**SEQUENTIAL ENZYMATIC SACCHARIFICATION AND FERMENTATION OF
IONIC LIQUID AND ORGANOSOLV PRETREATED AGAVE BAGASSE FOR
ETHANOL PRODUCTION**

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ABSTRACT

Agave bagasse (AGB) has gained recognition as a drought-tolerant biofuel feedstock with high productivity in semiarid regions. A comparative analysis of ionic liquid (IL) and organosolv (OV) pretreatment technologies in AGB was performed using a sequential enzymatic saccharification and fermentation (SESF) strategy with cellulolytic enzymes and the ethanogenic *Escherichia coli* strain MS04. After pretreatment, 86% of xylan and 45% of lignin were removed from OV-AGB, whereas IL-AGB reduced lignin content by 28% and xylan by 50% when compared to the untreated biomass. High glucan (>90%) and xylan (>83%) conversion was obtained with both pretreated samples. During the fermentation stage (48h), 12.1 and 12.7 kg of ethanol were produced per 100 kg of untreated AGB for IL and OV, respectively. These comparative analyses showed the advantages of SESF using IL and OV in a biorefinery configuration where a better understanding of AGB recalcitrance is key for future applications.

Keywords

Agave bagasse; fuel ethanol, ionic liquid, organosolv, sequential saccharification and fermentation, crystallinity

1. Introduction

Cellulosic ethanol has the potential to be a renewable biofuel that can be produced from lignocellulosic feedstocks as a promising alternative to liquid fossil fuels while reducing air pollution and greenhouse gas emissions (Trinh et al., 2015). Biomass recalcitrance is a major challenge in the realization of cost-effective cellulosic ethanol. Mainly due to cellulose association with hemicelluloses and lignin, including other factors such as crystallinity and/or porosity, these biopolymers confer limited accessibility to enzymes or microorganisms for hydrolysis. A pretreatment step is generally required to accomplish extensive modification on lignocellulose structural features and to remove substrate specific barriers for efficient saccharification and fermentation (Perez-Pimienta et al., 2016a). To date, numerous pretreatments have been developed using physical (milling and grinding), physicochemical (steam pretreatment), chemical (alkali, dilute acid, ionic liquid, oxidizing agents, and organic solvents), biological (microbial delignification), or a combination of these (e.g. thermochemical treatments) (Kumar et al., 2009). A suitable pretreatment technology must be adopted to cover the properties of each biomass feedstock, as this choice would have a great impact on subsequent steps in terms of glucan/xylan digestibility, possible generation of toxic compounds that are inhibitory for enzymes and ethanogenic microorganisms, stirring power requirements, energy demand in the downstream process and wastewater treatment demands, among others (Alvira et al., 2010). Recently, species from the genera *Agave* (Agavaceae) have gained recognition as a potential biofuel feedstock due to desirable characteristics, including high productivity in semiarid regions and drought resistant, besides of its low water requirements due to the Crassulacean Acid Metabolism (CAM) (Davis et al., 2011; Long, 2014; Yang et al., 2015) and low amount of fertilization requirements. During 2015, about 8.09×10^5 tons of agave

were consumed for Tequila production in Mexico, where 40% corresponds to bagasse (CRT, 2015; Davis et al., 2011). Some independent pretreatments studies have been conducted in agave bagasse (AGB) including alkali (Ávila-Lara et al., 2015; Hernández-Salas et al., 2009), ammonia fiber expansion (Perez-Pimienta et al., 2016a), dilute acid (Ávila-Lara et al., 2015; Mielenz et al., 2015; Saucedo-Luna et al., 2011), hydrothermal (Li et al., 2014; Perez-Pimienta et al., 2016a), ionic liquid (Perez-Pimienta et al., 2015, 2013) and organosolv (acetic acid and sulfuric acid) (Caspeta et al., 2014; Hernández-Hernández et al., 2016) in order to reduce lignocellulose recalcitrance for subsequent saccharification. Nevertheless, only a few reports exist in the open literature on ethanol production from agave hydrolysates, mainly via separate hydrolysis and fermentation (SHF) using yeast (*Pichia caribbica*, *Saccharomyces bayanus*, and *Saccharomyces cerevisiae*) (Caspeta et al., 2014; Corbin et al., 2015; Hernández-Salas et al., 2009; Mielenz et al., 2015; Saucedo-Luna et al., 2011). In addition to SHF, two other process configurations for ethanol fermentation exist, namely, simultaneous saccharification and fermentation (SSF) and sequential enzymatic saccharification and fermentation (SESF). The SHF is carried out in two separate steps: firstly, a saccharification at optimal enzyme (e.g. 50 °C and pH 4.8) and secondly, optimal fermentation conditions for the fermentation of sugars (e.g. 30-32 °C and pH 4.5-5). SSF process conditions are constant (e.g. 37 °C and pH 5) (Cotana et al., 2015). SESF is carried out using a pre-hydrolysis step (e.g. 4-24h) followed by SSF (Gonçalves et al., 2014). The SESF configuration offers advantages over SHF and SSF to produce higher ethanol concentration, yield and productivity, but it has not yet been evaluated in AGB (Zhang et al., 2014). Meanwhile, ionic liquid (IL) (using 1-ethyl-3-methylimidazolium acetate or [C₂C₁Im][OAc]) and organosolv (OV) (using ethanol and sulfuric acid) pretreatments

showed prominent results on AGB in terms of a high cellulose to glucose conversion (>90%) and component removal (delignification above 20% at 120°C for IL and xylan removal above 80% at 160°C for OV) (Caspeta et al., 2014; Perez-Pimienta et al., 2013). Ionic liquid pretreatment reduces cellulose crystallinity by overcoming the hydrogen bonding network (from cellulose I to II) while partially removing hemicellulose and lignin due to an efficient solubilization of the main components of the plant cell wall. With this pretreatment the biomass is more amenable for saccharification processing without generating inhibitory products (Cheng et al., 2011; Samayam and Schall, 2010). Process conditions are within 100–160 °C, short processing times (1–3 h) and atmospheric pressure (Li et al., 2011). ILs can be highly recycled using different methods (e.g. membrane filtration or vacuum evaporation) which can decrease total costs (Klein-Marcuschamer et al., 2011).

Organosolv pretreatment employs organic or aqueous organic solvents (methanol, ethanol or acetone, among others) and inorganic acids (HCl or H₂SO₄) catalysts to break the internal lignin and hemicellulose bonds (Kumar et al., 2009). da Costa Sousa et al. (2009) mentioned that “in this pretreatment, operating temperatures range between 90 and 120°C for grasses and 155–220°C for woods, with a residence time ranging from 25 to 100 min and depending on the biomass, catalyst concentration can vary from 0.83 to 1.67% and alcohol concentration from 25 up to 74% (v/v)”. Solvents removal from the system can be performed using convenient separation process (e.g. evaporation and condensation) and, as for ILs, a solvent recycle step reduces overall processing costs. Besides, solvents and ILs need to be separated as they could inhibit saccharification and fermentation microorganisms (Alvira et al., 2010).

The main objective of the present study was to evaluate IL and OV pretreated AGB using a SESF strategy for ethanol production using commercial cellulases and the ethanogenic *E. coli* strain MS04. In order to perform a comparative analysis, one source of biomass and enzymes were used as feedstock for both pretreatments and for the saccharification stage, respectively. Biomass characterization was performed using X-ray diffraction for cellulose crystallinity changes, Fourier Transform Infrared (FTIR) spectroscopy to track chemical changes at the molecular level, Confocal Fluorescence and Scanning Electron Microscopy for examination of supramolecular structure changes and mass spectrometry (MALDI-TOF) to obtain a semiquantitative fingerprint of particular cell wall polysaccharides. We conclude the study comparing the process flowsheets and mass balances of both pretreatment techniques and ethanol production based on 100 kg of untreated AGB, glucan/xylan conversion and compositional analysis.

2. MATERIALS AND METHODS

2.1. Materials and sample preparation

Bagasse from blue Agave *tequilana* was received as a donation from “Destilería” Rubio, based in Tequila, Jalisco, Mexico. This tequila plant employs defoliated agave plants using only the central section (stem or “piña”). The stems are cooked in an autoclave for 18 h, then milled and compressed to separate the liquid fraction from the solids (bagasse). The samples were prepared as reported by Perez-Pimienta et al. (2016b), “wet bagasse samples were collected, washed thoroughly with distilled water and dried in a convection oven at 40 °C and the samples were milled with a 20 mesh screen and stored at 4 °C prior to their use”.

Ionic liquid pretreatment was carried out at the Joint Bioenergy Institute and organosolv pretreatment at the Biotechnology Institute (Universidad Nacional Autónoma de México). The pretreated biomass was dried in a convection oven (40°C for 3 day) and stored at 4°C. Compositional analysis (glucan, xylan and acid-insoluble lignin) of the untreated and pretreated samples were determined accordingly to NREL standard analytical procedures by the two-step sulfuric acid hydrolysis method (Hames et al., 2008; Sluiter et al., 2008).

2.1.1. Chemicals and Enzymes

Betaine, citric acid, glucose, 1-ethyl-3-methylimidazolium acetate ([C₂C₁Im][OAc]), MOPS, sodium hydroxide, sulfuric acid and xylose were purchased from Sigma–Aldrich (St. Louis, MO). Commercial enzyme cocktails, CTec2 with 75 FPU/mL and HTec2 with 380 CBU/mL were obtained from Novozymes.

2.2. Biomass pretreatment

2.2.1. Ionic Liquid (IL)

The AGB was pretreated with [C₂C₁Im][OAc] at 120°C for 3 h (14.7 psi, ramp time: 25 min) using a 1-L Globe reactor (Syrris, UK) at 150 rpm with a Teflon overhead stirrer. 50 g of AGB (dry basis) and 450 g of [C₂C₁Im][OAc] were mixed to obtain 10% (w/w) biomass loading (Perez-Pimienta et al., 2016a). A washing step was performed after pretreatment to remove the IL as previously described (Perez-Pimienta et al., 2013). In short, 4500 g of DI water was added into the biomass/IL slurry to remove the residual IL from the solid fraction with a centrifuge.

2.2.2. Organosolv (OV)

Fifty grams of AGB (on a dry weight basis) was mixed with a 500 mL solution containing 74.5% water, 25% ethanol and 0.5% H₂SO₄ as catalyst. Pretreatment was carried out at 160 °C, 138 psi and 10min in a high-pressure chemical reactor (Model 4530 / Controller 4848, Parr Instruments, IL, USA)(Caspeta et al., 2014). A ten-minute holding time was used and the vessel was air-cooled to room temperature. The solid fraction was washed according to Caspeta et al. (2014), “the solid fraction was washed three times at 60 °C with 75 mL of an ethanol solution at the same concentration used in the process, then washed once with 225 mL of water to remove fermentable sugars released during pretreatment”.

2.3.Sequential enzymatic saccharification and fermentation (SESF)

Both saccharification (18h) and fermentation (48h) of pretreated AGB were performed sequentially for a 66 h total process time. In the first stage, the enzymatic hydrolysis of pretreated AGB was conducted at 50°C and 150 rpm in 50 mM citrate buffer (pH 4.8) in 200 mL (working volume) peg-mixer reactors (Caspeta et al., 2014) using a 10% (w/w) biomass loading and a low enzyme loading of 8 FPU/g biomass and 15 CBU/g biomass for CTec2 and HTec2, respectively. The pH of saccharification reactors was adjusted to 7 with 8 N KOH (technical grade), and the fermentation was performed using the ethanologenic *E. coli* strain MS04 (Fernández-Sandoval et al., 2012). Accordingly, to Vargas-Tah et al. (2015) “concentrated solutions of betaine (as osmoprotectant) and MOPS (as buffer) were added to final concentrations of 1 and 100 mM, respectively”. Inoculum preparation was performed as previously described (Vargas-Tah et al., 2015) and cells were harvested to start the fermentation with 3 g_{DCW}/L. The cultures were incubated at 37 °C, 60 rpm (with the peg-mixer) and the pH was controlled at 7 with the automatic addition of 2 N KOH. All assays were performed in duplicate. Reactions were followed by sampling 500 µL of the

supernatant at specific time intervals of saccharification and fermentation as shown in the results section.

2.4. Analysis

2.4.1. Sugars and ethanol

Glucose and xylose were measured using a biochemical analyzer (YSI 2700 Select, YSI Inc,) on the saccharified and fermented samples that were centrifuged at 10,000 x g for 5 min and then diluted to a proper measurable concentration. The standard error of duplicate measurements is shown in tables and figures. Ethanol production was quantified by gas chromatography (Agilent, 6850 series GC System, Wilmington, DE, USA), as reported elsewhere (Vargas-Tah et al., 2015).

2.4.2. Calculations

Glucose and xylose measurements at the final time of the saccharification stage (18 h) of pretreated AGB were used for conversion calculations. Glucan and xylan conversion were calculated as previously described (Perez-Pimienta et al., 2016a) and established as the overall individual mass before pretreatment, hence representing a global process conversion. Theoretical ethanol yield of pretreated biomass was determined based on the available glucose and xylose released during enzymatic saccharification, on sugar conversion factors and a 100kg untreated AGB basis, following Eq. (1).

$$TY_E = 0.51 * (C_G + C_X) \quad (1)$$

where TY_E = theoretical ethanol yield (g/L); C_G = glucose concentration (g/L) of glucose at saccharification (18h) and C_X = xylose concentration (g/L) at saccharification (18h).

Ethanol yield (%) was calculated as a percent ratio of the actual ethanol amount produced to the theoretical ethanol yield from a 100kg untreated AGB basis, following Eq. (2).

$$Y_E = \frac{\text{Ethanol produced (g)}}{TY_E(g)} * 100$$

(2)

where Y_E = ethanol yield (%).

2.5. Characterization

2.5.1. Crystallinity measurement

X-ray powder diffraction patterns of AGB samples were collected with a RigakuMiniflex 600 diffractometer equipped with a high-velocity Dtex ultra detector and operated at 40 kV and 15 mA using Cu-K α radiation ($\lambda = 1.54 \text{ \AA}$). In the incident beam a soller slit with a Ni filter was placed at 0.5mm gap. Patterns were collected in the 2θ range of 5 to 50°, the step size was 0.01°, and a velocity of 3°/min. The crystallinity index (CrI) of the biomass samples were determined according to Segal et al. (Segal et al., 1959) where “ I_{total} is the scattered intensity at the main peak, whereas I_{am} is the scattered intensity due to the amorphous portion” using the following equation:

$$CrI(\%) = \frac{I_{total} - I_{am}}{I_{total}} * 100 \quad (3)$$

2.5.2. Attenuated total reflectance (ATR)–FTIR spectroscopy

An infrared IR2 module coupled to a microscope of a HORIBA Jobin Yvon micro-Raman confocal spectrometer model LabRam HR-800 was used for ATR–FTIR. The samples were placed on-site in a low emissivity slide in the infrared over the equipment platen. Samples spectra from untreated and pretreated biomass were obtained with an average of 32 scans

(in triplicates) over the range between 800 cm⁻¹ and 4000 cm⁻¹ with a spectral resolution of 4 cm⁻¹ and processed as previously described (Perez-Pimienta et al., 2016b).

2.5.3. Scanning Electron Microscopy

Morphology of AGB solids (untreated and pretreated) were analyzed using a high-resolution scanning electron microscope (SEM) by a JEOL JSM-7800F equipment. The images were acquired with a 1 kV accelerating voltage and analysis using 20 kV. Energy dispersive X-ray spectroscopy (EDS) was used simultaneously with SEM imaging for the determination of element content.

2.5.4. Confocal Fluorescence Microscopy

Biomass (untreated and pretreated) images from confocal fluorescence microscopy were captured using a Carl Zeiss LSM 710 NLO coupled two laser sources (405 and 633 nm). All samples were labeled with Calcofluor white stain (0.1 %) for 5 min in order to show microstructure based on lignin and cellulose distribution (autofluorescence). Finally, the AGB samples were washed with distilled water and dried in the dark until analysis.

2.5.5. MALDI-TOF mass spectrometry

Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry was performed in untreated and pretreated AGB using an Autoflex Speed mass spectrometer (Bruker Daltonics, Bremen, Germany). One mg of the sample was dissolved in 1 mL of toluene and heated during 5 min. From this solution, 1.5 µL were mixed with 1.5 µL of a saturated solution of 2,5-dihydroxybenzoic acid and placed in a MALDI-TOF plate until analysis. After a delayed extraction time of 400 ns, the ions were

accelerated to a kinetic energy of 19kV. Hereafter, the ions were detected using the reflector mode.

3. RESULTS AND DISCUSSION

3.1. Compositional analysis and process mass balances

In order to understand the impact of IL and OV pretreatments in AGB, compositional analysis and SESF strategy were used to develop a process mass balance for each pretreatment technology. Using a normalized basis (100 kg of dry and untreated AGB) all process yields were established, including the calculation due to water gain during monosaccharides formation. A symbolic description in Figure 1 shows the different pretreatment conditions from three of the major plant cell wall components (glucan, xylan and lignin) before and after pretreatment (IL and OV). Untreated AGB showed a particularly low glucan (25.8%) and high lignin (28.0%) values than in previous reports, which are in the range of 31-45% and 15-20% for glucan and lignin, respectively (Caspeta et al., 2014; Davis et al., 2011; Perez-Pimienta et al., 2016a). A recent study investigated four different sources of untreated *Agave tequilana* bagasse with a glucan content ranging from 21.4 to 40.2% (Hernández-Meléndez et al., 2016). According to Perez-Pimienta et al. (2016a), “this relatively large difference between glucan content could be attributed to specific environmental conditions from the biomass origin, particle size, extraction, and post-harvest procedures, as well as process conditions mainly during the cooking step during tequila production”.

During IL pretreatment, a total of 9,000 kg of DI water was used per 100 kg of raw AGB to remove the residual IL in a washing process. A recycle step can be used to recover the IL in the wash stream that can be reused in further pretreatments. Based on the untreated solids a

total mass fraction of 71.2% was recovered after IL pretreatment. Sulfuric acid, and other mineral acids, are good catalysts that help to remove hemicellulose (Caspeta et al., 2014), and in the presence of a solvent, like ethanol, promotes lignin solubilization. The liquid hydrolysate resulting from OV after being properly detoxified contains a large fraction of xylose and lignin which can be transformed into ethanol or other value-add products (Caspeta et al., 2014). The recovered solid biomass after OV pretreatment was 53.8% from the raw AGB, mainly due to high xylan removal (86%) during the process. The removed xylan ratio of OV after pretreatment is higher than the values (62%) from a previous report where AGB was pretreated using autohydrolysis at 180 °C and 140 psi (Perez-Pimienta et al., 2016a). Currently, 450 kg of a 25% ethanol solution plus 450 kg of water is required after OV pretreatment to wash 100 kg of the biomass. Both pretreatments were able to remove a large fraction of lignin (~45%) when compared to the untreated biomass. The solids stream was subjected to the SESF strategy. The first stage of the SESF strategy, where only pure enzymatic hydrolysis occurred and lasted for 18 h. For IL-AGB, 20.9 kg of glucose and 8.3 kg of xylose were produced during the saccharification stage, whereas for OV-AGB 26.8 kg of glucose and 2.5 kg of xylose were obtained, per 100 kg of initial raw biomass. No significant differences were reached from the combined sugar production (29.2 kg vs. 29.3 kg) for both pretreatments. In the solid (pretreated biomass) and liquid (washed materials) streams, besides glucan, xylan and lignin, other components, such as ashes, extractives, protein, and other carbohydrates (e.g. arabinan and galactan) are present (Caspeta et al., 2014; Perez-Pimienta et al., 2013, 2016a). Nevertheless, in order to simplify the mass balance, we decided to only track the composition of the major cell wall components: glucan, xylan and lignin.

In the subsequent step of fermentation, all monomeric sugars available from the hydrolysates obtained during the saccharification stage from both pretreatments were consumed by the ethanologenic bacteria. The final ethanol production yields were 12.1 and 12.7 kg per 100 kg of untreated AGB for IL and OV, respectively. It can be shown that the ethanol values from the pretreatments evaluated are a direct effect of the process used. When the ethanol yields from both pretreatments are compared to that obtained after the untreated sample fermentation (5.7 kg; see Table 1) on the same basis, a significant difference is presented.

The solids residues after SESF process are composed of the non-consumed sugars during fermentation (3.5 kg from IL and 1.9 kg from OV of glucan plus xylan) and lignin, 15.0 and 15.3 kg of lignin for IL and OV pretreatments, respectively, per 100 kg of inlet biomass. Potentially, these solids could be used to generate electricity or phenolic derivatives in a biorefinery configuration.

From a biorefinery processing perspective, an OV-based process could use the xylose-rich wash stream generated after pretreatment (15.2 kg xylan per 100 of initial AGB) hence increasing the overall ethanol yield. In the same way, an IL-based biorefinery could use the liquid wash stream containing xylan plus lignin where they can be upgraded to value added products including new ILs derived from lignin such as the one presented by Socha et al. (Socha et al., 2014), "*N*-ethyl-*N*-(4-methoxybenzyl)ethanamine dihydrogen phosphate salt (*[p*-AnisEt₂NH][H₂PO₄])" that have shown significant potential to design a "closed loop" process for lignocellulosic biorefineries.

3.2. Effect of pretreatment on biomass crystallinity and chemical changes

Crystallinity index (CrI) of cellulose is acknowledged as an important characteristic that affects cellulose hydrolysis and its efficiency after biomass pretreatment (Sathitsuksanoh et al., 2011). The XRD spectra of untreated and pretreated AGB are shown in supporting information, Figure SI. The spectrum of untreated AGB shows distinctive and prominent peaks at $2\theta = 14.8^\circ$, 24.2° , 30.0° and 38.0° corresponding to calcium oxalate (CaOX) monohydrate as previously reported (José A. Perez-Pimienta et al., 2016; Perez-Pimienta et al., 2015). When compared to IL and OV-pretreated AGB, all these CaOX peaks decrease in intensity.

The cellulose CrI was estimated by separating crystalline and amorphous contributions to the diffraction spectrum. The CrI of untreated AGB was 39.0% whereas IL decreased to 22.6% and OV increased to 44.6%. In addition, the main peak at around 22.1° shifted to a lower angle and became broader after IL pretreatment up to 20.7° , which is clear evidence of its transformation to cellulose II (Cheng et al., 2011). By contrast, a broad and composite peak at around 16.0° dropped its intensity and turned into a weak shoulder peak. These effects, including crystallinity decrease, is consistent with a severe distortion of cellulose I lattice which did not occur during OV pretreatment where, in contrast, the crystallinity index increased due to the high removal of amorphous fractions (xylan) (Sathitsuksanoh et al., 2011).

Moreover, Fourier transform infrared spectroscopy was used to track the changes in chemical composition of untreated and pretreated AGB solids (supporting information, Figure SII). To monitor the chemical changes after pretreatment, a total of seven unique carbohydrate and lignin bands were used. Additionally, two bands related to calcium oxalate were used in the FTIR data. The relative changes of untreated and pretreated AGB samples from the assignment of band positions are shown in Table 2 (Kumar et al., 2009).

The peak near 3348 cm^{-1} for the latter represents O-H stretching, where IL pretreatment in this peak had a significant reduction in the intensity which indicated cellulose hydrogen bonds disruption (He et al., 2008). In contrast, the band position at 2900 cm^{-1} attributed to C-H stretching, where both (IL and OV) increase when compared to the untreated AGB that would suggest that the methyl and methylene portions of cellulose were not affected during pretreatment. Regarding the bands positions attributed to CaOX at 1622 and 1321 cm^{-1} , all materials exhibited these distinctive peaks with an intensities reduction in the pretreated samples (supporting information, Figure SII) (Perez-Pimienta et al., 2016b). In addition, these CaOX peaks are not found at these intensities in other common biofuel feedstocks (Kumar et al., 2009; Li et al., 2011). The band at 1745 cm^{-1} which is associated with carbonyl C=O stretching decreased to a major extent for IL than OV, indicating more cleavage of lignin removal and side chains (Kumar et al., 2009). Typically, the band at 1510 cm^{-1} (aromatic skeletal from lignin) is tracked, but a high CaOX peak overlaps the lignin peak which is a characteristic feature of AGB that are discussed elsewhere (Perez-Pimienta et al., 2016b). Furthermore, a major decrease is observed with IL than with OV in the intensity of the band at 1235 cm^{-1} (C-O stretching in lignin and hemicellulose) whereas in the 1027 cm^{-1} band (C-O stretching in cellulose and hemicellulose) OV increased to a higher extent than IL pretreatment, due to their particular process characteristics for lignin and xylan removal with IL and OV, respectively (Li et al., 2011). Finally, the peak at 900 cm^{-1} is observed to decrease in the pretreated samples due to lignin and xylan removal in IL and OV-treated biomass which is reflected in a relative glucan content increase. Furthermore, MALDI-TOF mass spectroscopy is a spectroscopic technique applied to investigate and generate a semiquantitative fingerprint of particular cell wall polysaccharides with the limitations of matrix signals which was appeared in the lower

mass ranges (Awal and Sain, 2011). MALDI-TOF mass spectrum of untreated and pretreated AGB are shown in supporting information, Figure SIII. The repeating unit of biomass samples corresponds to a hexose (glucose), which is characteristic of cellulosic materials. The samples could contain adducts that can be formed by H^+ , Na^+ and NH_4^+ ions. When compared, the observed average molecular weight of untreated AGB is similar to IL-AGB with 1670 m/z. In contrast, OV-AGB shows 1514 m/z, a lower average value than raw AGB, probably due to the high xylan removal after pretreatment.

3.3. Effect of pretreatment on biomass morphology

The confocal fluorescence and scanning electron microscopy high-resolution images from the AGB samples are shown in supporting information, Figure SIV. The intact plant cell wall structure of AGB is shown in supporting information, Figure SIV-A and SIV-D, with distinctive fluorescence intensities for lignin (blue) and cellulose (green). In contrast, the pretreated samples, shown evident morphological differences when compared to the untreated AGB. Ionic liquid pretreatment disrupts the lignin-carbohydrate linkages by removing lignin and during the process of cellulose dissolution and regeneration. Pretreatment with $[C_2C_1Im][OAc]$ in AGB resulted in a structural modification into the organization of microfibrils with a rough and swollen surface (Fig. SIV-B and Fig. SIV-E). Otherwise, organosolv pretreatment disarray the plant cell wall structure by removing hemicellulose in great extent, however, the main cellulose structures remain (Fig. SIV-C and Fig. SIV-F). In addition, elemental analysis (weight percentages) using EDS of untreated and pretreated AGB are shown in supporting material, Table SI. The measurable calcium content was removed during the OV pretreatment when compared to the untreated sample (1.2%). In contrast, IL-AGB showed a minor decrease in calcium concentration

(1.0%). Calcium content of AGB are within range when compared to others agave species (1.7-6.1%), but similar to a recent report (1.4%) (Ávila-Lara et al., 2015; Perez-Pimienta et al., 2016b). However, Perez-Pimienta et al. (Perez-Pimienta et al., 2016b) showed that calcium content in AGB would not cause an effect on pretreatment unless a large fraction of calcium is separated from the plant cell wall and an oxidative process is applied. The calcium concentration in agave bagasse is mostly located in the form of calcium oxalate in a monohydrated form, which has been reported that after ionic liquid pretreatment using [C₂C₁Im][OAc] does not liberate/solubilize from the plant cell wall (as in the organosolv pretreatment) but rather agglomerate as crystals of different sizes (Perez-Pimienta et al., 2015).

3.4. Effect of pretreatment on sequential enzymatic saccharification and fermentation (SESF)

The kinetic profiles of saccharification and fermentation using IL and OV pretreated agave bagasse are shown in Figure 2 and Table 1 summarizes the sugar conversion, ethanol yields and ethanol volumetric productivity. The highest sugar production was obtained with OV-pretreated AGB, reaching 67.7 g glucose/L and 5.6 g xylose/L at 18 h of saccharification. With the IL pretreated bagasse 36.3 g glucose/L and 14.4 g xylose/L were obtained at 18 h. Furthermore, the glucan to glucose conversion from both samples was above 90%, whereas xylan to xylose conversion were higher than 83%. These results showed a significant improvement compared to the saccharification of untreated samples in similar hydrolysis conditions, but in a longer time period (72h) (Perez-Pimienta et al., 2016a). In the same way, glucose and xylan conversions are comparable to previous reports using IL and OV in agave bagasse (Caspeta et al., 2014; Perez-Pimienta et al., 2016a). As

expected, differences between glucan and xylan conversion are derived from the
 physicochemical effects carried out by each pretreatment on the AGB. Wherein organosolv
 pretreatment removes a considerable amount of xylan and lignin, resulting in improved
 cellulose accessibility while ionic liquid pretreatment accomplish cellulose decrystallization,
 and a significant lignin removal leading to an increase of sugar conversion (Cheng et al.,
 2011; P. Kumar et al., 2009). Similarly, as observed in FTIR key recalcitrance-causing
 factors in the AGB can be decreased with component removal (lignin, xylan and/or calcium
 oxalate), crystallinity in IL-AGB, and increasing the accessible surface area contributed to
 improve overall process yields when compared to the untreated sample.

The results shown in Figure 2 indicate that both glucose and xylose produced during the
 saccharification stage from pretreated AGB were efficiently fermented to ethanol by *E. coli*
 MS04. The kinetic profiles had a similar pattern for glucose and xylose consumption in
 both samples, with a complete consumption of both sugars before 50 h of the whole process
 or 32 h of fermentation elapsed time. The ethanol yield for the syrup obtained from the
 saccharification of IL-AGB was 81.6% and the 24 h volumetric productivity of ethanol was
 0.68 g_{EtOH}/(L h) at 24 h of the fermentation stage; while for OV-AGB the obtained values
 were 85.3 % and 1.20 g_{EtOH}/(L h).

These values are in good concordance with previous studies in which similar process
 conditions were used to achieve high sugar conversion for agroindustrial biomass (e.g. corn
 stover and wheat straw) using [C₂C₁Im][OAc] in IL pretreatment and a combination of
 H₂SO₄, ethanol and water in OV pretreatment (Uppugundla et al., 2014). The SESF high
 fermentation efficiency can be explained by the simultaneous consumption of glucose and
 xylose by *E. coli* MS04 in the same reactor with no solids removal (where the process

temperature was modified accordingly to the enzyme/bacteria requirements)hence improving overall ethanol yields (Pedraza et al., 2016).

Moreover,in a previous report by Vargas-Tah et al.(2015) using *E. coli* MS04 for the fermentation of pretreated corn stover hydrolysates, the SESF was performed in the presence of solids where all sugars were consumed; indicating that when *E. coli* MS04 is used, solid removal is not necessary, in comparison to othersreports where solids were separated from the solid/liquid slurry(Saha et al., 2013).Additionally, even if the total ethanol production from AGB is lower than for some energy crops, the ethanol yield per land area could be higher when the high productivity of agave is considered (20 to 44 Mg/ha year) obtaining3067-7082 L/ha year, favorable when compared to others biomass feedstocks such as corn (2050 L/ha year) orsugarcane (4900 L/ha year) (Iddrisu and Bhattacharyya, 2015; Yan et al., 2011).

We have shown the potential of Agave as abiofuel feedstock that include different advantageous features (e.g. low water requirements and drought tolerant) which could be potentially allocated in semiarid regions (~20% world surface) and be introduced in abandoned or low productivity areas representing one major opportunity for biofuel feedstocks because this will minimize indirect land use change (Davis et al., 2011; Somerville et al., 2010).

To recap, this study reports the production of ethanol from pretreated agave bagasse with a high lignin content (28 % w/w), a bioenergy crop which has not been deeply studied; furthermore, a sequential saccharification and fermentation strategy was used to process the bagasse to bioethanol. The two pretreatments used, ionic liquid and organosolv, enabled cellulose morphological changes, an efficient lignin removal, and therefore glucan conversion during saccharification with a concomitant xylan hydrolysis. The C5 and C6

sugars, obtained from the saccharification process, were simultaneously and efficiently fermented to ethanol by the metabolic engineered *E. coli* strain MS04. Finally, agave bagasse was explored as a feedstock for ethanol production, and side-by-side mass balances and different characterization techniques, including one that is rare in biomass characterization such as MALDI-TOF (besides of typical ones, including XRD, FTIR and SEM), allowed to propose the use of this feedstock in a biorefinery configuration.

4. Conclusions

A SESF strategy was evaluated in IL and OV-pretreated AGB for ethanol production. IL and OV pretreatments removed a large fraction of xylan and lignin while modifying the structure and cellulose crystallinity of AGB. Both processes achieved a high glucan (>90%) and xylan (>84%) conversion in the hydrolysis stage and subsequent high ethanol yield for IL (82%) and OV (85%). Final ethanol production yields were 12.1 and 12.7 kg per 100 kg of untreated AGB for IL and OV, respectively. These comparative analyses showed the advantages of SESF in an Agave bagasse biorefinery configuration when compared to traditional SHF process.

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642

FIGURES CAPTIONS

No	Title
1	Mass balance per 100 kg of untreated AGB during sequential enzymatic saccharification and fermentation (SESF) for ionic liquid (top) and organosolv (bottom) pretreatment.
2	Kinetic profiles of SESF using ionic liquid (IL, left) and organosolv (OV, right) pretreated agave bagasse with commercial enzymes and ethanogenic <i>E. coli</i> strain MS04. Error bars show the standard error of duplicate measurements.

Figure 1

Figure 1.

Figure 2

Table 1. Comparison of sugar and ethanol yield/conversion of ionic liquid and organosolv pretreated agave bagasse using SESF.

Pretreatment method	18 h sugar conversion (%)		TY _E (kg)	Y _E (%)	Q _{EtOH 24h} (g _{EtOH} /L h)
	Glucan	Xylan			
Untreated	26.0*	13.1*	5.7*	-	-
Ionic liquid	89.8	83.8	14.9	81.6	0.68
Organosolv	93.3	89.7	14.9	85.3	1.20

TY_E = theoretical ethanol yield (kg) from a 100 kg basis.

Y_E = ethanol yield (%).

Q_{EtOH 24h} = volumetric productivity of ethanol at 24 h elapsed fermentation time.

* Sugar conversion at 72 h. Adapted from Perez-Pimienta et al. [2016a]

Table 2

Table 2. Relative changes in agave bagasse after ionic liquid (IL) and organosolv (OV) pretreatments.*

Band	Assignment	Pretreatment	
		IL	OV
900	Anti-symmetric out of plane ring stretch of amorphous cellulose	18.0	0.6
1027	C-O stretching in cellulose & hemicellulose	-1.0	-23.7
1235	C-O stretching in lignin & hemicellulose	44.0	17.4
1321	C=O stretching of calcium oxalate	4.6	-9.3
1375	C-H deformation in cellulose & hemicellulose	-16.9	-3.1
1622	C-O stretching of calcium oxalate	39.0	40.5
1745	Carbonyl (C=O) stretching	79.8	44.0
2900	C–H stretching (related to rupture of methyl/methylene group of cellulose)	-12.0	-29.0
3348	O–H stretching (indicates rupture of cellulose hydrogen bonds)	16.6	-30.4

* %Relative change = 100*[(intensity of untreated solids – intensity of pretreated solids) / intensity of untreated solids]; where positive numbers indicate reduction.