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Effective hydrothermal-enzymatic sequential process of agave bagasse enhances saccharification and methane production

Dendera Munguía-Aguilar [a], Héctor A. Ruiz [b], Elías Razo-Flores [a], César Nieto-Delgado [a], Edith Cadena-Chamorro [c], Felipe Alatriste-Mondragón [a,*]

[a] Instituto Potosino de Investigación Científica y Tecnológica A.C., División de Ciencias Ambientales, Camino a la Presa San José 2055, Lomas 4A, Sección, C.P. 78216 San Luis Potosí, SLP, Mexico
[b] Biorefinery Group, School of Chemistry, Universidad Autónoma de Coahuila, 25280 Saltillo, Coahuila, Mexico
[c] Facultad de Ciencias Agrarias, Universidad Nacional de Colombia, Sede Medellin, Carrera 65 N° 59A –110, Medellin, Colombia

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ABSTRACT

The efficiency of enzymatic saccharification of lignocellulosic biomass is a key step for biofuels production in the biochemical biorefineries. A novel sequential process, hydrothermal pretreatment followed by enzymatic hydrolysis, of agave bagasse was investigated to improve the release of sugars and the methane production. After optimization of the hydrothermal pretreatment by surface response methodology, a hemicellulose removal of 93.6% was obtained at 180 °C/50 min. Hemicellulose removal enhanced cellulose depolymerization during the enzymatic hydrolysis of the pretreated bagasse by 2.3 times as compared to the untreated bagasse. The single-stage batch methane production of the hydrothermal and enzymatic hydrolysates were 0.223 ± 0.02 NL CH_4/g COD_{add} and 0.305 ± 0.03 NL CH_4/g COD_{add} respectively, indicating a higher biodegradability of the enzymatic hydrolysate. The combined energy recovered from the hydrothermal and enzymatic hydrolysates was 3.4 times greater than that recovered from untreated agave bagasse hydrolysate. Also, the combined energy recovery was higher than the energy recovery reported in the literature for agave bagasse pretreated with other chemical and hydrothermal pretreatments. Overall, this study enhanced the saccharification efficiency and biodegradability of polysaccharides present in agave bagasse through efficient saccharification of cellulose and hemicellulose. This was reflected in a high value of combined energy recovery efficiency (54.1%) in the form of methane.

1. Introduction

Use of renewable natural resources are necessary for a transition to a green and circular economy and lignocellulosic biomass biorefineries could contribute to reach the goal. They have been recognized as an important feedstock for methane production due to their abundance and the sugar content in the form of hemicellulose and cellulose [1]. However, it is necessary to solubilize those sugars to improve the production of methane from lignocellulosic substrates. For this purpose, several biomass pretreatments have been reported in the literature, including physical, chemical, biological, and enzymatic treatments [2]. After processing the biomass with one or a combination of treatments, a liquid fraction known as hydrolysate is obtained, and contains the solubilized sugars used for biofuels production.

Two types of widely used pretreatments to remove hemicellulose are chemical (diluted or concentrated acid hydrolysis) and hydrothermal (steam explosion and autohydrolysis). Autohydrolysis is also called liquid hot water. These pretreatments are the most used to solubilize hemicellulose into its monomers and oligomers by breaking the ester and ether bonds between sugars present in hemicellulose [3]. They are used as an initial pretreatment to remove hemicellulose with the goal of enhancing cellulose availability, thereby increasing its depolymerization in a subsequent enzymatic hydrolysis [4]. Several commercial enzyme preparations are commonly used to carry out the saccharification of the sugars present in lignocellulosic biomass for biofuel production such as Celluclast 1.5 L, Cellic® CTec2 [5] and Cellic® CTec3 [6]. Agave bagasse (*Agave tequilana* Weber var. Azul) is a lignocellulosic biomass produced during the manufacture of tequila, composed by three main fractions hemicellulose (11–22% w/w), cellulose (31–43% w/w) and lignin (11–20% w/w) [2]. From 2021–2022, an average of 786,400 ± 21,080

* Corresponding author.
E-mail addresses: dendera.munguia@ipicyt.edu.mx (D. Munguía-Aguilar), hector_ruiz_leza@uadec.edu.mx (H.A. Ruiz), erazo@ipicyt.edu.mx (E. Razo-Flores), cesar.nieto@ipicyt.edu.mx (C. Nieto-Delgado), emcadenac@unal.edu.co (E. Cadena-Chamorro), falatriste@ipicyt.edu.mx (F. Alatriste-Mondragón).

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tons/year of agave bagasse were produced causing diverse environmental problems [7]. Valorization of this residual biomass is being investigated because final disposal is problematic and complex. It has a high potential to produce methane due to its abundance, renewability, and sugar content in the form of cellulose and hemicellulose [8,9].

The depolymerization of cellulose sugars can be achieved by enzymatic hydrolysis. Enzymatic saccharification of untreated agave bagasse has been studied, although saccharification yields lower than 60% were reported [5,10]. On the other hand, the enzymatic saccharification of pretreated bagasse has been studied to increase the saccharification yield. Two approaches to improve enzymatic saccharification have been studied. One of them is to remove the hemicellulose before enzymatic saccharification using chemical and hydrothermal pretreatments [11–13]. The other one is to remove the lignin first using alkaline hydrogen peroxide, organosolv and ionic liquids pretreatments among others [14–16].

Regarding hydrothermal pretreatments, autohydrolysis (hereinafter called hydrothermal pretreatment) is a thermal method that applies high temperatures and pressures using heated water to initiate biomass decomposition followed by slow decompression [17]. On the other hand, in a steam explosion treatment, high pressures and temperatures are achieved using steam followed by slow decompression [18]. Hydrothermal pretreatment has been reported for hemicellulose removal from agave bagasse with high removal yields of 99.9%, with a severity factor between 3.7 and 4.28, temperatures > 190 °C, and treatment times between 10 and 50 min [4,19]. Pino et al. [19] hydrothermally pretreated bagasse followed by enzymatic hydrolysis of the pretreated bagasse using Cellic® CTec2 obtaining enzymatic saccharification yields between 80% and 100% of glucans. The enzymatic hydrolysate was used for bioethanol production. The other pretreatment used to remove hemicellulose from agave bagasse is steam explosion. In this case, removals of 41% have been reached [20]. Regarding the pretreatment by acid hydrolysis of agave bagasse, there are several published reports [11, 13]. However, they do not report the efficiency of hemicellulose removal.

Regarding methane production there are some reports using steam explosion hydrolysate from agave bagasse. However, methane production from hydrothermal hydrolysates has not been reported. Valdez-Vazquez et al. [12] found a biomethane potential (BMP) of 0.225 NL CH_4 /g COD_{add}. Duran-Cruz et al. [21] evaluated the methane production from agave bagasse hydrolysates obtained from a sequential process where hemicellulose is first removed by a steam explosion treatment, followed by an enzymatic saccharification using Cellic® CTec2. They reported a BMP of 0.169 ± 0.03 NL CH_4 /g COD_{add} for the hydrothermal hydrolysate and a BMP of 0.284 ± 0.02 NL CH_4/ g COD_{add} for the enzymatic hydrolysate. Arreola-Vargas et al. [11] reported energy recovery efficiencies in form of methane of 10.52 kJ in a one-stage digestion process (only methane) of an enzymatic hydrolysate from agave bagasse. In the case of methane production from delignified agave bagasse there is a report using alkaline hydrogen peroxide [14] and ionic liquids [15]. In the case of ionic liquids Pérez-Pimienta et al. [15], reported a delignification of 45.4%, an enzymatic saccharification of 82% and BMP of 0.300 NL CH_4 /g COD_{add}.

Therefore, the improvement of sugars recovery from agave bagasse could increase both, the amount of methane production and the energy recovery efficiency. Thus, the objective of this work was to improve the release of sugars from both, the hydrothermal pretreatment and the enzymatic hydrolysis of agave bagasse and to evaluate the effect of this on the energy recovery by methane production. Optimization of hydrothermal pretreatment of agave bagasse by surface response methodology (SRM) was carried out to maximize hemicellulose hydrolysis followed by an effective enzymatic hydrolysis that included both, amorphous and crystalline cellulose, using Cellic® CTec2 enzyme. Biomass characterization was performed before and after hydrothermal treatment. The hydrolysates of the hydrothermal and enzymatic treatments were used to evaluate the methane production in batch conditions and the energy recovery efficiency was calculated. It is important to highlight that the proposed sequential process, hydrothermal treatment followed by enzymatic saccharification, for methane production from agave bagasse is a novel process that has not been studied. Also, a comprehensive comparison with other pretreatment process for agave bagasse reported in the literature is discussed.

2. Materials and methods

2.1. Agave bagasse

Bagasse from *Agave tequilana* Weber var. Azul was obtained from Casa Herradura, a tequila distillery located in Amatitán, Jalisco, México. The bagasse was sun-dried and ground with an agricultural grinder, then washed with tap water and sun-dried again. Subsequently, it was sieved to obtain a particle size of < 1 mm.

2.2. Hydrothermal pretreatment (autohydrolysis)

Hydrothermal pretreatment was done in a stainless-steel pressurized batch reactor with a total volume of 190 mL with a working volume capacity of 100 mL with a PID temperature controller that allowed to set the reactor temperature and the residence time (isothermal heating-stage) as described by Shiva et al. [22]. The agave bagasse was mixed with distilled water at a load of 10% (w/v). To optimize the hydrothermal pretreatment a response surface methodology (RSM) with a central composite design (CCD) was used. The significance of the model was evaluated by analysis of variance (ANOVA) [23]. The fit of the model was evaluated by determining the adjusted R_2. The effects of the independent variables on the response variable are presented in the 3D surface plot. A quadratic polynomial equation was obtained to describe the mathematical relationship between the response variable with the independent variables evaluated. After obtaining the best response predicted by the RSM, the best conditions were confirmed. An experimental assay of the hydrothermal pretreatment under the best conditions was performed. The results were analyzed using the Design Expert 7.0 software.

The CCD design is summarized in Table 1. It included three central points (assays 5–7) assays, and a 95% confidence level was used during analysis [23]. Two independent variables were evaluated in this design: temperature (150–180 °C) and residence time (10–50 min). The response variable was the percentage of hemicellulose removed in the agave bagasse. The severity factor was used to compare the different CCD assays and was calculated using Eqs. 1 and 2 [19].

$$\log \quad R_o = [R_o \quad heating] + [R_o Isothermal \quad processing] + [R_o \quad cooling] \tag{1}$$

$$\log \quad R_o = \left[\int_0^{t_{max}} \frac{T(t) - 100}{\omega} dt\right] + \left[\int_{ctrl}^{ctrf} \exp \quad \left[\frac{T(t) - 100}{\omega}\right] \quad dt\right] + \left[\int_0^{t_{max}} \frac{T'(t) - 100}{\omega} dt\right] \tag{2}$$

Where [log R_o] is the severity factor, t_{max} is the time (min) needed to attain the maximum hydrothermal temperature, *ctrl* and *ctrf* are the time (min) needed for the heating-cooling stages, respectively. The value 100 is the temperature of reference, T(*t*) y T′(*t*) (°C) are the temperature profiles in heating and cooling respectively, and ω is an empirical parameter (value of 14.75) related to activation energy.

The suspension obtained after hydrothermal pretreatment was filtered using Whatman No. 1 filter paper to separate the solid (called agave bagasse fiber hydrothermally pretreated, AB-HP) from the liquid phase (called hydrothermal hydrolysate, HH). The AB-HP was washed 3 times with distilled water and the moisture content was determined. The solid recovery yield was calculated as the percentage of initial bagasse solids recovered after hydrothermal pretreatment.

Table 1
Experimental conditions, response variable and the characterization of the remaining agave bagasse fibers for each assay evaluated in the CCD of the hydrothermal pretreatment[a].

Assay	Experimental conditions		Response variable	[log R_o]	pH[b]	Solids recovery	Composition of the remaining fiber (% dry weight)		
	Temperature (°C)	Time (min)	Hemicellulose removal (%)			(%)	Cellulose	Hemicellulose	Lignin
1	150	10	61.6	3.5	4.2	84	28.8 ± 1.3	6.4 ± 0.7	18.9 ± 0.9
2		30	69.7	3.9	4.1	80.9	31.2 ± 1.5	5.2 ± 0.7	20.1 ± 0.7
3		50	80.7	4.1	3.9	77.2	33.0 ± 1.9	4.4 ± 0.5	21.6 ± 0.9
4	165	10	70.6	3.6	4.1	82.2	31.5 ± 1.7	5.0 ± 0.8	19.9 ± 0.8
5[c]		30	80.7	3.8	3.8	70.7	34.2 ± 1.8	3.6 ± 0.5	21.1 ± 0.6
6[c]			80.3	4	3.9	70.8	33.5 ± 1.4	3.9 ± 0.9	22.1 ± 2.3
7[c]			80.4	4	3.7	70.1	34.9 ± 1.4	3.5 ± 0.4	21.4 ± 0.2
8		50	85.6	4.1	3.6	65.8	36.6 ± 1.8	3.2 ± 0.4	23.4 ± 0.9
9	180	10	82.9	3.7	3.7	67.8	34.7 ± 1.1	3.5 ± 0.3	23.3 ± 0.9
10		30	91.3	4.1	3.6	62.8	39.0 ± 0.9	2.1 ± 0.8	25.4 ± 1.1
11		50	96.2	4.4	3.5	59.6	43.5 ± 1.1	0.9 ± 0.8	26.9 ± 0.8
12[d]	180	50	94.5	4.4	3.5	58.4	42.6 ± 2.1	1.3 ± 0.5	25.4 ± 1.9

[a] Hydrothermal pretreatment was done in the 190-mL stainless steel reactor (ABHP-R_1)
[b] Final pH of the hydrothermal hydrolysate.
[c] Central points of central composite design (CCD).
[d] Experimental verification assay of best condition predicted by CCD.

2.3. Scale-up of hydrothermal pretreatment

The best condition for the hydrothermal pretreatment obtained with the CCD using the 190 mL pressurized stainless-steel reactor (ABHP-R_1) was replicated in a stained-steel reactor with a larger volume of 662 mL (ABHP-R_2) and a working volume of 300 mL, with a PID temperature controller and mechanical agitation at 120 rpm. The severity factor was also calculated according to Pino et al. [19].

2.4. Enzyme preparation

A commercial cellulase cocktail (Cellic® CTec2) from *Trichoderma resei* (Novozymes, USA) was used. Cellulase activity was measured as Filter Paper Units (FPU) per mL of enzyme (FPU/mL) as reported by Adney and Baker [24]. The initial enzymatic activity was 125 FPU/mL.

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis was carried out using the best performance condition reported by Aguilar et al. [4]. A solid load of 10% (w/v) of dry untreated or pretreated agave bagasse, using citrate buffer (50 mM) to maintain the pH at 4.8 and 15 FPU/g glucan of Cellic® CTec2 were used. Maximum cellulose sugar solubilization was determined by following the glucose concentration over time. For this, samples were taken at 0, 6, 12, 24, 48, and 72 h. Two experimental controls were included, an enzyme control (containing only the enzyme preparation in citrate buffer) and a bagasse control (containing only bagasse in citrate buffer); contribution of glucose from both controls were subtracted for each experiment to calculate the net amount of the saccharified sugars from the agave bagasse. The saccharification yield (%) of cellulose to glucose was calculated according to Eq. 3 reported by Shiva et al. [22].

$$Saccharification\quad yield\quad (\%) = \frac{[Glucose] + 1.053[Cellobiose]}{1.111f[biomass]}\quad x100 \qquad (3)$$

Where [*Glucose*] is the glucose concentration in the enzymatic hydrolysate (g/L), [*Cellobiose*] is the cellobiose concentration in the enzymatic hydrolysate (g/L), [*biomass*] is the dry agave bagasse, untreated or hydrothermally pretreated, used for the enzymatic saccharification (g/L), *f* is the fraction of cellulose in dry agave bagasse, untreated or hydrothermally pretreated (g/g), 1.111 is the factor used for the conversion of cellulose into glucose equivalent and 1.053 is a factor to convert cellobiose to equivalent glucose.

2.6. Bagasse fiber characterization

Untreated agave bagasse (AB-UT), AB-HP, and enzymatic hydrolyzed AB-HP fibers were characterized according to the National Renewable Energy Laboratory (NREL) technical reports. Extractives and ashes were analyzed using NREL/TP-510–42619 [25] and NREL/TP-510–42622 [26], respectively. Polysaccharides and acid soluble and insoluble lignin were analyzed by NREL/TP-510–42618 [27]. The sugars (glucose, xylose, cellobiose and arabinose) and chemical by-products (acetic and formic acids, furfural and hydroxymethyl furfural) present in the liquid hydrolysate of the bagasse fibers analyzed by NREL/TP-510–42618 were determined by HPLC. Hydrolysis samples were filtered through a 0.45-m nylon filter and analyzed using an Agilent 1260 Infinity II HPLC system. Details of analysis conditions are reported by Pino et al. [19].

Morphological characteristics of the agave bagasse fibers before and after the hydrothermal pretreatment as well as after enzymatic hydrolysis, were analyzed under the Environmental scanning electron microscope (SEM) model ESEM-Quanta 200, FEI.

The cellulose crystallinity index (CI) of bagasse fibers, before and after each treatment, was analyzed using a Rigaku, SmartLab X-Ray Diffractometer (XRD), with Cu Kα radiation (λ = 1.5818 Å) source. The lamp was excited at 40 keV and 44 mA. The samples were processed in an interval of 5–70° in 2θ with a step size of 0.01°. The CI was calculated using the deconvolution method reported by Ibbett et al. [28] The data was processed with the MagicPlot Student 2.9 software.

Functional groups were determined by attenuated total reflection Fourier transform infrared analysis (ATR-FTIR), using a Thermo Scientific spectrophotometer (Nicolet 6700, USA) with single reflection ATR in the range of 4000–400 cm^{-1}, with a spectral resolution of 4 cm^{-1} and 128 scans [29]. Each dry sample (10–20 mg) was placed in the ATR accessory.

Thermogravimetric analysis (TGA) of AB-UT and AB-HP dried fibers (60 ºC for 24 h), was made with a versa Therm HS model Cahn TGA analyzer (Thermo Fischer Scientific, NY, USA) at a heating rate of 10 °C min^{-1} under nitrogen supply (20 mL min^{-1}) and around of 25 mg of each dry sample. Dataset was plotted as both, weight (%) and derivate of thermogravimetric (DTG, %/°C) curve as a function of temperature. The analysis was made between 50 °C and 800 °C. Data was processed using SigmaPlot 11.0 software.

2.7. Chemical characterization of hydrolysates

The glucose concentration of samples from enzyme hydrolysis time-series experiments was determined by HPLC [30]. In the case of BMP assays sugar content of HH, enzymatic hydrolysate of untreated agave

bagasse (EH-ABUT) and enzymatic hydrolysate of hydrothermally pretreated agave bagasse (EH-ABHP) were measured as total sugar (TS) content according to the phenol-sulfuric method [31]. Also, the chemical oxygen demand (COD) was determined according to Standard Methods [32].

2.8. Biochemical methane potential test

2.8.1. Inoculum

Granular anaerobic sludge was obtained from a full scale UASB reactor treating tequila vinasses at Casa Herradura (Amatitán, Jalisco, Mexico). Total solids and volatile solids contents were determined as described by the Standard Methods [32]. The sludge had a total solids content of 0.15 g/g wet sludge and a volatile solids concentration of 0.14 g/g wet sludge.

2.8.2. Batch methane production experiments

The BMP tests were carried out on an automatic gas monitoring instrument AMPTS-II (Automatic Methane Potential Test System, Bioprocess Control, Lund, Sweden), according to Tapia-Rodríguez et al. [10]. The concentration of the substrate in each experiment was 5 g COD/L. In the case of the BMP enzyme controls, the concentration was 0.8 g COD/L for the enzymatic control of EH-ABUT and 0.4 g COD/L for the enzymatic control of EH-ABHP. BMP assays were carried out using three different substrates HH, EH-ABUT, and EH-ABHP. Also, a positive control (using glucose as model substrate), a negative control (endogenous, using only inoculum without substrate) and two BMP enzyme controls were prepared. For the BMP enzyme control-EHABHP and for the enzyme control-EHABUT, a volume of the enzyme hydrolysis control (described in the Section 2.5) equal to the volume of the EH-ABHP or EH-ABUT respectively, were used for the BMP assay. The BMP assays were carried out in triplicate. The BMP values for the EH-ABUT and EH-ABHP were corrected considering the BMP values of endogenous and enzymatic controls.

Anaerobic biodegradability was calculated by comparing each BMP of the different types of hydrolysates evaluated to the theoretical BMP, based on the calculations reported by Buitrón et al. [8]. Energy recovery efficiency (EFE) was calculated as the percentage of the calorific value contained in the dry bagasse recovered as methane using Eq. (4) [33].

$$ERE \quad (\%) = \frac{(COD \quad hydrolysate)(BMP \quad hydrolysate)(35.8)}{(biomass)(16.35)} \times 100 \qquad (4)$$

Where, ERE is the energy recovery efficiency (%), COD hydrolysate correspond to the COD concentration of each hydrolysate (g COD/L); BMP hydrolysate in the cumulative methane production (NL CH_4/g COD_{add}), biomass is the solid load of agave bagasse with or without pretreatment (g /L), used to obtain the different hydrolysates (HH, EH-ABUT, or EH-ABHP), 35.8 correspond to the methane energy equivalent (kJ/L CH_4) and 16.35 is the calorific value of agave bagasse (kJ/g AB).

3. Results and discussion

3.1. Composition of agave bagasse

The chemical composition of agave bagasse was 27.4 ± 3.7% cellulose, 13.9 ± 2.5% hemicellulose, 17.3 ± 0.3% lignin, 4.5 ± 0.9% total extractives (measured as water and ethanol soluble extractives) and 7.8 ± 1.2% ashes in dry weight basis. These values are like those reported by Pino et al. [19] with 20.6 ± 1.2% cellulose, 12.2 ± 1.1% hemicellulose, 17.3 ± 0.4% lignin, 8.4 ± 0.6% water extractives, 1.5 ± 0.1% acetone extractives, and 7.6 ± 0.5% ashes. The difference in the composition of agave bagasse can be attributed to different environmental conditions such as the origin of the biomass, the time and type of harvest, the particle size, and the type of processing during the production of tequila [34].

3.2. Effect of hydrothermal pretreatment on agave bagasse carbohydrate composition

CCD results for the optimization of hydrothermal pretreatment are shown in Table 1 and the corresponding three-dimensional response surface obtained is shown in Fig. 1. Hemicellulose removal was the response variable, and time and temperature were the independent variables. Quadratic equations were obtained from the regression analysis of the experimental data for hemicellulose removal (%) and cellulose content (%), respectively, with the p-value of both models below the significance level ($\alpha < 0.05$). The value of adjusted R^2 statistic suggests that the model explained 98.1% of the data for the hemicellulose removal in the remainder fiber of hydrothermal pretreatment. Those models were accepted because an adjusted R^2 value equal to or greater than 70% have acceptable prediction quality [23]. From the analysis of the CCD data, the following quadratic equations for each response variable were obtained:

$$Y_1 = +36.73914 - 0.47514X_1 + 1.70870X_2 - 6.74569E-003X_1X_2 + 3.93867 - 003X_1^2 - 3.69734E-003X_2^2 \qquad (1)$$

Where: Y_1 is hemicellulose removal (%), X_1 is temperature (°C) and X_2 corresponds to time (min).

The response surface (Fig. 1) shows that, when the temperature and the reaction time increased, the percentage of hemicellulose removal also increased. These two factors were statistically significant in the ANOVA (p-values <0.0001, data not reported), indicating that both factors influence the response variable. Additionally, as the temperature and the reaction time increased, the value of the severity factor ([log R_o]) increased and the pH of the hydrothermal hydrolysate decreased (Table 1). The observed decrease of pH is due to the presence of hydronium groups generated by the autoionization of water molecules when the temperature increase, as well as, the presence of acetic acid, and other degradation by-products produced during the hydrolysis of hemicellulose. The hydronium groups carry out the hemicellulose removal by cleaving the ether and ester bonds between lignin and hemicellulose, and the bonds present in the internal hemicellulose structure [18].

Table 1 shows the chemical composition of the remaining bagasse fiber after hydrothermal treatment. It can be observed that hemicellulose in the remaining fiber decreased as the temperature and reaction time of the experiments increased. At 150 °C for 10 min, the hemicellulose removal was 61.7% (pH 4.2 and log R_o 3.5), while under the most severe experimental conditions, 180 °C for 50 min (Table 1, assay 11), hemicellulose removal was 96.2% (pH 3.5 and log R_o 4.4). Table 1 also shows the solids recovery, which was estimated as the percent of initial

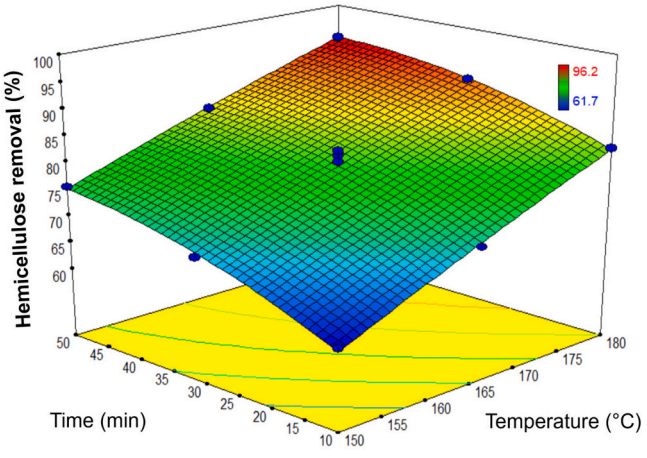

Fig. 1. Response surface for the CCD obtained for the hydrothermal pretreatment of agave bagasse using hemicellulose removal as a response variable.

Table 2
Chemical composition of AB-UT and agave bagasse in percentage (g/100 g of biomass) hydrothermally pretreated in reactors with volumes of 190 mL (ABHP-R_1) and 662 mL (ABHP-R_2).

Sample	[log R_o]	pH	Cellulose	Hemicellulose	Lignin	Solids recovery (%)	Hemicellulose removal (%)
AB-UT	-	-	27.4 ± 3.7^{a}	13.9 ± 2.5^{c}	17.3 ± 0.3^{e}	100	-
ABHP-R_1	4.4	3.5	42.6 ± 2.1^{b}	1.3 ± 0.5^{d}	25.4 ± 1.9^{f}	58.4	94.5
ABHP-R_2	4.2	3.4	46.2 ± 4.2^{b}	1.7 ± 0.3^{d}	27.6 ± 1.2^{f}	53.8	93.6

*AB-UT: untreated agave bagasse; ABHP-R_1: agave bagasse hydrothermally pretreated in reactor of 190 mL; ABHP-R_2: agave bagasse hydrothermally pretreated in reactor of 662 mL. $^{a, b, c, d, e, f}$ Different letters indicate significant differences at $p \leq 0.05$ level.

untreated biomass recovered after each treatment. The opposite trend was observed for the recovery of solids, since under more severe experimental conditions, the recovery percentage was low (59.6%) whereas at the less severe condition the solid recovery was high (84%). The percentage of cellulose and lignin in the remaining fiber increased in all the assays as compared to AB-UT.

The best conditions for hemicellulose removal predicted by the model were at 180 °C for 50 min with a 95.8% removal. Experimental verification of the predicted conditions showed a hemicellulose removal of 94.5% (Table 1, assay 12). This hemicellulose removal is close to the 99% removal found by Pino et al. [19] but at lower temperature (180 °C in this work vs temperatures >190 °C). To obtain enough pretreated bagasse for enzymatic hydrolysis, verification of the conditions predicted by the model was conducted in a larger reactor with a volume of 662 mL (ABHP-R_2). According to the chemical composition results (Table 2), the hemicellulose removal of bagasse pretreated in the ABHP-R_2 was 93.6%, which was like the hemicellulose removal found in the 190 mL-reactor (ABHP-R_1) of 94.5%. Furthermore, the solid recovery yield was similar for both reactors, 58.4% for ABHP-R_1 and 53.8% for ABHP-R_2 indicating that the scaling-up of the reactor did not have any effect.

3.3. Morphological and chemical changes in the solid fraction after hydrothermal pretreatment

The morphological changes of agave bagasse before and after hydrothermal pretreatment were analyzed by SEM (Figs. 2-A and 2-B). The main morphological difference between both fibers is related with the styloid-type calcium oxalates crystals -structures that have already been reported for agave bagasse [35]- and the parenchyma tissue removal. Both of these structures are observed in the AB-UT fiber (Fig. 2-A), but they are not present in the AB-HP (Fig. 2-B). The parenchyma tissue removal is attributed to the fact that one of its components is hemicellulose [36].

Changes in the chemical structure of the agave bagasse after hydrothermal pretreatment were analyzed by identifying FTIR-bands (Fig. 3-A) corresponding to the characteristic functional groups of hemicellulose. The identification of the functional groups was carried out based on what was reported by Louis and Venkatachalam [37] and Hernández et al. [38].

The main changes in FTIR signals (Fig. 3-A, in bold characters) are related to the disappearance of the bands at 1736 cm^{-1} (C═O conjugates of xylan) and at 1243 cm^{-1} (C-O) after hydrothermal pretreatment [39]. These changes corroborate the hemicellulose removal during hydrothermal pretreatment. On the other hand, the increase in the relative intensity of the band at 1456 cm^{-1}, as compared to the band between 3000 and 3300 cm^{-1}, in the AB-HP spectrum is attributed to the skeletal vibrations of the aromatic ring of the phenolic compounds present in the lignin-enriched fiber after hemicellulose removal. The band at 1302 cm^{-1} corresponds to the vibrations of the aromatic rings present in syringil and guayacil. The relative increase of these bands in the AB-HP spectrum is also due to the enrichment of this fiber with lignin after removing the hemicellulose. Furthermore, the increase in the relative intensity of bands at 1048 $cm^{-1}$and 1028 cm^{-1} (C-O stretch in cellulose as reported by Chávez-Guerrero et al. [40] in the AB-HP spectrum, can be attributed to a cellulose-enriched fiber. Overall, the observed spectral changes were consistent with the chemical composition analysis (Table 2).

Thermal stability of the bagasse before and after hydrothermal pretreatment was analyzed by TGA (Fig. 3-B) with the aim to correlate thermal stability changes with the changes in bagasse composition. The main difference between the thermograms of agave bagasse fibers with and without hydrothermal pretreatment was from 190° to 300°C with a weight loss of 18% for AB-UT and of 4% for AB-HP, values that closely correlate to the percentage of hemicellulose present in each fiber. Because the hemicellulose structure is a complex heteropolymer, its decomposition temperature is between 190 and 300 °C, since it has multiple branched units, as well as ester bonds that correspond to low activation energies [41].

3.4. Effect of hydrothermal pretreatment on the enzymatic saccharification of agave bagasse

Both, the AB-UT and AB-HP, were enzymatically hydrolyzed in order to evaluate the effect of the hydrothermal pretreatment on the enzymatic digestibility of agave bagasse cellulose. Fig. 4 shows the glucose concentrations obtained during the enzymatic hydrolysis for AB-UT and AB-HP. The glucose concentration at 72 h of the AB-HP hydrolysate (41.6 ± 1.9 g glucose/L) was 2.3 times higher than the concentration of the AB-UT hydrolysate (18.1 ± 2.2 g glucose/L). Furthermore, the saccharification yield for AB-UT was 47.9% while for AG-HP was 81.8%, indicating that hemicellulose removal favors the saccharification of cellulose. This is because hydrothermal pretreatment of agave bagasse produced a greater surface area and porosity, making cellulose more accessible for the enzymes, as is observed in Figs. 2-A and 2-B. The hydrolysis at 72 h allowed Cellic® CTec2 to saccharify both amorphous and microcrystalline cellulose [35]. This was corroborated by XRD (Fig. A.1) because the CI could not be determined due to the microcrystalline cellulose signals decreased considerably and only the signal from amorphous components was present. On the contrary, in the case of the enzymatic hydrolysis of the AB-UT, the CI decreased from 42.9% to 31.4% after 72 h of hydrolysis with Cellic® CTec2 indicating a partial removal of microcrystalline cellulose.

The micrographs after 72 h of enzymatic hydrolysis of the untreated and hydrothermally pretreated agave bagasse are shown in the Figs. 2-C and 2-D, respectively. The AB-UT after enzymatic hydrolysis for 72 h (Fig. 2-C), shows small orifices or cavities on the parenchyma tissue. In the case of the AB-HP after 72 h of enzymatic hydrolysis (Fig. 2-D), a thinner remnant fiber is observed with a greater number of cavities in the tissues. Furthermore, in the fiber that is in the upper part of the Fig. 2-D, spiral-shaped structures can be observed (indicated with an X) that correspond to the xylem tissue. Xylem is a lignified plant tissue responsible for the conduction and supply of liquids from one part to another in plants [42]. The chemical composition of this enzymatic hydrolyzed AB-HP fiber was $69.2 \pm 2.6\%$ lignin, $21.0 \pm 0.8\%$ of cellulose, $7.4 \pm 1.7\%$ of ash and a solid recovery of 28.8%. According to this characterization, 81.8% of the cellulose was removed during enzymatic hydrolysis of the AB-HP fiber and a lignin-enriched fiber was recovered. Overall, the enzymatic hydrolysis improved due to the hydrothermal pretreatment that drastically modified both the morphological and chemical structures of the agave bagasse.

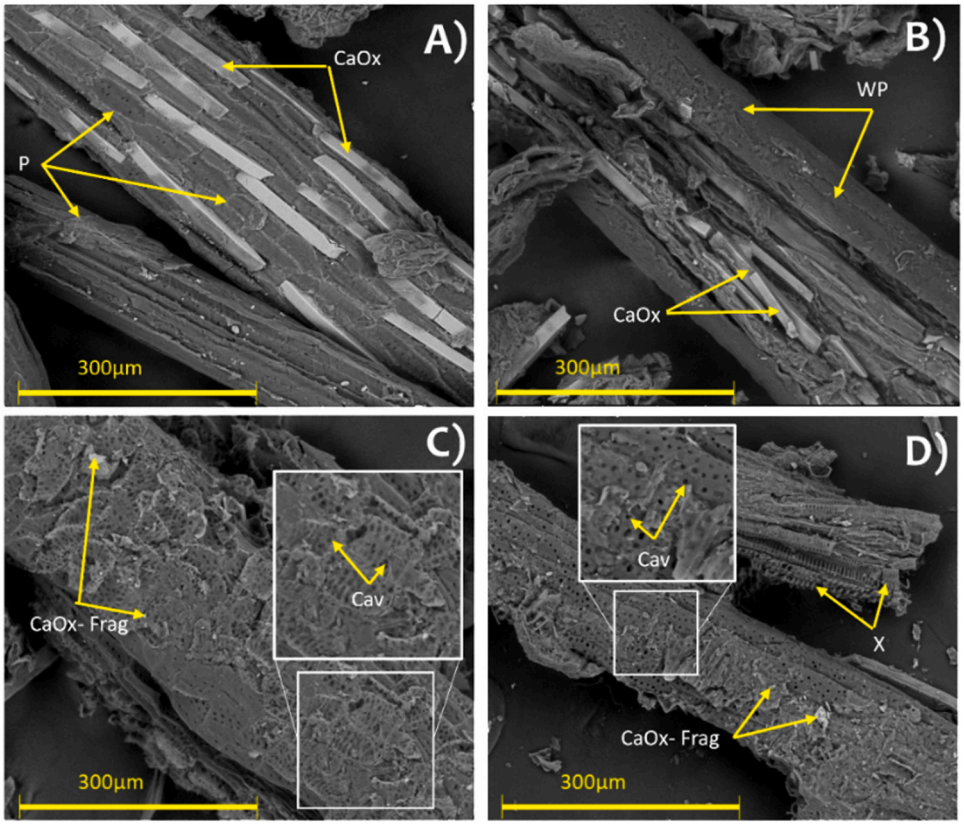

Fig. 2. SEM images of A) untreated agave bagasse; B) agave bagasse after hydrothermal pretreatment; C) untreated agave bagasse after 72 h of enzymatic hydrolysis; D) agave bagasse hydrothermally pretreated after 72 h enzymatic hydrolysis. The letters in the micrographs means: P: Parenchyma; CaOx: Calcium oxalates; CaOx-Frag: Calcium oxalates fragment; WP: Without parenchyma; Cav: Cavities; X: Xylem.

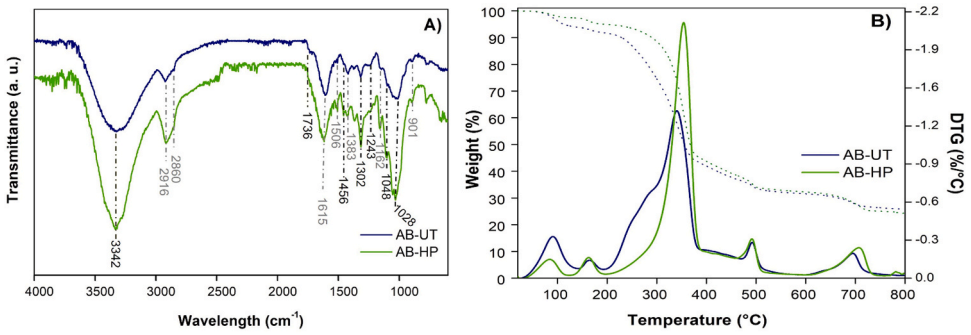

Fig. 3. Chemical changes in agave bagasse fibers untreated (AB-UT) and hydrothermally pretreated (AB-HP) determined by A) ATR-FTIR, and B) Weight (%, dotted lines) and DTG (%/°C, solid lines).

3.5. Chemical characterization of hydrothermal and enzymatic hydrolysates

The hydrolysates recovered from the hydrothermal pretreatment, and for both enzymatic hydrolysates from untreated and hydrothermally pretreated bagasse were characterized to be able to carry out the BPM assays as well as to compare with the enzymatic saccharification efficiency from other reports. Concentrations of COD and TS of these hydrolysates are presented in Table 3. The results indicate that the highest COD (61.4 ± 2.1 g COD/L) and TS (44.8 ± 2.5 g TS/L) values were obtained with the enzymatic hydrolysate of the AB-HP (EH-ABHP). These values are 1.9 times and 2.1 times higher than the COD and TS obtained for the enzymatic hydrolysate of AB-UT (EH-ABUT) respectively. This higher concentrations of COD and TS in the EH-ABHP were due to the higher cellulose saccharification of AB-HP (81.8%) as compared to cellulose saccharification of the AB-UT (47.9%). These results further confirmed that the removal of hemicellulose favored the cellulose enzymatic saccharification of the agave bagasse.

Valencia-Ojeda et al. [43] reported values of 30.2 ± 2 g COD/L using Cellulase 50XL and 36.9 ± 2 g COD/L using a mixture of Cellulase 50XL and Viscozyme for the enzymatic hydrolysate of untreated agave bagasse. Galindo-Hernández et al. [14] reported 29.0 ± 2.2 g COD/L for the enzymatic hydrolysates of untreated agave bagasse obtained with Celluclast 1.5 L and 26.7 ± 0.5 g COD/L for enzymatic hydrolysates of bagasse pretreated with alkaline hydrogen peroxide. These values are like the ones obtained in this work with the hydrolysate of untreated agave bagasse. It is important to highlight that the enzyme contribution of COD and TS to the hydrolysates was evaluated. The COD and TS contribution was higher for EH-ABUT (15.1% and 9.1%, respectively), than for EH-ABHP (7.8% and 4.2% respectively). On the other hand,

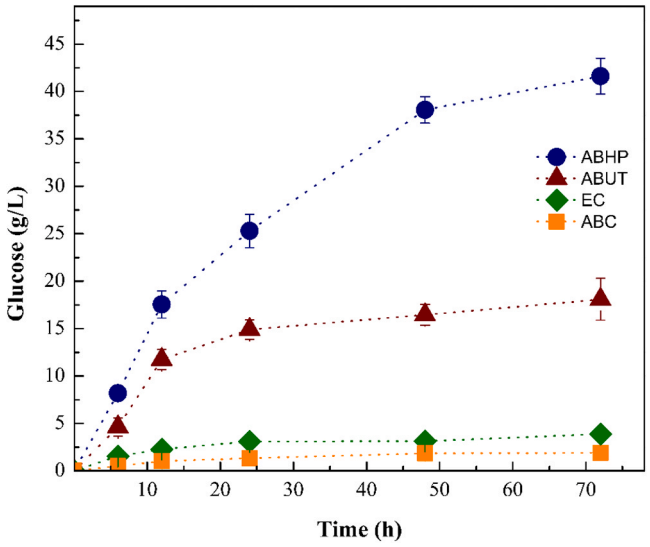

Fig. 4. Time-series of glucose concentration obtained during the enzymatic saccharification of untreated agave bagasse (AB-UT), hydrothermally pretreated agave bagasse (AB-HP), enzyme control (EC) and agave bagasse control (ABC).

COD and TS concentrations of the HH were the lowest (26.7 ± 0.5 g/L and 16.1 ± 0.1 g/L, respectively) since only sugars from hemicellulose were solubilized, which represents 14% of the chemical composition of agave bagasse.

3.6. BMP and biodegradability of hydrothermal pretreatment and enzymatic hydrolysates

Table 3 also shows the results of the BMP, anaerobic biodegradability and the ERE of the hydrothermal and enzymatic hydrolysates. The BMP of EH-ABHP was 1.3 times higher than the BPM of EH-ABUT indicating that the EH-ABHP was more biodegradable than the EH-ABUT. A similar effect was reported by Galindo-Hernández et al. [14] who observed an increase of 1.8 for the BMP of an enzymatic hydrolysate of delignified agave bagasse with an alkaline hydrogen peroxide pretreatment.

For and overall comparison, results obtained in this study for COD, BMP, and anaerobic biodegradability and the ERE are shown in Table 4, along with data reported in the literature. The BMP of the HH was 0.223 ± 0.02 NL CH_4/ g COD_{add} which correspond to a biodegradability of 63.7% as calculated according to Buitrón et al. [8]. These values are higher than the BMP of 0.178 ± 0.01 L CH_4/g COD_{add} and biodegradability of 59.1% reported by Buitrón et al. [8] for a hydrolysate recovered from agave bagasse treated by steam explosion. The low biodegradability values in both cases are probably due to the presence of bacterial inhibitors present in the hydrolysate as suggested by Buitrón et al. [8]. On the other hand, the BMP of the EH-ABUT was 0.229 ± 0.02 NL CH_4/g COD_{add}, which is lower than 0.287 NL CH_4/g COD_{add} reported by Tapia-Rodríguez et al. [10] for the enzymatic hydrolysate of untreated bagasse, using the Cellulase 50XL enzyme. This difference is due to the higher COD contribution to the BMP of the Cellulase 50XL enzyme (54%) used by Tapia-Rodríguez et al. [10] as compared to Cellic® CTec2 (15.1% for the enzyme control of EH-ABUT) used in this study. In the case of Tapia-Rodríguez et al. [10] the BMP value was not corrected for the COD contribution of Cellulase 50XL, while for the Cellic® CTec2 the COD correction was included. The BMP corrected value is shown in Table 4 (0.132 NL CH_4/g COD_{add}) which is much lower than the one obtained in this study. Regarding the BMP for EH-ABHP a value of 0.305 ± 0.03 NL CH_4/g COD_{add} was found which is higher than the BMP of 0.284 ± 0.02 NL CH_4/g COD_{add} reported by Duran-Cruz et al. [21] for an enzymatic hydrolysate obtained with Cellic® CTec2, from bagasse pretreated with steam explosion. Based on these BMP values, the biodegradability for both hydrolysates are similar, since the biodegradability of the EH-ABHP is only 1.1 times higher than the one of the enzymatic hydrolysates reported by Duran-Cruz et al. [21].

Regarding the specific methane yield (Table 3), a higher value was obtained for the EH-ABHP than for the EH-ABUT (2.5 times higher). This difference was because a higher percentage of cellulose was saccharified from the AB-HP. In the case of the EH-ABUT and the hydrothermal hydrolysate similar methane yields were obtained since both hydrolysates have a similar COD content.

3.7. Energy recovery efficiency of hydrothermal and enzymatic hydrolysates

Table 4 shows ERE values of this study as well as the ERE values reported in the literature to compare them. When the ERE value was not reported it was calculated from data provided in its reference. The HH has the lowest ERE value of 13.1% followed by the EH-ABUT (16.1%) and the highest recovery of 41% for EH-ABHP. These results correlate

Table 3
Chemical characterization and methane production from agave bagasse hydrolysates obtained with different treatments.

Sample	COD (g/L)[a]	Total sugars (g/L)	Saccharification yield (%)[b]	COD consumption efficiency (%)	BMP (NL CH_4/g COD_{add})[c]	Anaerobic biodegradability (%)[d]	Specific methane yield (NL CH_4/g AB)	Energy recovery efficiency (%)
Hydrothermal hydrolysate (HH)	26.7 ± 0.5	16.1 ± 0.1	-	72.6	0. 223 ± 0.02	63.7	0.060	13.1
Enzymatic hydrolysate of untreated agave bagasse (EH-ABUT)	32.1 ± 1.5	21.0 ± 0.4	47.9	85.2	0. 229 ± 0.02	65.4	0.074	16.1
Enzyme control for EH-ABUT	4.8	1.9	-	99.9	0.038 ± 0.01	-	-	-
Enzymatic hydrolysate of hydrothermally pretreated agave bagasse (EH-ABHP)	61.4 ± 2.1	44.8 ± 2.5	81.8	85.8	0.305 ± 0.03	87.1	0.187	41
Enzyme control for EH-ABHP	4.8	1.9	-	99.9	0.020 ± 0.01	-	-	-
Glucose	-	-	-	99.0	0.337 ± 0.02	96.3	-	-

[a] COD: chemical oxygen demand.
[b] Saccharification yield (%): calculated only for cellulose.
[c] The BMP results were corrected by subtracting the methane production obtained with the enzyme and endogenous control.
[d] Anaerobic biodegradability was calculated considering the theoretical value for BMP of 0.350 NL CH_4/g COD_{add}.

Table 4
Biomethane potential, biodegradability, and energy recovery from different types of agave bagasse hydrolysates with and without pretreatment.

Type of pretreatment	Pretreatment conditions	Enzymatic hydrolysis conditions		COD (g/L)	BMP (NL CH_4/g COD $_{add}$)	Anaerobic biodegradability (%)	Energy recovery (kJ/g AB)	Energy recovery efficiency (%)	Reference
Hydrothermal	Hydrothermal or autohydrolysis	180 °C, 50 min, 100 g AB/L	-	26.7	0.223	63.7	2.1	13.1	This study
			CTec2 15 FPU/g AB, 50 °C, 72 h, 100 g AB/L	61.4	0.305	87.1	6.7	41.0	
	Steam explosion	0.98 MPa, 178 °C, 24 min, 50 g AB/L	-	25.9	0.230	65.7[c]	4.3[c]	26.0[c]	[12]
		45 psig, 150 °C, 40 min, 12 g AB/L	-	10.5	0.178	50.9[c]	5.7[c]	35.0[c]	[8]
Chemical	Acid hydrolysis	HCl 1.8% wt/v, 119 °C, 133 min, 50 g AB/L	-	20.6	0.170	48.6[c]	2.5[c]	15.3[c]	[13]
		HCl 2.74% wt/v, 124 °C,78 min, 50 g AB/L	-	30.2	0.160	45.7[c]	3.5	21.4	[11]
	Ionic liquid	[Ch][Lys], 124 °C, 205 min, 200 g AB/L	CTec2 8 FPU/g AB, 50 °C, 72 h, 40 g AB/L	35.4	0.300	85.7[c]	6.3	38.3[c]	[15]
	Alkaline hydrogen peroxide	H_2O_2 2%, 50 °C, 90 min, pH 11.5, 50 g AB/L	Celluclast 1.5 L +Viscozyme 1.84 mg P/mL+ 0.1 mg P/mL (respectively), 40 °C, 50 g AB/L	55.4	0.200[a]	57.1[c]	n.c.	n.c.	[14]
Untreated			Cellulase 50XL 60.31 mg Protein/mL, 57.94 °C, 23.09 h, 67.46 g AB/L	11.9	0.132[b]	40.7[c]	0.9[c]	5.5	[10]
			Celluclast 1.5 L 40 FPU/g AB, 40 °C, 12 h, 40 g AB/L	40.1	0.090	25.7[c]	2.5	15.5	[11]
			CTec2 15 FPU/g AB, 50 °C, 72 h, 100 g AB/L	32.1	0.229	65.4	2.6	16.1	This study

[a] Methane yield reported as NL CH_4/g $COD_{removed}$.
[b] BMP reported in the reference was corrected for the COD enzyme contribution using de original data.
[c] Biodegradability, energy recovery and ERE values were calculated from the original data reported in the references.
n.c. ERE value was not calculated because lack of enough information in the report.

with the anaerobic biodegradability for each hydrolysate since the hydrothermal hydrolysate has the lowest value (63.7%) whereas the EH-ABHP as the highest value (87.1%) of biodegradability.

The ERE value of 16.1% for EH-ABUT found in this work is similar to the ERE value reported by Arreola-Vargas et al. [11] (15.5%) from methane production using an enzymatic hydrolysate of untreated agave bagasse. However, the ERE of EH-ABUT was 2.9 times higher than the ERE obtained in the work of Tapia-Rodríguez et al. [10], using an enzymatic hydrolysate of untreated agave bagasse using Cellulase 50XL. It is worth noting that this last ERE value (5.5%, Table 4) was calculated using the BMP corrected value for COD enzyme contribution (0.132 NL CH_4/g $CH_{4\ add}$).

The ERE value of HH was lower as compared to other hydrothermal hydrolysates (Table 4), such as the steam explosion hydrolysates reported by Buitrón et al. [8] (2.7 times lower) and Valdez-Vazquez et al. [12] (2 times lower). The difference between the ERE value of HH and the ERE values calculated for the steam explosion hydrolysates (Table 4) is due to the lower solid load used in the case of Buitrón et al. [8] (12 g AB/L) and Valdez-Vazquez et al. [12] (50 g AB/L) as compared to the solid load used for HH used in this study (100 g AB/L). Thus, according to the ERE definition, the ERE values are higher when the solid load is lower, although those hydrolysates had lower or similar BMP values to the HH. The acid hydrolysates (other type of pretreatment used to remove hemicellulose) have the same effect of solid load in ERE values. Breton-Deval et al. [13] and Arreola-Vargas et al. [11] reported higher ERE values, using an acid hydrolysate with a lower solid load (50 g AB/L) as compare the HH, although they reported a lower BMP and anaerobic biodegradability values. The lower biodegradability is due to the formation inhibitory compounds during both treatments [44].

It is important to mention that the higher ERE values of the acid and steam explosion hydrolysates as compared to the ERE of HH, are also due to the contribution of the COD yield per gram of bagasse of each hydrolysate as expected from the ERE definition. For example, COD yields of 0.3 g COD/g AB for HH is smaller than the COD yields (0.4 g COD/g AB and 0.6 g COD/g AB) calculated from acid hydrolysates [11, 13], and smaller than COD yields (0.5 g COD/g AB and 0.9 g COD/g AB) calculated for the steam explosion hydrolysates [8,12]. These values shows that the higher the COD yield, the higher the ERE values.

On the other hand, steam explosion and acid hydrolysis treatments of the agave bagasse (Table 4) were not efficient to remove the sugar fraction, because part of the cellulose and hemicellulose are left in the remaining fiber. If the remaining fibers of these two treatments were enzymatically hydrolyzed the methane production and ERE for the combined process would improve. On the contrary, hydrothermal pretreatment effectively removed hemicellulose which was used for methane production and the fiber recovered from hydrothermal pretreatment were subsequently used for an efficient enzymatic hydrolysis which allowed a higher total energy recovery.

Regarding the ERE values of pretreated bagasse enzymatic hydrolysates reported in Table 4, the highest value was obtained with the EH-

ABHP (41%) in this work, followed by the ERE of the enzymatic hydrolysate of delignified bagasse pretreated with ionic liquids (38.3%) as reported by Pérez-Pimienta et al. [15]. Therefore, these two pretreatments before enzymatic hydrolysis increased the anaerobic biodegradability of sugars as well as the ERE values as compared with the values obtained for EH-ABUT. However, during the ionic liquid pretreatment, part of the hemicellulose is removed along with the lignin, so the sugars in this hemicellulose are not used for methane production. On the contrary, during hydrothermal pretreatment, hemicellulose was effectively removed and used for methane production. It is important to mention that Galindo-Hernández et al. [14] studied the production of methane from delignified agave bagasse pretreated with alkaline hydrogen peroxide, achieving a methane yield of 0.200 NL CH_4/g COD_{rem}, and a biodegradability of 57.1% but they did not report enough data to calculate the ERE value.

Consequently, considering the ERE for HH (13.1%) and for EH-ABPH (41%) a total ERE of 54.1% was obtained, which is higher than the ERE obtained from the enzymatic hydrolysis of agave bagasse pretreated with ionic liquid (38.3%) [15]. Another advantage of hydrothermal pretreatment is that the use of ionic liquids makes the methane production process more expensive. Overall, the sequential process proposed in this work is more efficient because a higher percentage of sugars from agave bagasse is used for methane production, which improves the total ERE (54.1%) as compared to the ERE showed for all the pretreatments reported in Table 4.

4. Conclusions

This work demonstrated that the sequential process, hydrothermal followed by enzymatic hydrolysis, enhanced the saccharification efficiency, the biodegradability of the agave bagasse, and the total energy recovery, as compared with the enzymatic hydrolysis of untreated agave bagasse. It also demonstrates that the initial hemicellulose removal is as effective as the initial lignin removal to improve the enzymatic saccharification of cellulose. Moreover, this sequential process can be easily upgraded to full-scale level because hydrothermal and enzymatic treatments, and anaerobic digestion are currently used at industrial level. Overall, the combined energy recovery efficiency reported in this work, was higher than the energy recovery values reported in the literature, for other pretreatments that include removal of hemicellulose or lignin before enzymatic hydrolysis.

E-supplementary data for this work can be found in e-version of this paper online.

CRediT authorship contribution statement

Dendera Munguía-Aguilar: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Héctor A. Ruiz:** Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing, Supervision, Funding acquisition. **Elías Razo-Flores:** Resources, Writing – review & editing, Funding acquisition. **César Nieto-Delgado:** Formal analysis, Writing – review & editing. **Edith Cadena-Chamorro:** Writing – review & editing. **Felipe Alatriste-Mondragón:** Resources, Supervision, Project administration, Funding acquisition, Conceptualization, Formal analysis, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2023.110644.

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