane press mud was hydrothermally pretreated at 200 °C for 5 min [34], while even higher concentration (5.13 g/L) has been produced after LHW treatment of corn cobs [35]. In the case of beach wood, furfural and 5-HMF concentrations of 0.03–2.73 g/L and 0.03–0.84 g/L were reported for LHW pretreatment at 130–220 °C and for 15–180 min [36].

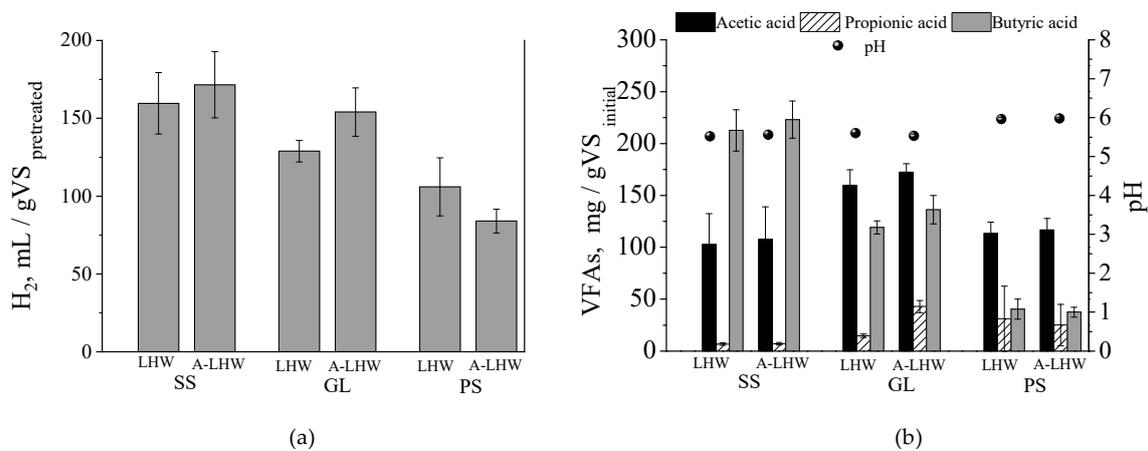
**Table 2.** Concentration of formic and acetic acids as well as of 5-hydroxymethylfurfural (5-HMF) and furfural (expressed as % of initial TS) contained in the hydrolysates obtained after liquid hot water (LHW) and acid LHW (A-LHW) pretreatments of all types of biomass.

		Formic Acid	Acetic Acid	5-HMF	Furfural
SS	LHW	1.98 ± 0.08	1.52 ± 0.07	0.05 ± 0.00	0.15 ± 0.03
	A-LHW	2.20 ± 0.06	1.56 ± 0.05	0.07 ± 0.01	0.27 ± 0.04
GL	LHW	0.00 ± 0.00	1.09 ± 0.06	0.02 ± 0.00	0.51 ± 0.04
	A-LHW	0.00 ± 0.00	1.02 ± 0.04	0.15 ± 0.01	0.92 ± 0.08
PS	LHW	0.23 ± 0.01	0.62 ± 0.01	0.05 ± 0.01	0.22 ± 0.01
	A-LHW	0.25 ± 0.02	0.65 ± 0.01	0.12 ± 0.03	0.75 ± 0.04

### 3.2. Biofuel Generation

#### 3.2.1. BHP of Pretreated Samples

The rich in cellulose solid fractions obtained after A-LHW and LHW pretreatments, as well as the raw biomasses, were used for BHP experiments in an SSF concept using 40 FPU cellulase/g TS (and glycosidase at a ratio 3:1 (*v/v*)). In Figure 4, the hydrogen yields of the pretreated solid fractions of SS, GL, and PS (in mL H<sub>2</sub>/g VS<sub>pretreated</sub>; VS<sub>pretreated</sub>: VS of pretreated biomass) are presented, while in Figure 4, the pH (right axis) and the dominant VFAs (acetic, propionic, butyric acid; left axis) at the end of the BHP experiments are presented. The BHP of the raw (non-pretreated) substrates with SSF was 74.9 ± 5.2, 86.3 ± 7.8, and 89.3 ± 4.3 mL H<sub>2</sub>/g VS<sub>initial</sub> (VS<sub>initial</sub>: VS of initial biomass) for GL, SS, and PS, respectively.



**Figure 4.** (a) Hydrogen yields and (b) pH and volatile fatty acids (VFAs) of liquid hot water (LHW) and acid-LHW (A-LHW) pretreated sunflower straw (SS), grass lawn (GL), and poplar sawdust (PS).

From Figure 4a, it is obvious that despite the differences in the lignocellulosic content of the substrates, the LHW and A-LHW pretreatments applied in this study enhanced hydrogen production under SSF. A-LHW pretreatment led to higher BHP yield compared to the LHW (expressed as mL/g VS<sub>pretreated</sub>) for SS and GL, contrary to the case of PS. As can be seen from Figure 4b, acetic, butyric, and propionic acids were the only metabolites detected at the end of fermentations. For both pretreatment methods, acetate and butyrate were the dominant metabolic products, indicating that hydrogen was generated mainly from the typical acetate–butyrate pathway during DF [37]. The concentration of acetate was higher in the case of GL and PS, while butyric acid was the dominant metabolite in the case of SS. In the case of PS, where low BHPs were observed, the concentration of butyrate was low and the pH culture at the end of the experiment was high (5.96 for LHW and 5.98 for A-LHW). In general, a correlation of butyrate with enhanced BHP yields has also been reported in previous studies when different pretreatment methods were applied in SS [6,38] or GL [2].

In Table 3, the BHP expressed as mL/g VS<sub>initial</sub> (based on Equation (4)) and the energy recovery are presented. For comparison, the BHP of the raw, untreated substrates is also presented. It can be seen that both pretreatments enhanced the hydrogen yields of SS (106% and 102% for LHW and A-LHW pretreatment, respectively) compared to the BHP of the raw SS with SSF. LHW pretreatment was advantageous for PS, causing a 5.8% increase of the BHP, while in the case of GL, LHW and A-LHW pretreatment enhanced 19.8% and 32.6% the yield of hydrogen. Based on the BHP yields and the energy density of hydrogen [39], the recoverable energy in MJ/kg TS<sub>initial</sub> (TS<sub>initial</sub>: TS of initial biomass) was estimated. The energy is comparable with that of other studies in which LHW pretreatment was applied for enhancing the BHP of wheat straw, rice straw, sorghum biomass, and corn stover [3].

**Table 3.** Biological hydrogen production (BHP) (mL/g VS<sub>initial</sub>) and energy recovery of the raw biomasses and of the solid fractions obtained after liquid hot water (LHW) and acid LHW (A-LHW) pretreatment methods.

		H <sub>2</sub> (mL/g VS <sub>initial</sub> )	Energy (MJ/kg TS <sub>initial</sub> )
SS	Raw	74.9 ± 5.2	0.8
	LHW	154.1 ± 15.1	1.6
	A-LHW	151.4 ± 15.0	1.5
GL	Raw	86.3 ± 7.8	0.9
	LHW	103.4 ± 4.6	1.1
	A-LHW	114.4 ± 9.6	1.2
PS	Raw	89.3 ± 4.3	1.0
	LHW	94.6 ± 15.4	1.1
	A-LHW	70.5 ± 5.9	0.8

### 3.2.2. BMP of Pretreated Samples

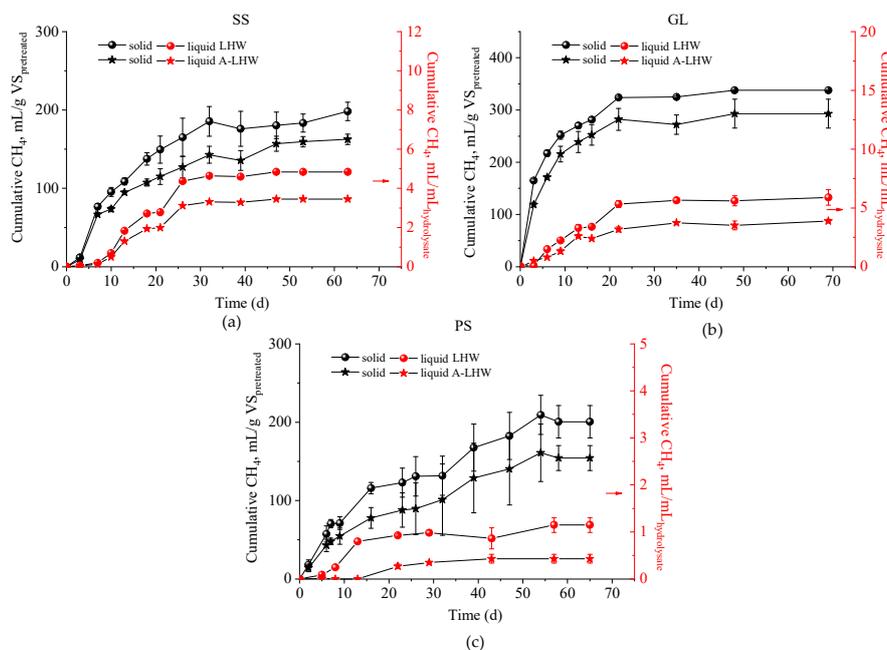
The BMP of the raw GL, SS, and PS was  $339.9 \pm 1.2$ ,  $272.9 \pm 16.3$ , and  $140.2 \pm 13.08$  mL CH<sub>4</sub>/g VS<sub>initial</sub>, respectively. As anticipated, the low lignin and high holocellulose content of GL (Figure 1) corresponded to the highest methane yield compared to the other substrates, with PS being the most difficult to degrade. The BMP of GL is consistent with the literature ( $372 \pm 52$  mL CH<sub>4</sub>/g [40] and  $402.5$  mL CH<sub>4</sub>/g [41]). In the case of SS, similar BMP (260 mL CH<sub>4</sub>/g) was reported by Antonopoulou et al. [42], but lower yields ( $192 \pm 2$  mL CH<sub>4</sub>/kg VS) have also been reported [43] due to the differences in the lignocellulosic content.

In Figure 5, the effect of LHW and A-LHW pretreatment on the BMP of the solid (left axis; in mL/g VS<sub>pretreated</sub>) and the liquid (right axis) fraction (in mL/mL hydrolysate) obtained after pretreatments of all substrates is presented. It is obvious that for all types of biomass, A-LHW pretreatment led to lower methane yields compared to the case of LHW pretreatment. The lower methane yields in the case of A-LHW treated samples could be attributed to the lower holocellulose content that remained in the solid fraction and that was available for biodegradation by the anaerobic bacteria. Since A-LHW pretreatment led to higher hemicellulose degradation towards xylose or arabinose, which were present in the hydrolysate, the biodegradable solid fraction of all substrates was reduced.

The highest methane yield was produced for GL ( $337.7 \pm 1.2$  and  $292.9 \pm 27.5$  L CH<sub>4</sub>/kg VS<sub>pretreated</sub> for LHW and A-LHW pretreatment, respectively). These values were close to the corresponding values obtained after alkali pretreatment of GL and higher compared to the acid pretreated solid fraction of GL [17]. This fact indicates that although the holocellulose was not affected significantly by hydrothermal pretreatment, the surface structure was changed enough, so as the cellulose surface area increased and became available for enzymatic attack, giving high methane yields. This is also evident in SEM images (Figure 2e,f) and ATR spectra (Figure 3b), and is consistent with other studies [22]. For SS,  $198.2 \pm 11.6$  and  $162.5 \pm 6.6$  mL CH<sub>4</sub>/g VS<sub>pretreated</sub> were produced after LHW and A-LHW pretreatment, while the respective values for PS were  $200.6 \pm 20.1$  and  $154.3 \pm 15.8$  L CH<sub>4</sub>/kg VS<sub>pretreated</sub>.

Regarding the BMP of the hydrolysates obtained after pretreatments, the acid addition (A-LHW) seems to have a negative effect on methane yields compared to LHW treated samples. For instance,  $5.9 \pm 0.7$  and  $3.9 \pm 0.2$  mL/mL<sub>hydrolysate</sub> were produced after LHW and A-LHW pretreatment, respectively, of GL. This could be attributed to a partial inhibition of anaerobic sludge caused by the release of compounds at high concentrations during A-LHW pretreatment. Specifically, under those conditions, production of high concentrations of furfural ( $0.92 \pm 0.08$  g/100 g TS), 5-HMF ( $0.15 \pm 0.01$  g/100 g TS), and acetic acid ( $1.02 \pm 0.04$  g/100 g TS) were observed (Table 2). Although such concentrations were not reported as inhibitory for mixed anaerobic bacteria, when furans or acetic acid are added separately [44], their co-existence might have a synergistic toxic effect [31], causing a reduction of the methane yields. In general, hydrolysates obtained from pretreatments at moderate conditions (low pretreatment severity factor) might lead to high methane yields due to the high sugar content (due to hemicellulose

solubilization) and minimum release of inhibitors [22]. For instance, Fernandez-Cegri et al. [44] obtained a BMP of  $310 \pm 4$  mL/g COD for sunflower oil cake that was hydrothermally pretreated at  $100^\circ\text{C}$ , and this yield was reduced to  $247 \pm 10$  mL/g COD when the temperature of pretreatment increased to  $200^\circ\text{C}$ . In general, it is difficult to make predictions for the methane potential of the hydrolysate due to the transference of inhibitors in this fraction. Comparing the BMP of the liquid fractions of the three substrates, the higher value was observed for GL after LHW pretreatment due its high sugar content (Table 1) compared to the other biomasses.



**Figure 5.** Cumulative methane yields of the solid (left axis) and liquid (right axis) fractions of liquid hot water (LHW) and acid-LHW (A-LHW) pretreated (a) sunflower straw (SS), (b) grass lawn (GL), and (c) poplar sawdust (PS).

In Table 4, the BMP of solid and liquid fractions (expressed as mL/g VS<sub>initial</sub>) obtained from both pretreatment methods is presented. From the values of the table it is evident that LHW pretreatment enhanced the BMP of all substrates compared to the raw, untreated samples. In the case of PS, a 45.5% increase of the BMP was achieved, while in the case of GL and SS, a slight increase was observed (11.7% and 3.6% enhancement, respectively). In the case of A-LHW of GL, AD of the separated fractions did not enhance the methane production, as also observed for other acid pretreatment methods applied in GL [17].

Based on the methane yield of each fraction and the energy density of methane [39], the recoverable energy might be estimated in MJ/kg TS<sub>initial</sub>. Due to the high BMP values, AD of the hydrolysate obtained after LHW pretreatment of GL led to a high energy recovery of 5.1 MJ/kg TS<sub>initial</sub>.

For the determination of the total produced energy that could be gained for each substrate, two possible configurations that were developed in the present study after hydrothermal pretreatment might be compared: LHW or A-LHW pretreatment and separation of both fractions as well as (a) AD for CH<sub>4</sub> production for both fractions (CH<sub>4</sub>-CH<sub>4</sub>) and (b) DF for H<sub>2</sub> production for the rich in cellulose solid fraction (under the SSF mode) and AD for CH<sub>4</sub> production for the liquid fraction (H<sub>2</sub>-CH<sub>4</sub>). The energy gained through the aforementioned processes is presented in the last column of Table 4, from which it could be concluded that, for all substrates, the concept of CH<sub>4</sub>-CH<sub>4</sub> is more preferable in terms of energy, and that LHW pretreatment led to higher energy production than the A-LHW pretreatment.

**Table 4.** Biochemical methane potential (BMP; mL/g VS<sub>initial</sub>) and energy yield (MJ/kg TS<sub>initial</sub>) of the separated fractions obtained after liquid hot water (LHW) and acid-LHW (A-LHW) pretreatment of all substrates as well as the total energy gained through two process schemes: (a) anaerobic digestion (AD) for CH<sub>4</sub> production of both fractions (CH<sub>4</sub>-CH<sub>4</sub>) as well as (b) dark fermentation (DF) for H<sub>2</sub> production of the solid fraction and AD for CH<sub>4</sub> production of the liquid fraction (H<sub>2</sub>-CH<sub>4</sub>).

		Solid Fraction		Liquid Fraction		Total
		BMP (mL/g VS <sub>initial</sub> )	Energy (MJ/kg TS <sub>initial</sub> )	BMP (mL/g VS <sub>initial</sub> )	Energy (MJ/kg TS <sub>initial</sub> )	Energy (MJ/kg TS <sub>initial</sub> ) H <sub>2</sub> -CH <sub>4</sub> /CH <sub>4</sub> -CH <sub>4</sub> Concept
	Raw <sup>a</sup>	272.9 ± 16.3	8.6			0.8 <sup>b</sup> /8.6 <sup>c</sup>
SS	LHW	161.2 ± 9.4	5.1	105.1 ± 0.4	3.3	4.9/8.4
	A-LHW	121.4 ± 4.9	3.8	75.1 ± 0.3	2.4	3.9/6.2
	Raw <sup>a</sup>	339.9 ± 1.2	11.2			0.9 <sup>b</sup> /11.2 <sup>c</sup>
GL	LHW	225.9 ± 0.8	7.4	153.6 ± 17.6	5.1	6.2/12.5
	A-LHW	181.2 ± 17.0	5.6	101.0 ± 3.9	3.3	4.5/8.9
	Raw <sup>a</sup>	140.2 ± 13.08	5.1			1.0 <sup>b</sup> /5.1 <sup>c</sup>
PS	LHW	177.2 ± 17.8	6.5	26.8 ± 3.7	1.0	2.1/7.5
	A-LHW	123.4 ± 12.7	4.5	10.1 ± 1.0	0.4	1.2/4.9

<sup>a</sup> The BMP of raw substrates. <sup>b</sup> Based on the biochemical hydrogen potential (BHP) from raw biomass. <sup>c</sup> Based on the BMP from raw biomass.

For GL, AD of the separated LHW pretreated fractions led to 12.5 MJ/kg TS<sub>initial</sub>, which is almost 11.6% higher than the energy that could be gained if untreated GL were to be directly used for methane production in an AD process (11.2 MJ/kg TS<sub>initial</sub>). Based on a previous study, the BMP and the recoverable energy in the form of methane were at their maximum when GL was pretreated with 20 g NaOH/100 g TS, and, specifically, AD of the whole biomass led to the production of 413.5 mL CH<sub>4</sub>/g VS<sub>initial</sub> or 13.7 MJ/kg TS<sub>initial</sub>, while the respective values after separation of both fractions and AD of the separated ones were 427.1 mL CH<sub>4</sub>/g VS<sub>initial</sub> or 14.1 MJ/kg TS<sub>initial</sub> [17].

In the case of PS, the AD of the separated LHW pretreated fractions led to 7.5 MJ/kg TS<sub>initial</sub>, which was almost 47% higher than the energy that might be gained through AD of untreated PS (5.1 MJ/kg TS<sub>initial</sub>) or 53% higher than the corresponding value after A-LHW pretreatment (AD of both separated fractions (4.9 MJ/kg TS<sub>initial</sub>)). For SS, the energy gained through the AD of the LHW pretreated fractions was comparable with the energy from AD of the untreated substrate. The recoverable energy from all the substrates is comparable with the results of other studies in which LHW pretreatment was applied for enhancing the BMP of different substrates, such as wheat straw, rice straw, sorghum biomass, and corn stover [2].

However, the final selection of the process scheme will depend not only on the energy yield of the final products, but also on the upstream processes that are involved. Based on this, the cost of the LHW pretreatment concept comprises the operational cost for temperature and pressure increase in the microwave digester (210 °C for 15 min) as well as the cost of separation of both fractions and the AD of liquid and solids in different vessels. For A-LHW pretreatment, additional cost for chemicals (sulfuric acid) should be taken into account. Taking into account the lower methane and hydrogen production yields, the higher inhibitor generation, the higher process cost due to chemicals, and the lower energy recovery from the A-LHW pretreated substrates, it could be concluded that the addition of acid does not improve biofuel generation from SS, GL, and PS in the conditions tested. A lower concentration than 0.1% w/v of H<sub>2</sub>SO<sub>4</sub> (2 g H<sub>2</sub>SO<sub>4</sub>/100 g TS) might be beneficial for the process by enhancing BMP and BHP. Using a lower acid concentration, a lower concentration of inhibitors might be generated, which could possibly be nontoxic for anaerobic sludge. In case other acids, such as nitric, phosphoric, or hydrochloric acid, at different concentrations are used, different BMP and BHP values will be expected. In the latter case, the combined severity factor ( $R'_0$ ) will be different due to the

different value of the final pH. This is also confirmed by recent studies, where different concentrations of sulfuric, hydrochloric, and phosphoric acid were used to enhance hydrogen, ethanol, and methane from GL [2,17] or hydrogen and ethanol from SS [6]. In those studies, it was shown that there was an optimum acid concentration and an optimum type of acid for each process, which were not necessary the same for all biofuels. In any case, optimization of LHW pretreatment conditions is needed in order to explore the optimum operational conditions (in terms of temperature, time, and acid concentration) for enhancing saccharification and ensuring lower release of compounds. In order to decide for the optimum scenario for each substrate, the individual costs, on one hand and the energy produced, on the other, should be taken into account for the final process design.

#### 4. Conclusions

The experimental results obtained showed that LHW pretreatment was beneficial for hydrogen and methane production from SS, GL, and PS compared to the A-LHW pretreatment. Both pretreatment methods led to hemicellulose solubilization, but A-LHW resulted in the release of more potential inhibitors (furans and acids) than the LHW pretreatment. For all types of biomass used, the higher energy recovery was obtained when LHW pretreated biomass was separated and used for methane production through AD, and these values were 8.4, 12.5, and 7.5 MJ/kg  $TS_{initial}$  for SS, GL, and PS, respectively.

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