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Recalcitrance Assessment of the Agro-industrial Residues from Five *Agave* Species: Ionic Liquid Pretreatment, Saccharification and Structural Characterization

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Abstract

Agave has recently shown its potential as a bioenergy feedstock with promising features such as higher biomass productivity than leading bioenergy feedstock while at the same time being drought-resistant with low water requirements and high sugar to ethanol conversion using ionic liquid (IL) pretreatment. IL pretreatment was studied to develop the first direct side-by-side comparative recalcitrance assessment of the agro-industrial residues from five *Agave* species [*Agave americana* (AME), *A. angustifolia* (ANG), *A. fourcroydes* (FOU), *A. salmiana* (SAL), and *A. tequilana* (TEQ)] using compositional analysis, X-ray diffraction, and the lignin syringyl/guaiacyl subunit ratio (S/G) by pyrolysis molecular beam mass spectrometry (PyMBMS). Prominent calcium oxalate peaks were found only in unpretreated AME, SAL, and TEQ. The S/G ratios of all five unpretreated *Agave* species were between 1.27 and 1.57 while the IL-pretreated samples were from 1.39 to 1.72. The highest overall sugar production was obtained with IL-pretreated FOU with 492 mg glucose/g biomass and 157 mg xylose/g biomass at 120 °C and 3 h using 1-ethyl-3-methylimidazolium acetate ($[C_2C_1Im][OAc]$). An estimated theoretical ethanol yield from the studied agro-industrial residues from the five *Agave* species was in the range of 1060 to 5800 L ethanol/ha/year. These comparison results demonstrate the potential of the *Agave* spp. as a suitable biofuel feedstock which can be employed within a biorefinery scheme.

Keywords Agave · Biofuels · Characterization · Crystallinity · S/G · Saccharification

Introduction

Renewable feedstocks for the production of biofuels and value-added products have become an important resource to reduce the dependency on fossil fuel [1]. In this scenario, lignocellulosic biomass is a promising alternative to fossil resources because of its abundance, renewability, and versatility [2]. *Agave* has recently shown its potential as a bioenergy feedstock with positive features including low water requirements, high productivity in semiarid lands, adaptability to high temperatures, and drought resistance [3]. Nevertheless, a pretreatment step is required to overcome the biomass recalcitrance making polysaccharides easily available for enzymatic saccharification and fermentation. In the past few decades, different pretreatment strategies have been developed using physical, chemical, biological, or a combination (physicochemical) with unique systems applied (chemical loading, temperature, time, among other characteristics) [4].

In 2017, 1.68 million tons of different *Agave* species was produced in Mexico including species of agro-industrial interest (*Agave americana*, *A. angustifolia*, *A. fourcryodes*, *A. salmiana*, and *A. tequilana*) from diverse sectors (fabrics, spirits, and syrup) where *A. tequilana* leads its utilization with 0.96 million tons in the same year [5, 6]. For example, the *A.*

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tequilana, which is employed for spirit production that costs $6–35 USD/L or higher depending on the spirit category [7], has caused a large demand for the whole plant with prices ranging from ~ $100 to $970 USD/ton [8] while its bagasse value has been estimated at $39 USD/ton [9]. In the recent years, a number of pretreatment studies (dilute acid, alkali, AFEX, ionic liquid, among others) have been conducted on the residues (bagasse and/or leaves) from the *Agave* industries mainly with *A. tequilana* species with fewer reports in other species (e.g., *A. americana* and *A. salmiana*) [3]. However, it is very difficult to make any meaningful comparison between the studies as they applied different pretreatment conditions, biomass sources, enzyme activities, and saccharification/ fermentation strategies.

In the past years, ionic liquid (IL) pretreatment using 1-ethyl-3-methylimidazolium acetate ($[C_2C_1Im][OAc]$), 1-butyl-3-methylimidazolium acetate ($[C_4C_1Im][OAc]$), and 1-butyl-3-methylimidazolium chloride ($[C_4C_1Im][Cl]$) has been successfully applied in *A. tequilana* bagasse [10–12]. Ionic liquid pretreatment has shown to be a promising technology due to its ability to solubilize biomass by overcoming the hydrogen bonding within cellulose (cellulose I to II), while partially removing hemicellulose and lignin from the plant cell wall [13]. This pretreatment does not generate inhibitory products and leads to a more amenable saccharification stage with mild process conditions within 100–160 °C and processing times from 1 to 3 h at atmospheric pressure [14, 15]. Furthermore, ILs can be highly recyclable with demonstrated recovery yields > 99.9 wt% $[C_2C_1Im][OAc]$ from aqueous solutions (≤ 20 wt% IL) using pervaporation systems hence lowering final total costs [16, 17].

To date, the scientific literature available suggest that $[C_2C_1Im][OAc]$ at 120 °C for 3 h in *A. tequilana* bagasse presents the best overall results in terms of a high cellulose to glucose conversion (> 90%), hemicellulose to xylose conversion (> 83%), and delignification (> 20%) [13], including an ~ 82% ethanol yield using a semi-simultaneous saccharification and fermentation (S-SSF) strategy from IL-pretreated *A. tequilana* bagasse to obtain 12.7 kg ethanol from the initial 43.4 kg glucan + xylan in the unpretreated biomass [18]. The main objective of this work was to evaluate the agro-industrial residues from five *Agave* species (*A. americana*, *A. angustifolia*, *A. fourcryodes*, *A. salmiana*, and *A. tequilana*)

and estimate their feasibility for downstream biofuel production. In order to address a comparative analysis, one source of chemicals and enzymes was used for the IL pretreatment (using $[C_2C_1Im][OAc]$) and the saccharification stage, respectively. Biomass characterization was performed using wet chemistry methods, X-ray diffraction for cellulose crystallinity changes, and the syringyl (S) and guaiacyl (G) lignin substructures ratios were determined by pyrolysis molecular beam mass spectrometry (PyMBMS). We also carried out a hydrothermal (HT) pretreatment on all five unpretreated *Agave* samples to compare its sugar release response versus the IL-pretreated samples in the saccharification stage. Finally, the sugar yields from the IL-pretreated samples along with the annual mass productivity were used to estimate the theoretical ethanol yields from the evaluated *Agave* species.

Methods

Experimental Design

An ionic liquid pretreatment at one process condition (120 °C and 3 h) was carried out in samples of the agro-industrial residues from five unpretreated *Agave* species to determine the effect of a single pretreatment on the saccharification yield and biomass recalcitrance with three replicates plus controls. Unpretreated samples were used as controls.

Materials and Sample Preparation

Agro-industrial residues from five *Agave* species were provided and collected from different regions of Mexico (Table 1). Samples from the spirit industry were *Agave americana* (AME) var. *oaxacensis* and *Agave tequilana* (TEQ) F.A.C. Weber obtained from defoliated *Agave* plants leaving only the central section (stem or "piña") which were cooked in brick ovens (AME) and diffusers (TEQ) then milled and compressed to separate the liquid fraction from the solids. On the other hand, samples from the fabric industry named *Agave angustifolia* (ANG) Haw and *Agave fourcroydes* (FOU) Lemaire used the leaves from these *Agave* species where a defibration procedure is applied and a variable fraction of the fiber remains complete for further processing while

Table 1 Sources of the agro-industrial residues used in the study

Genus species	Location*	Industry	Industrial process	Growth potential
A. americana	Oaxaca	Spirits	Thermal extraction	High
A. angustifolia	Coahuila	Fabrics	Defibrillation	Low
A. fourcroydes	Yucatan	Fabrics	Defibrillation	Low
A. salmiana	Nayarit	Syrups	Diffuser extraction	Medium
A. tequilana	Jalisco	Spirits	Diffuser extraction	High

*by Mexican State

another considered as residue is usually employed as fertilizer [19]. Finally, the remaining sample named *Agave salmiana* (SAL) was collected from the *Agave* syrup industry where a diffuser is applied to obtain must (where the solid fraction is separated) which is concentrated using different procedures according to the syrup specification. All samples were collected, washed thoroughly with distilled water, and dried in a convection oven at 40 °C for 3 days, then milled to pass a 20-mesh screen and stored at 4 °C prior to their use [18]. Compositional analysis including glucan, xylan, and acid-insoluble lignin of the unpretreated and IL-pretreated samples were determined using the standard analytical procedures of the National Renewable Energy Laboratory (NREL) by the two-step sulfuric acid hydrolysis method (NREL/TP-510-42618) [20, 21]. Xylan or lignin removal after pretreatment was calculated using the following equation:

$$\text{Xylan or lignin removal}(\%) = \frac{I-F}{I} * 100 \tag{1}$$

where:

I = xylan or lignin content in the unpretreated sample.
F = xylan or lignin content in the pretreated sample.

Ionic Liquid Pretreatment

All *Agave* samples were pretreated with $[C_2C_1Im][OAc]$ at 120 °C for 3 h using a 10% (*w/w*) biomass suspension using 1 g of biomass and 9 g of IL in a 50-mL autoclave vial. A washing step was performed after pretreatment to remove the IL using 90 g of DI water per gram of biomass to obtain a biomass with less than 0.2% IL content measured by Fourier transform infrared (FTIR) spectroscopy [22]. The pretreated solids were recollected after separating the supernatant containing IL with a centrifuge at 10,000*g* for 25 min. All experiments were performed in triplicate.

Hydrothermal Pretreatment

Hydrothermal pretreatment was conducted on all *Agave* samples at NREL by mixing 5.0 ± 0.5 mg of 20-mesh milled *Agave* samples and 250 μL of water, held at 180 °C for 17.5 min using custom designed and built 96-well Hastelloy microtiter reactor plates, and sealed with silicone adhesive and Teflon tape [23]. After pretreatment time, the steam was vented, and cooling water was allowed to flow directly into the reactor and when temperature dropped below 50 °C, water was drained, and the sealed plate stack removed. After pretreatment, the plate was separated from the stack and centrifuged for 10 min at 1000 rpm. These solids without solid/liquid separation were used in the saccharification step. All experiments were performed in triplicate.

Enzymatic Hydrolysis

Using 5.0 ± 0.2 mg of unpretreated and pretreated (IL and HT) samples, digestion was conducted applying 40 μL of CTec2 (Novozymes; 70 mg protein/g biomass) and 250 μL of 0.05 M citrate buffer (pH 5.0) for 70 h at 50 °C in 96-well reactor plates [23]. The hydrolysates were analyzed for glucose and xylose using glucose oxidase/peroxidase and xylose dehydrogenase assays, respectively [24]. Results are calculated using standard curves created from mixtures of glucose and xylose. All experiments were performed in triplicate.

Crystallinity Index

The crystallinity indexes (CrI) of both unpretreated and IL-pretreated *Agave* samples were measured by X-ray diffraction (XRD) using a Rigaku (Tokyo, Japan) Ultima IV diffractometer with CuKα radiation having a wavelength of λ (Kα1) = 0.15406 nm generated at 40 kV and 44 mA. The diffraction intensities of freeze-dried samples placed on a quartz substrate were measured in the range of 8 to 42° 2θ using a step size of 0.02° at a rate of 2°/min. The CrI of the cellulose samples were calculated according to the method described by Segal et al. [25] and calculated using Eq. (2) as follows:

$$\text{CrI} = \frac{I_{200}-I_{Am}}{I_{200}} \tag{2}$$

where I_{200} and I_{Am} are the maximum and minimum intensity of diffraction at approximately $2\theta = 22.4$–22.5° and $2\theta = 18.0$–19.0°, respectively.

Pyrolysis Molecular Beam Mass Spectrometry

A commercially available molecular beam mass spectrometer (MBMS) designed specifically for biomass analysis was used for pyrolysis vapor analysis [26–28]. Approximately 4 mg of air-dried 20-mesh biomass was introduced into the quartz pyrolysis reactor via 80-μL deactivated stainless steel Eco-Cups provided with the autosampler. Mass spectral data from *m/z* 30–450 were acquired on a Merlin Automation data system version 3.0 using 17-eV electron impact ionization. S/G ratios were determined by summing the syringyl peaks 154, 167, 168, 182, 194, 208, and 210 and dividing by the sum of guaiacyl peaks 124, 137, 138, 150, 164, and 178 (Table 2).

Theoretical Ethanol Annual Yield

The theoretical ethanol annual yield (*Y*) of the agro-industrial residues from five *Agave* species was estimated according to Eq. (3):

$$Y = (P \times e_R) \times C \text{ [}L \text{ ethanol/ha/year]} \tag{3}$$

Table 2 Peak and precursor assignments in mass spectra of the *Agave* samples [26]

m/z	Assignment	Type of lignin precursor
124	Guaiacol	G
137*	Ethylguaiacol, homovanillin, coniferyl alcohol	G
138	Methylguaiacol	G
150	Vinylguaiacol, coumaryl alcohol	G
154	Syringol	S
164	Allyl ± propenyl guaiacol	G
167*	Ethylsyringol, syringyl acetone, propiosyringone	S
168	4-Methyl-2,6-dimethoxyphenol	S
178	Coniferyl aldehyde	G
182	Syringaldehyde	S
194	4-Propenylsyringol	S
208	Sinapylaldehyde	S
210	Sinapyl alcohol	S

*Fragment ion

where P is the annual dry mass (DM) productivity from the evaluated *Agave* species (ton DM/ha/year) and e_R is the average residue-to-process *Agave* plant ratio (0.4 ton bagasse/ton DM and 0.46 ton leaves/ton DM) [29, 30] and the ethanol conversion constant, C, was calculated as follows:

$$e_S = (e_G + e_X) \left[\frac{\text{kg sugars}}{\text{ton Agave bagasse or leaves}} \right] \quad (4)$$

$$C = (e_S \times e_C)/e_D \left[\frac{\text{L ethanol}}{\text{ton Agave bagasse or leaves}} \right] \quad (5)$$

where:

e_G is the glucose yield (kg glucose/ton *Agave* bagasse or leaves)

e_X is the xylose yield (kg xylose/ton *Agave* bagasse or leaves)

e_S is the sugar yield (kg sugars/ton *Agave* bagasse or leaves)

e_C is the ethanol conversion efficiency (0.511 kg ethanol/kg sugars) [31]

e_D is the ethanol density (0.789 kg/L) [32].

Statistical Analysis

The software Minitab 18.1 was used to analyze the saccharification yields and the S/G ratios between unpretreated and pretreated *Agave* samples. The data were analyzed for statistical significance by a one-way analysis of variance (ANOVA) and the Tukey test ($p < 0.05$).

Results and Discussion

Chemical Composition Before and After Pretreatment

In recent years, different studies have found that the ionic liquid [C_2C_1Im][OAc] solubilize the plant cell wall, regenerate cellulose, and separate lignin after antisolvent addition and an effective process conditions for *Agave* bagasse has been identified as 120 °C for 3 h [12, 30, 33]. The IL pretreatment conditions used in this study are known to produce a high sugar conversion and delignification in the TEQ species, but according to the open literature, there are no reports with other *Agave* species. It should be addressed that one challenge within a viable biorefinery scheme is the selection of pretreatment conditions for different types of feedstocks. Hence, rather than optimizing the process parameters for each species, this study investigated the impact of a well-known pretreatment procedure with proven results at 120 °C for 3 h using [C_2C_1Im][OAc] on all five *Agave* species including TEQ.

Table 3 shows the compositional analysis of the unpretreated *Agave* species where the glucan content of the unpretreated ANG, TEQ, and SAL was rather similar (38.6–42.6%) while AME was relatively lower (35.0%) and FOU was the highest (63.9%). The xylan content of all five species was within 11.2–17.6% while the lignin content was 12.1–17.4%. Within the five species, TEQ has the highest xylan (17.6%) and lignin (17.4%) content values. AME has the lowest glucan plus xylan content (46.2%) whereas FOU has the highest glucan plus xylan content (81.5%). From the five studied *Agave* species, only for the ANG bagasse is there no available data on the compositional analysis as it only exists for ANG leaves [43] while from process materials of the other species (AME, FOU, SAL, and TEQ), analyses are reported as can be verified in Table 3 [11, 33–42]. In addition to these structural polymers, the *Agave* spp. could contain other

Table 3 Compositional analysis of unpretreated *Agave* species and comparison to previous reports (dry basis)

Biomass	Component (%)			References
	Glucan	Xylan	Lignin	
AME	35.0 ± 0.8	11.2 ± 1.1	15.9 ± 0.5	This study
	26–38	13–14	7–18	[34, 35]
ANG	38.6 ± 1.9	12.6 ± 1.3	15.6 ± 0.8	This study
	–	–	–	No data
FOU	63.9 ± 1.8	17.6 ± 1.3	12.1 ± 0.5	This study
	58–78	5–30	6–13	[36, 37]
SAL	41.6 ± 0.7	14.8 ± 1.5	15.0 ± 0.3	This study
	39–47	14–16	10–16	[38, 39]
TEQ	42.6 ± 1.1	17.6 ± 1.0	17.4 ± 1.0	This study
	26–46	15–23	13–20	[11, 22, 33, 40–42]

compounds such as proteins, extractives (including calcium oxalate), starch, soluble sugars, fructans, waxes, ash, and other extraneous materials [11, 44, 45].

Results shows that after IL pretreatment, all five *Agave* species have a higher glucan content (41.8–68.1%) and lower lignin content (10.5–15.3%) when compared to the unpretreated samples (Table 4). Overall, FOU has the highest glucan content (78.1%) and lowest lignin (10.5%) from the pretreated materials. In previous reports by our group, IL pretreatment of TEQ bagasse with $[C_2C_1Im][OAc]$ at 120 °C for 3 h removed more lignin ranging from 16.4 to 25.0% which is relatively higher than the values observed in this study (12.0%) [30, 33]. We hypothesized that this difference in lignin removal after IL pretreatment could be attributed to the selection of the cooking process during spirit production where the *Agave* bagasse is obtained and, hence, could be applied to AME and TEQ. It is important to point out the difference of the origin of agro-industrial residues from *Agave* when compared to traditional feedstocks (e.g., corn stover or wood residues) that do not undergo any process under relatively high pressure and temperature. The *Agave* for spirit production can be cooked using either three possibilities, (1) brick oven using pressurized steam (90 °C and 36 to 48 h) [46, 47], (2) autoclave (121 °C and 6–18 h) [18, 48], or (3) diffuser (80–90 °C and 6–7 h) [49] which the latter is also called soft sugar extraction which, after this stage, a syrup with a high sugar concentration (> 10% in weight) is obtained. Recently, the spirit factories due to larger demand are switching to the diffuser option due to its milder conditions which prevent overcooking and have more control over the final product.

Based on the unpretreated solids from all five samples, a total mass fraction from 73.3 to 82.5% was recovered after IL pretreatment. The solid fraction present after pretreatment was lower for ANG (73.3%) when compared to AME (82.5%) per 100 kg of inlet biomass. The weight loss mainly came from the solubilization of particular components from each *Agave* species such as lignin, xylan, glucan, and other soluble extractives such as calcium oxalate. A minor loss of the glucan fraction was achieved < 3.1% in all five feedstocks. Based on the recovered biomass and calculated using Eq. (1), the xylan removal had a

relatively large variation within *Agave* species from 6.8% with FOU to 32.4% with TEQ when compared to the unpretreated samples, whereas the delignification observed was 28.9 and 37.8% for AME and ANG, respectively.

Within a biorefinery processing perspective, an IL-based biorefinery could potentially employ the liquid wash stream containing xylan and lignin where they can be processed to value-added products including new ILs derived from xylan [e.g., *N*-ethyl-*N*-(4-methoxybenzyl) ethanamine dihydrogenphosphate, H_3PO_4 salt ($[p$-AnisEt$_2$NH][H$_2$PO$_4$])] which have demonstrated to shown a "closed loop" process for lignocellulosic biorefineries [50].

Lignin S/G Ratio Using Pyrolysis Molecular Beam Mass Spectrometry

A high throughput tool capable of lignin characterization has been developed by the National Renewable Energy Laboratory (NREL) named pyrolysis molecular beam mass spectrometry (pyMBMS). The lignin substructures syringyl (S) and guaiacyl (G) ratio (S/G) have been proven to be an important parameter for gauging lignin recalcitrance which affects cell wall deconstruction during biomass pretreatment and further downstream processing [51, 52]. Table 5 presents a comparison between the S/G ratios of the unpretreated and IL-pretreated *Agave* samples measured by pyMBMS. This is the first report that shows the S/G ratios of unpretreated and IL-pretreated AME, ANG, FOU, and SAL samples, whereas the S/G ratio of unpretreated and IL-pretreated TEQ using $[C_2C_1Im][OAc]$ has been estimated by two-dimensional ^{13}C-^{1}H heteronuclear single-quantum coherence nuclear magnetic resonance spectroscopy (HSQC-NMR) [33]. The S/G ratios of all five unpretreated *Agave* species were between 1.27 and 1.57 while the IL-pretreated samples were from 1.39 to 1.72. An increased S/G ratio is observed in the pretreated samples when compared to the unpretreated except for TEQ which decreased. Additionally, only SAL and TEQ were not statistically different ($p < 0.05$) between the S/G ratios of the unpretreated and IL-pretreated samples.

Table 4 Compositional analysis of IL-pretreated *Agave* species (dry weight)*

Biomass	Recovered biomass (%)	Component (%)		
		Glucan	Xylan	Lignin
AME	82.5 ± 2.7 (82.5 g)	41.8 ± 1.0 (34.5 g)	10.8 ± 1.6 (8.9 g)	13.7 ± 0.2 (11.3 g)
ANG	73.3 ± 3.4 (73.3 g)	51.0 ± 0.6 (37.4 g)	11.9 ± 0.2 (8.7 g)	13.3 ± 1.9 (9.7 g)
FOU	79.9 ± 0.9 (79.9 g)	68.1 ± 1.9 (54.4 g)	20.5 ± 1.2 (16.4 g)	10.5 ± 0.7 (8.4 g)
SAL	76.4 ± 1.8 (76.4 g)	53.8 ± 0.9 (41.1 g)	16.2 ± 2.0 (12.4 g)	13.1 ± 0.5 (10.0 g)
TEQ	79.1 ± 3.9 (79.1 g)	53.5 ± 0.9 (42.3 g)	15.1 ± 0.5 (11.9 g)	15.3 ± 0.2 (12.1 g)

*Values in parentheses represent the amount of each component recovered from 100 g of total biomass

Table 5 Comparison of syringyl (S) and guaiacyl (G) lignin substructures determined by PyMBMS from unpretreated and IL-pretreated *Agave* samples from different species

Biomass	Treatment	S/G ratio
AME	Unpretreated	1.27 ± 0.04^a
	IL	1.39 ± 0.02^b
ANG	Unpretreated	1.29 ± 0.11^a
	IL	1.72 ± 0.14^b
FOU	Unpretreated	1.40 ± 0.03^a
	IL	1.52 ± 0.00^b
SAL	Unpretreated	1.33 ± 0.13^a
	IL	1.45 ± 0.04^a
TEQ	Unpretreated	1.57 ± 0.05^a
	IL	1.47 ± 0.13^a
	Unpretreated	4.3*
	IL	4.7*

Different lowercase letters indicate statistically significant different among row means ($p < 0.05$)

*Determined by HSQC-NMR from Perez-Pimienta et al. [33]

The highest increase of S/G ratio was obtained for FOU from 1.29 to 1.72 after pretreatment. An increase in S/G measured by pyMBMS after other biomass pretreatments such as hydrothermal was also observed in other biomass feedstocks such as switchgrass [53]. Unpretreated switchgrass material had an S/G ratio of 0.64 and increased to 0.83 after pretreatment. This increase in S/G ratio is favorable for biological processing as more β–O–4 bonds are present which can be easily deconstructed by biocatalysts for lignin valorization [54]. In a recent report, the S/G ratio of unpretreated and IL-pretreated TEQ bagasse was measured to be 4.3 and 4.7, respectively, determined by HSQC-NMR [33]. While the HSQC-NMR S/G value for the IL-pretreated TEQ is much higher than the result found by pyMBMS in this study, this is not unusual. These two methods for S/G ratio determination generally agree on relative rankings (high/low) of S/G concentration, but not on absolute S/G ratio values.

During biomass pretreatments, recalcitrance characteristics such as the ratio of S/G subunits in the plant cell wall have to define the degradability of lignin, where some studies have reported that higher S-lignin led to increased delignification with concomitant increases in sugar yields as can be related with the corresponding sugar production section of this manuscript [52].

Most publications centered in IL pretreatment have shown that G-type lignin was more easily degraded and collected than the S-type lignin during biomass pretreatment at 110–120 °C [55]. Furthermore, the S/G ratios from the reported *Agave* species are within range of other succulent plants from different species of *Cactaceae* such as *Ariocarpus retusus* (1.1), *Lophocereus marginatus* (1.1), and *Pilosocereus alensis* (1.3) as well as some leading bioenergy feedstocks [sugarcane bagasse (1.4), switchgrass (1.2), or wheat straw (1.2)] [52, 56].

Biomass Crystallinity After Pretreatment

The crystallinity index (CrI) of cellulose is an important recalcitrance characteristic that affects saccharification efficiency after biomass pretreatment where the crystal structure of cellulose can be modified after IL pretreatment by disrupting inter- and intra-chain hydrogen bonding of cellulose fibril [57, 58]. The XRD spectra of all five unpretreated and pretreated *Agave* species were examined and are presented in Fig. 1. By separating the crystalline and amorphous contributions of the diffraction spectrum, the cellulose CrI was

Fig. 1 XRD spectrum of unpretreated and ionic liquid (IL)-pretreated *Agave* species. CrI crystallinity index
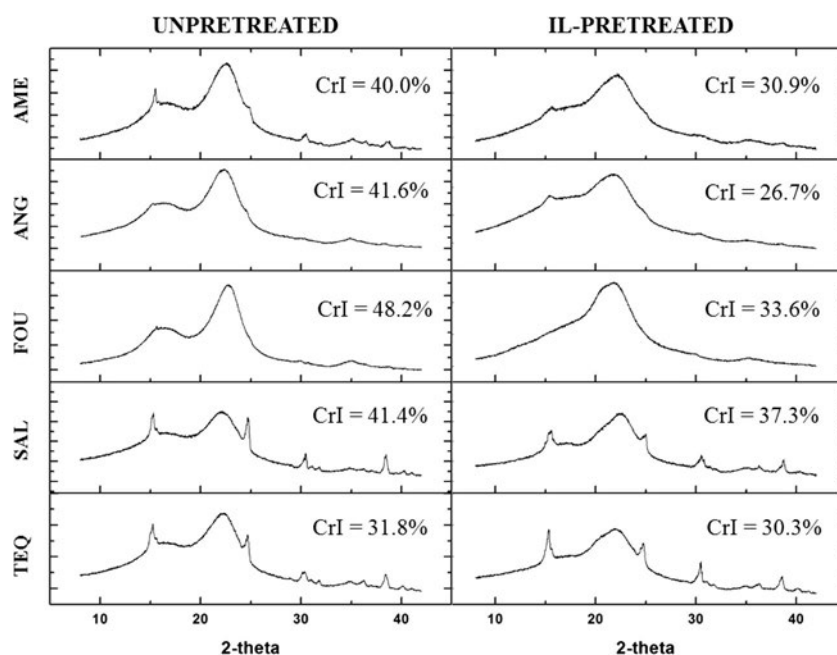
estimated. From the unpretreated samples, FOU has the highest initial CrI with 48.2% while TEQ has the lowest (31.8%). After IL pretreatment, all samples show a decrease in CrI in which ANG shows the highest relative reduction followed by FOU from 41.6 to 26.7% and 48.2 to 33.6%, respectively. Reduction of cellulose crystallinity from 31.8 to 30.3% in TEQ was the lowest compared to all samples analyzed while in previous reports, the difference was significantly higher [e.g., 39% (unpretreated) and 22.6% (IL-pretreated)] [18]. The low decrease of cellulose crystallinity of the IL-pretreated TEQ correlates to its low delignification presumably due to TEQ recalcitrance which is higher than in previous reports with likely difference being the older cooking process (autoclave vs. diffuser); hence, it can be inferred that a high pretreatment temperature is necessary to achieve a higher lignin removal and lower CrI. Moreover, the main peak at around 22.1° became broader after IL pretreatment, which it is clear evidence of significant reduction in cellulose crystallinity [14]. In addition, the peak at around 16.0° became broader and lower in intensity which is more evident in AME, ANG, and FOU and is consistent with a severe distortion of cellulose I lattice [58]. As expected, unpretreated AME, SAL, and TEQ show distinctive and prominent peaks at $2\theta = 14.8°$, $24.2°$, $30.0°$, and $38.0°$ corresponding to calcium oxalate (CaOX) monohydrate as previously reported [11, 12, 22]. Nevertheless, these peaks were found in neither ANG nor FOU as these samples come from the leaves where the CaOX has a lower concentration than in the bagasse samples. When compared to the pretreated samples, all the CaOX peaks decrease in intensity.

Sugar Release from Pretreated Biomass

Enzymatic saccharification is a key parameter to measure the biomass digestibility after pretreatment. We have hydrothermally (HT) pretreated a new batch of the raw agro-industrial residues of the five *Agave* species to compare their cellulose digestibility with the IL-pretreated samples in terms of glucose yield using commercial enzyme cocktails in a 96-well multiplate reactor system develop by NREL (Table 6). Besides, the xylose releases from the enzymatic saccharification of unpretreated and IL-pretreated samples from all *Agave* species are shown in Table S1. When compared to the unpretreated samples, both pretreatments (HT and IL) demonstrate a high glucose yield and were statistically different ($p < 0.05$) for all five *Agave* species likely due to its specific effects on plant cell wall recalcitrance such as an increase in the accessible surface area and the partial removal of xylan and lignin for HT and IL, respectively.

Glucose production was higher with IL pretreatment when compared to HT samples. The highest glucose production was obtained with IL-pretreated FOU with 491.9 mg glucose/g biomass followed by ANG IL pretreated with 396.4 mg

Table 6 Glucose release per g of biomass from unpretreated and pretreated samples from the evaluated *Agave* species*

Biomass	Treatment	Glucose yield (mg/g biomass)
AME	Unpretreated	30.8 ± 2.3^a
	HT	169.5 ± 20.7^b
	IL	284.7 ± 39.9^c
ANG	Unpretreated	63.3 ± 3.2^a
	HT	292.1 ± 20.2^b
	IL	396.4 ± 10.4^c
FOU	Unpretreated	184.7 ± 9.6^a
	HT	482.9 ± 8.5^b
	IL	491.9 ± 64.4^b
SAL	Unpretreated	64.5 ± 0.3^a
	HT	176.8 ± 12.5^b
	IL	233.3 ± 7.9^c
TEQ	Unpretreated	50.7 ± 1.2^a
	HT	293.5 ± 21.7^b
	IL	310.2 ± 13.6^b

Different lowercase letters indicate statistically significant different among row means ($p < 0.05$)

*UNT untreated, HT hydrothermal, IL ionic liquid. Error bars show the standard deviation of triplicate measurements

glucose/g biomass at 70 h of saccharification. After 70 h of enzymatic saccharification of the pretreated samples, the glucose production for FOU and TEQ was not statistically different between pretreatments ($p < 0.05$) while the IL-pretreated AME, ANG, and SAL were significantly higher than the HT-pretreated samples. In terms of xylose production, the highest production was achieved by IL-pretreated ANG (173.3 mg xylose/g biomass) followed by FOU IL with 173.3 mg xylose/g biomass with a three- to tenfold increase when compared to the unpretreated samples. Per species, the highest glucose plus xylose yield occurred with FOU (491.9 mg glucose/g biomass and 156.7 mg xylose/g biomass) while the lowest was obtained with SAL (233.3 mg glucose/g biomass and 117.6 mg xylose/g biomass).

The differences in biomass digestibility between the agro-industrial residues from the five *Agave* species studied could be attributed to the following: (1) leaves (ANG and FOU) have higher glucan and xylan content than bagasse (AME, SAL, and TEQ) and (2) *Agave* samples come from different industrial applications (fabrics, spirits, and syrup) yielding different specific recalcitrance properties prior to pretreatment. It is worth to mention that significant levels of free sugars and depolymerized fructans have been removed from the *Agave* stem due to the agro-industrial processing of AME, SAL, and TEQ [48]. The processing of the *Agave* leaves (ANG and FOU) removes the sugars contained in the liquid fraction while the solids could be treated either by mechanical

Table 7 Theoretical ethanol yield from the agro-industrial residues of the evaluated *Agave* species (*A. americana*, *A. angustifolia*, *A. fourcroydes*, *A. salmiana*, and *tequilana*) after IL pretreatment

Parameter	Unit	AME	ANG	FOU	SAL	TEQ
Glucose yield (e_G)[a]	kg/ton DM	285	396	492	233	310
Xylose yield (e_X)[a]	kg/ton DM	124	173	157	118	136
Ethanol production (*C*)	L ethanol/ton DM	264	368	419	227	288
Dry mass productivity (*P*)	ton/ha/year	10–34 [35]	10–34 [35]	15–30 [64, 65]	26–42 [65, 66]	25–44 [67, 68]
Theoretical ethanol annual yield (*Y*)[b]	L ethanol/ha/year	1058–3596	1694–5759	2893–5786	2359–3811	2883–5074

[a] Yield of glucose and xylose after ionic liquid pretreatment and enzymatic saccharification

[b] Theoretical ethanol annual yield calculated from sugar yield and ethanol constants (Eq. (3–5))

or chemical defibration process (using mild sodium hydroxide or sodium peroxide to soften the fibers) giving specific physicochemical characteristics to the biomass [59]. Furthermore, the IL pretreatment process parameters for each individual *Agave* species have to be optimized to ensure the highest sugar yield due to the observed difference previously described.

While HT pretreatment removes xylan and relocates lignin with a resulting higher cellulose accessibility, the IL pretreatment results in cellulose decrystallization, and partial lignin removal of these pretreatment processes impacts biomass differently but both lead to a high sugar production [33]. In accordance with delignification and crystallinity, FOU and ANG IL-pretreated samples obtained a higher sugar production than the rest of the *Agave* species.

In addition, there are a few reports where HT pretreatment was applied on either AME or TEQ. Li et al. [60] used a customized 96-well plate reactor for HT pretreatment of raw and fresh AME obtaining the highest glucose (~76%) and xylose + galactose (~64%) yields at 160 °C and 17.1 min. Another paper reported HT pretreatment on TEQ in a pressurized batch reactor at 180 °C and 43 min obtaining a relatively high glucan (~80%) and xylan (83%) conversion [33]. In addition, Mielenz et al. screened different *Agave* species using the whole raw and fresh plant with HT pretreatment at 180 °C and 7.5 min obtaining for TEQ and three AME varieties, 328 and 278–328 mg sugars/g biomass, respectively, where the sugars reported are the combination of glucose, xylose, galactose, arabinose, mannose, and fructose [61].

Theoretical Ethanol Yield from the *Agave* Species

The results obtained from the glucose and xylose obtained in the saccharification stage from all the studied *Agave* species after IL pretreatment reaffirmed its potential as a biofuel feedstock. In Table 7, the theoretical ethanol yield from the five evaluated *Agave* agro-industrial residues is shown, where the structural carbohydrates (glucan + xylan) in the studied biomass residues ranging from ~46% (AME) to ~82% (FOU) are highly comparable with the composition of raw energy crops such as corn stover, sugarcane bagasse, or switchgrass

[62, 63]. Another important characteristic of the evaluated *Agave* species is their high dry mass productivity, ranging from 10 to 44 ton/ha/year with appropriate spacing and irrigation [34, 64–68]. Even so, SAL and TEQ are capable to generate ~25 ton/ha/year without irrigation and in semiarid conditions [69]. Otherwise, leading bioenergy feedstocks such as corn stover, sugarcane bagasse, and switchgrass could produce only 3, 10, and 15 ton/ha/year, respectively [70, 71].

The highest theoretical annual ethanol yield value was obtained with FOU ranging from 2893 to 5786 L ethanol/ha/year while AME obtained the lowest values ranging from 1058 to 3596 L ethanol/ha/year. Hence, the theoretical annual ethanol yield from the studied agro-industrial *Agave* species was estimated to be within the range of 1060–5800 L ethanol/ha/year. These values are comparable to estimates for other biofuel feedstocks (based on their ethanol production and biomass productivity) such as corn stover (1283 L ethanol/ha/year) or sugarcane bagasse (4221 L ethanol/ha/year) [72].

At the end, FOU can be referred as the most useful *Agave* species when compared to the other four studied in terms of lignin removal, decrease crystallinity, sugar production, and potential ethanol productivity. However, consideration should be taken with the expanding market of spirit production especially on TEQ even in some cases outstripping TEQ production when compared to the much smaller fiber and syrup industries. Finally, this side-by-side analysis demonstrates the potential of the *Agave* spp. as a suitable feedstock which can be employed within a biorefinery scheme.

Conclusions

The agro-industrial residues from five *Agave* species (*A. americana*, *A. angustifolia, fourcroydes*, *A. salmiana*, and *tequilana*) were assessed using IL pretreatment and characterization methods. IL pretreatment decreases the lignin fraction and crystallinity of the five *Agave* species. The S/G ratio from most of the IL-pretreated samples was higher when compared to the unpretreated biomass. HT-pretreated *Agave* samples were used as comparison during the saccharification. The

highest overall sugar production was obtained with IL-pretreated FOU with 492 mg glucose/g biomass and 157 mg xylose/g biomass at 70 h of saccharification. An estimated theoretical ethanol yield from the evaluated agro-industrial residues from the five *Agave* species were in the range of 1060–5800 L ethanol/ha/year. The *Agave* spp. have favorable agronomic features (low water requirements, high productivity in semiarid lands, and drought resistance), and its high carbohydrate content and sugar to ethanol conversion yields have proven potential as a bioenergy feedstock. If the *Agave* industry can optimize its cultivation systems, this could increase the productivity into a biorefinery scheme for the production of biofuels and value-added products.

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