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Batch biohydrogen production from dilute acid hydrolyzates of fruits- and-vegetables wastes and corn stover as co-substrates

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ABSTRACT

Fruits-and-vegetables wastes (FVW) and corn stover (CS) are two of the most recurred lignocellulosic biomasses used for biofuel production. In this work, the co-processing of FVW and CS for biohydrogen production was proposed and evaluated through a set of experimental designs. First, a 5×2 general factorial was applied on the dilute acid pretreatment at five levels of FVW:CS ratios (0:1, 1:3, 1:1, 3:1 and 1:0 dry mass basis) and two levels of the type of catalyst (HCl or H₂SO₄ at 0.5% in volumetric basis). Then, biohydrogen production using the dilute acid hydrolyzates was carried out in batch mode at 35 °C in a 3² factorial design, the factors being the inoculum to substrate ratio (0.8, 1.0, and 1.2 g g⁻¹) and the initial concentration of reducing sugars (10, 13 and 16 g L⁻¹). The effects of the type of acid catalyst and the FVW:CS ratio were significant in terms of sugars production and yield. The best catalyst was HCl for the 3:1 FVW:CS ratio, which produced monomeric sugars concentrations of 10.0, 3.7 and 2.9 g L⁻¹ for glucose, xylose and arabinose, respectively. The acid hydrolyzates proved to be suitable for biohydrogen production, reaching yields of 2.31 mol H₂ mol⁻¹glucose and hydrogen production rates of 8.83 mL H₂ h⁻¹. An economic prospection at lab scale demonstrated that production of hydrogen presented net revenues of 0.009 USD per kg of co-substrates (wet basis), resulting in 24 % profitability of hydrogen production over its production costs. Therefore, this co-processing is an interesting proposal with further applications on biorefinery models.

Keywords: dark fermentation, dilute acid, factorial experimental design, hydrolyzates, overliming.

1. Introduction

Dark fermentation biohydrogen production has become a promising technology as it may be used to process various types of organic wastes (solid or liquid). It does not require luminous energy, it has high hydrogen production rates in comparison to other biological methods and it may be carried out at non-sterile conditions [1]. Lignocellulosic wastes have been highly esteemed for their abundance and bioprocessing potential [2]. Corn stover (CS) is a worldwide important lignocellulosic waste; high amounts are produced in countries such as Mexico where corn production reaches 21.1 million tons yearly along with the production of up to 38 million tons of corn cobs and CS [3,4].

Due to the highly stable and organized microstructure of its lignocellulosic components, pretreatments are required to reduce its recalcitrance and the cellulose crystallinity to facilitate the carbohydrates depolymerization [5]. Cellulose and hemicellulose can be broken-down to its mono- and disaccharides constituents by dilute acid hydrolysis. Yet the resultant sugar concentration depends on the operational conditions (*i.e.* reaction time, temperature, acid concentration) and type of feedstock [1,6]. Moreover, acid hydrolyzates influence dark fermentation by many factors, such as the type and concentration of inoculum, sugars and inhibitors concentration, temperature, and pH [7]. An excessive loading of carbohydrates may hinder the biohydrogen due to the excessive production of organic acids that lower the fermentation pH and promote solventogenic lactic-acid oriented metabolism [2]. Therefore, the determination of an adequate inoculum to substrate ratio (ISR), which relates the amounts of inoculum fed to the bioreactor in comparison to the substrate loadings, is important to foster biohydrogen production. Moreover, ISR may help reduce the inhibitory characteristic of acid hydrolysis. On the other hand, at higher proportion of substrate (e.g. 1:6 ISR), hydrogen production has been inhibited due to increase of osmotic pressure that affects microbial growth [7], and the excessive production and accumulation of volatile fatty acids (VFA) that cause pH decrease.

CS dilute acid hydrolyzates are mainly composed of xylose since its hemicellulose content present easier hydrolysis [8]. Indeed, this represents a challenge for the microbial metabolism preference of hexoses over pentoses. An opportunity to increase the hexoses content would be to combine the acid hydrolysis pretreatment with that of a substrate with easier degradability, such as the fruits-and-vegetables wastes (FVW) another carbohydrates rich lignocellulosic material [9].

Indeed, combination of substrates has been previously assayed through the co-digestion processes. These are characterized by the simultaneous processing of an incompletely degraded and microbiologically rich substrate (e.g. sewage sludge, pig manure, cow manure, waste activated sludge) and a carbohydrate rich substrate (e.g. sugarcane bagasse, rice straw, corn stover, corn stalk, wheat straw, cassava stillage) [10]. Co-digestion has been resorted for its advantages, such as dilution of toxic compounds, improvement of nutrients balance (carbohydrates/proteins) [11] and microbial synergies [10]. Recently, co-digestion of vegetable and food wastes with cow manure has been associated to process stability, but not necessarily with improved biomethane production [12].

Even though lignocellulosic biomasses have been successfully co-digested with microbial rich sludges or manures for hydrogen production (e.g. cassava stillage and food waste) [10,13], the co-processing of the CS and FVW has not been previously assayed elsewhere. Researching this kind of alternatives would be useful in the development of future biorefineries by avoiding mono-substrates implicit hurdles, such as seasonality and lack of nutrients, and improving supply chain management through the reduction of delivery costs and harmful impacts related to climate change and fossil depletion [14,15].

In this work, the main aim was to evaluate how the mix ratio of FVW and CS affected the production of hydrolyzates used in biohydrogen production. Specifically, design of experiments were developed for insightful determination of the effects of acid catalyst on reducing sugars production, and thereafter the effects of reducing sugars concentration and the inoculum to substrate ratio on biohydrogen production. As it is becoming of utter importance to assess the

economics of bioenergetics (hydrogen, methane, bioethanol), an economic prospection at the laboratory level was also developed.

2. Material and Methods

2.1. Feedstocks

The CS feedstock obtained from Cuencamé, Durango, Mexico, was dried at 85 °C during 24 h. Subsequently, dried CS was grinded to a particle size of 180 µm using a manual mill.

The characterization of the CS was made following methods previously reported [2]: pH 7.54, total solids (TS) 94.59%, volatile solids (VS) 89.78%_{dry basis} and ashes 10.22%_{dry basis}. The main composition of the CS in dry basis (db) was 33.25%_{db} cellulose, 24.35%_{db} hemicellulose, 24.74%_{db} lignin, 3.25%_{db} protein and 10.19%_{db} extractives. The elemental composition of CS was C-43.84%, H-15.74%, O-39.98% and N-0.44%.

The FVW were collected from a local cafeteria (Facultad de Ciencias Químicas, Universidad Autónoma de Nuevo León, Nuevo León, México). FVW were shredded (Hamilton Beach, Food Processor) and dried at 85 °C during 24 h, and grinded to a particle size of 180 µm using a mortar. FVW consisted of (wet weight basis): papaya peels (16.94%), squash peels (15.57%), potato peels (17.67%), spinach stems (20.26%); parsley stems (6.71%), cucumber peels (5.72%); melon peels (10.20%) and apple residues (6.92%). FVW presented pH 5.52, total solids 10.19%, volatile solids 87.66%_{db} and 12.34%_{db} of ashes. Further characterization of this material showed as composition of 12.8%_{db} cellulose, 23.4%_{db} hemicellulose, 10.26%_{db} lignin, 12.63%_{db} protein and 38.11%_{db} extractives. The elemental composition of FVW was C-51.69%, H-3.43%, O-42.69% and N-2.19%.

2.2. Dilute acid hydrolysis pretreatment

Dilute acid hydrolysis was evaluated using a 5 × 2 general factorial experimental design [16]. The factors were the FVW to CS ratio (0:1, 1:3, 1:1, 3:1, 1:0 FVW:CS_{db}) and the type of acid catalyst (HCl or H₂SO₄, 0.5% in volumetric basis). The acid hydrolysis was carried out by triplicate in a

block digester (DRB 200, HACH, U.S.A.); the co-substrates preparations were put into HACH
134 tubes at 6.6% of total solid content and the reaction volume was 5 mL. The hydrolysis time and
temperature were set at 120 min and 120 °C [17,18]. After the hydrolysis pretreatment, solid
136 residues were removed by centrifugation at 10 000 g, 10 min. Liquid hydrolyzates (LH) were used
for sugar analysis (reducing and monomeric sugars) and for the determination of degradation
138 products (furfural; 5-hydroxymethyl-furfural, HMF; total phenolic compounds, TPC; formic acid;
acetic acid; propionic acid; succinic acid; lactic acid) according to the method reported by Muñoz-
140 Páez *et al* [19].

After obtaining the LH, these were processed to remove inhibitory compounds (i.e. HMF,
142 furfural, TPC). For that, the pH was adjusted to pH 10 by adding powder $\text{Ca}(\text{OH})_2$ and continuous
stirring for an hour. The formed precipitate was separated by centrifugation at 10 000 g, 15 min;
144 supernatants were decanted and neutralized to pH 7 by HCl 6 M addition [20].

146 2.3. Dark fermentation of LH

Hydrogenogenic inoculum was obtained from an anaerobic digester fed with FVW at 30 days of
148 hydraulic retention time, started-up according to the Poggi-Varaldo *et al.* [21]. To inhibit methane-
producing microflora, the anaerobic sludge was heat-shock treated in boiling water at 96 °C for 2
150 h [22].

The effects of inoculum to substrate ratio ($\text{ISR} = \text{g VS}_{\text{inoculum}} \text{g VS}_{\text{substrate}}^{-1}$) and initial reducing
152 sugars concentration ($C_{\text{RS},i}$) on the hydrogen production were evaluated using a 3^2 factorial design.
The levels for ISR and $C_{\text{RS},i}$ were 0.8, 1.0, 1.2 and 10, 13, 16 g L⁻¹, respectively.

154 Batch dark fermentation experiments were carried out by duplicate in 120 mL serum bottles
with 70 mL of working volume. The $C_{\text{RS},i}$ was adjusted by dilution of concentrated LH (ca. 21.13 g
156 L⁻¹) according to the experimental design. The fermentation was supplemented with 0.4 mL of 200
fold concentrated mineral medium [22]. After inoculation, each bottle was flushed during 3 min
158 with nitrogen to promote anaerobic conditions and sealed with a rubber stopper and aluminum

rings. The serum bottles were incubated at 35 °C in a controlled temperature incubator with orbital
160 agitation (150 rpm).

162 2.4. Analytical methods

The pH of co-substrates was determined according to proceeding described by NMX-AA-25-1984
164 [23]; 5 g of CS or FVW were diluted with 25 ml of distilled water, stirred for 10 min, and finally
measure using a pH-meter (Conductronic PC45, Mexico). Total solids (TS), volatile solids (VS)
166 and ashes were measured according standard methods [24]. Cellulose and insoluble lignin
analysis were determined by gravimetric method according to AOAC [25]. The hemicellulose
168 amount was determined by difference between holocellulose and cellulose content. Holocellulose
was determined by lignin oxidation using NaClO according to AOAC modified proceeding [26].
170 Extractives were measured by water extraction in a water bath at 60°C during 24 h [27]. Elemental
quantities of carbon, hydrogen, oxygen and nitrogen were determined by organic elemental
172 analyzer (Thermo Scientific Flash 2000, U.S.A.).

The content of total reducing sugars (RS) in LH was determined by 3,5-dinitrosalicylic acid
174 method (dextrose as the standard) [28]. Monomeric sugars were quantified by high performance
liquid chromatography (LDC Analytical, U.S.A.) with a Rezex RHM-Monosacharide (300mm x 7.8
176 mm) column and a refractive index detector (Varian Prostar, U.S.A.) with H₂O as mobile phase.
The column temperature and mobile phase flow rate were 65 °C and 0.6 mL min⁻¹, respectively;
178 glucose, xylose and arabinose were used as standards.

The content of total phenolic compounds (TPC) was determined by colorimetric method using
180 Folin Ciocalteu reactive (tannic acid as standard) proposed by Blainski *et al.* [29]. The subproducts
of the acid hydrolysis (furfural, HMF, formic acid, acetic acid, propionic acid, succinic acid, and
182 lactic acid) were determined against standards bought from Sigma Aldrich. These compounds
were analyzed using a gas chromatograph (Varian CP 3380, U.S.A.) equipped with a column ZB-
184 FFAP (15m x 0.53 x 1 µm) and flame ionization detector (FID). The temperatures of the injector

and the detector were 230 and 280 °C, respectively. The column was first heated to 90 °C for 3 min, then the temperature was raised gradually to 200 °C with the step rate of 20 °C min⁻¹, the temperature was set at 200°C for 3 min and finally raised to 250 °C at 30 °C min⁻¹, which was then kept for 4 min. Calibration curves were previously developed using pure components.

Hydrogen gas content was determined using a gas chromatograph (Thermo Scientific Trace 1310, U.S.A.) equipped with a molecular sieve column (TG-BOND Msieve 5A, 30 m x 0.33 mm) and thermal conductivity detector (TCD). The temperatures of the oven, injector and detector were 100 °C, 150 °C and 200°C, respectively. Nitrogen gas was used as carrier gas with a flow rate of 3 mL min⁻¹. The amount of total gas produced was determined by acid-brine displacement method.

Volatile fatty acids (i.e acetic acid, propionic acid, butyric acid) and solvent (ethanol) composition in the liquid phase were determined by using a gas chromatograph (Thermo Scientific Trace 1310, U.S.A) as described in our previous publication [22] .

2.5. Calculations and statistical analysis

Hydrolysis potential (*HP*, g mol⁻¹ H₃O⁺) was considered as the quotient of RS concentration, *C_{RS}* in g L⁻¹, and the theoretical hydronium concentration of the acid catalyst, *C_{H3O+}* as mol H₃O⁺ L⁻¹, as described in Eq. 1. According to the concentration 0.5% volumetric basis for H₂SO₄ and HCl used for the hydrolysis experiments, their respective theoretical hydronium ion concentrations were 0.193 and 0.447 mol H₃O⁺ L⁻¹.

$$HP = \frac{C_{RS}}{C_{H_3O^+}} \quad (1)$$

Reducing sugars production yield, *Y_{RS}* (%), was calculated comparing the *C_{RS}* and the total carbohydrates content in the co-substrates (Eq. 2):

$$Y_{RS} = \frac{C_{RS} \cdot V_r}{m_H \cdot (H_{CS} \cdot C_{cs} + H_{FWW} \cdot C_{FWW})} \cdot 100 \quad (2)$$

where V_r is the hydrolysis volume (L), m_H is the co-substrates mass (g), H_{CS} and H_{FVW} are the total carbohydrates content (considered as the sum of cellulose, hemicellulose and extractives) in CS and FVW in dry basis (g g⁻¹), respectively, whereas C_{CS} and C_{FVW} are the CS and FVW mass concentration in each co-substrate preparation (g g⁻¹), respectively.

The hydrogen cumulative hydrogen production, $H(t)$ (mL H₂), was fitted by the modified Gompertz equation (Eq. 3), to estimate maximum cumulative H₂ production, H_{max} (mL H₂), maximum H₂ production rate, R_{max} , (mL H₂ h⁻¹), and lag time, λ (h) [30]:

$$H(t) = H_{max} \cdot \exp \left\{ -\exp \left[\frac{R_{max}}{H_{max}} \cdot e (\lambda - t) + 1 \right] \right\} \quad (3)$$

where t is any time (h) and e is 2.718.

The hydrogen molar yield in terms of glucose equivalents consumed (Y_{H_2} mol H₂ mol⁻¹_{glucose}) was determined according Eq. 4:

$$Y_{H_2} = \frac{H_{max} \cdot (MW_{glucose})}{V_R \cdot (C_{RS_o} - C_{RS_f}) \cdot (V_M) \cdot 1000} \quad (4)$$

where V_R is the fermentation volume (L), $C_{RS,i}$ and $C_{RS,f}$ are the initial and final RS concentrations (g L⁻¹), respectively, $MW_{glucose}$ is the glucose molar weight (180.16 g mol⁻¹), V_M is the molar volume at standard reference conditions (22.4 L mol H₂⁻¹) and 1000 is a volume conversion factor (mL L⁻¹).

Analysis of variance (ANOVA) was performed for the experimental designs in the hydrolyzates and hydrogen production, using Design-Expert 6.0 (Design-Ease Inc. Co., Minneapolis, USA).

The main effects of the factors evaluated in the experimental designs were calculated according to Montgomery [16]. In the dilute acid hydrolysis experimental design, reducing sugars yield (Y_{RS}), HMF concentration, furfural concentration and TPC were selected to evaluate the main effects, whereas for the dark fermentation experimental design the maximum cumulative hydrogen production (H_{max}) and hydrogen molar yield (Y_{H_2}) were selected. The standard error of the

experimental designs was calculated from the square root of the mean square of the error divided
by the number of experimental repetitions.

2.6. Economic prospection

The economic prospection accounted the expenses from the main operation stages, and the substances and compounds required for the process. The prospection considered the processing of 1 kg preparation of 3:1 FVW:CS, including the size reduction, acid hydrolysis and dark fermentation. The size reduction accounted the shredding of FVW in a food processor whereas CS was milled in a vibratory mill. Afterwards, the co-substrates mixture was acid hydrolyzed using HCl (0.5% volume basis, 2 h, 120 °C). The LH were recovered by centrifugation (10 000 g, 10 min) and were overlimed with Ca(OH)₂ (1 h, room temperature). After centrifugation (*ibid.*), LH were neutralized using HCl (6 M). The LH were fed to a bioreactor with hydrogenogenic inoculum (35 °C). The same mineral composition as in the experiments was considered. Hydrogen content in biogas was 50.1% and hydrogen yield was 1.91 mol H₂ mol⁻¹_{glucose}.

Collection costs of the substrates were retrieved from literature. Thompson and Tyner [31] calculated the collection, baling and transport costs of CS at farms. For the cost of collection and transport of FVW, an average was obtained after the works of Yepes *et al.* [32] and Mattsson *et al.* [33], who evaluated the management for FVW valorization in production fields and cities, respectively. Operation costs for the processing of FVW and CS, and hydrogen production, were calculated from energy balances and the Mexican electricity rates at industry [34]. Water cost was taken from Mexican National Water Commission [35]. Chemicals for acid hydrolysis, overliming and mineral medium were taken from different market sources.

The energy calculations from shredding, milling and overliming were obtained as previously described [36]. The energy consumption of acid hydrolysis was determined according to Mafe *et al.* [37]. The energy balance in dark fermentation reactor was carried out as described by Lübken

et al. [38]. The total process revenue was determined from the market price of H₂ and CO₂ [39] and their productivities.

3. Results and Discussion

3.1. Diluted acid hydrolysis pretreatment

The highest concentrations of RS were obtained at 1:0 FVW:CS ratio, being 19.32 and 27.32 g L⁻¹ for H₂SO₄ and HCl, respectively (Fig. 1A), probably as a consequence of a number of factors such as: a greater digestibility of FVW over CS, heterogeneous composition of FVW, lower lignin content and lower degradation temperature [9]. Regarding the co-substrate preparations, the maximum RS were 24.69 and 17.99 g L⁻¹ for HCl and H₂SO₄, respectively, obtained from the 3:1 FVW:CS ratio.

PLEASE INSERT FIGURE 1

The HCl as acid catalyst presented higher RS production than H₂SO₄ at each co-substrate ratio, probably influenced by its 2.32 times higher concentration of hydronium ion than H₂SO₄ treatments. However, when observing the *HP* (Fig. 1B), H₂SO₄ was better than HCl in achieving a *HP* of 100.33 g mol⁻¹ H₃O⁺ at 1:0 FVW:CS ratio, versus 61.13 g mol⁻¹ H₃O⁺ for HCl at same ratio. Hilpmann *et al.* [40] observed that hydronium concentration is one of the several factors that have influence on the efficiency of the acid hydrolysis of xylan. They found that hydronium concentrations lower than 0.1 M presented yields below 86%, whereas hydronium concentrations higher than 0.1 M promoted yields close to 100%. It is worth noticing that due to the lower corrosivity of H₂SO₄ compared to HCl or HNO₃, it has been the most used catalyst in hydrolysis processes of lignocellulosic wastes [41,42].

The main effects analysis (Fig. 2) showed that the maximum Y_{RS} was observed at 1:0 FVW:CS ratio, which corroborated the ease of degradation of FVW and also demonstrated a correlation

amidst the contents of FVW and Y_{RS} (Fig. 2A). Regarding the type of acid catalysts influence on Y_{RS} , HCl was superior over H_2SO_4 (Fig 2B), thus the combination of high contents of FVW and HCl is advisable to promote high Y_{RS} . The ANOVA (Table S1) of Y_{RS} showed that the model, the co-substrate ratios, the acid catalyst and their interaction were significant ($p < 0.0053$). The R^2 and R^2_{adj} were 0.8411 and 0.7696, respectively.

PLEASE INSERT FIGURE 2

Regarding the production and distribution of monosaccharides, noticeable differences were observed amidst the type of catalyst as shown in Fig. 3. When using HCl as catalyst (Fig. 3A), maximal concentrations of glucose were obtained at 3:1 FVW:CS ratio (10.02 g L^{-1}), and at 1:3 FVW:CS for xylose (8.41 g L^{-1}). No particular trends were appreciated after H_2SO_4 treatment (Fig. 3B). In the experiments with HCl, it was observed that the ratios with higher CS content presented higher xylose concentrations, whereas those ratios with higher FVW presented higher glucose concentrations. This difference is interesting and has also been reported elsewhere. For instance, Zu *et al.* [43] evaluated the CS hydrolysis with HCl as acid catalyst and found that xylose yield was also higher than that of glucose (20.44 and $1.82\text{ g per }100\text{ g}_{\text{raw material}}$, respectively) at $120\text{ }^\circ\text{C}$, 40 min and 1% HCl. Similarly, Cao *et al.* [44] found higher xylose concentrations than glucose (6.25 and 1.68 g L^{-1} , respectively) when assessing the CS acid hydrolysis with H_2SO_4 at $121\text{ }^\circ\text{C}$, 105 min, and 0.25% H_2SO_4 , ascribing this phenomenon to the easier hemicellulose solubilization than cellulose by acid catalyst in this particular substrate.

PLEASE INSERT FIGURE 3

An undesirable trait about the acid hydrolysis processes is the concomitant degradation of hydrolyzed carbohydrates into compounds such as furfural and HMF, and the lignin degradation

into acetic acid and phenolic compounds, which altogether act as inhibitors to microbial growth [5,41]. In our experiments, there were significant differences ($p < 0.0085$) of both the acid catalysts and the FVW:CS ratio on HMF, furfural and TPC production (Table S2, Table S3 and Table S4, respectively). Fig. 4 shows that for HMF and TPC, their concentrations were higher for HCl and as FVW increased its proportion. Regarding furfural, HCl also produced higher concentrations than H_2SO_4 , although FVW effect was not as evident as in HMF and TPC showing a maximum at 1:3 and a minimum at 3:1 FVW:CS ratios. These observations have some relation to that observed for Y_{RS} , and the respective explanation is also applicable to this case: FVW is a substrate much easier to degrade, and HCl provides higher H^+ concentration than H_2SO_4 when both substances are assayed at the same volumetric concentration.

PLEASE INSERT FIGURE 4

The concentrations of HMF, furfural and TPC in our experiments (Table 1) were lower than the inhibitory concentrations ($<1 \text{ g L}^{-1}$) reported for dark fermentation of mixed cultures, except for the hydrolyzates at 1:0 FVW:CS ratio and HCl [45]. Moreover, when two or more inhibitors are present in the substrate medium, inhibition concentrations may be considerably lower, yet not consistent data has been found amidst literature. For instance, Kumar *et al.* [46] found that at $0.69 \text{ g HMF L}^{-1}$ and $12 \text{ g formic acid L}^{-1}$ concentrations, microbial growth and hydrogen production were inhibited. On the other hand, Zheng *et al.* [18] reported that up to 4 g L^{-1} of HMF or furfural and $6 \text{ g acetic acid L}^{-1}$ inhibited microbial metabolism. On their behalf, Muñoz-Páez *et al.* [19] observed that individual concentrations of furfural and HMF up to 1 and 0.09 g L^{-1} , respectively, were even beneficial for biohydrogen production; yet when concomitantly present, these have inhibited 10% the hydrogen production even at low concentrations as 0.10 and 0.02 g L^{-1} for furfural and HMF, respectively. In general, the most important inhibitor has been suggested to be HMF, and it should be considered that concentrations above 1 g L^{-1} in hydrolyzates have reduced the yield in

hydrogen bioproduction [5]. Indeed, except for one of our assays, such value was not reached, pointing out that our hydrolyzates would be suitable for dark fermentation or anaerobic digestion [45].

PLEASE INSERT TABLE 1

It is important to note that for both HCl and H₂SO₄ at 1:0 FVW:CS assays (Fig. 3), no xylose content was detected, most likely due to its conversion into furfural and subsequent transformation into formic, lactic and succinic acids [47]. Indeed, the sum of these latter compounds was around 9.5 g L⁻¹, a concentration comparable to that of xylose at FVW:CS ratio of 1:3.

Since the acid hydrolysis of 3:1 FVW:CS with HCl catalyst (Fig.3) showed a balanced distribution of glucose, xylose and arabinose, as well as high RS concentration, dark fermentation was developed in the next section using the hydrolyzates from such conditions. Despite the low concentration of inhibitors, the liming treatment was applied to the hydrolyzates to avoid possible interactions of any kind during the hydrogen production. The overliming treatment decreased both the reducing sugars by 11.71% (24.00 to 21.19 g L⁻¹) and the TPC by 26.4% (1.06 to 0.78 g L⁻¹) in 26.41%. This diminution of RS was comparable with the 9.1% reduction observed by Chang *et al.* [20] when rice husk HCl hydrolyzates were overlimed. The removals of TPC and HMF over 20% are according to expected, whereas it is common that some organic acids might not be reduced by overliming [48].

3.2. Dark fermentation of LH

The highest H_{max} (212 mL H₂) and R_{max} (8.83 mL H₂ h⁻¹) were obtained for ISR 1.2 and $C_{RS,i}$ of 13 g L⁻¹ (Fig. 5, Table 2). Comparing the cumulative hydrogen production against literature is intricate due to the different reported operation conditions. For mixed cultures, Datar *et al.* [8] demonstrated a cumulative hydrogen volume of 4138 mL when using CS hydrolyzates (10 g L⁻¹) in dark

fermentation by anaerobic heat-treated sludge (105 °C, 2h) in a 1250 mL CSTR. Their volumetric hydrogen productivity, 3310 mL H₂ L⁻¹_{reactor} was 13% higher than our best result of 2933 mL H₂ L⁻¹_{reactor} at ISR 1.2 and 13 g RS L⁻¹ (Table 3). However, in their experiments the longer lag time obtained, 38 h, was ascribed to the presence of inhibitory compounds such as HMF and furfural obtained after extreme hydrolysis conditions (acid steam-explosion 1.2% H₂SO₄, 200 °C, 1 min). The longest lag time registered in our experiments was 22.5 h for the assay with the lowest inoculum load (ISR 0.8) and the highest substrate amounts (RS 16 g L⁻¹), which could have been an indicative of a slower adaptation of hydrogen-microbial producers to substrate [20].

PLEASE INSERT FIGURE 5

PLEASE INSERT TABLE 2

PLEASE INSERT TABLE 3

The ANOVA (Table S5 and Table S6) of this experiment showed that $C_{RS,i}$ had significant effects on both H_{max} and Y_{H_2} , whereas the ISR did not present significant effects on neither response variables in the studied operation range. The main effects of the $C_{RS,i}$ on the H_{max} (Fig. 6A) demonstrated that 10 g L⁻¹ presented the lowest average hydrogen production (183 mL H₂), whereas using 13 or 16 g L⁻¹ presented higher values (ca. 202 mL H₂). Oppositely, the main effects on Y_{H_2} (Fig. 6B) were higher at $C_{RS,i}$ 10 g L⁻¹ (2.2 mol H₂ mol glucose⁻¹) than at 13 and 16 g L⁻¹ (1.8 and 1.9 mol H₂ mol glucose⁻¹, respectively). This was in accordance to the reducing sugars consumption (Table 3): at $C_{RS,i}$ of 10 and 13 g L⁻¹ the RS consumption was higher than 94.9%, whereas at 16 g L⁻¹, it was close to 81%, except for the experiment with ISR 0.8 that presented ca. 70% consumption, indicating the possible inhibition by substrate or metabolites. Therefore, in terms of H_{max} and Y_{H_2} , It may be drawn that $C_{RS,i}$ of 10 and 13 g L⁻¹ may be the best options for conducting dark fermentation of hydrolyzates. Fangkum *et al.* [6] reported an increase in cumulated hydrogen production (42 to 92 mL H₂) when the initial concentration of fermentable

sugars increased from 5 to 10 g L⁻¹; yet when initial concentration of sugars exceeded 20 g L⁻¹, cumulated hydrogen production decreased mainly due to the accumulation of organic fatty acids, which was reflected as a drop in pH, causing bacterial growth inhibition. In our experiments, the final pH dropped as $C_{RS,I}$ increased, being the lowest of 5.17 when using RS 16 g L⁻¹ at ISR 0.8 (Table 3).

PLEASE INSERT FIGURE 6

Comparing our highest molar yields of 1.9-2.3 mol H₂ mol glucose⁻¹ amidst the range 0.8-1.2 ISR (Table 3) to literature (Table 4), ours were higher than some using pure cultures as in the case of Pattra *et al.* [49] who reported 1.73 mol H₂ mol glucose⁻¹ by *Clostridium butyricum* when fermenting sugarcane acid hydrolyzates at higher concentration (20 g L⁻¹); or as in the case of Cao *et al.* [44] who obtained 2.24 mol H₂ mol glucose⁻¹ by *Thermoanaerobacterium thermosaccharolyticum* W16 fermenting 12 g L⁻¹ of sugars from corn hydrolyzates at thermophilic temperatures (60 °C). Using microbial consortia, Zhang *et al.* [50] obtained low hydrogen yields (0.35 mol H₂ mol glucose⁻¹) with an anaerobic granular sludge at 37 °C, whereas at 55 °C the hydrogen yield was increased to 1.39 mol H₂ mol glucose⁻¹. The main difference from our experiments against those listed in Table 4 was our noticeably higher ISR. Indeed, the next highest ISR, 0.4, was that from Datar *et al.* [8], who indeed obtained the highest Y_{H_2} . Our results have demonstrated that it was feasible to hydrolyze preparations of FVW and CS, and to use the sugars released to achieve competent batch hydrogen production by microbial consortia.

PLEASE INSERT TABLE 4

Regarding the metabolites produced, in all the experiments butyrate was the most abundant, followed by acetate and lastly by propionate. High butyrate concentrations are indicative of the

predominance of the butyrogenic pathway in this dark fermentation hydrogen production [5]. On the other hand, low propionate concentrations is advantageous since it may indicate low presence of propionogenic microorganisms that use H_2 as electron donor [51]. Low ethanol concentrations are attributed to a low partial pressure effect due to the frequent hydrogen depressurization. Since a high variation in the partial pressure may change the route of metabolites production (i.e. solvents such as ethanol, acetone or butanol instead of VFA) [7,52].

3.3. *Economic prospection*

A net benefit of 0.009 USD per kg of co-substrates (wet basis) processed was observed (Table 5). The total costs were 0.029 USD/kg (including energy, water and chemicals), whereas the revenue for H_2 and CO_2 produced was 0.038 USD/kg. The costs were distributed as 54% related to FVW and CS supply, 26% due to substances and compounds, and the remaining 20% to electricity consumption. From this analysis, it is evident that waste biomass long before considered to be low cost [53] is not necessarily so. Different alternatives have been implemented to reduce such related production costs, such as government funding and subsidies [39].

The unit costs for H_2 and CO_2 considering a revenue-based allocation were 2.05 and 0.3 USD/ m^3 , respectively. The market price of H_2 and CO_2 are 2.7 and 0.3 USD/ m^3 , which indicates the profitability in hydrogen production of 24%. Indeed, hydrogen cost was comparable to other works in literature using different substrates. For instance, Han *et al.* [54] reported a cost of 2.29 USD/ m^3 H_2 from the techno-economic analysis of hydrogen production from food waste by integrated solid state fermentation and dark fermentation. The plant capacity was proposed to be 10 ton/d, and yield was 52.4 mL H_2 /g food waste. Using bread waste, Han *et al.* [39] reported a production cost of 1.34 USD/ m^3 for the continuous hydrogen production in a 2 ton/d CSTR.

The production of hydrogen from hydrolyzates of FVW and CS has shown a promissory alternative for the development of biorefinery models. Still, as hydrogen production finds alternatives of production from different substrates, biorefinery configurations and operation

conditions, it is becoming necessary to improve economic evaluations. We envisage areas of opportunity that will have consequences both for revenues and costs. For instance, the process profitability can be improved if the sub-products as organic acids and solvents from dark fermentation are further processed (e.g. hydrogen production by the photofermentation or methane production by anaerobic digestion) or marketed, as in the case of un-solubilized biomass that could be sold as biofertilizer. On the other hand, equipment, construction, and other operating costs such as gas compression, salaries and taxes will increase production costs [55].

PLEASE INSERT TABLE 5

4. Conclusion

The main conclusions of this work were several and noteworthy. They arranged according to the principal sections of the research, as follows:

- i) Hydrolysis. HCl as acid catalyst was superior than H_2SO_4 in terms of amount and quality of hydrolyzates, and the 3:1 FVW:CS ratio using HCl resulted in the highest production of reducing sugars in a balanced distribution of the monosaccharides glucose, xylose and arabinose.
- ii) Dark fermentation. The FVW:CS sugar-rich hydrolyzates were efficiently fermented. Moreover, the initial concentration of reducing sugars had important and significant effects both on maximum hydrogen production and yield. H_2 production was not dependent by inoculum to substrate ratio in the range 0.8 to 1.2 g $\text{VS}_{\text{inoculum}}$ g $\text{VS}_{\text{substrate}}^{-1}$. Concentrations of 10 and 13 g L^{-1} are suitable for conducting competent dark fermentation of hydrolyzates.
- iii) Economic prospection. Hydrogen production presented economic benefits, such as net revenues of 0.009 USD per kg of co-substrates (wet basis) processed and H_2 unit cost

below its market price, resulting in 24 % profitability of hydrogen production. The main costs were associated to FVW and CS supply.

Finally, the production of hydrogen from hydrolyzates of FVW and CS, as alternative to mono-substrates fermentation, has shown a promissory option for the development of biorefinery models.

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Abbreviations

CS	corn stover
C_{CS}	corn stover concentration in acid hydrolysis ($\text{g g}^{-1}_{\text{dry base}}$)
C_{FVW}	fruits-and-vegetables wastes concentration in acid hydrolysis ($\text{g g}^{-1}_{\text{dry base}}$)
C_{RS}	reducing sugars concentration in acid hydrolysis (g L^{-1})
$C_{RS,i}$	reducing sugars initial concentration in dark fermentation (g L^{-1})
$C_{RS,f}$	reducing sugars final concentration in dark fermentation (g L^{-1})

666	$C_{H_3O^+}$	theoretical hydronium concentration of the acid catalyst ($\text{mol H}_3\text{O}^+ \text{L}^{-1}$)
	db	dry basis
668	FVW	fruits-and-vegetables wastes
	H_{cs}	carbohydrates content in corn stover ($\text{g g}^{-1}_{\text{db}}$)
670	H_{FVW}	carbohydrates content in FVW ($\text{g g}^{-1}_{\text{db}}$)
	HMF	hydroxymethyl-furfural
672	$H(t)$	cumulative hydrogen production at time 't' (mL H_2)
	H_{max}	maximum cumulative hydrogen production (mL H_2)
674	HP	hydrolysis potential ($\text{g mol}^{-1} \text{H}_3\text{O}^+$)
	ISR	inoculum substrate ratio
676	LH	liquid hydrolyzates
	m_H	co-substrates mass (g)
678	MW_{glucose}	glucose molar weight (g mol^{-1})
	R_{max}	maximum hydrogen production rate ($\text{mL H}_2 \text{h}^{-1}$)
680	RS	reducing sugars
	t	time (h)
682	TPC	total phenolic compounds
	VFA	volatile fatty acids
684	VS	volatile solids
	V_r	hydrolysis volume (L)
686	V_R	fermentation volume (L)
	Y_{H_2}	hydrogen molar yield ($\text{mol H}_2 \text{mol}^{-1}_{\text{glucose}}$)
688	Y_{RS}	reducing sugars yield (%)
690	Greek symbols	
	λ	lag time (h)
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696 Fig. 1. Influence of fruits-and-vegetables wastes to corn stover ratios, FVW:CS, and acid catalysts, HCl or H₂SO₄, on (A) reducing sugars production, RS, and (B) hydrolysis potential, *HP*.

698 Fig. 2. Main effects on reducing sugars production yield, Y_{RS} , by (A) fruits-and-vegetables wastes to corn stover ratios, FVW:CS, and (B) acid catalysts, HCl or H₂SO₄.

700 Fig. 3. Monomeric sugars production at different fruits-and-vegetables wastes to corn stover ratios, FVW:CS, by acid hydrolysis using (A) HCl, and (B) H₂SO₄.

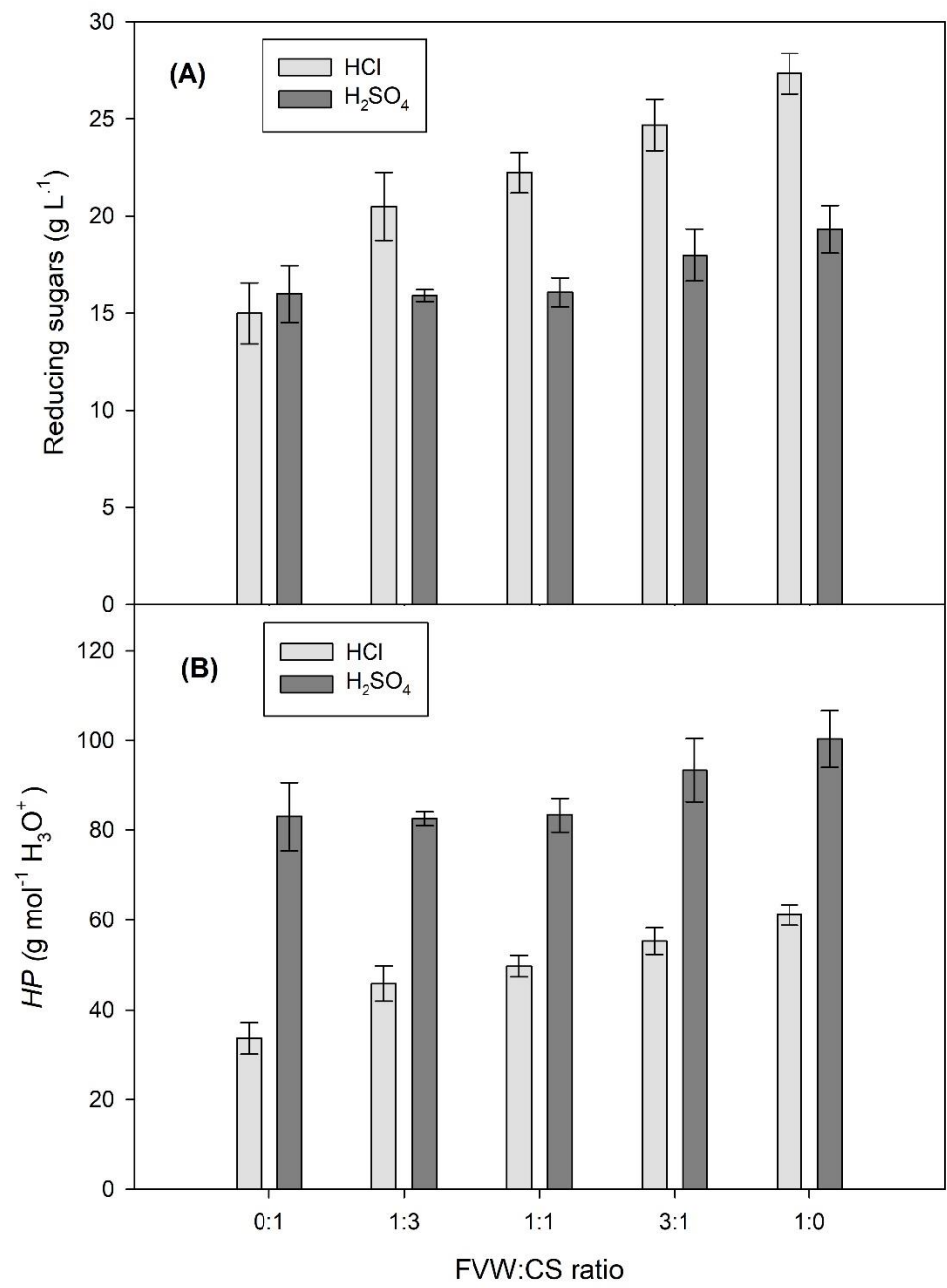
702 Fig. 4. Main effects by fruits-and-vegetables wastes to corn stover ratios, FVW:CS, and acid catalysts, HCl or H₂SO₄ on the production of HMF (A and B), furfural (C and D), and TPC (E and F).

704 Fig. 5. Cumulative hydrogen production by dark fermentation of overlimed HCl hydrolyzates from FVW:CS preparation at ISR equal to: 1.2 (A), 1.0 (B), and 0.8 (C). Reducing sugars initial concentration, $C_{RS,i}$, were 10 (triangle), 13 (circle), and 16 g L⁻¹ (square).

708 Fig. 6. Main effects by the reducing sugars initial concentration, $C_{RS,i}$, on the (A) maximum cumulative hydrogen production, H_{max} , and (B) the hydrogen molar yield, Y_{H_2} .

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716 **Fig. 1. Influence of fruits-and-vegetables wastes to corn stover ratios, FVW:CS, and acid**
718 **catalysts, HCl or H₂SO₄, on (A) reducing sugars production, RS, and (B) hydrolysis**
potential, HP.

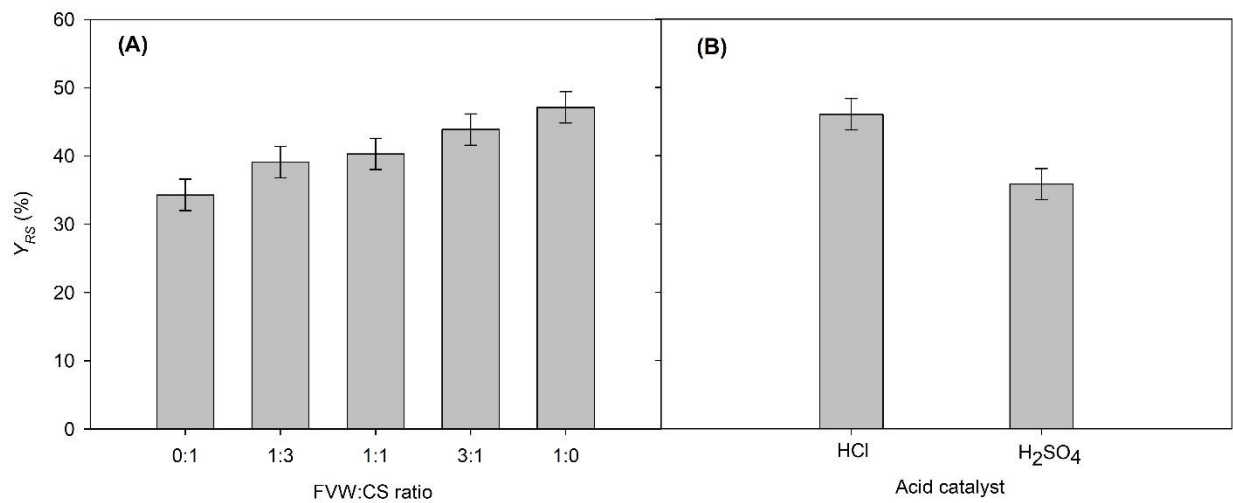


Fig. 2. Main effects on reducing sugars production yield, Y_{RS} , by (A) fruits-and-vegetables wastes to corn stover ratios, FVW:CS, and (B) acid catalysts, HCl or H_2SO_4 .

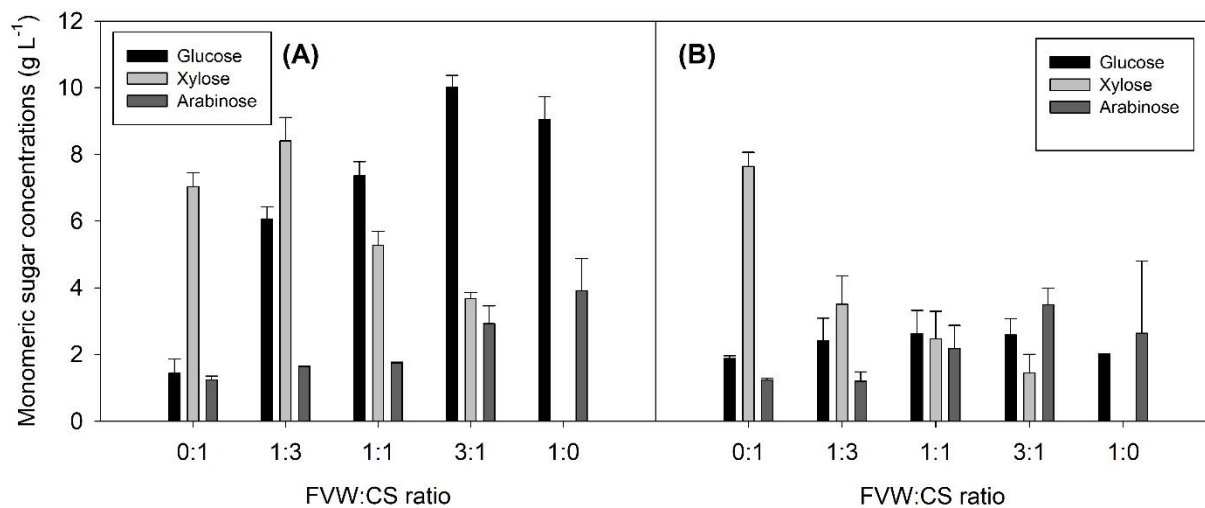
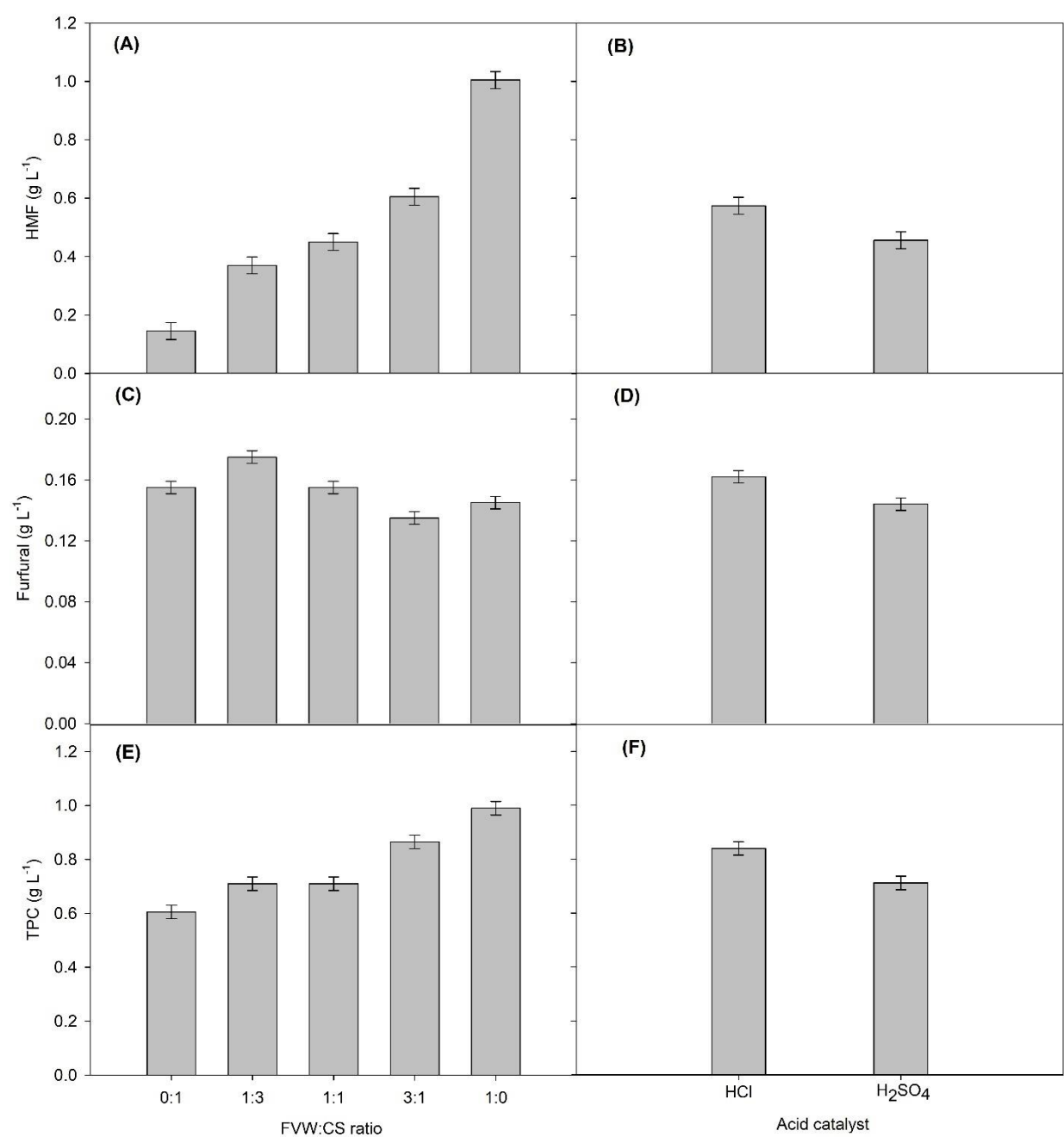


Fig. 3. Monomeric sugars production at different fruits-and-vegetables wastes to corn stover ratios, FVW:CS, by acid hydrolysis using (A) HCl, and (B) H₂SO₄.



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Fig. 4. Main effects by fruits-and-vegetables wastes to corn stover ratios, FVW:CS, and acid catalysts, HCl or H₂SO₄ on the production of HMF (A and B), furfural (C and D), and TPC (E and F).

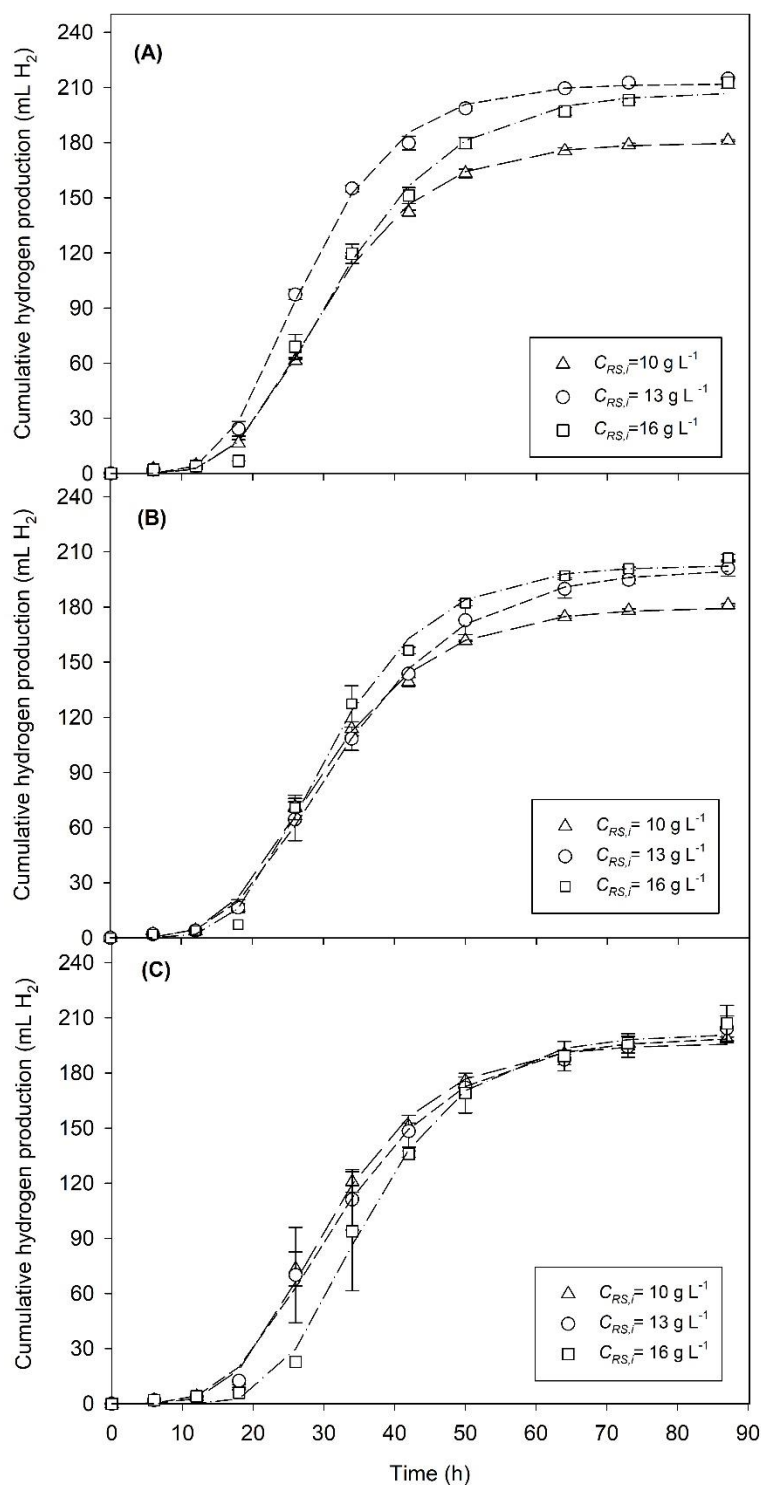


Fig. 5. Cumulative hydrogen production by dark fermentation of overlimed HCl hydrolyzates from FVW:CS preparation at ISR equal to: 1.2 (A), 1.0 (B), and 0.8 (C). Reducing sugars initial concentration ($C_{RS,i}$) were 10 (triangle), 13 (circle), and 16 g L⁻¹ (square)

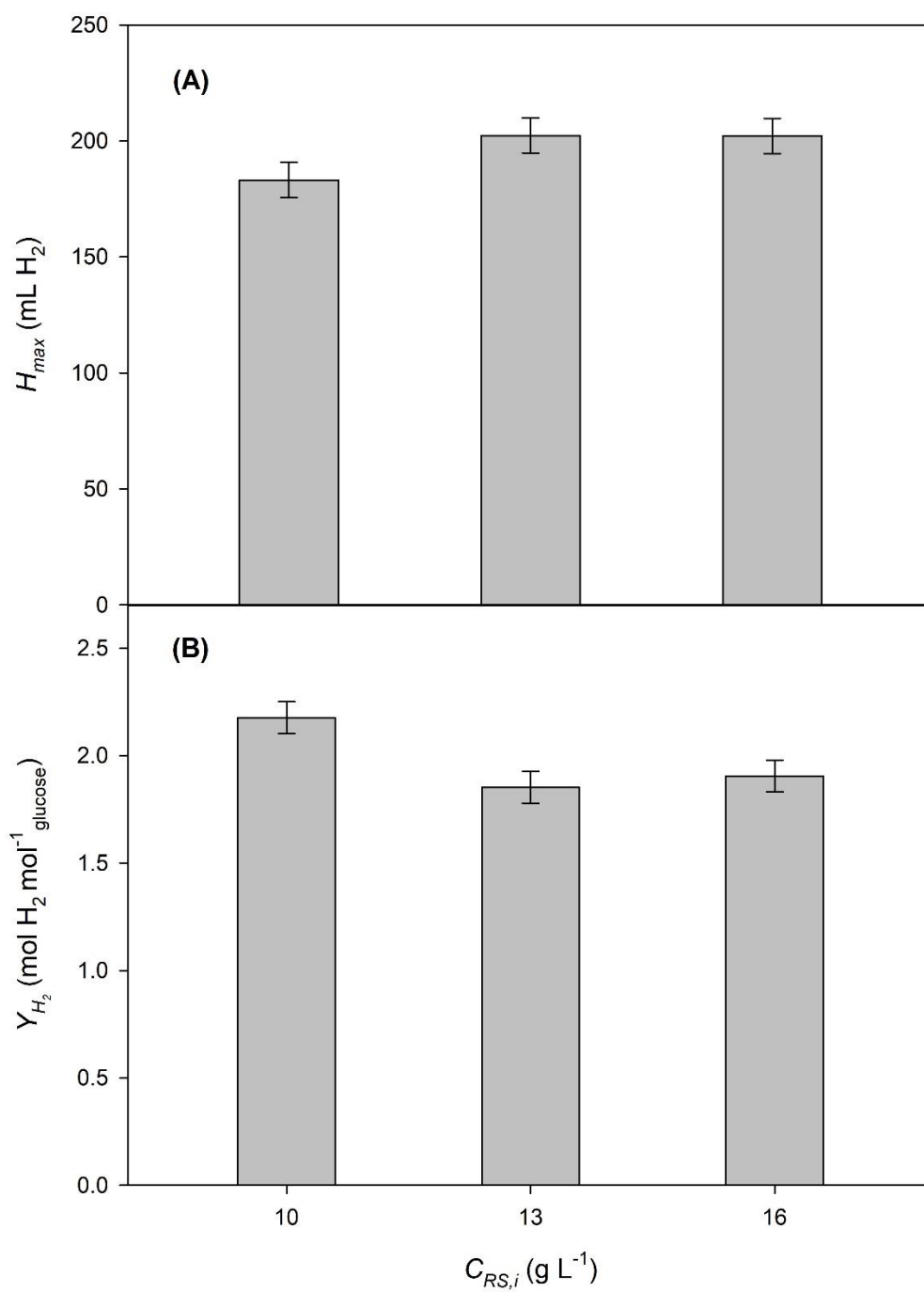


Fig. 6. Main effects by the reducing sugars initial concentration, $C_{RS,i}$, on the (A) maximum cumulative hydrogen production, H_{max} , and (B) the hydrogen molar yield, Y_{H_2} .

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760 **Table 1. Secondary products concentration from acid hydrolysis of fruits-and-vegetables wastes to corn stover ratios, FVW:CS.**

FVW:CS ratio and acid catalyst	HMF (g L ⁻¹)	Furfural (g L ⁻¹)	TPC (g L ⁻¹)	Formic acid (g L ⁻¹)	Acetic acid (g L ⁻¹)	Propionic acid (g L ⁻¹)	Succinic acid (g L ⁻¹)	Lactic acid (g L ⁻¹)
0:1 HCl	0.14 ±0.00	0.14 ±0.00	0.63 ±0.01	6.13 ±0.07	0.74 ±0.16	0.86 ±0.00	0.18 ±0.00	0.18 ±0.03
0:1 H ₂ SO ₄	0.15 ±0.00	0.17 ±0.00	0.58 ±0.02	5.80 ±0.49	0.77 ±0.08	0.75 ±0.43	0.17 ±0.00	0.17 ±0.00
1:3 HCl	0.47 ±0.02	0.21 ±0.01	0.79 ±0.02	5.37 ±0.30	0.84 ±0.24	1.09 ±0.45	0.17 ±0.01	0.83 ±0.24
1:3 H ₂ SO ₄	0.27 ±0.04	0.14 ±0.01	0.63 ±0.01	3.10 ±0.86	0.45 ±0.09	0.76 ±0.21	0.16 ±0.01	0.54 ±0.12
1:1 HCl	0.45 ±0.04	0.17 ±0.00	0.77 ±0.02	3.42 ±0.87	0.51 ±0.09	1.05 ±0.34	0.16 ±0.01	0.57 ±0.49
1:1 H ₂ SO ₄	0.45 ±0.04	0.14 ±0.01	0.65 ±0.02	4.29 ±0.44	0.46 ±0.07	1.64 ±0.15	0.17 ±0.00	1.15 ±0.09
3:1 HCl	0.65 ±0.01	0.14 ±0.00	0.90 ±0.04	4.02 ±0.15	0.53 ±0.01	1.80 ±0.06	0.16 ±0.00	1.39 ±0.05
3:1 H ₂ SO ₄	0.56 ±0.01	0.13 ±0.00	0.83 ±0.12	3.83 ±0.38	0.48 ±0.03	2.10 ±0.23	0.17 ±0.00	1.43 ±0.17
1:0 HCl	1.16 ±0.01	0.15 ±0.01	1.11 ±0.03	6.30 ±0.72	0.79 ±0.12	3.78 ±0.37	0.21 ±0.01	2.99 ±0.30
1:0 H ₂ SO ₄	0.85 ±0.14	0.14 ±0.01	0.87 ±0.01	6.59 ±0.58	0.77 ±0.19	4.27 ±0.28	0.21 ±0.01	3.06 ±0.53

762 Notes: FVW:CS of fruits-and-vegetables wastes to corn stover ratios; HMF, Hydroxymethyl-furfural; TPC, Total phenolic compounds.

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Table 2. Parameters of the modified Gompertz equation of the factorial design 3² from the HCl treatment at assayed reducing sugars initial concentrations, C_{RS,i}, and inoculum to substrate ratios, ISR.

Assay	Real values		Coded values		<i>H</i> _{max} (mL H ₂)	<i>R</i> _{max} (mL H ₂ h ⁻¹)	λ (h)
	C _{RS,i} (g L ⁻¹)	ISR	C _{RS,i}	ISR			
1	10	1.2	-1	1	180	6.70	16.5
2	13	1.2	0	1	212	8.83	15.4
3	16	1.2	1	1	208	6.92	17.0
4	10	1.0	-1	0	180	6.19	15.2
5	13	1.0	0	0	201	6.13	16.1
6	16	1.0	1	0	203	7.61	17.3
7	10	0.8	-1	-1	196	7.03	16.6
8	13	0.8	0	-1	200	6.34	16.1
9	16	0.8	1	-1	201	7.52	22.5

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Note: For all experiments, R² was greater than 0.99; *H*_{max}, maximum cumulative hydrogen production; *R*_{max}, maximum hydrogen production rate; λ , lag time (h).

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Table 3. Secondary metabolites and response variables of hydrogen production of HCl hydrolyzates, at assayed reducing sugars initial concentrations, $C_{RS,i}$, and inoculum to substrate ratios, ISR.

$C_{RS,i}$ -ISR	Final pH	Ethanol (g L ⁻¹)	Acetic acid (g L ⁻¹)	Propionic acid (g L ⁻¹)	Butyric acid (g L ⁻¹)	TVFA (g L ⁻¹)	Volumetric productivity (mL H ₂ L ⁻¹ _{reactor})	RS consumption (%)	Y_{H_2} (mol H ₂ mol ⁻¹ _{glucose})
10-1.2	6.02	0.06	2.30	0.29	4.34	6.93	2474	95.36	2.14
13-1.2	5.94	0.06	2.42	0.41	5.29	8.12	2933	96.33	1.91
16-1.2	5.25	0.05	1.30	0.13	1.61	3.04	2905	80.38	1.81
10-1.0	5.96	0.04	1.92	0.29	3.78	5.99	2473	95.27	2.08
13-1.0	5.90	0.05	2.01	0.27	5.09	7.37	2747	95.94	1.82
16-1.0	5.26	0.07	2.00	0.12	3.16	5.28	2753	78.04	1.85
10-0.8	5.71	0.13	2.10	0.36	3.61	6.07	2712	95.33	2.31
13-0.8	5.67	0.06	2.09	0.30	5.37	7.76	2789	94.92	1.82
16-0.8	5.17	0.06	1.88	0.13	2.73	4.74	2827	69.96	2.05

Notes: RS: reducing sugars; TVFA, total volatile fatty acids measured as the sum of acetic, propionic and butyric acids; Y_{H_2} , hydrogen molar yield.

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Table 4. Hydrogen production by dark fermentation of organic wastes.

Inoculum	Substrate (initial concentration)	Operational conditions	ISR	Hydrogen production and Gompertz kinetic parameters	Hydrogen molar yield (Y_{H_2}) and volumetric productivity (P_v)	Ref
<i>Thermoanaerobacterium thermosaccharolyticum</i> W16	Corn stover acid hydrolyzates (11.84 g L ⁻¹)	V ₀ = 50 mL T=60 °C t= 30 h	0.0123	H_{max} =165 mL R_{max} =5.51 mL h ⁻¹ λ =4 h	2.24 mol H ₂ mol glucose ⁻¹ P _v =3305 mL L ⁻¹	[44]
<i>Clostridium butyricum</i>	Sugarcane bagasse hydrolyzates (20 g L ⁻¹)	V ₀ = 70 mL T=37 °C t= 60 h	0.001	H_{max} =180 mL R_{max} =3 mL h ⁻¹ λ =5 h	1.73 mol H ₂ mol glucose ⁻¹ P _v =2571.43 mL L ⁻¹	[49]
Anaerobic sludge	Food waste and sewage sludge (15.5 g L ⁻¹)	V ₀ = 415 mL T=35 °C t=216 h	0.041	H_{max} =359 mL R_{max} =14.93 mL h ⁻¹ λ =6.48 h	0.98 mol H ₂ mol glucose ⁻¹ P _v =796.39 mL L ⁻¹	[13]
Anaerobic sludge	Corn stover steam-explosion (10.98 g L ⁻¹)	V ₀ = 1250 mL T=35 °C t=78 h	0.4	H_{max} = 4138 mL R_{max} =206 mL h ⁻¹ λ =38 h	2.42 mol H ₂ mol glucose ⁻¹ P _v =3310 mL L ⁻¹	[8]
Activated sludge	Corn stover acid hydrolyzates (5 g L ⁻¹)	V ₀ = 50 mL T=37 °C t= 60 h	0.032	H_{max} = 14 mL R_{max} =0.22 mL h ⁻¹ λ =15.3 h	0.44 mol H ₂ mol glucose ⁻¹ P _v = 275 mL L ⁻¹	[50]
Granular anaerobic sludge	Corn stover acid hydrolyzates (5 g L ⁻¹)	V ₀ = 50 mL T=37 °C t= 60 h	0.192	H_{max} = 11 mL R_{max} =0.19 mL h ⁻¹ λ =15.4 h	0.35 mol H ₂ mol glucose ⁻¹ P _v = 223.6 mL L ⁻¹	[56]
Anaerobic sludge	Organic fraction of municipal solid waste (150 g kg ⁻¹)	V ₀ = 500 g T=55 °C t= 470 h	0.068	H_{max}^a = 331 mL R_{max}^a =0.70 mL h ⁻¹ λ^a =0 h	0.29 mol H ₂ mol glucose ⁻¹ P _v = 662 mL g ⁻¹	[2]
Anaerobic sludge	FVW:CS acid hydrolyzates at 3:1 ratio (13 g L ⁻¹)	V ₀ = 70 mL T=35 °C t= 87 h	1.2	H_{max}^a = 212 mL R_{max}^a =8.83 mL h ⁻¹ λ^a =15.35 h	1.91 mol H ₂ mol glucose ⁻¹ P _v = 2933 mL L ⁻¹	This work

Notes: ^a Experimental data; CS, corn stover; FVW, fruits and vegetables wastes; H_{max} , maximum cumulative hydrogen production; ISR, inoculum to substrate ratio; P_v, volumetric productivity (hydrogen production/oper); R_{max} , maximum hydrogen production rate; T, temperature (°C); t, time (h); V₀, operational volume (mL); Y_{H_2} , hydrogen molar yield; λ , lag time (h).

Table 5. Economic prospection of hydrogen production from FVW:CS (3:1 ratio) at lab scale.

Stage	Component	Price (USD/kg)	Quantity	Unit	Price (USD)	Ref
Raw materials	CS	0.088	0.035	kg	-0.003	[31]
	FVW	0.013	0.965	kg	-0.013	[32,33]
Co-substrates size reduction	Electricity	0.014	0.089	MJ	-0.001	[34]
Acid hydrolysis	HCl (30%)	0.029	0.039	kg	-0.001	[57]
	Water	0.0006	1.079	kg	-0.001	[35]
	Electricity	0.014	0.322	MJ	-0.004	[34]
Mechanical separation	Electricity	0.014	0.012	MJ	-0.002	[34]
Overliming pretreatment	Ca(OH) ₂	0.12	0.013	kg	-0.002	[58]
	Electricity	0.014	0.009	MJ	-0.0001	[34]
Dark fermentation	NH ₄ Cl	0.11	0.003	kg	-0.0004	[59]
	Buffer media	0.3	0.008	kg	-0.0023	[60]
	Water	0.0006	0.016	kg	-0.00001	[35]
	ZnCl ₂	0.9	0.0002	kg	-0.0002	[61]
	MgCl ₂ ·6H ₂ O	0.1	0.0003	kg	-0.00003	[62]
	MnCl ₂ ·4H ₂ O	2	0.00004	kg	-0.00007	[63]
	FeCl ₃ ·6H ₂ O	2	0.00009	kg	-0.00017	[64]
	CuCl ₂ ·2H ₂ O	2.5	0.00001	kg	-0.00003	[65]
	NiCl ₂ ·6H ₂ O	3	0.0003	kg	-0.001	[66]
	H ₂	0.0027	13.353	L	0.036	[39]
	CO ₂	0.0003	6.663	L	0.002	[39]
Total system cost					-0.029	
Total revenue					0.038	
Total Net					0.009	