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Biorefinery concept comprising acid hydrolysis, dark fermentation and anaerobic digestion for co-processing of fruit and vegetable wastes and corn stover

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Biorefinery concept comprising acid hydrolysis, dark fermentation and anaerobic digestion for co-processing of fruit and vegetable wastes and corn stover

Abstract

A new biorefinery conceptual process is proposed for biohydrogen and biomethane production from a combination of fruits and vegetables wastes (FVW) and corn stover (CS). The objective of this work was to perform the acid hydrolysis (HCl 0.5 % v/v, 120 °C, 2 h) of the FVW and CS at 3:1 dry basis ratio, and to process its main physical phases, liquid hydrolyzates (LH) and hydrolyzed solids (HS), by mesophilic dark fermentation (DF) and anaerobic digestion (AD), respectively. In DF of LH as carbon source, hydrogen was produced at maximum rate of 2.6 mL H₂/(g_{glucose} h) and maximum accumulation of 223.8 mL H₂/g_{glucose}, equivalent to 2 mol H₂/mol_{glucose}, in a butyric-pathway driven fermentation. HS were digested to methane production assessing inoculum to substrate ratios in the range 2 – 4 g_{inoculum}/g_{vs}. The main results in AD were, 14 mmol CH₄/g_{vs}. The biorefinery demonstrated the feasibility to integrate the acid hydrolysis as pretreatment and subsequently use the LH for hydrogen production, and the HS for methane production, with an energy yield recovery of 9.7 kJ/g_{vs}, being the energy contribution from anaerobic digestion 8-fold higher than of dark fermentation.

Keywords: Biohydrogen; Furfural; Hydrochloric acid; Lignocellulosic waste; Methane; Multisubstrate; Phenolic compounds

Introduction

50 Mexico is committed to reducing its greenhouse gases (GHG) by the year 2030 to 22%, whereas emissions
of short-lived climate pollutants (black carbon) are aimed to 51% reduction (Gobierno de México 2015).
52 The main proposals for obtaining energy from renewable sources are bioenergy, solar energy, geothermal
energy, hydroelectric energy, wind energy, and tidal energy. The biomass as a source for bioenergies, i.e.
54 biohydrogen, biomethane, bioethanol, and biobutanol (Mahlia et al. 2019), is most promising in the
energetic share it could reach; according to some estimates, in Mexico it could supply ca. 46% of the
56 annual energy, yet currently, only 5% is supplied (International Energy Agency 2016).

Residual biomass can be classified by its origin in forestry residues, agricultural residues, agro-
58 industrial wastes and organic fraction of municipal solid waste (Li et al. 2016). Residual biomass
exhaustive use has led to the development of the biorefinery of residues.

60 The biorefinery concept is associated with the intensive conversion of organic matter into added-value
products (Escamilla-Alvarado et al. 2016; Moncada-Botero et al. 2016). Biorefineries are outlined by the
62 following sections: *i*) pretreatment section to fractionate biomass, *ii*) section of bioprocesses (e.g.
fermentation, digestion), *iii*) purification of bioproducts to discard impurities and pollutants, and *iv*) energy
64 conversion section (Giuliano et al. 2016; Hernández-Flores et al. 2017).

The pretreatment with the highest efficiency of fermentable sugars release is diluted acid hydrolysis
66 (Gonzales et al. 2016; Joglekar et al. 2019). It employs high temperatures and acids in low concentrations
to hydrolyze macromolecules such as cellulose and hemicellulose into monomeric sugars such as glucose,
68 xylose, arabinose, and others (Kumar et al. 2015). Such soluble hydrolyzate is prone to any kind of
fermentation, yet the insoluble fraction remaining from the pretreatment (hydrolyzed solids) is also
70 susceptible for downstream processing. In the biorefinery perspective, the use of both hydrolyzates would
be very attractive to increase the productivities of the installation.

72 Fermentable sugars in liquid hydrolyzates (HL) can be used as a carbon source to produce hydrogen
through dark fermentation (DF) bioprocess (Roy and Das 2016). On the other hand, the insoluble biomass,
74 or hydrolyzed solids (HS), may be converted into a bioenergetic such as methane through anaerobic
digestion (AD) (Oliwit et al. 2019). Actually, only few works have dealt with the either DF or AD of
76 hydrolyzates with interesting results. For instance, Datar et al. (2007) applied steam explosion hydrolysis
followed by the separated fermentation of the liquid hydrolyzates and the solid hydrolyzates for hydrogen
78 production. Curiously, the dark fermentation of solid hydrolyzates did not produce hydrogen during the
first 21 d, which was attained only after 20 h since the addition of cellulases. On the other hand, Tapia-
80 Rodríguez et al. (2019) evaluated the parallel production of hydrogen and methane from
enzymatic hydrolyzates of agave bagasse, however the HS were not included in the biorefining process.

82 The sources of biomass for biorefineries are numerous and in order to not compromise the food supply,
biowastes should be mainly used as its substrates (Romero-Cedillo et al. 2016). Considering that Mexico
84 was the 11th agriculture producer worldwide with an annual production of 210 million tons in 2018
(SADER 2019), its wastes generation along the food supply chains (production, postharvest, processing,
86 distribution, consumption) are also considerable. Indeed, as corn is the second most important crop in
Mexico, the wastes associated to the over 26×10^6 metric tons production (SADER 2019) are corn stover
88 (CS) and corn cobs. Since close to 82% of the CS generated is used for animal feed, the remaining 18%
does not have a defined use (ca. 4.7×10^6 metric tons in dry base) and is therefore underused (Hernández
90 et al. 2019). Currently, CS is the most promising lignocellulosic waste for biofuels production due to its
high cellulose (23-40%) and hemicellulose (12-32%) contents, and its low-cost abundant biomass (Kim
92 et al. 2019; Tan et al. 2019).

Another underused biowaste in Mexico are the fruits and vegetable wastes (FVW), which compose *ca.* 44% of municipal solid wastes and that are disposed in landfills and open dumps (Taboada-González et al. 2011; Díaz et al. 2017; Gavilán et al. 2018).

Even though worldwide distribution of biowaste has raised attractiveness on biorefineries, some traits of these substrates such as its recalcitrant nature and seasonal availability are some of the most important challenges for the development of biorefineries at large scale (Sultana and Kumar 2011; Giuliano et al. 2016). It has been proposed that the combination of multiple lignocellulosic biomass would be a feasible solution as it also improves biofuels yields, complements the nutritional balance and reduces delivery costs (Sultana and Kumar 2011; Wang et al. 2011). Therefore, this work aimed to evaluate a biorefinery concept for biohydrogen and biomethane production, as well as the resulting energy potential, from a combination of fruits and vegetables wastes (FVW) and corn stover (CS). The biorefinery concept (*h-H-M*) integrated the diluted acid hydrolysis (*h*) as pretreatment and the parallel production of hydrogen by DF (*H*) from liquid hydrolyzates and methane (*M*) from hydrolyzed solids.

Materials and methods

Biorefinery h-H-M set up

The FVW and CS were used as substrates to test the *h-H-M* biorefinery concept as shown in Fig 1. The co-substrates were combined in dry basis (db) at the ratio 3:1 FVW:CS according to Rodríguez-Valderrama (2018). The co-substrates were subjected to hydrolysis pretreatment (*h-stage*) using HCl as the catalyst acid. After the dilute acid hydrolysis, the liquid and solid phases were separated by centrifugation. Liquid hydrolysates (LH) were limed to decrease the concentration of inhibitory compounds, and used as a carbon source in an H₂-producing dark fermentation stage (*H-stage*) at mesophilic temperature. The hydrolyzed solids (HS) were washed to remove the catalyst acid excess and

used as carbon source in a methane producing anaerobic digestion bioprocess (*M-stage*). Each of the three stages will be described in detail in the following sections.

INSERT FIG. 1

Co-substrates

Corn stover (collected from Cuencamé, Durango, Mexico) and FVW (collected from a local cafeteria Chemical Sciences Faculty, Universidad Autónoma de Nuevo León, Nuevo León, Mexico) were separately dried in an oven at 85 °C for 24 h, grinded to 180 µm particle size of using a manual mill, and stored in distinct tightly closed plastic bags at room temperature. FVW and CS were physico-chemically characterized (Table 1). The empirical molecular formulas and the heat power value were $\text{CH}_{4.31}\text{O}_{0.68}\text{N}_{0.01}$, 3606 cal/g_{db}, and $\text{CH}_{0.8}\text{O}_{0.62}\text{N}_{0.04}$, 2712 cal/g_{db} for CS and FVW, respectively.

INSERT TABLE 1

Diluted acid hydrolysis and overliming

The acid hydrolysis was carried out using dilute HCl (0.5% v/v) in 0.5 L Schott bottles. The reaction volume was 0.3 L and the solid content was 6.6% of reaction volume. The co-substrates ratio was 3:1 FVW:CS (g:g). The hydrolysis reaction was performed in an autoclave (121 °C) for 120 min (Yan et al. 2009; Kumar et al. 2015). After the hydrolysis, liquid hydrolyzates (LH) were separated by centrifugation (10 000 g, 10 min) and characterized in terms of reducing and monomeric sugars and the inhibitory

138 compounds, i.e. acetic acid, formic acid, furfural, 5- hydroxymethyl-furfural (HMF), and total phenolic
compounds (TPC).

140 Overliming treatment was used applied to the LH in a two-step approach (Chang et al. 2011). Firstly, the
pH of LH was adjusted to 10 by adding powder $\text{Ca}(\text{OH})_2$ in continuous stirring for one hour. Secondly,
142 the pH was reduced to 7 by 6 M HCl addition, following a centrifugation (10 000 g, 15 min) and the
separation of liming precipitates and supernatant. Sample were retrieved from the LH to analyze the
144 removal of inhibitory compounds and sugars.

146 *Dark fermentation*

DF was carried out by duplicate in 0.5 L Schott bottles with 0.4 L of reaction volume. The methanogenic
148 anaerobic sludge was heat-treated to inhibit methane-producing microflora in water bath at 96° C for 2 h.
The initial reducing sugars (RS) were adjusted to 13 g/L and the amount of substrate and inoculum were
added according to the inoculum to substrate ratio (ISR) of 1.2 (VS basis). The fermentation volume was
150 supplemented with 4 mL of 200 fold mineral medium previously reported by Rodríguez-Valderrama et
al. (2019). The medium was supplemented with nitrogen source (1 g $\text{NH}_4\text{Cl}/\text{L}$) and buffer medium (3 g
152 $\text{K}_2\text{HPO}_4/\text{L}$, 1.5 g $\text{KH}_2\text{PO}_4/\text{L}$). The experimental units were stirred at 150 rpm in a multiple magnetic stirrer
154 inside an incubator at 35 °C.

156 *Anaerobic digestion*

HS from the separation of LH were washed twice with distilled water (0.03 L of water per 10 g_{wb} of HS),
158 vortexed and sedimented for 10 min, to remove the residual acid catalyst. Afterwards, HS were recovered

by centrifugation (15 min at 10 000 g), dried at 80 °C and characterized (Table 1). HS empirical molecular
160 formula based on elemental composition was $\text{CH}_{2.30}\text{O}_{0.42}\text{N}_{0.03}$.

AD was carried out in 0.120 L serum bottles with 0.08 L of work volume. The inoculum was anaerobic
162 sludge from a semi-continuous digester fed with FVW at 30 d of hydraulic retention time. The inoculum
was degassed during 3 d and then used for HS anaerobic digestion. The inoculum had the following
164 characteristics: 7.81 pH, 93.64%_{wb} moisture, 6.36%_{wb} TS, 51.46%_{db} VS, 48.54%_{db} ashes. The alkalinity
and total VFA were 12 300 mg CaCO_3/L and 8 040 mg VFA/L, respectively. Its empirical molecular
166 formula based on elemental composition was $\text{CH}_{1.34}\text{O}_{2.02}\text{N}_{0.10}$.

Inoculum to substrate ratios (ISR) were assayed in batch mode: 2, 2.5, 3, 3.5 and 4 g_{vs} inoculum/g_{vs}
168 HS. A control was run with fresh FVW at ISR 3. A blank was loaded only with inoculum and water to to
determine the methane production by the organic matter present in the inoculum. The methane production
170 (2.16 mmol CH_4) from blanks was subtracted from the methane produced by HS. All the experimental
units were kept constant in its inoculum content at 2.5 g_{vs}, the substrate amounts were adjusted according
172 to each ISR. The anoxic environment in each bottle was promoted by flushing N_2 during 3 min.
Afterwards, the bottles were sealed with a rubber stopper and aluminum rings. The operation temperature
174 and stirred velocity were 35 ± 1 °C and 150 rpm, respectively. All the experiments were carried out by
duplicate.

Analytical methods

178 The pH was determined according to the procedure described by NMX-AA-25-1984 (1992). Solids profile
was measured according to standard methods (APHA/AWWA/WEF 2005). Cellulose and acid-insoluble
180 lignin contents were determined by the gravimetric method base on AOAC methods (AOAC 1992).
Hemicellulose amount was determined subtracting the cellulose content from holocellulose content after

182 by lignin oxidation by NaClO (Escamilla-Alvarado et al. 2015). The extractives in CS and FVW were
determined by differential weight after extraction in a water bath at 60 °C 24 h (Sluiter et al. 2008). The
184 elemental characterization (C, H, O, N) was determined by an elemental analyzer (Thermo Scientific Flash
2000, U.S.A), O₂ was used as combustion gas and He was used as carrier gas.

186 The reducing sugars (RS) in hydrolyzate were determined by the 3,5-dinitrosalicylic acid method (DNS)
using dextrose for the calibration curve (Miller 1959). Glucose, xylose, and arabinose were quantified by
188 high performance liquid chromatograph (LDC Analytical, U.S.A.) equipped with a Rezex RHM-
Monosaccharide (300mm X 7.8 mm) column and a refractive index detector (Varian Prostar, U.S.A.). The
190 column temperature was 65 °C, whereas the mobile phase (H₂O) flow rate was maintained at 0.6 mL/min.

The total amount of biogas produced in DF and AD was determined by the acid-brine displacement
192 method (Escamilla-Alvarado et al. 2013). Hydrogen and methane gas contents were determined in a gas
chromatograph (Thermo Scientific Trace 1310, U.S.A.); the gas chromatograph was equipped with a
194 thermal conductivity detector and a molecular sieve column (TG-BOND Msieve 5A, 30 m x 0.33 mm).
The operating temperatures were 100 °C, 150 °C, and 200°C for the oven, injector, and detector,
196 respectively. Nitrogen gas was used as a carrier gas with a flow rate of 3 mL/min.

The total phenolic compounds (TPC) were determined by the Folin-Ciocalteu method proposed by
198 Blainski et al. (2013) using tannic acid as the standard. Furfural, HMF, formic acid, acetic acid, propionic
acid, succinic acid, and lactic acid were determined by gas chromatography (Varian CP 3380, U.S.A.) with
200 a column ZB-FFAP (15m x 0.53 x 1 µm) and flame ionization detector. The injector and detector
temperatures were 230 and 280 °C, respectively. The temperature program for the column initiated at 90
202 °C for 3 min, then increased to 200 °C at 20 °C/min rate and maintained for 3 min, and finally raised to
250 °C at 30 °C/min, which was maintained for 4 min.

Total volatile fatty acids (TVFA) and alkalinity concentration were determined by a titration method (Anderson and Yang 1992). Acetic acid, propionic acid, butyric acid, and ethanol were quantified using a gas chromatograph according to the method described in our previous work (Rodríguez-Valderrama et al. 2019).

Calculations

A set of response variables was calculated according to Table 2 to analyze the production of hydrogen, methane and energy potential. The cumulative specific hydrogen production $H(t)$ (mL H₂/g_{glucose}) was used for describing the accumulated production of hydrogen in time and to obtain kinetic parameters from fitting the results by the Gompertz equation (Eq. 1). Thus, the maximum cumulative specific hydrogen production H_{max} (mL H₂/g_{glucose}), the maximum specific hydrogen production rate $r_{max,H}$ (mL H₂/(g_{glucose} h)) and the adaptation time λ (h) were determined.

The hydrogen molar pseudoyield, Y'_{H_2} (mol H₂/mol_{glucose}) in Eq. 2 was obtained from the H_{max} (mL H₂/g_{glucose}) as a means to compare the system to the maximum theoretical hydrogen yield (2 and 4 mol H₂/g_{glucose} for butyrogenic and acetogenic pathways, respectively).

Other variables in the equations listed in Table 2 used in Eq. 1 or Eq. 2 are t is fermentation time (h), e is 2.718, $C_{RS,0}$ and $C_{RS,f}$ (g/L) are the concentration of RS at the beginning and at the end of DF, and $MW_{glucose}$ is the glucose molar weight (180.16 g/mol).

Regarding the AD equations and parameters (Table 2), the cumulative methane production $B(t)$ (mmol CH₄) and cumulative specific methane production $b(t)$ (mmol CH₄/g_{vs}) were used to calculate the kinetic parameters by two methods: an adaptation of the Gompertz equation (Lo et al. 2010, Eq. 3 and Eq. 4), and the first-order model proposed by Hashimoto (1989, Eq. 5 and Eq. 6). Through Eq. 3 and Eq. 5 the maximum cumulative methane production B_{max} is obtained, whereas through Eq. 4 and 6 the maximum

cumulative specific methane production b_{max} is estimated. The other parameters estimated through these equations are the maximum methane production rate $R_{max,M}$ (mmol CH₄/ d), the maximum specific methane production rate $r_{max,M}$ (mmol CH₄/(g_{vs} d)), λ , and the methane production rate k (1/d).

The specific gross energy potential $\hat{E}_p$ (kJ/g_{vs}) was used to compare our *h-H-M* biorefinery against other biorefinery models in the literature, either serial where DF is followed by AD (Eq. 7), or in parallel DF and AD systems (Eq. 8). In these equation, the hydrogen high heating value HHV_{H_2} is 282.8 kJ/mol, the methane high heating value HHV_{CH_4} is 889.9 kJ/mol, V_M is the molar volume of an ideal gas at standard conditions (22.4 L/mol), 1000 is the mL to L conversion factor, η_{DF} is the quotient of VS consumed and VS fed in the DF units, and η_{AH} is the quotient of glucose released and VS fed in acid hydrolysis experiments.

INSERT TABLE 2

Results and Discussion

Diluted acid hydrolysis and overliming

The RS concentration after co-substrates hydrolysis was 23.49 g/L, containing high amounts of glucose (10.36 g/L) followed by xylose (8.61 g/L) and arabinose (0.39 g/L). The RS production yield was 48.54% (calculated as the amount of RS produced divided by the sum of volatile solids added) for the 3:1 FVW:CS combination. This yield is comparable to those reported in the literature for acid hydrolysis of either FVW or CS. For instance, Datar et al. (2007) obtained a hydrolysis yield of 47% in the steam-explosion treatment of acid impregnated CS. Cao et al. (2009) managed to extract the 35.20% of sugars in the CS acid hydrolysis. On the other hand, Díaz et al. (2017) extracted 35.9% of the reducing sugars available in tomato wastes through acid hydrolysis. Additionally, one of the main benefits of the co-substrates acid

250 hydrolysis is the improvement of the monomeric sugars distribution. For instance, in our 3:1 FVW:CS
experiment the monosaccharide distribution in the liquid hydrolyzates was 53.5% glucose, 44.5% xylose
252 and 2% arabinose (Table 1). In contrast, the main monomeric sugars distribution from hydrolysis of only
CS were 9.08% of glucose, 83.08% of xylose and 7.84% of arabinose (Datar et al. 2007), whereas FVW
254 were only composed of 100% hexoses (Del Campo et al. 2006).

The overliming treatment of acid hydrolyzates successfully reduced the inhibitory compounds as TPC
256 in 33.86%, although 10.05% RS were also lost (Table 3). RS loss is commonly expected in such treatments
(Saha et al. 2005), for instance, Chang et al. (2011) reported 9% RS loss after the overliming of rice husk
258 hydrolyzates, and Purwadi et al. (2004) had 8.42% RS loss from detoxification by $\text{Ca}(\text{OH})_2$ of Swedish
forestry residues hydrolyzates. The concentration of TPC, HMF, furfural, and acetic acid, did not surpass
260 the concentrations known to inhibit hydrogen production, which are in the following ranges: 0.8-2.28 g/L
for TPC, 0.86-1.89 g/L for HMF, 0.8-3.41 g/L for furfural and 0.6-7.80g/L for acetic acid (Ren et al. 2008;
262 Gonzales et al. 2016).

264 INSERT TABLE 3

266 *Dark fermentation*

After overliming pretreatment, the LH were used for hydrogen production at initial pH of 7 and 35 °C.
268 After 150 h of fermentation, maximum experimental cumulative biogas and maximum cumulative specific
hydrogen production were 2717 mL and 223.8 mL $\text{H}_2/\text{g}_{\text{glucose}}$, respectively (Fig. 2). The hydrogen average
270 content in biogas was 50.89%, whereas methane was not detected. According to the Gompertz parameters
(Table 4), the production of hydrogen from the acid hydrolyzates of the co-substrates mixtures (3:1
272 FVW:CS) showed a $r_{\text{max},H}$ of 2.60 ($\text{mL H}_2/(\text{g}_{\text{glucose}} \text{ h})$) and 19.25 h of adaptation time.

$r_{max,H}$ is comparable to other studies presented in literature where hydrogen production from acidic

hydrolyzates of organic waste has been studied. Zhang et al. (2015) reported a $r_{max,H}$ of 0.92 (mL H_2 /(g_{glucose} h)) in a 100 mL serum vials with acid hydrolyzates (1.7% v/v H_2SO_4 , 120 min, 120 °C) from CS produced by activated sludge. The reason for their low $r_{max,H}$ compared to our study might be attributed to the low initial sugars concentration (5 g/L against 13 g/L, respectively) and the low initial ISR (0.19 against 1.2, respectively), as such parameters are directly related to hydrogenogenic performance (Fan et al. 2006; Ozmihci et al. 2011). In addition, the hydrogen batch fermentation at ISR lower than 0.16 could have presented inhibition due to the high amounts of substrate (Wong et al. 2014).

INSERT FIG. 2

The maximum productivity of hydrogen from LH was 2909 mL H_2 /L_{reactor}, which can be compared with other studies also using LH for hydrogen production. In the case of Datar et al. (2007), they reported hydrogen productivity of 3310 mL H_2 /L_{reactor} from CS hydrolyzates in a CSTR reactor using a pre-treated anaerobic sludge as an inoculum; the volumetric productivity reported in our work was 12.1% lower than theirs. However, the adaptation time reported by Datar et al. (2007) was 49.5% higher due to the initial concentrations of inhibitors present in their hydrolyzates.

INSERT TABLE 4

The molar pseudoyield obtained at 35°C was 2.02 mol H_2 /mol_{glucose}, comparable to those using pure and mixed cultures from acid hydrolyzates. Biohydrogen production from starch hydrolyzates led to a maximum Y_{H_2} of 1.28 and 0.85 mol H_2 /mol_{glucose} by either *Clostridium pasteurianum* CH5 or by

consortium from dark fermentation sludge, respectively (Chen et al. 2008). Both experiments were carried out at 37 °C and initial sugars concentration of 26.7 g/L. Using acid hydrolyzates from wheat starch (121 °C, 15 min, pH 2.5, H₂SO₄), Cakr et al. (2010) obtained a Y'_{H_2} of 2.4 mol H₂/mol_{glucose} at 55 °C and initial sugars concentration of 18.5 g/L using heat-treated anaerobic sludge. This value was 1.2-fold higher than ours, however, the use of thermophilic temperatures may decrease the energy gains from biofuels production (Rodríguez-Valderrama et al. 2019).

The final pH in hydrogen production was 5.34 (Table 4), an adequate value for hydrogen production as reported in literature (Braguglia et al. 2018). In our experiments, the pH₀ was adjusted to 7 as commonly done in free fermentation experiments. For instance, Liu et al. (2013) observed a considerable hydrogen yield increase from 0.02 mol H₂/mol_{sugars} at pH₀ 5, to 0.44 mol H₂/mol_{sugars} at pH₀ 7 when using rice straw acid hydrolyzates.

The main metabolites in DF were butyric acid (6.13 g/L), acetic acid (1.11 g/L), propionic acid (0.36 g/L) and a small amount of ethanol (0.07 g/L), which indicates that the fermentation was carried out in greatly by butyric acid metabolic pathway (Chen et al. 2006; Wang and Wan 2009). These results demonstrated the good performance of dark fermentation from acid hydrolyzates obtained from organic substrates mixtures by anaerobic sludge.

Anaerobic digestion

The cumulative methane production and specific methane production as a function of time for different ISR are shown in Fig 3. The best results for each parameter were obtained at ISR 2.5 and 4, respectively. Indeed, all the experiments evaluated with HS presented methane productions and specific methane productions higher than the experiments with FVW (control) during the first five days due to the readily

318 biodegradable organic matter in the HS. Still, at the end the control produced as good results as most of
them due to the RIS used and the good biodegradability of FVW at high digestion times (Li et al. 2011).
320 The cumulative methane production (B_{max}) at the different studied ISR (2-4) was in the range 6.37 to 8.78
mmol CH₄. The best results were obtained in the order ISR 2.5>4>3>2>3.5 (Table 5, Fig 3A). On the
322 other hand, the cumulative specific methane production (b_{max}) was in the range 5.84-13.64 mmol CH₄/g_{vs},
obtained from highest to lowest in the order ISR 4>3>3.5>2.5>2. This was in agreement to reports in
324 literature that have ascribed this phenomenon to the fact that a greater inoculum to substrate ratio allows
a more exhaustive conversion of biomass into methane (Hashimoto 1989).

326 The predicted maximum cumulative methane production (B_{max} , 8.90 mmol CH₄) and the lowest
deviation in respect to the experimental value (1.30%) were presented with the first-order kinetic model
328 for ISR=2.5, whereas Gompertz prediction had a deviation of 3.36% respect to the measured production.
The coefficient of determination for this experiment (ISR = 2.5) was 0.99, slightly higher than the obtained
330 by the Gompertz model (0.99). The maximum methane rates (k) for each ISR evaluated were in the range
of 0.19 to 0.28 (1/d). The maximum k (0.28 1/d) corresponded to ISR of 2.5, which could have reflected
332 a positive interaction of a rapid substrate biodegradability due to pretreatment and an adequate inoculum
load. Similarly, at 2.5 ISR Moset et al. (2015) registered a k of 0.16 (1/d) in the anaerobic digestion of
334 corn stover silage (1 year), and 0.08 (1/d) for wheat straw without any previous treatment.

The predicted specific methane productions (b_{max}) by the first-order kinetic and Gompertz models
336 were 14 mmol CH₄/g_{vs} and 13.2 mmol CH₄/g_{vs}, respectively (Table 6). However, the first-order kinetic
model presented the best fit to the experimental data since its error (2.62%) was lower than the respective
338 of the Gompertz model (3.07%). These errors are usual when comparing the fits of mathematical models.
As reported by Zhang et al. (2014) who obtained deviations from 1.5% for the first-order kinetic model

and 3.7% for the Gompertz model when they evaluated the anaerobic digestion of dewatered sewage sludge at ISR of 1 and 37 °C of fermentation temperature.

INSERT FIG. 3

INSERT TABLE 5

The maximum predicted b_{max} (14 mmol CH₄/g_{vs} or 342.3 L CH₄/kg_{vs}) is comparable to the maximum methane specific productions in literature, only 8% lower than the value of 372.4 L CH₄ /kg_{vs}, from alkaline pretreated (5% NaOH) CS on solid-state (Zhu et al. (2010). This difference may be mainly due to the positive traits of alkaline treatment and its less severity compared to acid treatments. Besides lignin solubilization, alkaline pretreatments also provide neutralization of the different acids in lignocellulosic compounds degradation. However, the maximum methane production rate (9.3 L CH₄/(kg_{vs} d)) found by Zhu et al. (2010) after 40 d, was 6.2 fold lower than that obtained from HS during 15 d (2.35 mmol CH₄/(g_{vs} d); 57.4 L CH₄/(kg_{vs} d)), which indicates the rapid substrate degradation due to the acid pretreatment process.

The b_{max} and the $r_{max,M}$ obtained in the control experiment with fresh untreated FVW were 10.01 mmol CH₄/g_{vs} (244.5 L CH₄/ kg_{vs}) and 1.29 mmol CH₄/ (g_{vs} d) (31.6 L CH₄/(kg_{vs} d)), respectively. This b_{max} was 28.63% lower than that obtained with HS (342.3 L CH₄/kg_{vs}), because when pretreating a substrate, either by alkaline pretreatment or acidic pretreatment, the methane production is raised, since soluble organic matter was increased and then easily used by microorganisms (Abudi et al. 2016). The increase in methane production (28.63%) due to substrate pretreatment was also observed in the literature. For instance, Abudi et al. (2016) registered the increment of 6.69% in the methane production from rice straw without

pretreatment and 45.15% from alkali rice straw organic fraction of municipal solid wastes (OFMSW). On the other hand, both substrates (rice straw and pretreated rice straw) showed higher methane production when compared to OFMSW digestion, but this phenomenon seems to be related to the nature of the substrate rather than to the pretreatment applied.

INSERT TABLE 6

Energy evaluation of the h-H-M concept biorefinery

The energy yield from *h-H-M* biorefinery model and its comparison with two-stage systems from different biomasses as substrates are shown in Table 7. The energy yield for *h-H-M* biorefinery based on gaseous biofuels production was 9.7 kJ/g_{vs}. The stage with the greatest contribution to the energy yield was the methane production (*M*) stage with a contribution near to 89.4% (8.7 kJ/g_{vs}), whereas the contribution of hydrogen produced in dark fermentation (*H*) was the remaining 10.6% (1.0 kJ/g_{vs}). Ghimire et al. (2015) calculated a maximum energy yield of 4.4 kJ/g_{vs} for hydrogen and methane production from food waste. Energy contribution of DF was 29.6% (1.3 kJ/g_{vs}) when the food wastes were fermented in a 1.5 L semi-continuous reactor at 55°C, whereas the maximum energy yield contribution was presented by AD (70.4%, 3.1 kJ/g_{vs}). The relatively low energy contribution of DF is ascribed to the inherent energy loss in the form of metabolites in the effluents (Xia et al. 2013).

INSERT TABLE 7

Conclusions

This work demonstrated that a new biorefinery approach coined as *h-H-M*, was technically feasible for treating a preparation of fruits and vegetables wastes and corn stover through acid hydrolysis, dark

fermentation and anaerobic digestion. Acid hydrolysis of the preparation 3:1 fruit and vegetable wastes to corn stover produced hydrolyzates containing up to 10.4 g/L of glucose, 8.61 g/L of xylose and 0.39 g/L of arabinose.

Dark fermentation from liquid hydrolyzates performed competitively at inoculum to substrate ratio 1.2, reaching productivity of 2909.7 mL H₂/L_{reactor} and pseudoyield 2.02 mol H₂/mol_{glucose}.

The hydrolyzed solids were adequately used as carbon source for anaerobic digestion, promoting higher initial methane production when compared to compared to fresh substrate. The maximum cumulative methane production, 8.9 mmol CH₄, and the highest methane specific production, 13.6 mmol CH₄/g_{vs}, were obtained at the inoculum to substrate ratios of 2.5 and 4, respectively.

The total energy potential from the *h-H-M* biorefinery concept in the form of hydrogen and methane reached 9.7 kJ/g_{vs}, of which hydrogen contributed to 10.6 %.

Using a combination of co-substrates such as FVW and CS opens up the possibility of interesting configurations for real biorefineries, as this approach may provide an alternative to mono-substrate drawbacks.

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576

Abbreviations

578	AD	Anaerobic digestion
	$B(t)$	Cumulative methane production (mmol CH ₄)
580	B_{max}	Maximum cumulative methane production (mmol CH ₄)
	$b(t)$	Cumulative specific methane production (mmol CH ₄ /g _{vs})
582	b_{max}	Maximum cumulative specific methane production (mmol CH ₄ /g _{vs})
	CS	Corn stover
584	$C_{RS,0}$	Initial reducing sugars concentration (g/L)
	$C_{RS,f}$	Final reducing sugars concentration (g/L)
586	DF	Dark fermentation
	e	Euler number (2.718)
588	$\hat{E}_p$	Specific gross energy potential (kJ/g _{vs})
	FVW	Fruits and vegetables wastes
590	h	Diluted acid pretreatment stage
	H	Hydrogen production stage by DF
592	$H(t)$	Cumulative hydrogen specific production (mL H ₂ /g _{glucose})
	H_{max}	Maximum cumulative specific hydrogen production (mL H ₂ /g _{glucose})
594	h - H - M	Biorefinery model
	HHV_{H_2}	High hydrogen heating value (282.8 kJ/mol)
596	HHV_{CH_4}	High methane heating value (889.9 kJ/mol)
	HMF	5-hydroxymethylfurfural
598	HS	Hydrolyzed solids
	ISR	Inoculum to substrate ratio
600	k	Methane production rate (1/d)
	LH	Liquid hydrolyzates
602	M	Methane production stage by AD
	$MW_{glucose}$	Glucose molar weight (180.16 g/mol)
604	ND	Not determined
	pH ₀	Initial pH
606	$r_{max,M}$	Maximum specific methane production rate (mmol/(g _{vs} d))
	$r_{max,H}$	Maximum specific hydrogen production rate (mL/(g _{glucose} h)),
608	$R_{max,M}$	Maximum methane production rate (mmol/d)
	R ²	Coefficient of determination

610	RS	Reducing sugars
	T	Operational temperature
612	t	Time
	TPC	Total phenolic compounds
614	TS	Total solids
	TVFA	Total volatile fatty acids
616	V_M	Molar volume at standard conditions (22.4 L/mol H ₂ or CH ₄)
	V_O	Operational volume
618	VS	Volatile solids
	Y'_{H_2}	Hydrogen molar pseudoyield (mol H ₂ /mol _{glucose})
620		

622 Subindices

	db	dry basis
624	wb	wet basis

626 Greek characters

628	λ	adaptation time (h or d)
	η_{AH}	Acid hydrolysis efficiency (g _{glucose} /g _{vs})
630	η_{DF}	Dark fermentation efficiency (g _{vs} consumed/g _{vs} added)

632   **List of Tables**

634   **Table 1** Characteristic of co-substrates (FVW and CS) and HS

634   **Table 2** Equations used to describe the *H* and *M* stages and the biorefinery performance

636   **Table 3** Main sugar production and secondary products from acid hydrolysis pretreatment of co-substrates
(3:1 FVW:CS)

638   **Table 4** Gompertz coefficients and performance parameters from dark fermentation of LH

640   **Table 5** Kinetic parameters comparison between the first-order model and Gompertz model on the
maximum cumulative methane production ($B_{max.}$) during anaerobic digestion of HS and FVW

642   **Table 6** Kinetic parameters comparison between the first-order model and Gompertz model on the
maximum cumulative specific methane production ($b_{max.}$) during anaerobic digestion of HS and FVW

642   **Table 7** Biorefinery process conditions and energy yields

644

646

648

650

652 **Table 1** Characteristic of co-substrates (FVW and CS) and HS

Parameter	FVW	CS	HS
pH	5.52	7.54	3.66
Moisture (%wb)	89.81	5.41	84.00
TS (%wb)	10.19	94.59	16.00
VS (%db)	87.66	89.78	93.43
Ashes (%db)	12.34	10.22	6.57
Alkalinity (mg CaCO ₃ /L)	475	450	200
Total Volatile fatty acids (mg VFA/L)	570	420	220
Cellulose (%db)	12.80	33.25	24.86
Hemicellulose (%db)	24.40	24.35	17.81
Lignin (%db)	10.26	24.74	16.29
Protein (%db)	12.63	3.25	12.14
Extractives (%db)	38.11	10.19	ND
C (%db)	51.69	43.84	56.19
H (%db)	3.43	15.74	10.74
O (%db)	42.69	39.98	31.15
N (%db)	2.19	0.44	1.92

654 Abbreviations: CS, corn stover; db, dry base; FVW, fruits and vegetables wastes; ND, not determined; TS, total solids; VFA, volatile fatty acids; VS, volatiles solids; wb, wet base.

656

658

Table 2 Equations used to describe the H and M stages and the biorefinery performance

Response variable	Equation	Equation number
Cumulative hydrogen specific production	$H(t) = H_{\max} \cdot \exp \left\{ -\exp \left[\frac{R_{\max, H} \cdot e}{H_{\max}} (\lambda \cdot t) + 1 \right] \right\}$	(1)
Hydrogen molar pseudoyield	$Y'_{H_2} = \frac{H_{\max} \cdot C_{RS,0} \cdot (MW_{glucose})}{(C_{RS,0} - C_{RS,f}) \cdot (V_M) \cdot 1000}$	(2)
Cumulative methane production (Gompertz-modified model)	$B(t) = B_{\max} \cdot \exp \left\{ -\exp \left[\frac{R_{\max, M} \cdot e}{B_0} (\lambda \cdot t) + 1 \right] \right\}$	(3)
Cumulative specific methane production (Gompertz-modified model)	$b(t) = b_{\max} \cdot \exp \left\{ -\exp \left[\frac{r_{\max, M} \cdot e}{b_0} (\lambda \cdot t) + 1 \right] \right\}$	(4)
Cumulative methane production (first-order kinetic model)	$B(t) = B_{\max} \cdot (1 - \exp^{-k \cdot t})$	(5)
Cumulative specific methane production (first-order kinetic model)	$b(t) = b_{\max} \cdot (1 - \exp^{-k \cdot t})$	(6)
Specific gross energy potential for serial hydrogen and methane production	$\hat{E}_P = \frac{1}{V_M \cdot 1000} \cdot (H_{\max} \cdot HHV_{H_2} + (1 - \eta_{DF}) \cdot b_{\max} \cdot HHV_{CH_4})$	(7)
Specific gross energy potential for parallel hydrogen and methane production	$\hat{E}_P = \frac{1}{V_M \cdot 1000} \cdot (\eta_{AH} \cdot H_{\max} \cdot HHV_{H_2} + (1 - \eta_{AH}) \cdot b_{\max} \cdot HHV_{CH_4})$	(8)

662 **Table 3** Main sugar production and secondary products from acid hydrolysis pretreatment of co-substrates
(3:1 FVW:CS)

Parameter	LH before overliming	LH after overliming
RS (g/L)	23.49	21.13
Glucose (g/L)	10.36	9.65
Xylose (g/L)	8.61	8.36
Arabinose (g/L)	0.39	0.07
HMF (g/L)	0.65	ND
Furfural (g/L)	0.14	ND
TPC (g/L)	1.14	0.76
Formic acid (g/L)	4.02	ND
Acetic acid (g/L)	0.53	ND

664 Abbreviations: HMF, 5-hydroxymethylfurfural; ND, not detected; LH, liquid hydrolyzates; RS, reducing sugars; TPC, total
phenolic compounds.

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Table 4 Gompertz coefficients and performance parameters from dark fermentation of LH

Parameter	Value
H_{max} (mL H ₂ /g _{glucose})	223.8 ± 5.2
$R_{max,H}$ (mL H ₂ /(g _{glucose} h))	2.60 ± 0.08
λ (h)	19.25 ± 0.52
R ²	0.99
Final pH	5.34 ± 0.60
Acetic acid (g/L)	1.11 ± 0.007
Propionic acid (g/L)	0.36 ± 0.011
Butyric acid (g/L)	6.13 ± 0.265
Ethanol (g/L)	0.07 ± 0.003
Sugars consumption (%)	89.30 ± 0.4
Volumetric productivity (mL H ₂ /L _{reactor})	2909 ± 67.8
Y'_{H2} (mol H ₂ /mol _{glucose})	2.02 ± 0.05

670 Abbreviations: H_{max} , maximum cumulative specific hydrogen production; $R_{max,H}$, maximum specific hydrogen production rate; R², coefficient of determination ; Y'_{H2} , hydrogen molar pseudoyield; λ , adaptation time.

Table 5 Kinetic parameters comparison between the first-order model and Gompertz model on the maximum cumulative methane production (B_{max}) during anaerobic digestion of HS and FVW

Model	ISR-substrate type					
	2-HS	2.5-HS	3-HS	3.5-HS	4-HS	3-FVW
Experimental B_{max} (mmol CH ₄)	7.59	8.78	7.70	6.37	8.19	7.74
<i>First-order</i>						
B_{max} (mmol CH ₄)	7.85	8.89	8.09	6.84	8.40	NA
k (1/d)	0.23	0.28	0.23	0.19	0.25	NA
R^2	0.99	0.99	0.99	0.99	0.99	NA
Error (%)	3.47	1.29	5.09	7.36	2.62	NA
<i>Gompertz</i>						
B_{max} (mmol CH ₄)	7.39	8.49	7.69	6.32	7.93	8.01
$R_{max,M}$ (mmol CH ₄ /d)	1.18	1.67	1.14	0.82	1.41	1.03
λ (d)	0.00	0.00	0.00	0.00	0.00	1.46
R^2	0.99	0.99	0.99	0.99	0.99	0.99
Error (%)	2.61	3.36	0.11	0.87	3.07	3.49

Abbreviations: B_{max} , maximum cumulative methane production; k , methane production rate; HS, hydrolyzed solids; FVW, fruits and vegetables wastes; NA, not applicable; $R_{max,M}$, maximum methane production rate; R^2 , coefficient of determination; λ , adaptation time.

Note: range of final pH values 7.53-7.72.

Table 6 Kinetic parameters comparison between the first-order model and Gompertz model on the maximum cumulative specific methane production (b_{max}) during anaerobic digestion of HS and FVW

Model	ISR-substrate					
	2-HS	2.5-HS	3-HS	3.5-HS	4-HS	3-FVW
Experimental b_{max} (mmol CH ₄ /g _{VS})	5.84	8.78	9.63	9.10	13.64	9.67
<i>First-order</i>						
b_{max} (mmol CH ₄ / g _{VS})	6.04	8.89	10.12	9.77	14.00	NA
k (1/d)	0.23	0.28	0.23	0.19	0.25	NA
R ²	0.99	0.99	0.99	0.99	0.99	NA
Error (%)	3.47	1.29	5.09	7.36	2.62	NA
<i>Gompertz</i>						
b_{max} (mmol CH ₄ / g _{VS})	5.68	8.49	9.62	9.02	13.22	10.01
$r_{max,M}$ (mmol CH ₄ / (g _{VS} d))	0.90	1.67	1.43	1.18	2.35	1.29
λ	0.00	0.00	0.00	0.00	0.00	1.46
R ²	0.99	0.99	0.99	0.99	0.99	0.99
Error (%)	2.61	3.36	0.11	0.87	3.07	1.46

Abbreviations: b_{max} , maximum cumulative specific methane production; k , methane production rate; HS, hydrolyzed solids; FVW, fruits and vegetables wastes; NA, not applicable; $r_{max,M}$, maximum specific methane production rate; R², coefficient of determination; λ , adaptation time.

Note: range of final pH values 7.53-7.72.

Table 7 Biorefinery process conditions and energy yields

Substrate	Substrate pretreatment	Process	Dark fermentation	Anaerobic digestion	$\hat{E}_p$ (kJ/g _{VS})	Reference
Rapeseed straw	Alkaline peroxide (3% v/v) and steam (18 °C, 10 min)	Serial two-stage: DF (batch) + AD (batch)	V ₀ =60 mL T=55 °C pH ₀ =5.5 H _{max} =91 mL H ₂ /g _{VS}	V ₀ =60 mL T= 55 °C pH ₀ =7.5 b _{max} =370 mL CH ₄ /g _{VS}	13.2	Luo et al. (2011)
<i>Nannochloropsis oceanica</i>	Microwave (140 °C, 15 min, 1.0% H ₂ SO ₄ , 50 g/l	Serial two-stage: DF (batch) + AD (batch)	V ₀ =250 mL T=35 °C pH ₀ =6.0 H _{max} = 39 mL H ₂ /g _{VS}	V ₀ =250 mL T= 35 °C pH ₀ =8.0 b _{max} = 114 mL CH ₄ /g _{VS}	4.7	Xia et al. (2013)
Food waste	NA	Serial DF (semi-continuous) and AD (batch)	V ₀ = 1500 mL T= 55 °C pH ₀ = 7.0 H _{max} =104 mL H ₂ /g _{VS}	V ₀ = 600 mL T= 34 °C pH ₀ = 7 b _{max} = 99.3 mL CH ₄ /g _{VS}	4.4	Ghimire et al. (2015)
Organic fraction of municipal solid wastes	NA	Serial two-stage DF (semi-continuous) + AD (semi-continuous)	V ₀ = 500 g T= 55 °C pH ₀ = 8.0 H _{max} = 33.3 mL H ₂ /g _{VS}	V ₀ = 500 g T= 55 °C pH ₀ = 8.0 b _{max} = 353 mL CH ₄ /g _{VS}	13.5	Escamilla-Alvarado et al. (2014)
Sugarcane bagasse and water hyacinth (1:2 ratio)	Alkaline peroxide (2.5% v/v, 50°C, 150 min) and enzymatic hydrolysis (<i>Cellulase</i> , 24 U/g _{db} , 37 °C, 180 rpm, 48 h	Serial two-stage DF (continuous) + AD (continuous)	V ₀ = 500 mL T= 37 °C pH ₀ =6.5 H _{max} = 303 mL H ₂ /g _{VS}	V ₀ = 4000 mL T= 37 °C pH ₀ = 7.5 b _{max} = 142 mL CH ₄ /g _{VS}	6.0	Kumari and Das (2019)
Water hyacinth	NA	Serial two-stage DF (semi-continuous) + AD (semi-continuous)	V ₀ = 500 mL T= 37 °C pH ₀ = 6.5 H _{max} = 93 mL H ₂ /g _{VS}	V ₀ = 500 mL T= 37 °C pH ₀ = 7.5 b _{max} = 270.5 mL CH ₄ /g _{VS}	2.6	Varanasi et al. (2018)
Corn stover	Steam-explosion (220 °C, 3 min) water-impregnated)	Parallel DF	V ₀ = 1250 mL T= 35 °C pH ₀ = 5.5 H _{max} = 304.3 mL H ₂ /g _{VS}	V ₀ = 1250 mL T= 35 °C pH ₀ = 5.5 H _{max} = 152.3 mL H ₂ /g _{VS}	2.24	Datar et al. (2007)
Organic wastes (FVW-40%, WAS-40%, OMW-10%, CM-10%)	NA	Serial two-stage DF (continuous) and AD (continuous)	V ₀ = 800 mL T= 37 °C pH ₀ = 5.2 H _{max} = 79.4 mL H ₂ /g _{VS}	V ₀ = 1200 mL T= 37 °C pH ₀ = 7.2 b _{max} = 410 mL H ₂ /g _{VS}	14.69	Farhat et al. (2018)
Preparation of FVW:CS 3:1	Acid hydrolysis (0.5% HCl, 120 °C, 120 min)	Parallel DF and AD	V ₀ = 400 mL T= 35°C pH ₀ =7.0 H _{max} = 223.8 mL H ₂ /g _{VS}	V ₀ = 80 mL T= 35 °C pH ₀ = 7.6 b _{max} = 342.3 mL CH ₄ /g _{VS}	9.7	This work

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Abbreviations: AD, anaerobic digestion; b_{max} , Maximum cumulative specific methane production; DF, dark fermentation; CM, cattle manure NA, not applicable; FVW, fruit and vegetable waste; H_{max} , maximum cumulative specific hydrogen production; OMW, olive mill wastewater; pH₀, initial pH; T, operational temperature; V₀, operational volume; WAS, waste-activated sludge.

List of Figures

Fig. 1 The *h-H-M* biorefinery concept

Fig. 2 Cumulative specific hydrogen production using LH as substrate

Fig. 3 Anaerobic digestion performance using HS or FVW as substrate in terms of (A) cumulative methane production and (B) cumulative specific methane production

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The prediction lines for ISR 2 to 4 correspond to the first order model;

The prediction line for control corresponds to the Gompertz model.