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Hydrothermal Pretreatment of Water-Extracted and Aqueous Ethanol-Extracted Quinoa Stalks for Enzymatic Saccharification of Cellulose

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Abstract: Auto-catalyzed hydrothermal pretreatment (A-HTP) and sulfuric-acid-catalyzed hydrothermal pretreatment (SA-HTP) were applied to quinoa stalks in order to reduce their recalcitrance towards enzymatic saccharification. Prior to pretreatment, quinoa stalks were extracted with either water or a 50:50 (*v*/*v*) ethanol–water mixture for removing saponins. Extraction with water or aqueous ethanol, respectively, led to removal of 52 and 75% (*w*/*w*) of the saponins contained in the raw material. Preliminary extraction of quinoa stalks allowed for a lower overall severity during pretreatment, and it led to an increase of glucan recovery in the pretreated solids (above 90%) compared with that of non-extracted quinoa stalks (73–74%). Furthermore, preliminary extraction resulted in enhanced hydrolysis of hemicelluloses and lower by-product formation during pretreatment. The enhancement of hemicelluloses hydrolysis by pre-extraction was more noticeable for SA-HTP than for A-HTP. As a result of the pretreatment, glucan susceptibility towards enzymatic hydrolysis was remarkably improved, and the overall conversion values were higher for the pre-extracted materials (up to 83%) than for the non-extracted ones (64–69%). Higher overall conversion was achieved for the aqueous ethanol-extracted quinoa stalks (72–83%) than for the water-extracted material (65–74%).

Keywords: hydrothermal pretreatment; quinoa stalks; enzymatic saccharification; saponins; extraction

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1. Introduction

Concerns associated with declining availability of fossil resources, and the effects of fossil fuel combustion on the climate have increased the interest for using renewable sources for production of energy carriers and chemicals. Biofuels and other biocommodities can provide alternatives to most of the current petrochemical products [1]. Nowadays, the production of biofuels and other bio-based products is based on the fermentation of sugars obtained mostly from corn and sugar cane [2], as well as from some other crops, such as wheat, sugar beet and cassava [3,4]. However, to meet the increasing demands and to avoid conflicts between food and energy production, it is necessary to investigate other biomass sources, such as lignocellulosic materials [5,6]. Deconstructing lignocellulose and processing it by the sugar platform route is a solid base for developing biorefineries to produce advanced biofuels, specialty chemicals and biomaterials [7,8].

Quinoa (*Chenopodium quinoa* Willd.) is a major crop in Bolivia, where the production reached around 70,000 tons in 2019 [9]. Quinoa harvest generates large amounts of stalks, which have no practical use and are usually discarded. Since quinoa stalks are rich in carbohydrates, and they are a cheap and abundant renewable feedstock, they can be considered as an attractive feedstock for the production of biofuels and other sugar platform-based bio-products in Bolivia and some other countries [10,11].

As for other lignocellulosic feedstocks, the bioconversion of quinoa stalks is challenged by the intimate interconnection between carbohydrates and lignin [12] and other intrinsic factors that hinder cellulose saccharification. In order to separate the lignin–carbohydrate complex and to reduce the recalcitrance of biomass to enzymatic hydrolysis, a pretreatment needs to be implemented. By different mechanisms, such as removal of lignin and/or hemicelluloses, increasing the porosity and surface area of the fiber fraction, or reducing cellulose crystallinity, pretreatment clears away the physical and chemical barriers that impede bioconversion of native biomass. That makes cellulose amenable to enzymatic hydrolysis, and facilitates fractionating the feedstock for processing it according to a biorefinery approach [13].

Among many pretreatment methods that have been proposed, hydrothermal processing is one of the most effective ones for increasing the reactivity of cellulose towards enzymes [13]. Hydrothermal pretreatment is a relatively clean technology that uses water as the only chemical, and it does not require externally added catalysts. Consequently, there are no major problems related to the recovery of chemicals or to equipment corrosion. Hydrothermal pretreatment, which is based on heating moist biomass for a certain time period at around 200 °C, is an auto-catalyzed process, where the catalytical power is provided by hydronium ions resulting from water auto-ionization. A further improvement of hydrothermal pretreatment can be achieved by adding an acid catalyst to the system [14]. Sulfuric acid is the most commonly used acid, but other acids, as well as sulfur dioxide, can also be used. Dilute sulfuric acid pretreatment is a widely used method for different kinds of lignocellulosic biomass [15,16], and it is the option of choice in many current commercial-scale efforts for the development of cellulosic ethanol production [17].

A particular challenge of the pretreatment of quinoa stalks is the presence of saponins, which can undergo hydrolytic reactions under acidic conditions, resulting in compounds that are inhibitory to certain carbohydrate-degrading enzymes [18]. Saponins are antinutritional factors contained in quinoa seeds [19], but they can also be found in other parts of quinoa plants [20], including the stalks [21]. Therefore, in order to avoid further inconveniences, if quinoa stalks are going to be pretreated using an acidic method, removal of saponins has to be implemented before pretreatment.

An acid-assisted hydrothermal processing approach has already been reported for pretreating quinoa stalks [10]. In that study, the biomass material was water-washed for removing saponins, impregnated with sulfur dioxide and subjected to steam pretreatment. However, water washing is not a very efficient method for removing saponins [19], and recent studies have shown that using ethanol is a better option [22]. Therefore, in the current study, ethanol was used as an alternative extracting solvent. There have been no previous reports on using ethanol for removing saponins from quinoa stalks prior to hydrothermal pretreatment. On the other hand, the use of sulfuric acid in a non-explosive hydrothermal pretreatment and the use of solvents other than water for saponin removal has not been reported for quinoa stalks. In the current work, quinoa stalks were extracted with either water or aqueous ethanol, and the resulting materials were pretreated using either auto-catalyzed (A-HTP) or sulfuric-acid-catalyzed hydrothermal pretreatment (SA-HTP) under similar temperature and time settings for both approaches. The effectiveness of the pretreatment is assessed by enzymatic saccharification of cellulose using two different loads of pretreated solids.

2. Materials and Methods

2.1. Raw Material

Around 10 kg of white royal quinoa (*C. quinoa* Willd.) stalks were collected from different plantations in Oruro (Huancani, −18.709615°, −66.874864°; Santiago de Quillacas, −19.261023°, −6.820049°; Huari, −19.137280°, −66.771995°; Sevarullo, −19.366673°, −66.866977°, and Orinoca, −18.974068°, −67.241646°) and Potosí (Salinas de Garci Mendoza, −19.889402°, −67.589835°), Bolivia. All the material was collected during the dry season (in May 2017), and transferred to IIDEPROQ laboratories in La Paz, where it was

dried at room temperature for 48 h, hammer-milled, sieved to a particle size of 1.7 mm and stored at 4 °C.

2.2. Saponin Removal

Saponins were removed by extraction with either water or aqueous ethanol according to previously developed procedures [10,23]. For the water extraction, two batches of approximately 330 g of quinoa stalks were suspended each in six liters of water at 44 °C in a 20-L plastic cylindrical container, and the suspension was mixed by stirring at 300 rpm for one hour. After that, the liquid phase was separated by vacuum filtration, and the extraction procedure was repeated. For the aqueous ethanol extraction, two batches of 107 g of quinoa stalks were mixed with one liter of a 50:50 (v/v) ethanol–water mixture, in a 2000-mL one-neck round bottom flask, equipped with a reflux condenser, and placed in a water bath on a hot plate. The mixture was boiled for 90 min under reflux. After that, the suspension was vacuum filtered, and the solids were subjected to a new extraction cycle. The water extraction was repeated three times, and the ethanol was repeated twice. The solid material resulting from both extractions was washed thoroughly with warm distilled water (40 °C), and then air dried for five days at room temperature. A sample of dry solids, with dry matter content above 90% (w/w), was saved for compositional analysis, and the rest was used in hydrothermal pretreatment experiments.

2.3. Hydrothermal Pretreatment

Pre-extracted quinoa stalk biomass was pretreated by either auto-catalyzed (A-HTP) or sulfuric acid-catalyzed (SA-HTP) hydrothermal pretreatment. For A-HTP, 37.5 g (dry matter, DM) of biomass was suspended in water, giving a 300-g suspension with a 12.5% (w/w) solids load. For SA-HTP, the biomass was suspended in a dilute sulfuric acid solution, whose concentration was calculated for having a load of 0.2 g H_2SO_4 per 100 g suspension. Fresh quinoa stalks were also pretreated by both A-HTP and SA-HTP, following the same experimental procedure as that applied to pre-extracted stalks. A 1-L pressurized stainless steel reactor equipped with magnetically driven impellers, electrical heating and PID temperature control (Parr Instrument Co., Moline, IL, USA) was used. The suspension was loaded into the reactor, heated to either 200 or 215 °C and held at the work temperature for either 1.8 or 5 min (Table 1). The pretreatment time and temperature were chosen based on previous results [10], but they were combined so that the severity factor (log R_0) of the isothermal period was always the same. The severity factor was calculated following the method developed by Overend and Chornet [24] (Equations (1) and (2)); the combined severity (CS) was also calculated (Equation (3)):

$$Severity\ Factor = Log\ R_0 \tag{1}$$

$$R_0 = t \times exp\left(\frac{Tr - 100}{14.75}\right) \tag{2}$$

$$CS = Log\ R_0 - pH \tag{3}$$

where t is the pretreatment time in minutes, Tr is the pretreatment temperature in °C and pH is the final pH of the pretreatment slurry.

Upon elapsing of the pretreatment time, the heating was stopped, and the reactor was cooled down by circulation of a controlled flow of cold water using a solenoid valve module (Parr Instrument Co.). The pretreated slurry was separated by vacuum filtration, and both fractions were processed separately. The pretreated solids were washed with abundant water, air dried until reaching at least 90% (w/w) DM content, and then used for determining the gravimetric yield and for compositional analysis. An aliquot was separated before drying and stored frozen for analytical enzymatic saccharification. After determining the volume and pH, the pretreatment liquid was stored frozen until analysis and further use.

Table 1. Operational conditions used for the hydrothermal pretreatment of quinoa stalk biomass.

Codification [1]	Solvent	Temperature, °C	Time, min	H_2SO_4 Load, % (*w*/*w*)	Log $R_{0(IT)}$ [2]
N/A-HTP 215	None	215	1.8	-	3.6
N/SA-HTP 215	None	215	1.8	0.2	3.6
N/SA-HTP 200	None	200	5.0	0.2	3.6
W/A-HTP 215	H_2O	215	1.8	-	3.6
W/SA-HTP 215	H_2O	215	1.8	0.2	3.6
W/SA-HTP 200	H_2O	200	5.0	0.2	3.6
E/A-HTP 215	Aq. ethanol	215	1.8	-	3.6
E/SA-HTP 215	Aq. ethanol	215	1.8	0.2	3.6
E/SA-HTP 200	Aq. ethanol	200	5.0	0.2	3.6

[1] The codification includes the extraction solvent (N, none; W, water; E, aqueous ethanol), the pretreatment approach (A-HTP and SA-HTP) and the temperature (200 and 215). [2] Severity factor calculated using the temperature and time of the isothermal period.

2.4. Determination of the Composition of the Raw Material and Processed Solids

The compositional analysis of raw, extracted and pretreated quinoa stalks was performed by analytical acid hydrolysis [25]. The content of polysaccharides was calculated from the concentrations of monosaccharides in the hydrolysate, and the monosaccharides were analyzed by high-performance anion-exchange chromatography (HPAEC). Acid-insoluble (Klason) lignin was determined gravimetrically, and acid-soluble lignin was determined spectrophotometrically at 240 nm with a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The saponin quantification was performed by the afrosimetric method [26] and backed with a spectrophotometric procedure adapted from a previously reported protocol [27]. All the analyses were performed in triplicates.

2.5. Enzymatic Hydrolysis

The cellulose susceptibility to hydrolytic enzymes was evaluated by analytical enzymatic saccharification [2]. Pretreated solids were suspended in 50 mM sodium citrate buffer (pH 5.2) in 15-mL Falcon tubes, and the cellulase blend Cellic CTec2, procured from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), was added at a load of 100 CMCase units per gram (DM) of biomass. The overall weight of each suspension was 5 g, and the solids load was either 5 or 10% (*w*/*w*) of dry matter. The tubes with the reaction mixtures were placed in an Ecotron orbital incubator (INFORS HT, Bottmingen, Switzerland), and the hydrolysis was run at 45 °C and 180 rpm for 72 h. After that, the tubes were centrifuged, and glucose was determined in the supernatants. Glucose concentrations were used for calculating the enzymatic digestibility of pretreated cellulose (ED, Equation (4)) and the overall conversion of initial cellulose (OC, Equation (5)):

$$ED\ (\%) = \frac{Hydrolyzed\ cellulose}{Cellulose\ in\ pretreated\ solids} \times 100 \quad (4)$$

$$OC\ (\%) = \frac{ED \times Cellulose\ recovery\ in\ pretreated\ solids}{100} \quad (5)$$

2.6. Analysis of Liquid Samples

The concentration of monosaccharides in the pretreatment liquids and in the analytical acid hydrolysates was determined using HPAEC with pulsed amperometric detection (PAD). A Dionex ICS-5000 system (Sunnyvale, CA, USA) equipped with a 3 × 30 mm^2 guard column and a 3 × 150 mm^2 separation column (CarboPac PA20, Dionex) was used. The samples were diluted with ultra-pure water and filtered through a 0.2 µm nylon membrane (Millipore, Billerica, MA, USA) before injection. Elution was performed with a 2 mM solution of sodium hydroxide for 25 min, followed by regeneration with a mixture consisting of 200 mM sodium hydroxide and 68 mM sodium acetate for 5 min, addition of

a solution of 200 mM sodium hydroxide for 5 min and equilibration with a 2 mM solution of sodium hydroxide for 25 min. The flow rate was always 0.4 mL/min.

High-performance liquid chromatography (HPLC) was used for determining the concentrations of glucose in the analytical enzymatic hydrolysates, and the furan aldehydes furfural and 5-hydroxymethylfurfural (HMF) in the pretreatment liquids. A Dionex UltiMate 300 system (Thermo Fisher Scientific, Germering, Germany), fitted with an autosampler, a binary pump and a degasser, was used. For glucose, an RI (refractive index) detector was used, and the separation was performed on an Aminex HPX-87H column (Bio-Rad Laboratories AB, Solna, Sweden) operating at 60 °C. The mobile phase was a 5 mM aqueous solution of sulfuric acid supplied at a flow rate of 0.6 mL/min. For furfural and HMF, diode-array detection at 282 nm was used, and their separation was performed on a 3 × 50 mm^2, 1.8 µm Zorbax RRHT SB-C18 column (Agilent Technologies, Santa Clara, CA, USA) held at 40 °C. As mobile phase, aqueous 0.1% (*v*/*v*) formic acid (A) and acetonitrile with 0.1% (*v*/*v*) formic acid (B) were supplied at a flow rate of 0.5 mL/min using a previously described gradient profile [28].

Acetic acid, formic acid and levulinic acid in the pretreatment liquids were determined by HPAEC-PAD (Dionex ICS-5000) using a 4 × 50 mm^2 AG15 guard column and a 4 × 250 mm^2 AS15 separation column (Dionex) and a conductivity detector set at 35 °C. A 10 mM aqueous solution of sodium hydroxide at a flow rate of 1.2 mL/min was used as eluent, as previously indicated [28].

3. Results

3.1. Saponin Removal

Extensive washing of quinoa stalks with either water or aqueous ethanol was performed before pretreatment in order to remove a large part of saponins and other extractives, and thus, avoiding undesirable reactions during pretreatment conditions. Water washing was used because it is the traditional method for removing saponins from quinoa seeds for improving their palatability [19]. However, since it has been shown that aqueous extraction is not fully efficient for removing saponins [19], it was decided to use ethanol as an alternative extracting solvent. Water extraction was performed according to a previously described procedure [10], while the conditions for aqueous ethanol extraction were based on a recently developed protocol (Carrasco, unpublished). The saponin content of raw quinoa stalks was 5.2% (*w*/*w*) (Table 2), which is higher than the value that has been reported previously for quinoa stem [20]. As a result of extraction, the saponin content in the resulting solids was reduced to 1.3% and 2.5% in the materials extracted with aqueous ethanol and water, respectively. That corresponds to a removal of 52% of the initial saponins in the water extraction and 75% in the extraction with aqueous ethanol. The highest saponin removal with the aqueous ethanol extraction is in agreement with published results comparing ultrasound-assisted extraction of saponins from quinoa and other edible seeds using either ethanol, ethanol:water or water [22].

In addition to saponins, other extractives were removed as indicated by the yields of solids resulting from the extraction (Table 2). Comparing the yields of solids for both extraction procedures, it is evident that while aqueous ethanol was effective for the removal of saponins, water removed a larger fraction of other extractives. As a result of the removal of extractives, the extracted solids were enriched compared to the raw material with regard to main constituents. The glucan content in the raw quinoa stalks was 34.6%, which is within the range of previously reported results [10,29], but it increased to 36.8% and 39.0% after extraction with, respectively, aqueous ethanol and water. Water extraction resulted also in increase of the content of xylan, mannan and lignin in the solids, while for the ethanol-extracted material, that increase was less remarkable. No clear trend was observed with respect to the contents of other hemicellulosic constituents (galactan and arabinan).

Table 2. Yield of solids after extraction, and composition of raw and extracted quinoa stalks, % (*w*/*w*).

Extracting Solvent	Yield of Solids	Saponins	Glucan	Xylan	Arabinan	Galactan	Mannan	Lignin
None	-	5.2 (0.2)	34.6 (1.3)	22.7 (<0.1)	2.2 (0.1)	2.0 (<0.1)	1.5 (<0.1)	19.5 (0.1)
Water	86.2	2.5 (<0.1)	39.0 (0.5)	25.7 (5.0)	1.9 (0.3)	2.0 (0.3)	1.8 (0.2)	22.0 (0.7)
Ethanol	92.3	1.3 (0.1)	36.8 (0.6)	23.2 (0.5)	2.1 (0.2)	2.0 (<0.1)	1.7 (0.1)	20.6 (0.8)

Mean values from at least two replicates. The standard deviations are shown in parentheses.

3.2. Pretreatment of Pre-Extracted Quinoa Stalks

The solids resulting from the extraction procedure, as well as the raw quinoa stalks, were subjected to hydrothermal pretreatment, either auto-catalyzed (A-HTP) or catalyzed with sulfuric acid (SA-HTP). For each material, two SA-HTP runs and one A-HTP run were performed (Table 1). The pretreatment conditions were based on a previous study, where quinoa stalks were pretreated by SO_2-assisted steam explosion under different conditions in the severity range between log R_0 = 3.35 and log R_0 = 3.94 [10]. Here, a severity factor in the middle of that range (log R_0 = 3.6) was applied, and the time and temperature were selected in such a way that the severity factor calculated for the isothermal period was the same for all the pretreatments.

The severity factor calculations reported in the literature are often restricted to the isothermal period of the pretreatment, and therefore, they do not cover the time required for reaching the work temperature and the cooling period at the end of the process. Since in our experiments the heating (25–35 min) and cooling (17–25 min) times were rather long (Figure 1), it was reasonable to consider them in the severity calculation. By integration of the temperature and time values registered by accurate measurements at two-minute intervals along the whole process, an overall severity factor (log $R_{0(O)}$) was calculated. The overall severity factor (3.7–4.1, Table 3) was always higher than the severity factor calculated for the isothermal period (log $R_{0(IT)}$ = 3.6, Table 1). The divergence between log $R_{0(O)}$ and log $R_{0(IT)}$ was more remarkable for the pretreatments at 215 °C than for those at 200 °C, which can be explained by the longer time required for reaching the isothermal period when working at higher temperature. It was also observed that at 215 °C, the log $R_{0(O)}$ values fluctuated between 3.9 and 4.1, while at 200 °C, it was always 3.7. In the pretreatments with sulfuric acid addition, the log $R_{0(O)}$ was always higher for the experiments performed at a higher temperature and shorter time than for those performed at a lower temperature and longer time. The same trend was observed for the combined severity (CS), which was calculated as recommended by Chum et al. [30] for pretreatments involving acids.

The experiments run at 215 °C revealed some differences between the log $R_{0(O)}$ values of the two hydrothermal pretreatment approaches, and those differences were affected by preliminary extraction. For the non-extracted quinoa stalk, the overall severity factor was considerably higher for the auto-catalyzed pretreatment (N/A-HTP 215, log $R_{0(O)}$ = 4.1) than for the sulfuric acid-catalyzed one (N/SA-HTP 215, log $R_{0(O)}$ = 4.0), while for the water-extracted solids, the values were comparable, and for the ethanol-extracted material, log $R_{0(O)}$ was slightly lower for A-HTP than for SA-HTP (Table 3). All the same, when the pretreatment was run at a lower temperature, no major effect of the extraction step on the log $R_{0(O)}$ was observed.

When comparing the effect of the preliminary extraction on the severity of each pretreatment approach, clear differences in the log $R_{0(O)}$ and CS are revealed. For the auto-catalyzed pretreatments, log $R_{0(O)}$ was higher for the non-extracted material (4.1) than for those extracted with any of the solvents (3.9–4.0; Table 3). On the other hand, for the SA-HTP experiments, the preliminary extraction led to lower pH of pretreated slurries, which resulted in higher CS compared with that of the non-extracted stalks.

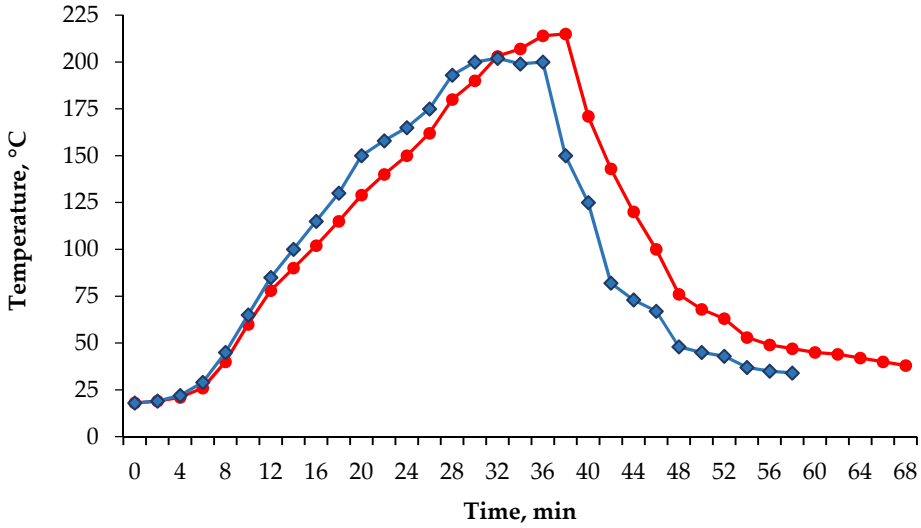


Figure 1. Example of temperature profiles for pretreatments performed at 200 °C (blue rhombs) and 215 °C (red circles). The plotted data correspond to SA-HTP of water-extracted stalks.

Table 3. Immediate [1] results of the pretreatments.

Pretreatment Codification [2]	Log $R_{0(O)}$ [3]	pH	CS [4]	Yield of Pretreated Solids, % (*w*/*w*)	Volume of the Pretreatment Liquid, mL
N/A-HTP 215	4.1	3.5	0.6	59.6	98
N/SA-HTP 215	4.0	2.7	1.3	57.0	196
N/SA-HTP 200	3.7	2.6	1.1	56.8	187
W/A-HTP 215	4.0	3.5	0.5	60.0	123
W/SA-HTP 215	4.0	2.2	1.8	58.4	162
W/SA-HTP 200	3.7	2.2	1.5	59.9	140
E/A-HTP 215	3.9	3.0	0.9	61.7	75
E/SA-HTP 215	3.9	2.2	1.7	59.5	151
E/SA-HTP 200	3.7	2.2	1.5	60.9	129

[1] These parameters were determined right after the pretreatment run. [2] The pretreatment codification given in the first column is the same as in Table 1. [3] The severity calculation covers the whole process. [4] Combined severity.

3.3. Yield and Composition of the Pretreated Solids

For all the experimental conditions, the amount of solids recovered after pretreatment was considerably lower than the initial biomass load, which is a consequence of the partial solubilization of lignocellulosic constituents. The gravimetric yield of pretreated solids was higher for the preliminary extracted material than for non-extracted quinoa stalks, and it was larger for the auto-catalyzed pretreatment than for the sulfuric acid-catalyzed one (Table 3). For the SA-HTP of the pre-extracted material, the yield of solids was higher for experiments at 200 °C than for those at 215 °C, while in SA-HTP of raw quinoa stalks, comparable yields were achieved for both pretreatment temperatures. The recovered volumes of pretreatment liquid were higher for SA-HTP, especially for the pretreatments at 215 °C, than for A-HTP. With the exception of the experiment W/A-HTP 215, the liquor volumes were lower for the pretreatment of the pre-extracted materials than for those of raw stalks.

Compared with the composition of raw quinoa stalks and pre-extracted materials (Table 2), a remarkable decrease of the hemicellulosic constituents was observed for the pretreated solids (Table 4). That is explained by the hydrolysis of hemicelluloses that typically happens at different degrees during hydrothermal processing and other acidic pretreatments [13]. A high degree of hydrolysis of hemicelluloses was achieved in a previous study on SO_2-catalyzed steam pretreatment of water-washed quinoa stalks, where up to 80% of the initial xylan was hydrolyzed [10]. Hydrolysis and solubilization of

hemicelluloses are also major reasons for the decrease in the gravimetric yield of solids after pretreatment, and for the increase in the mass fractions of glucan and lignin in the pretreated solids. The highest values for glucan content were achieved for the water-extracted material, while the highest lignin contents were observed for the non-extracted stalks. No clear effect of the preliminary extraction on the hemicellulose components was found. The recovery of glucan after the pretreatment of the extracted materials was above 90% of the initial amount, while for the non-extracted quinoa stalk, only around 73–74% was recovered (Figure 2a). It is evident that the preliminary extraction contributed to a better preservation of cellulose during pretreatment.

Table 4. Main components of the pretreated solids, g per 100 g DM.

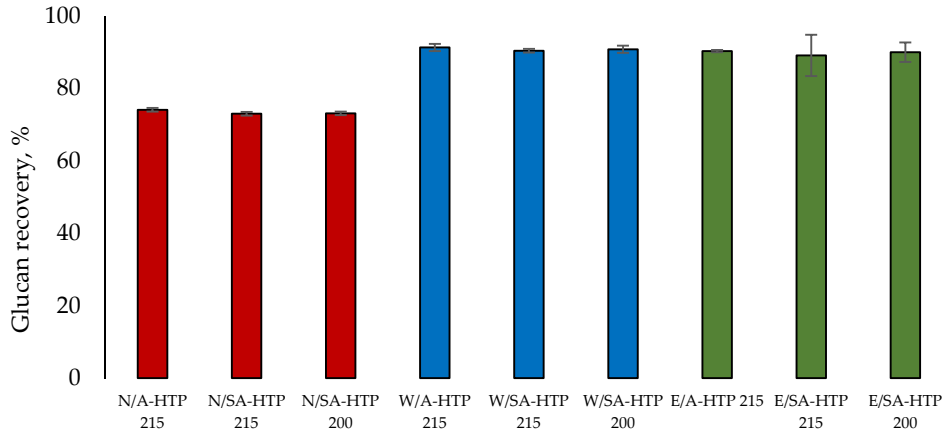
Pretreatment Codification [1]	Glucan	Xylan	Arabinan	Galactan	Mannan	Lignin [2]
N/A-HTP 215	49.5 (0.3)	2.9 (0.6)	0.7 (<0.1)	0.8 (0.1)	1.3 (0.1)	44.0 (1.8)
N/SA-HTP 215	52.1 (0.4)	2.1 (0.2)	ND	0.6 (<0.1)	0.9 (0.1)	44.7 (0.3)
N/SA-HTP 200	52.3 (0.2)	2.7 (0.5)	ND	0.6 (<0.1))	1.0 (0.3)	43.0 (<0.1)
W/A-HTP 215	56.0 (1.2)	3.1 (0.2)	ND	0.7 (0.1)	1.2 (0.1)	40.1 (0.9)
W/SA-HTP 215	57.0 (0.9)	3.2 (0.3)	ND	0.6 (<0.1)	1.1 (0.1)	41.1 (1.9)
W/SA-HTP 200	56.2 (1.0)	2.2 (0.1)	ND	ND	0.8 (<0.1)	41.1 (2.6)
E/A-HTP 215	48.3 (0.3)	3.2 (0.1)	0.7 (<0.1)	0.8 (<0.1)	1.6 (<0.1)	43.6 (0.9)
E/SA-HTP 215	51.8 (5.7)	2.6 (0.1)	ND	0.7 (<0.1)	1.2 (<0.1)	42.6 (<0.1)
E/SA-HTP 200	50.3 (2.7)	2.4 (<0.1)	ND	0.7 (<0.1)	1.0 (<0.1)	44.0 (1.8)

[1] The pretreatment codification given in the first column is the same as in Table 1. [2] Sum of Klason lignin and acid-soluble lignin. ND, not detected.

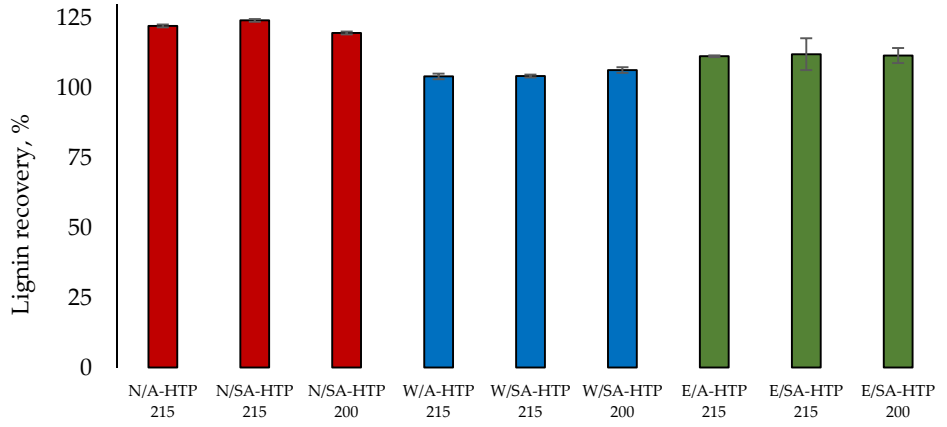
For all the materials, the glucan content in the pretreated solids was higher for SA-HTP solids than for A-HTP solids, while for the hemicelluloses, it was the other way around (Table 4). For SA-HTP, the glucan content was comparable for pretreatments at 200 and 215 °C. The xylan content was also comparable in the solids of SA-HTP at both temperatures investigated.

The lignin content was higher in the solids resulting from pretreatment of non-extracted quinoa stalks than in those from pretreatment of preliminary extracted stalks (Table 4). No evident trend was found for the lignin content depending on the hydrothermal pretreatment approach or the temperature. Material balances revealed that the amount of lignin recovered in the pretreated solids was higher than the input amount (Figure 2b). The lignin recovery after pretreatment of non-extracted stalks was around 120%, while it was 104–112% in the solids resulting from pretreatment of pre-extracted materials. The apparent lignin increase can be explained by formation of pseudo-lignin, which is a by-product resulting from the partial thermal degradation of carbohydrates [31]. Evidently, pseudo-lignin formation was higher in the pretreatment of fresh quinoa stalks than in that of pre-extracted stalks. Pseudo-lignin formation in pretreatment of quinoa stalks has not been reported previously.

Saponin content in the pretreated solids of non-extracted quinoa stalks was around two to three times higher than that of the pretreatment of pre-extracted materials (data not shown). No evident saponin removal occurred during the pretreatment.



(**a**)



(**b**)

Figure 2. Recovery of glucan (**a**) and lignin (**b**) in the pretreated solids resulting from A-HTP and SA-HTP of non-extracted (red bars), water-extracted (blue bars) and aqueous ethanol-extracted (green bars) quinoa stalks. The codification used in the horizontal axis is the same as in Table 1.

3.4. Composition of the Pretreatment Liquids

Sugar, furan aldehydes and aliphatic acids were detected in the pretreatment liquids. Based on the concentrations found, the amounts were expressed as yields from the initial material, and they are presented in Tables 5 and 6.

Table 5. Yields of sugars in the pretreatment liquids, g/100 g DM.

Pretreatment Codification [1]	Glucose	Xylose	Arabinose	Galactose	Mannose
N/A-HTP 215	0.2	1.1	0.1	0.1	0.1
N/SA-HTP 215	0.8	3.0	0.3	0.5	0.2
N/SA-HTP 200	1.0	4.5	0.5	0.4	0.6
W/A-HTP 215	0.1	1.4	0.1	0.1	0.2
W/SA-HTP 215	0.7	5.9	0.4	0.4	0.8
W/SA-HTP 200	0.5	8.2	0.6	0.7	1.0
E/A-HTP 215	0.1	1.2	0.1	0.1	0.2
E/SA-HTP 215	0.6	4.8	0.4	0.4	0.7
E/SA-HTP 200	0.5	8.4	0.6	0.6	0.9

[1] The pretreatment codification given in the first column is the same as in Table 1.

Xylose, resulting from xylan hydrolysis, was the main sugar found in the pretreatment liquids. Up to 8.4 g of xylose per 100 g initial material was detected in the pretreatment liquids from pre-extracted quinoa stalks, whereas the maximum amount detected for those from non-extracted materials was clearly lower (Table 5). The same trend was also observed for other hemicellulosic sugars. That suggests that the preliminary extraction of quinoa stalks enhanced the hydrolysis of hemicelluloses during pretreatment. That was especially remarkable for the SA-HTP, where the sum of hemicellulosic sugars found for each condition of the pretreatment of pre-extracted material was approximately twice as high as in the corresponding pretreatment of non-extracted stalks. For all the three materials, the release of all hemicellulosic sugars in the SA-HTP liquid was higher than in the A-HTP liquid, and it was higher for the experiments at 200 °C than for those at 215 °C. A similar response has been reported before for hydrothermal pretreatment of sugarcane bagasse [32] and wheat straw [33] under different acidity and temperature. It should be mentioned that even if the extraction enhanced the hydrolysis of hemicelluloses, the sugar yields are below those reported previously for SO_2-catalyzed steam pretreatment of water-washed quinoa stalks under similar severity, where the xylose yield was above 14 g/100 g [10]. The lower yield in this study might be attributed to sugar degradation phenomena, as can be interpreted from the rather high formation of sugar-degradation products, such as furan aldehydes and aliphatic acids (Table 6), as well as pseudo-lignin.

Table 6. Yields of furan aldehydes and aliphatic acids in the pretreatment liquids, g/100 g DM.

Pretreatment Codification [1]	Furfural	HMF	Formic Acid	Levulinic Acid	Acetic Acid
N/A-HTP 215	0.9	0.2	0.9	0.5	1.6
N/SA-HTP 215	3.3	0.8	2.5	2.2	3.9
N/SA-HTP 200	1.6	0.4	2.4	1.3	3.7
W/A-HTP 215	1.0	0.1	0.5	0.4	1.8
W/SA-HTP 215	2.6	0.3	0.6	0.5	2.4
W/SA-HTP 200	1.9	0.2	0.6	0.5	2.2
E/A-HTP 215	0.6	0.1	0.2	0.1	0.6
E/SA-HTP 215	3.1	0.4	0.4	0.2	1.2
E/SA-HTP 200	1.6	0.2	0.4	0.2	1.2

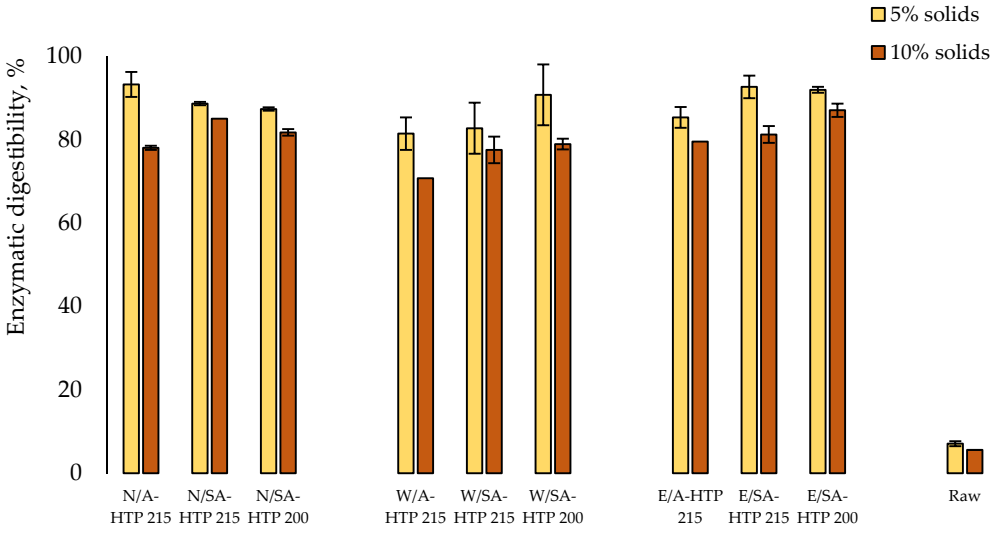
[1] The pretreatment codification given in the first column is the same as in Table 1.

The formation pattern of the furan aldehydes furfural and HMF reveals more remarkable sugar degradation for SA-HTP, especially for the high temperature runs, than for A-HTP (Table 6), which is in agreement with the literature [13], but it does not show remarkable differences between the pre-extracted and non-extracted materials. However, differences in sugar degradation for the different materials are clearly reflected by the released amounts of formic and levulinic acids. Formation of formic and levulinic acids was considerably higher in the pretreatment liquids of non-extracted quinoa stalks than in those of pre-extracted materials, and it was lower for the aqueous ethanol-extracted material than for the water-extracted ones. The higher formation HMF formation point towards a major degradation of hexoses in the pretreatment of non-extracted quinoa stalks. Together with the slightly higher glucose yield in the pretreatment liquids (Table 5), this is in good agreement with the lower cellulose recovery observed in the pretreated solids (Figure 2a).

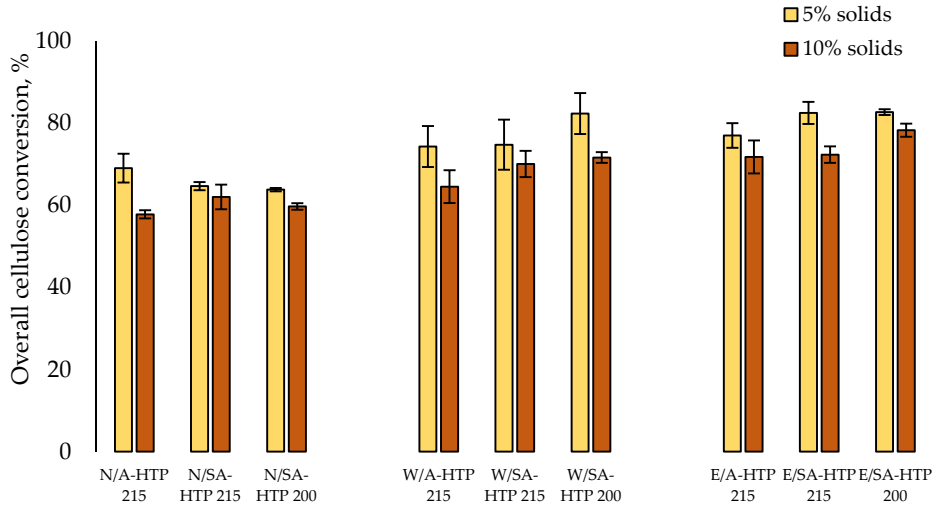
Table 6 also includes acetic acid, which is derived from the hydrolysis of acetyl groups in hemicelluloses. The formation of acetic acid, during the pretreatment of pre-extracted stalks (0.6–2.4 g/100 g), was comparable with previously reported value for SO_2-catalyzed steam pretreatment of water-washed quinoa stalks under comparable severity (1.98 g/100 g) [10]. For pretreatment of non-extracted quinoa stalks, acetic acid formation was higher than that, and reached 3.9 g/100 g in the experimental run N/SA-HTP 215.

3.5. Enzymatic Hydrolysis of Cellulose

Analytical enzymatic saccharification of raw and pretreated quinoa stalks was performed, and the enzymatic digestibility of pretreated glucan and overall conversion of initial glucan were calculated. The enzymatic digestibility increased from 6–7% in raw stalks to 71–93% in the pretreated material (Figure 3a), indicating that hydrothermal pretreatment, both auto-catalyzed and sulfuric-acid catalyzed, was effective for achieving a considerable enhancement of the enzymatic saccharification of glucan contained in quinoa stalks.



(**a**)



(b)

Figure 3. Enzymatic digestibility of the pretreated solids (**a**) and overall conversion of glucan after enzymatic hydrolysis (**b**). The codification used in the horizontal axis is the same as in Table 1.

The positive effect of pretreatment on the enzymatic digestibility of the fibers was noticeable in saccharification assays with both 5% and 10% solids. The digestibility ranged between 81% and 93% in the experiments with 5% solids and between 71% and 87% in those with 10% solids, covering the results of all the pretreated materials (Figure 3a). The relatively poorer performance observed when running the saccharification assays at 10% solids loading compared to 5% solids loading can be associated with the so-called

"high-solids effect," resulting in low cellulose conversions when enzymatic hydrolysis is performed with high solids loadings. The high-solids effect has generally been attributed to mass-transfer limitations [34], and it is both enzyme- and substrate-dependent [35] with a possible connection to enzyme saturation at higher substrate concentration [36] or to the increase of competitive adsorption of cellulases on lignin due to the higher load [37].

The enzymatic digestibility was comparable for solids resulting from pre-extracted stalks and for the material that had not been subjected to preliminary extraction (Figure 3a). That suggests that the saponins remaining in the pretreated solids were not an impediment to the enzymatic saccharification. This is not a surprising outcome, since previous results have shown that the addition of quinoa saponins enhances the enzymatic saccharification of cellulose/lignin mixtures and pretreated lignocellulose, which was attributed to the reduction of non-productive binding of cellulases to lignin [38].

Although the enzymatic digestibility of the pretreated fibers was not significantly affected by the preliminary extraction of quinoa stalks, the overall conversion, which takes into account the glucan fraction that was lost during pretreatment, was evidently influenced by the extraction itself and by the specific solvent (Figure 3b). Better overall conversion values were achieved for the preliminary extracted stalks than for the non-extracted materials. For instance, in the hydrolysis assays with 5% solids, the overall conversion ranged between 74% and 83% for the pre-extracted materials, while it was only 64–69% for the non-extracted ones (Figure 3b). These overall conversion results are well correlated with the previously observed trend of glucan recovery in the pretreated solids (Figure 2a).

The overall conversion values achieved for the material resulting from pretreatment of aqueous ethanol-extracted quinoa stalks (72–83%) were higher than those observed for the water-extracted pretreated material (65–74%) (Figure 3b). This provides an indication of the importance of the solvent system for the final result of the enzymatic saccharification, with aqueous ethanol extraction being more effective than water extraction.

The choice of the hydrothermal pretreatment approach, either SA-HTP or A-HTP, was also important for the enzymatic saccharification independently of the solvent used in the extraction. For the pretreated solids resulting from materials that had been preliminary extracted, better digestibility was achieved for SA-HTP (78–93%) than for A-HTP (71–85%) (Figure 3a), whereas for the non-extracted material, no clear differences were observed between the two hydrothermal pretreatment approaches. The overall conversion of glucan contained in the pre-extracted materials was also better for SA-HTP than for A-HTP, and pretreatments at 200 °C resulted in higher values than pretreatments at 215 °C. These results reveal the importance of the hydrothermal pretreatment approach and temperature to be applied to pre-extracted quinoa stalks in order to effectively enhance the enzymatic digestibility of glucan.

Although there are not many papers in the literature that can be used for assessing the value of the presented results, a worthy benchmark is the previously mentioned work on sulfur dioxide-catalyzed steam pretreatment of water-washed stalks [10]. The overall glucose yield obtained in the most favorable conditions in that study (up to 70%) is within the same range as the overall conversion values obtained in the current work for A-HTP and SA-HTP of non-extracted quinoa stalks (62–69%, Figure 3b). However, our results show that if the stalks are preliminarily extracted with water or ethanol, A-HTP and SA-HTP can result in higher conversion than those values obtained in the SO_2-catalyzed steam pretreatment [10]. In any case, these results should be interpreted with moderation, considering that different sorts of quinoa stalks were used in both studies, and that different cellulase preparations and slightly different protocols were applied for enzymatic hydrolysis.

4. Conclusions

The current study is an attempt to contribute with fundamental knowledge about factors that affect the efficiency of pretreatment for biorefining of quinoa stalks. An efficient

pretreatment is a prerequisite for bioconversion of quinoa stalks through sugar platform processes to fuels, novel materials and high-valuable chemicals. Our results show that having an extraction step with either water or aqueous ethanol as a first process step allows running the hydrothermal pretreatment at lower overall severity, which leads to higher cellulose recovery in the pretreated solids, higher yields of sugars in the pretreatment liquids with lower release of bioconversion inhibitors, such as aliphatic acids, and lower pseudo-lignin formation. Furthermore, extraction of saponins prior to pretreatment opens the possibility for their recovery for further processing into high-value products according to the biorefinery concept.

Hydrothermal pretreatment, either auto-catalyzed or externally catalyzed with sulfuric acid, considerably enhances the reactivity towards enzymatic hydrolysis of glucan in quinoa stalks.

Preliminary extraction of quinoa stalks is favorable for improving the overall hydrolytic conversion of cellulose, and the choice of solvent is an important issue, with aqueous ethanol being more effective than water.

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