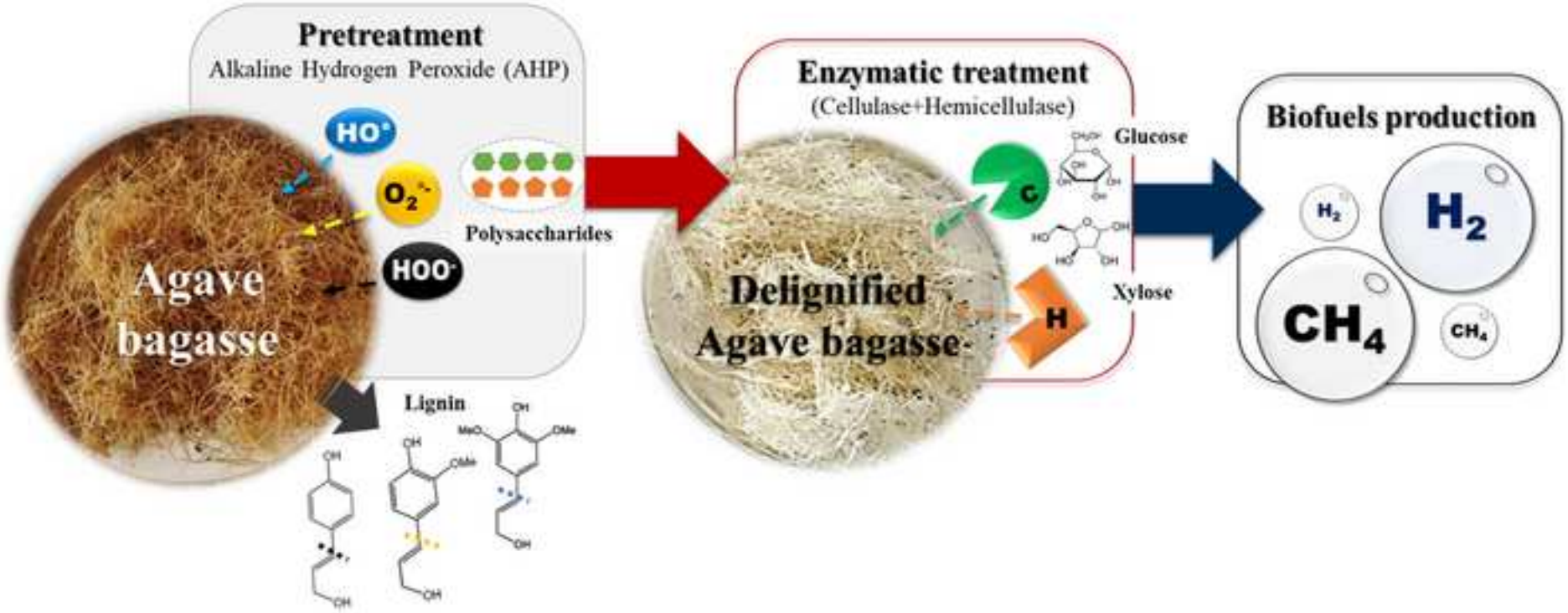
Graphical Abstract (for review)



Highlights

- Alkaline hydrogen peroxide removed 97% of lignin from agave bagasse
- A mixture of cellulases and hemicellulases showed a synergic activity
- Using a mixture of enzymes increases saccharification productivity by 2-fold
- Hydrolyzates increase H_2 and CH_4 production by 1.5 and 3.6-times, respectively

Enhancing saccharification of *Agave tequilana* bagasse by oxidative delignification and enzymatic synergism for the production of hydrogen and methane

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ABSTRACT

Agave tequilana bagasse is a suitable lignocellulosic residue for energy production. However, the presence of lignin and the heterogeneous structure of hemicellulose may hinder the availability of polysaccharides. In this work, the pretreatment of *A. tequilana* bagasse with alkaline hydrogen peroxide (AHP) followed by enzymatic saccharification was assessed. Results of the AHP pretreatment indicated that it is possible to attain up to 97% delignification and recover 88% of cellulose and hemicellulose after only 1.5 h of treatment. Regarding the saccharification process, the total sugar yield and productivity were both increased by 2-fold using an enzymatic mixture (cellulases + hemicellulases) compared to single enzyme hydrolysis (cellulases), evidencing synergism. Further evaluation of the hydrolyzates as substrate for hydrogen and methane production, resulted in yields 1.5 and 3.6-times (215.14 L H_2 and 393.4 L CH_4 per kg bagasse, respectively) superior to those obtained with hydrolyzates of non-pretreated bagasse processed with a single enzyme. Overall, using AHP pretreatment and subsequent hydrolysis with enzymatic mixtures improves the saccharification of *A. tequilana* bagasse enhancing the production of hydrogen and methane.

Keywords

Agave bagasse; anaerobic digestion; dark fermentation; delignification; enzymatic synergism; lignin

1. INTRODUCTION

The overreliance on fossil fuels for global energy production has contributed to the emergence of environmental issues, such as global warming. This has led to the development of sustainable energy sources, where hydrogen and biogas constitute important alternatives for energy transition [1]. Hydrogen has the highest energy content per unit weight (122-142 kJ/g) compared to other types of fuel such as methane, ethanol, biodiesel, etc., and it can be directly used to produce electricity through fuel cells, as well as for industrial, domestic and transportation purposes [2, 3]. Biogas, typically composed of 50-71% (v/v) CH_4 and 29-50% (v/v) CO_2, can be used for the generation of heat and electricity or, once refined, as a substitute for natural gas and biofuel for vehicles [4]. Both energy vectors can be obtained by dark fermentation and anaerobic digestion of organic wastes, which are less energy-intensive processes than water electrolysis, solar electrolysis and bio-photolysis of water in the case of dark fermentation [1–4].

Recently, the bagasse of *A. tequilana* (*Agave tequilana* Weber var. azul) emerged as a potential feedstock for hydrogen and methane production [5–7]. This waste is highly available in Mexico because it is the main solid waste generated from tequila manufacturing and is composed mainly by polysaccharides [5]. However, the presence of lignin hinders the availability of hemicellulose and cellulose from this residue [8]. It is well known that lignin constitutes a physical barrier that restricts the accessibility of cellulases or hemicellulases to their respective target substrates [9–11]. In addition, cellulases are non-specifically adsorbed to lignin, which reduces the efficiency of the saccharification process [12–14]. In previous studies, Arreola-Vargas *et al.* [15] and Contreras-Dávila *et al*. [6], reported low sugar yields (222–312 mg total sugars/g bagasse) conducting direct

63 saccharification over the bagasse of *A. tequilana*, evidencing the relevance of a
64 delignification before the saccharification step.

65 Among the various pretreatment methods available for lignin removal, oxidative
66 delignification with alkaline hydrogen peroxide (AHP) represents a promising pretreatment
67 due to the potential removal of high amounts of lignin, as well as the high cellulose and
68 hemicellulose (holocellulose) recovery from several lignocellulosic substrates [16–20]. The
69 mechanism by which AHP delignification takes place is not clearly understood yet.
70 However, Wilkinson *et al*. [21], reported that it consists in the saponification of the α-
71 benzyl ester bonds that bind the lignin and the hemicellulose, achieving their solubilization.
72 The saponification is probably carried out by the formation of highly oxidative hydroxyl
73 (°OH) and superoxide (O_2-•) radicals, and the hydroperoxide anion (-OOH), generated from
74 the dissociation of hydrogen peroxide (H_2O_2) in the presence of sodium hydroxide (NaOH)
75 at pH 11.5-13.1 [21, 22].

76 Concerning the saccharification process, it is commonly performed using cellulases only [6,
77 7, 15, 23, 24]. However, some authors such as Selig *et al*.[25] and Gao *et al*. [26] reported a
78 significant increase in conversion of glucans (of about 80%) adding xylanases or
79 hemicellulases to the saccharification process, suggesting that simultaneous hydrolysis
80 with hemicellulose and cellulose increases the yield of enzymatic saccharification. In this
81 context, *enzymatic synergism* is a term used to describe the cooperative action between
82 enzymes during saccharification to attain an efficient process. It occurs when the total
83 degree of hydrolysis achieved by an enzyme mixture is greater than the sum of the degree
84 of hydrolysis observed with individual enzymes [27]. The understanding of enzymatic
85 synergism is of considerable interest at industrial level, as it could represent a potential

minimization of the enzyme concentrations to achieve economic savings in the related processes.

Enzymatic synergism can be calculated through the method suggested by Andersen *et al*. [27], in which the degree of synergism is expressed as the ratio between the activity of the mixture and the sum of the individual activities on the same substrate. Thus, a quotient greater than 1 indicates that synergism is taking place; otherwise there is antagonistic activity or no synergism [27]. Currently, very few studies have been reported on the synergism or antagonism displayed by mixtures of enzymes used in the saccharification of cellulose and hemicellulose. Some of those studies have reported a synergistic interaction between cellulases/hemicellulases on complex substrates: sugarcane bagasse [28], corncob, corn stover and rice straw [29], and agave bagasse [20].

In this sense, this work aimed to evaluate the enzymatic synergism of cellulase (Celluclast 1.5 L) and hemicellulase (Viscozyme L) over the saccharification of *A. tequilana* bagasse previously pretreated with alkaline hydrogen peroxide. Additionally, the enzymatic hydrolyzates were evaluated as substrate for hydrogen and methane production via dark fermentation and anaerobic digestion in batch assays.

2. Materials and methods

2.1 Agave bagasse

A. tequilana bagasse was supplied by Casa Herradura distillery, located in Amatitan, Jalisco, Mexico. Prior to the assays of pretreatment and hydrolysis the bagasse was sun-dried and then grinded to reduce the fiber size between 1 - 5 cm in length.

2.2 Alkaline hydrogen peroxide pretreatment

For the delignification of *A. tequilana* bagasse, the methodologies established by Su *et al.* [19] and Munguía-Aguilar [30] were applied. Briefly, a solution of 2% w/v of AHP was prepared by diluting 66 mL of H_2O_2 (30% w/w) in 1000 mL of distilled water. The pH was adjusted to 11.5 with 5M NaOH [19]. Subsequently, the bagasse was placed in 2% w/v AHP solution to achieve a 1:20 ratio (w/v). The solid/liquid suspension was adjusted to pH 11.5 with 5 M NaOH and incubated at 50°C and 120 rpm at two reaction times, 1.5 or 6 h. Subsequently, the suspension was filtered (#16-mesh sieve) obtaining two fractions: a liquid (lignin and hemicellulose removed) and a solid (fibers enriched in cellulose and hemicellulose). The solid fraction was washed with distilled water until attaining neutral pH and dried at 60°C. Each experiment was performed in triplicate. Reaction times used in this study were selected according to previous studies reporting percentages of delignification greater than 90% and recovery of holocellulose greater than 85% from the pretreatment of corn cob [19] and agave penca [30] with AHP for 1.5 or 6 h.

The solid and liquid fractions from AHP pretreatments at 1.5 and 6 h were characterized in terms of total organic carbon (TOC) for mass balances. In addition, the bagasse with and without pretreatment was analyzed by thermogravimetry (TGA); microcrystalline cellulose and lignin were used as standards. Based on the thermograms obtained, the percentages of delignification and holocellulose recovery were calculated; t-student statistical test was performed to determine if there was significant difference between both pretreatment reaction times.

2.3 Enzymatic hydrolysis

During all enzymatic hydrolysis assays, enzymes with cellulolytic and hemicellulolytic activities were used. The enzyme with cellulolytic activity was the commercial mixture Celluclast 1.5L® from *Trichoderma reesei* (Novozymes, Denmark) – designated as Enzyme C for this study – while the enzyme with hemicellulolytic activity was the commercial mixture Viscozyme L® from *Aspergillus sp.* (Novozymes, V2010 Sigma-Aldrich) – designated as Enzyme H. These enzymes were diluted in citrate buffer (6.7 g of citric acid and 5.3 g of sodium citrate in 1000 mL of distilled water). The buffer pH was adjusted to the corresponding one in each enzymatic hydrolysis experiments adding 5 M NaOH or 5 M HCl, before adding the enzyme.

2.3.1 Impact of alkaline hydrogen peroxide pretreatment over saccharification

The effect of the pretreatment with AHP over the saccharification efficiency was evaluated using only Enzyme C in the saccharification step. The *A. tequilana* bagasse without pretreatment was used as control. Hydrolysis conditions were previously reported by López-Gutiérrez, [24]. Briefly, 3.5% w/v of total solids were incubated at 120 rpm, 40°C, for 12 h with Enzyme C at a concentration of 0.7 mg protein/mL citrate buffer pH 5.5. Samples of the hydrolyzate were taken for chemical oxygen demand (COD) and total sugars (TS) determinations as described in *Analytical methods* section.

2.3.2 Evaluation of enzymatic synergism

To evaluate the enzymatic synergism between enzymes C and H, the following experiments were carried out: simultaneous saccharification with both enzymes and individual

150 saccharification with each enzyme. In addition, the sequential saccharification using
151 enzyme C first and then enzyme H, and vice versa, were also evaluated.

152 For the simultaneous saccharification experiment, the hydrolysis conditions were: 5% w/v
153 solids, pH 5, 12 h, 40°C and 120 rpm. In these experiments, enzyme C was used at a
154 concentration of 1.84 mg protein/mL citrate buffer and enzyme H at a concentration of 0.1
155 mg protein/mL buffer. It should be stated that these conditions were obtained after two
156 experimental designs (*i.e.* Plackett-Burman and Central Composite Design), as shown in
157 the Supplementary Information (Tables S.1 through S.3).

158 For the individual hydrolysis procedures and the sequential hydrolysis experiments, the
159 hydrolysis conditions for enzyme C were as previously described in section 2.3.1. For
160 enzyme H the hydrolysis was performed at the following conditions 6% w/v total solids,
161 incubation at 120 rpm, 40°C for 12 h, with a concentration of 1.3 mg protein/mL citrate
162 buffer pH 4.5 [31]. In the first sequential hydrolysis experiment, a sequence with enzyme C
163 and then enzyme H was used. Whereas in the second sequential hydrolysis experiment the
164 enzyme H was firstly used and then enzyme C. All the hydrolysis experiments were done in
165 triplicate with their respective controls of bagasse and enzyme, to elucidate the contribution
166 of total sugars obtained from both elements in the saccharification procedure. Samples of
167 the hydrolyzates were taken for COD and TS determinations as described in *Analytical*
168 *methods* section.

169 The comparison of saccharification yields, percentages of saccharification and
170 productivities among the different hydrolysis assays was achieved with the equations
171 shown in Table 1.

172

Table 1. Equations used to calculate the saccharification yield, saccharification percentage and productivity for the evaluation of the enzymatic hydrolysis.

$SY = \frac{[TS]}{[S]} x1000$	Eq. 1	Where: ▪ SY: Saccharification yield (mg TS/g bagasse) ▪ TEH: Time of enzymatic hydrolysis treatment (h) ▪ [TS]: Concentration of total sugars released (g/L) ▪ TS: Mass of total sugars released (g) ▪ [S]: Initial substrate concentration, agave bagasse (g/L) ▪ 0.9 is a correction factor to compensate for the addition of a water molecule during hydrolysis ▪ 1000 is a conversion factor from g to mg ▪ 100 is a factor to get the percentage
$\text{Percent of saccharification} = \frac{TS \times 0.9 \times 100}{[S]}$	Eq. 2	
$\text{Productivity} = \frac{SY}{TEH}$	Eq. 3	

2.4 Hydrogen and methane production

2.4.1 Inoculum and mineral media

The inoculum used for the hydrogen and methane production batch tests was mesophilic anaerobic granular sludge from the vinasse treatment plant of Casa Herradura, located in Amatitan, Jalisco. The total and volatile solids (VS) content were 0.12 g/L and 0.11 g/L, respectively.

The granular sludge was thermally pretreated before using it in the hydrogen production assays, to eliminate the methanogenic archaea and conserve the hydrogenogenic bacteria. For this purpose, the anaerobic granular sludge was disaggregated using a No. 20 mesh sieve and heat-treated in an oven at 105°C for 24 h. Subsequently, the inoculum was grounded until a powder was obtained. Hydrogen production batch assays were performed

with the mineral phosphate medium reported by Arreola-Vargas *et al*. [31], with the following composition (g/L): 4.5 $NH_4H_2PO_4$, 11.9 Na_2HPO_4, 0.125 K_2HPO_4, 0.1 $MgCl_2 \cdot 6H_2O$, 0.015 $MnSO_4 \cdot 6H_2O$, 0.025 $FeSO_4 \cdot 5H_2O$, 0.005 $CuSO_4 \cdot 5H_2O$, 0.075 $ZnCl_2$.

For the batch methane production assays, fresh anaerobic granular sludge was used as inoculum. The modified anaerobic basic medium of Angelidaki & Sanders [32] was used, with the following composition (g/L): 1 NH_4Cl, 0.1 NaCl, 0.1 $MgCl_2 \cdot 6H_2O$, 0.05 $CaCl_2 \cdot 2H_2O$, 0.4 $K_2HPO_4 \cdot 3H_2O$.

2.4.2 Batch assays

Hydrogen and methane production batch assays were carried out in an automatic methane potential test system (AMPTS II, Bioprocess Control, Lund, Sweden), using glass bottles of 600 mL, with a 360-mL working volume and a 240-mL headspace purged with N_2 gas for 90 and 10 seconds, respectively, to guarantee anaerobic conditions. The operational conditions applied were 37°C and 120 rpm / pH 7.5 and 150 rpm / pH 7.0 for hydrogen and methane production assays, respectively [33, 34].

For hydrogen production assays, a substrate/inoculum ratio of 2.7 g TS/g VS and a substrate concentration of 5 g TS/L was used. The enzymatic hydrolyzate obtained from the best saccharification procedure (in terms of TS productivity) was used as substrate. An additional assay containing only thermally-treated inoculum and mineral phosphate medium was used as endogenous control.

For methane production tests, a 1:2 substrate/inoculum ratio was used, for which 5 g COD/L of substrate and 10 g VS/L of inoculum were used. The substrate used was the enzymatic hydrolyzate obtained from the best treatment evaluated in the enzymatic hydrolysis stage. An assay containing only granular sludge inoculum and anaerobic mineral

medium was used as endogenous control for this assay. All the assays were evaluated in triplicates.

The kinetic parameters of the hydrogen and methane production tests were calculated using the modified Gompertz equation (Equation 4), which was adjusted through the Matlab R2014a software (8.3) [35, 36]. The equations used to calculate the kinetic parameters and their description are shown in Table 2.

Table 2. Equations used for the evaluation of the kinetic parameters of the hydrogen and methane production stage using the modified Gompertz model.

$H(t) = Hmax * \exp\left\{-\exp\left[\frac{2.71828\ Rmax}{Hmax}\right](\lambda\text{-}t)+1\right]\right\}$	Eq. 4	Where: H(t): Total hydrogen or methane produced at the end of the assay (mL/L) Hmax: Maximum cumulative production (L H_2/L or L CH_4/L) Rmax: Maximum production rate (L H_2/L-h or L CH_4/L-h) λ: Lag phase or acclimation time of the microorganisms (h) t: Time span of the experiment (h) HMY: Hydrogen molar yield (mole H_2/mole glucose consumed) VHPR: Volumetric hydrogen production rate (mL H_2/L-h) Process yield: Production of hydrogen or methane per kg of biomass (L H_2 or CH_4 /kg bagasse) MY: Methane yield (L CH_4/g COD consumed) VMPR: Methane production rate (L CH_4/L-d) Vt: Total reaction volume
$HMY = \frac{\text{Moles of hydrogen produced}}{\text{Moles of glucose consumed}}$	Eq. 5	
$MY = \frac{Hmax}{\text{Substrate consumed}}$	Eq. 6	
$VMPR = \frac{Rmax}{Vt} * 24h$	Eq. 7	

2.5 Analytical methods

Thermogravimetric analysis was carried out in the TGA Setaram Analyzer model Setsys Evolution (France). Samples of 25 mg were analyzed at a heating rate of 10°C/min. The temperature range used was 25-800°C with a nitrogen atmosphere at a flow rate of 20 mL/min [30]. Prior to the analysis, samples were dried in an oven at 60°C for 24 h. A thermogram was obtained from each analysis, form which the weight loss percentage (% w/w) and the weight loss rate (% w/w/°C) were obtained. Total organic carbon determinations, were performed in a Shimadzu model TOCVSS/TNM-1 (Japan) equipped with a solid samples module (SSM-5000A). The samples of bagasse with and without pretreatment were powdered with a Retsch Mixel Mill model MM200 (Germany) equipment up to a particle size of 500 μm; 40 mg of this powder were used and processed in triplicate for 6 min at 900°C. Volatile fatty acids (VFA) were by capillary electrophoresis (Agilent model G1600A, Waldbronn, Germany), as previously described [34]. Furan derivatives (*i.e.* hydroxymethylfurfural and furfural) as well as the phenolic compounds (*i.e.* vanillin and syringaldehyde) were determined by HPLC according to the method described by Arreola-Vargas *et al*. [31]. COD, total solids, total suspended solids, volatile solids and volatile suspended solids were carried out through standard methods [37]. TS were determined by the phenol-sulfuric method [38].

3. RESULTS AND DISCUSSION

3.1 Delignification by alkaline hydrogen peroxide pretreatment

To assess the effect of the AHP pretreatment on the delignification of *A. tequilana* bagasse, two reaction times were used, 1.5 or 6 h. Figure 1 shows that after 1.5 h, 97% of the lignin was removed and 88% of holocellulose was recovered, both with respect to the initial mass

241 of these fractions contained in the non-pretreated bagasse. On the other hand, after 6 h, it
242 was possible to recover 83% of holocellulose and up to 93% of the lignin was removed.
243 The high level of delignification reached by the AHP pretreatment suggests that a
244 significant oxidation of lignin occurred due to the action of hydroxyl free radicals ($OH^{\bullet}$)
245 and superoxide ($O_2^{-\bullet}$) and hydroperoxide anions (HOO^-) formed in the alkaline medium,
246 which break the ester and ether bonds between lignin and hemicellulose, and directly
247 oxidize the side chains of lignin, as previously proposed [19, 22].

248 The influence of the exposure time over the organic matter removal, mainly lignin, was also
249 evaluated applying a t-student statistical test (with a confidence level of 95%) to the TOC
250 concentrations of the liquid fractions (Figure 1). This test indicated that there were no
251 significant differences between the reaction times evaluated in the AHP pretreatment.
252 Therefore, the shortest reaction time (1.5 h) was selected for further assays given that less
253 energy would be needed for the pretreatment.

254 The percentages of delignification obtained in this work are greater than the percentage
255 reported by Velázquez-Valadez *et al.* [20] of 82.6% using a sequential process with NaOH
256 and H_2O_2 at 6%. The differences observed in comparison to the present work suggest that,
257 in the work of Velázquez-Valadez, during the second step of the sequential pretreatment,
258 there was not sufficient release of hydroxyl ($OH^{\bullet}$) and hydroperoxyl (OOH^-) radicals to
259 attack lignin, since the pH was not adjusted to alkaline values. On the other hand, in
260 comparison with reports using other lignocellulosic substrates and where delignification
261 with AHP was carried out in the same conditions as in the present work, i.e. processes in a
262 single stage with alkaline pH, it was found in the present work that a higher percentage of
263 lignin was eliminated.

For instance, Su et al. [19] and Sun et al. [22] reported lignin removals of 74 and 80% by pretreating corn cob and rice straw, respectively. A possible explanation for the greater delignification achieved with *A. tequilana* bagasse is the origin of this residue, since it is produced after the thermal treatment of agave heads, which might be considered as an *in-situ* pretreatment.

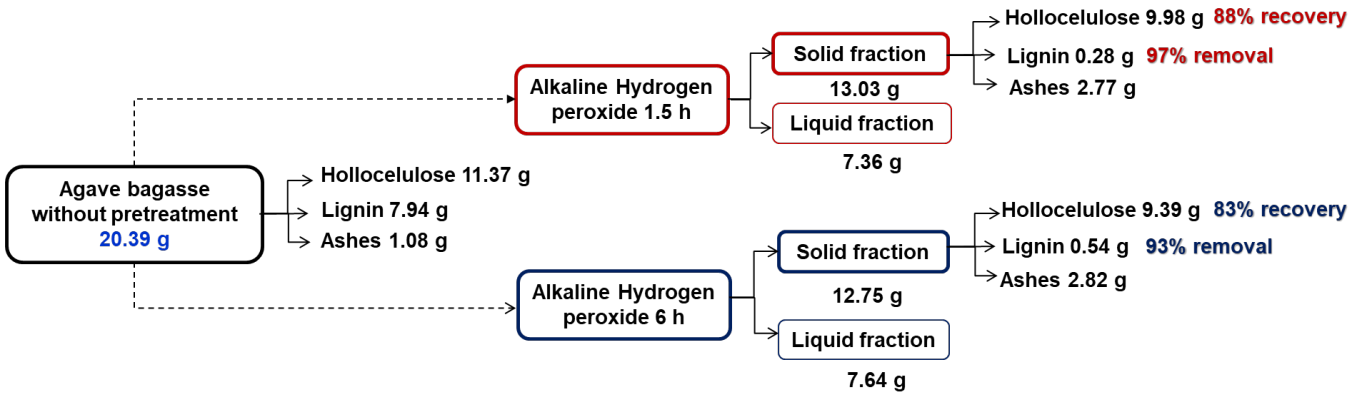

Figure 1. Mass balances obtained by the integration of thermogravimetric and total organic carbon analyses before and after pretreatment of *A. tequilana* bagasse with alkaline hydrogen peroxide (pH 11.5) at 1.5 h and 6 h reaction times.

3.2 Saccharification assays

Once the appropriate reaction time was selected for the AHP pretreatment (1.5 h), it was implemented to pretreat *A. tequilana* bagasse for the saccharification assays. Table 3 shows the results obtained from the saccharification experiments with pretreated and non-pretreated bagasse and using only enzyme C. In addition, a comparison with previous studies is also shown. Overall, the results show that a 2-fold increase in the yields and percentages of saccharification was attained using the AHP-pretreated bagasse, therefore it can be inferred that the polysaccharide structure became more exposed to the enzymatic attack. This increase is consistent in terms of the results obtained in other studies, as shown

282 in Table 3 [7, 15]. Nevertheless, Contreras-Dávila *et al*. [6] reported a higher
283 saccharification performance (in terms of productivity, and saccharification and hydrolysis
284 yields) compared to those achieved in this study, even when an additional pretreatment
285 (AHP) was performed. These differences may be due to the fact that other hydrolysis
286 conditions, such as the reactor configuration, type of agitation or the working volume, were
287 used and favored a better contact between the substrate and the enzyme [10].

288 Table 3. Summary of the saccharification performance parameters obtained for the
289 enzymatic hydrolysis experiments with bagasse without and with alkaline pretreatment and
290 using only cellulase (enzyme C).

Assay	Total sugars (g/L)	COD (g/L)	Hydrolysis yield (mg TS/g bagasse)	Productivity (mg TS/g bagasse-h)	Saccharification (%)	Reference
Agave bagasse without pretreatment + enzyme C	4.72 ± 0.2	29.03 ± 2.2	134.8 ± 5.2	11.2 ± 0.5	12.1 ± 0.5	This study
	8.9 ± 1.2	40.1 ± 5.8	~222.5	~22.3	~20.02	Arreola-Vargas et al. [15]
	12.5 ± 2.5	41.5 ± 3.1	~312.5	~31.25	~28.1	Contreras-Dávila et al. [6]
	5.3 ± 0.8	25 ± 0.9	~151.4	~12.6	~13.6	Montiel-Corona & Razo-Flores, [7]
Agave bagasse pretreated with AHP + enzyme C	10.3 ± 0.9	26.7 ± 0.5	190.1 ± 16.4*	17.1 ± 1.5*	26.7 ± 2.3*	This study

*Results obtained were multiplied by 0.64, to account for the fact that the pretreated bagasse represents 64% of the untreated bagasse.

291

3.3 Enzymatic synergism between cellulases and hemicellulases

To evaluate the level of enzymatic synergism, different hydrolysis conditions were assessed with enzyme C, enzyme H, and their combinations: sequential enzyme C first then enzyme H, sequential enzyme H then enzyme C, and enzyme mixture C + H. As previously stated, the results that allowed defining the best conditions for the simultaneous saccharification with the enzyme mixture (*i.e.* cellulase + hemicellulase) are shown in Supplementary Information (Tables S.2 and S.3).

Table 4 summarizes the yields, productivities and percentages of saccharification obtained in the five different experiments evaluated to determine the degree of enzymatic synergism. The best sugar yield and saccharification percentage was observed with the sequential hydrolysis using enzyme H first and then enzyme C, followed by the assay with the mixture of enzymes C and H. Comparing these experiments with the individual hydrolysis with only enzyme C or enzyme H, a significant increase in the yield and saccharification percentage is observed. These results agree with those reported by Selig *et al.* [25], that observed an increase in the conversion of cellulose and hemicellulose in glucans and xylans once the lignocellulosic material was treated with cellulases and hemicellulases. Therefore, it is suggested that by using sequential enzymatic hydrolysis and enzymatic mixtures with cellulolytic and hemicellulolytic activities, the cleavage of various bonds in the structure of hemicellulose is attained with the consequent increase in the accessibility of hydrolytic enzymes as well as a higher conversion of glucose and xylose [10].

It is also worth noting that when sequential hydrolysis with enzymes C and H was used, the amount of total sugars obtained was less than that obtained in the sequential hydrolysis with enzymes H and C. This result suggests that when hemicellulases were used first it was

315 possible to segregate the structure of xylan into shorter oligosaccharide sections allowing to
316 remove the fraction of hemicellulose that was still attached to cellulose and that hindered
317 the access of the cellulases [10, 11].

318 Table 4. Summary of the saccharification performance parameters obtained in the
319 enzymatic hydrolysis experiments with pretreated bagasse using a mixture of enzymes and
320 sequential hydrolysis.

Assay	Total sugars (g/L)	COD (g/L)	Hydrolysis yield (mg TS/g bagasse)*	Productivity (mg TS/g bagasse-h)*	Saccharification (%)*	Synergism
Cellulase (C)	10.3 ± 0.9	26.7 ± 0.5	188.5 ± 17.1	15.7 ± 1.5	16.9 ± 1.5	NA
Hemicellulase (H)	9.3 ± 0.1	21.98 ± 0.1	99.56 ± 0.9	8.3 ± 0.1	8.9 ± 0.1	NA
Mixture of enzymes C+H	24.9 ± 0.1	55.36 ± 1.01	318.7 ± 1.8	26.6 ± 0.2	28.68 ± 0.2	1.67
Sequential hydrolysis C-H	19.0 ± 0.3	49.21 ± 0.5	281.1 ± 14.9	11.7 ± 0.6	25.3 ± 1.3	NA
Sequential hydrolysis H-C	26.6 ± 0.4	64.43 ± 1.6	391.75 ± 7.4	16.3 ± 0.3	35.26 ± 0.7	NA

321 NA=Not applicable. *The pretreated bagasse used represents 64% of the untreated bagasse.

322

323 Even though the sequential hydrolysis with enzymes H and C attained the highest
324 percentage of saccharification, it is important to note that the incubation time required was

325 twice-fold (24 h) compared to assays with individual enzymes and in mixtures, for which
326 the incubation period was 12 h. Therefore, when performing an analysis of the
327 productivities, the experiment with the enzymatic mixture resulted more effective (26.6 ±
328 0.2 mg TS/g bagasse-h) than the sequential hydrolysis with enzymes H and C (16.3 ± 0.3
329 mg TS/g bagasse-h). Overall, comparing the results obtained in this study with those
330 previously reported, it was observed that a significant increase in the sugar productivity was
331 achieved as indicated in Table 5.

332 Table 5. Comparison of the percentages of delignification and sugar productivities reported
333 previously using agave bagasse.

Description of pretreatment	Delignification (%)	Enzymes	Productivity (mg TS/g bagasse-h)	Applied studies
$NaOH/H_2O_2$	82.6	Cellulase/ Hemicellulase	12.2 ± 3	Velázquez-Valadez *et al*. [20]
No delignification	-	Cellulase	6.3 ± 2	Saucedo-Luna *et al*. [23]
Alkaline H_2O_2	97	Cellulase/ Hemicellulase	26.6 ± 0.2	This study

334

335 The differences in productivity between this work and the others are attributed to the higher
336 percentage of delignification achieved during the pretreatment stage (with AHP) and to the
337 use of a mixture of enzymes, cellulases and hemicellulases that acted synergically (Table
338 4). A higher percentage of delignification promotes that the polysaccharide fractions are
339 more available or exposed to enzymatic attack. In addition, by using a mixture of specific
340 enzymes, such as cellulases and hemicellulases, the simultaneous attack of the different

bonds that make up the polysaccharides is promoted resulting in the improvement of yields, percentages and productivities of saccharification [10, 11, 25].

3.4 Potential of enzymatic hydrolyzates for hydrogen and methane production

To evaluate the biofuel production potential of the hydrolyzates obtained with the enzymatic mixture, hydrogen and methane production assays were performed. The kinetic profile of hydrogen production shows that the accumulated hydrogen reached 1370.6 ± 39.3 mL H_2/L in 64 h (Fig. 2A). During the experiment, only two metabolites were detected: acetic (3.45 ± 0.5 g/L) and butyric (3.33 ± 0.03 g/L) acids, which are fermentation products closely related to hydrogen production pathways [5].

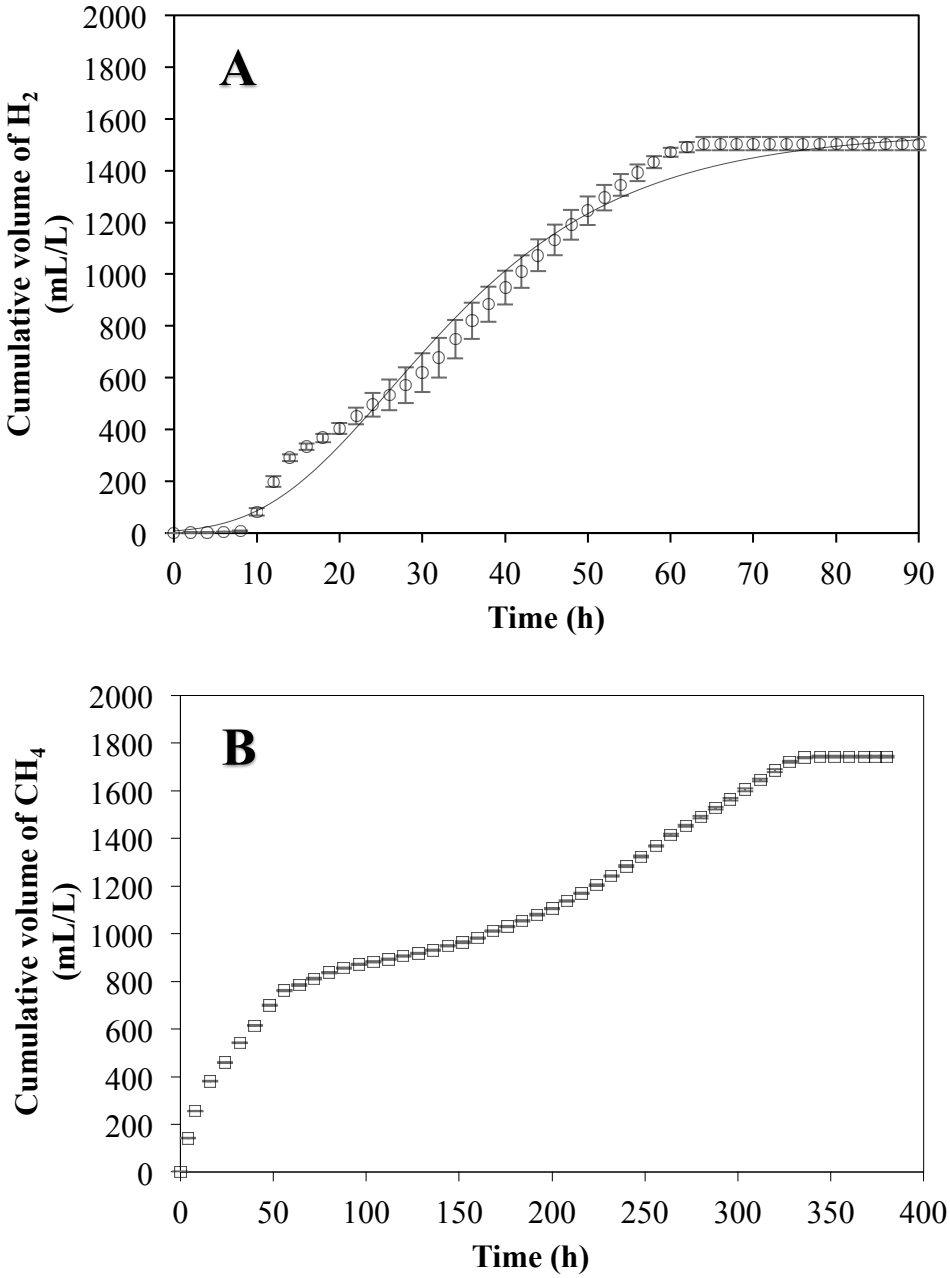

Figure 2. Time course of the cumulative hydrogen production (A) and methane production (B) in batch experiments with the hydrolyzates obtained from the alkaline pretreatment of agave bagasse and further hydrolysis with a mixture of cellulases and hemicellulases. Open symbols represent the experimental volumetric production data, and the continuous black line represents the modified Gompertz model adjustment for hydrogen production.

The corresponding kinetic parameters obtained (calculated from the modified Gompertz model equation) are presented in Table 6. The results indicated that it was possible to increase the hydrogen yield 1.5-times (kg of bagasse basis) by using the enzymatic hydrolyzate obtained with a mixture of enzymes, compared to the hydrolyzate from an assay with a single type of enzyme or to the untreated bagasse [15]. Likewise, it was possible to reach high molar yields of hydrogen compared to the maximum theoretical molar yield (4 mol H_2/mol hexose), which is consistent with previous results found by Arreola-Vargas *et al.* [15].

Table 6. Kinetic parameters obtained in the hydrogen and methane production potential tests of the hydrolyzates obtained by with an enzyme mixture, and their comparison with a previous report.

Hydrogen batch production (from a modified Gompertz model analysis)					
Hydrolyzate	**Hmax (mL H_2/L)**	**VHPR (mL H_2/L-h)**	**HMY (mol H_2/ hexose)**	**Process yield (L H_2/kg bagasse)**	**Ref.**
Untreated bagasse + Enzyme C	~3375	~75	3.4	~140.83	Arreola-Vargas et al. [15]
Pretreated bagasse + enzyme mixture (C + H)	1551.6 ± 13.0	38.7 ± 0.9	3.0 ± 0.1	215.14 ± 13	This study
Methane batch production (from experimental data analysis)					
Hydrolyzate	**Hmax (mL CH_4/L)**	**VMPR (L CH_4/L-d)**	**MY (L CH_4/g $COD_{consumed}$)**	**Process yield (L CH_4/kg bagasse)**	**Ref.**
Untreated bagasse + Enzyme C	~833	~0.48	0.11	~108.5	Arreola-Vargas et al. [15]
Pretreated bagasse + enzyme mixture (C + H)	1743.3 ± 3.7	0.67 ± 0.01	0.20	393.4 ± 13	This study

Hmax: maximum cumulative production of hydrogen or methane, VHPR: volumetric hydrogen production rate, HMY: hydrogen molar yield, VMPR: methane production rate, MY: methane yield.

To our knowledge, there are no previous reports of hydrogen production from agave bagasse pretreated with AHP and hydrolyzed with enzymatic mixtures. However, other studies have been carried out using agave bagasse pretreated with acid (2.7% w/w HCl concentration) and enzymatic hydrolysis separately. Such is the case of the work performed by Arreola-Vargas *et al.* [15], where acid and enzymatic agave bagasse hydrolyzates were used as substrate after a sequential pretreatment. In that study, the highest overall hydrogen yield was 140.83 L of H_2/kg of agave bagasse, which is low compared to our results (215.14 L of H_2/kg of agave bagasse). These differences may be due to the fact that during acid hydrolysis inhibitory compounds such as furans and weak acids can be formed, hindering the hydrogen production [15]. These results suggest that delignification of *A. tequilana* bagasse and subsequent hydrolysis with a synergistic enzymatic mixture had a beneficial effect on hydrogen production at laboratory scale.

Regarding methane production, the kinetic profile is displayed in Figure 2B. An accumulated methane volume of 1743.3 ± 3.7 mL CH_4/L was observed after 380 h. In this case, no volatile fatty acids were detected at the end of the kinetic assays, indicating that an efficient anaerobic digestion process occurred. The corresponding process parameters are shown in Table 6, after an analysis of the experimental data. The results showed that when using an enzymatic hydrolyzate produced from an enzyme mixture the methane production potential increased 3.6-times per kg of bagasse and the methane yield improved 2-fold, compared to a hydrolyzate coming from a treatment with a single type of enzyme [15]. This

latter study reports methane yields as high as 0.24 L CH_4/g COD-consumed in a process involving two stages (acidogenic and methanogenic), avoiding VFA accumulation. In the present work, the attained methane yield was 0.20 L CH_4/g COD-consumed in a single-stage process, which demonstrates the potential advantage of integrating a delignification pretreatment and the use of synergistic enzymatic mixtures before the anaerobic digestion processes.

Other studies have been carried out using lignocellulosic biomass pretreated with alkaline hydrolysis. As reported by Mancini *et al.* [36], wheat straw pretreated with a 1.6% alkaline solution (w/w) reached a biogas production yield of 241.54 mL CH_4/g wheat straw. These results show the importance of attaining the highest yield of sugars during the saccharification stage, and thereby maximizing the high potential of the lignocellulosic residue for the production of gaseous biofuels.

4. CONCLUSIONS

In this work, the delignification process with alkaline hydrogen peroxide contributed to obtain readily available fractions of cellulose and hemicellulose for a subsequent enzymatic attack. Therefore, it was possible to increase the yield and saccharification productivity 2-fold by applying a mixture of cellulases and hemicellulases, due to the synergy achieved with both enzymes. Furthermore, the hydrolyzate obtained from the saccharification process with a synergistic enzyme mixture improved the overall yield of hydrogen and methane production by 1.5 and 3.6-times, respectively, compared to the obtained with enzymatic hydrolyzates of agave bagasse without pretreatment and hydrolyzed with a single type of enzyme. Overall, considering the integration of a delignification pretreatment step along with the use of synergistic enzymatic mixtures for agave bagasse

saccharification could be of high relevance for taking advantage of this lignocellulosic residue for the production of energy biofuels.

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