


Role of xylose from acidic hydrolysates of agave bagasse during biohydrogen production

Karla María Muñoz-Páez^a and Germán Buitrón^{b*} 

^a CONACYT – Instituto de Ingeniería, Unidad Académica Juriquilla, Universidad Nacional Autónoma de México, Querétaro, México

^b Laboratory for Research on Advanced Processes for Water Treatment, Unidad Académica Juriquilla, Instituto de Ingeniería, Universidad Nacional Autónoma de México, Blvd. Juriquilla 3001, 76230 Querétaro, México

*Corresponding author. E-mail: gbuitronm@iingen.unam.mx

 GB, 0000-0003-3975-7644

ABSTRACT

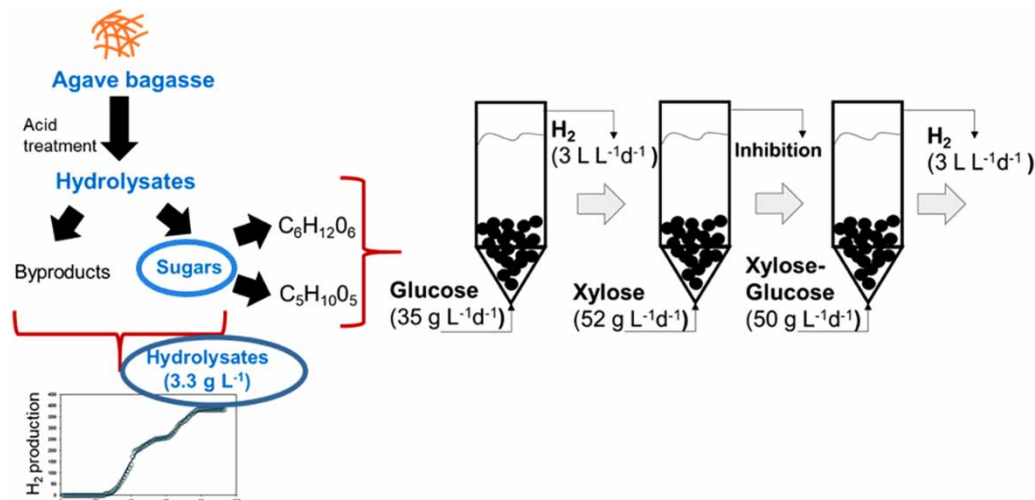
This study compares the H₂ production from glucose, xylose, and acidic hydrolysates of *Agave tequilana* bagasse as substrates. The fermentation was performed in a granular sludge reactor operated in two phases: (1) model substrates (glucose and xylose) and (2) acidic hydrolysates at 35 °C, pH 4.5 and a hydraulic retention time of 5.5 h with glucose (10 g L⁻¹) and xylose (12 g L⁻¹). A sequencing batch reactor was used to acclimate the biomass between the glucose and xylose continuous fermentation (with a mixture of xylose-glucose) and acidic hydrolysates. During the discontinuous acclimating step, the xylose/glucose ratio increment negatively affected the H₂ productivity. Although the continuous H₂ production with xylose was negligible, the co-fermentation with glucose (88–12%) allowed H₂ productivity of 2,889 ± 502 mL H₂ L⁻¹d⁻¹. An acidic hydrolysate concentration of 3.3 g_{carbohydrate} L⁻¹ showed a three-fold higher H₂ productivity than with a concentration of 10 g L⁻¹. The results indicated that xylose, as the only substrate, was challenging to metabolize by the inoculum, and its mixture with glucose improved the H₂ productivity. Therefore, the low H₂ productivity with hydrolysates could be related to the presence of xylose.

Key words: acidic hydrolysates, glucose, hydrogen, xylose

HIGHLIGHTS

- The H₂ productivities with glucose, xylose and acidic hydrolysates were contrasted.
- H₂ production was not observed with xylose as substrate in continuous operation.
- H₂ from acidic hydrolysates is affected by the xylose/glucose ratio and inhibitors.

GRAPHICAL ABSTRACT

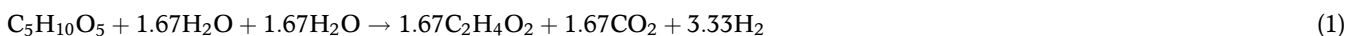


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INTRODUCTION

The lignocellulosic biomass can be used for hydrogen production through dark fermentation (Kumar *et al.* 2015). This kind of biomass is composed mainly of 35–45% of cellulose, 25–40% of hemicellulose, and 20–30% of lignin, forming a matrix interlinked with several chemical bonds, making it difficult to be used by the microorganisms (Prakasham *et al.* 2009a, 2009b). Due to its complex structure, it is necessary to apply a pretreatment to improve the lignocellulose hydrolysis efficiency (Ren *et al.* 2016). The diluted acidic pretreatment releases sugars from the biomass and other degradation products, negatively affecting the lignocellulosic fermentation (Chen *et al.* 2017). There is no consensus on how the by-products' characteristics may cause inhibition on hydrogen production by fermentation, related, for example, to the inhibitor concentration, the presence of other inhibitors, or the inoculum's tolerance (Kumar *et al.* 2015; Muñoz-Páez *et al.* 2020a). The *Agave tequilana* Weber bagasse is an agro-industrial waste from the tequila industry, which has been used for biohydrogen production with a diluted acidic pretreatment, observing the necessity of a detoxification process due to the presence of inhibitory compounds (Arreola-Vargas *et al.* 2013). The detoxification with activated carbon allowed improved H₂ productivity from 58 mL H₂ L⁻¹ h⁻¹ to 63 mL H₂ L⁻¹ h⁻¹ in batch experiments (Valdez-Guzmán *et al.* 2019).

Besides the presence of potentially inhibitory compounds, the type of sugar released could affect the fermentation, considering that xylose and glucose are the main sugars detected in the acidic hydrolysates, and pentoses could be more challenging to be used than hexoses (Ren *et al.* 2016; Valdez-Guzmán *et al.* 2019). Acidic hydrolysates from *Agave tequilana* Weber bagasse are mainly composed of xylose (Valdez-Guzmán *et al.* 2019). When xylose is used as a substrate for dark fermentation, the theoretical production of 3.33 mol H₂ mol-xylose⁻¹ occurs concomitant with the generation of 1.67 mol of acetate (Equation (1)). However, almost half of the amount of hydrogen produced from xylose is related to the generation of butyric acids (1.67 mol H₂ mol-xylose⁻¹); see Equation (2) (Khamtib & Reungsang 2012).



The acetic acid pathway should be favored to obtain the maximum hydrogen production from xylose. However, xylose's fermentation depends on the type of inoculum, substrate concentration used, and other sugars in batch fermentation (Ren *et al.* 2008). The co-fermentation of glucose and xylose in dark fermentation has been addressed by evaluating the pH effect and the xylose/glucose ratio and using mixed culture and pure culture (Calli *et al.* 2008; Ren *et al.* 2008; Prakasham *et al.* 2009b; Zhao *et al.* 2019). Nevertheless, only a few attempts have been made to study xylose's dark fermentation in continuous fermentation using mixed culture (Lin & Cheng 2006; Temudo *et al.* 2009). In light of the above developments, the main objective of this study was to compare the hydrogen production from glucose, xylose, its mixtures, and acidic hydrolysates of *Agave tequilana* Weber bagasse under comparable process conditions to elucidate the effect on the type of sugar present in the acidic hydrolysates during the biohydrogen production.

METHODS

Inoculum and substrates

The inoculum was methanogenic granular sludge from a brewery wastewater treatment plant. The sludge was seeded into the reactor at 7.3 g of volatile solids (VS) L⁻¹ and was acclimated to hydrogen production in continuous mode with glucose as substrate (5 g L⁻¹d⁻¹) and acidic conditions (pH of 4.5), according to Hernández-Mendoza & Buitrón (2014). The substrates used were glucose, xylose, a mixture of xylose-glucose (88–12%), and acidic hydrolysates of *Agave tequilana* bagasse. The acidic hydrolysates were obtained from *Agave tequilana* Weber bagasse (var. Azul) using diluted acidic treatment. Air-dried bagasse of *Agave tequilana* Weber was solubilized in HCl (1.4%v/v) at 124 °C, 2.1 h using 5% of total solids (Arreola-Vargas *et al.* 2015; Muñoz-Páez *et al.* 2020b). Several batches of hydrolysates were prepared using the same process mentioned before. The average total carbohydrate composition was 13–19 g L⁻¹, and concentrations of furfural and hydroxymethylfurfural were 0.027–0.097 and 0.0025–0.010 g L⁻¹, respectively. A more detailed description of the acidic hydrolysates' composition can be found in Muñoz-Páez *et al.* (2020b). A nutrient solution was added at all operational stages, according to Hernández-Mendoza & Buitrón (2014).

Reactor set-up and operating conditions

The dark fermentation was carried out in an expanded granular sludge bed reactor (EGSB) with 2.2 L of working volume. The EGSB was operated at controlled pH of 4.5, 35 °C, hydraulic retention time (HRT) of 5.5 h, and 5 m h⁻¹ of up-flow velocity. The pH was adjusted by adding 0.1N NaOH. The experimental design was divided into two operational phases (Table 1). In phase 1, the reactor was fed with model substrates: glucose, xylose, and mixtures. At first, the EGSB was operated with glucose at an organic loading rate (OLR) of 35 g L⁻¹d⁻¹. Then, the H₂ production from xylose started; first, the inoculum was acclimated to H₂ production from xylose in a sequencing batch mode with a mixture of xylose-glucose (10 g L⁻¹), in which the xylose concentration was increased in a stepwise manner from 25% to 100%. Once H₂ production with sole xylose was detected, the reactor was operated at continuous mode at OLR of 52 g L⁻¹d⁻¹ of xylose. Finally, the substrate was changed to a mixture of xylose-glucose (88–12%), maintaining the same OLR. During phase 2, the reactor was operated at discontinuous mode with acidic hydrolysates of *Agave tequilana* Weber bagasse (3.3 to 10 g_{carbohydrates} L⁻¹), followed by a continuous operation using 3.3 g_{carbohydrates} L⁻¹ at HRT of 11 h (Muñoz-Pérez *et al.* 2020b).

Analytical methods

Gas production was measured with a Ritter meter (MGC-1 V3.2 PMMA, Germany) when the model substrates were tested and with a μflow flowmeter (Microflow bioprocess, Lund Sweden) during the hydrogen production from acidic hydrolysates. The biogas composition (H₂, CO₂ and CH₄) and the volatile fatty acid concentration were determined by gas chromatography according to Hernández-Mendoza & Buitrón (2014). Gas volume is reported at standard temperature and pressure (0 °C and 101.325 kPa). The carbohydrate concentration was determined following the phenol-sulfuric acid method (DuBois *et al.* 1956) using glucose as the standard. The granules' diameter was measured using a stereoscopic microscope (Stemi DV4 Stereo Microscope, Carl Zeiss, Germany; Hernández-Mendoza & Buitrón 2014).

Calculations

The kinetics parameters in the biphasic fermentation of the sequential batch hydrogen production with hydrolysates (3.3 g L⁻¹) were calculated using the modified Gompertz equation according to Kim *et al.* (2003).

$$H(t) = P1 * \exp\left(-\exp\left(\frac{R1 * 2.71}{P1}(\lambda1 - t) + 1\right)\right) + P2 * \exp\left(-\exp\left(\frac{R2 * 2.71}{P2}(\lambda2 - t) + 1\right)\right) \quad (3)$$

where $H(t)$ is the total hydrogen production (mL H₂ L); $P1$ and $P2$ are the maximum H₂ production (mL-H₂ L⁻¹); $R1$ and $R2$ are the maximum H₂ production rate (mL H₂ L⁻¹ h⁻¹); and $\lambda1$ and $\lambda2$ are the lag phases (h).

RESULTS AND DISCUSSION

Hydrogen production from model substrates

The methanogenic inoculum acclimation to H₂ production in continuous mode lasted 21 days, and the reactor was operated for another 19 days to reach a steady-state performance. After that, in the first phase, the reactor was fed with 35 g L⁻¹d⁻¹ of

Table 1 | Experimental conditions

Phase	Substrate (g _{carbohydrate} L ⁻¹)	Operational conditions: HRT (h); OLR (g L ⁻¹ d ⁻¹)
1 Model substrates	Glucose (8)	5.5 h; 35 g L ⁻¹ d ⁻¹
	25% Xylose–75% glucose (10)	Sequencing batch mode
	50% Xylose–50% glucose (10)	
	75% Xylose–25% glucose (10)	
	100% Xylose (10)	
	Xylose (12)	
2 Acidic hydrolysates	88% Xylose–12% glucose (12)	5.5 h; 52 g L ⁻¹ d ⁻¹
	Acidic hydrolysates (10)	Sequencing batch mode
	88% Acidic hydrolysates–12% glucose (10)	
	Acidic hydrolysates (3.3)	
	Acidic hydrolysates (3.3)	

glucose to address the H_2 production with an easy to biodegrade carbohydrate. Operating at this OLR, the reactor reached an H_2 productivity and H_2 yield of $3,218 \pm 540 \text{ mL } H_2 \text{ L}^{-1} \text{d}^{-1}$ and $0.97 \pm 0.01 \text{ mol } H_2 \text{ mol gluc}_{\text{consumed}}^{-1}$, respectively (data not shown). During this phase, the carbohydrate and chemical oxygen demand (COD) removals were 71 and 13%, respectively.

Once the H_2 production with glucose was accomplished, the microorganisms' acclimation to xylose initiated, starting with an adaptation in sequencing batch mode. Maximum H_2 productivity of $1,450 \text{ mL } H_2 \text{ L}^{-1} \text{d}^{-1}$ was observed when the mixture with the lower content of xylose was used (Figure 1). Subsequent increments of the xylose content showed adverse effects on the H_2 productivity, decreasing it almost 55% ($655 \text{ mL } H_2 \text{ L}^{-1} \text{d}^{-1}$) with the mixture 50–50, and 75% ($356 \text{ mL } H_2 \text{ L}^{-1} \text{d}^{-1}$) with the mixture 75–15%. A linear negative relationship between the H_2 productivity and the xylose content ($R^2 = 0.9356$) was found. On the contrary, the H_2 yield was not directly related to the increment of xylose in the substrate. The maximum yield was obtained with only xylose and 25% xylose–75% glucose mixture (1.38 and $1.3 \text{ mol } H_2 \text{ per mol}_{\text{carbohydrate}}$). Whereas the carbohydrate removal was higher with the mixtures 50% xylose–50% glucose and 75% xylose–50% glucose than with the mixture with the lower proportion of xylose, this could suggest that part of the substrate could be used to generate other metabolic products besides hydrogen. In literature, the H_2 production from a mixture of xylose-glucose has been addressed using a pure culture of *Thermoanaerobacter thermosaccharolyticum* W16, reporting no significant effects of the xylose/glucose ratio on the H_2 production (Ren *et al.* 2008; Zhao *et al.* 2019).

Nevertheless, the effects on H_2 yield and H_2 production rate with the different xylose/glucose ratios have been already reported. Zhao *et al.* (2019) performed a batch hydrogen production from a mixture of xylose-glucose at 60°C and initial pH of 6.5, reporting a decrease of the H_2 yield and an increase of the H_2 production rate with the increments of the xylose proportion in the substrate. On the other hand, Ren *et al.* (2008) mentioned no effect of the xylose/glucose ratio on the yield; nevertheless, they observed a slight increase in the H_2 production rate. These different effects of the xylose to glucose ratio might be justified by the influence of some operational parameters of the fermentation, such as inoculum, pH and operational mode. *Thermoanaerobacter thermosaccharolyticum* W16 has been described as a microorganism capable of using xylose as a carbon source. However, hydrogen production from mixed cultures has been reported, mainly using anaerobic granular sludge from UASB reactors submitted to previous pretreatments to enrich the mixed culture with H_2 producing microorganisms (Maintinguer *et al.* 2011; Mockaitis *et al.* 2020). Mockaitis *et al.* (2020) discussed that the improvement in H_2 that was obtained using acidic pretreatment was related to the enhanced growth of members of the family *Peptostreptococcaceae* and the genera *Truepera* and *Kurthia*. Therefore, the selected microorganisms in that study played a fundamental role in the xylose degradation. Another factor is the pH at which the reactor is operated. Ren *et al.* (2008) reported a lower H_2 production from xylose by the pure culture of *Thermoanaerobacter thermosaccharolyticum* W16 when the reactor was operated at pH between 4.5 and 5.5 compared to the fermentation at a pH of 6.5. In the present work, the acidic conditions applied to avoid methane production (pH 4.5) could negatively affect the performance of the process.

Interestingly, when xylose was used alone, the reactor operation presented the maximum yield observed in this part of the experiment ($1.38 \text{ mol } H_2 \text{ mol gluc}_{\text{consumed}}^{-1}$). The carbohydrates removal with xylose alone was 73%, lower than that obtained with xylose-glucose mixtures (86%–96%); it seems that higher amounts of the removed carbohydrates were used for H_2 production than for other cellular activities in the latter mixture. The productivity and yield reported by Arreola-Vargas *et al.*

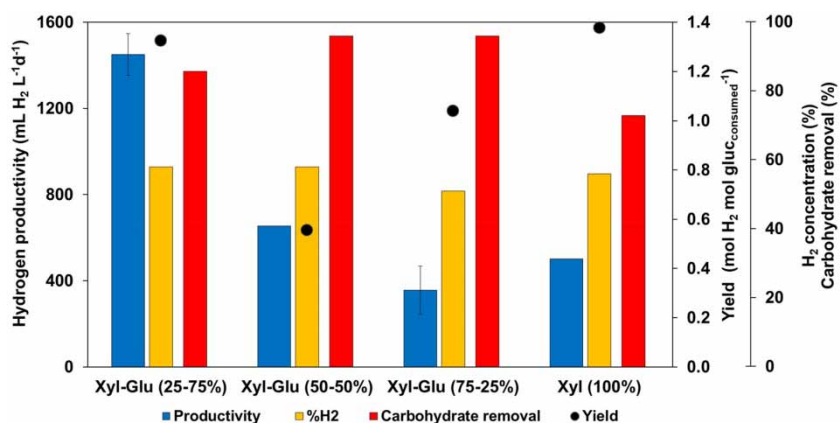


Figure 1 | Performance of the EGSB reactor during the sequential batch operation with a mixture of xylose-glucose (10 g L^{-1}).

(2013), with a thermally treated anaerobic granular sludge, using similar pH and temperature conditions (Table 2), were 4.8- and 1.5-fold lower than the results obtained in the present work. The differences in the behavior could be related to the type of microorganisms present in both inocula. The heat treatment allowed fewer H₂-consuming microorganisms that could not sporulate, whereas, in our work, the biokinetic control (low pH and HRT) affected mainly the methanogenic archaea.

After the adaptation with xylose in sequencing batch mode, the continuous operation was initiated at OLR of 52 g L⁻¹d⁻¹. The H₂ productivity progressively decreased after five days (13 ± 4 mLH₂L⁻¹d⁻¹) as well as the carbohydrate removal ($6.15 \pm 5.6\%$). The same trend was observed for the H₂ concentration, which decreased from $53.3 \pm 3\%$ to $18 \pm 0\%$ after five days. Those results suggest a possible inhibition of the microorganism under the specific operational conditions applied. The inhibition could be a combined effect of the high OLR and the low pH at which the EGSB was operated. Lin & Cheng (2006) reported H₂ production continuously from xylose (20 g L⁻¹) in a continuous stirred tank reactor (CSTR) at pH of 7.1 and HRT of 12 h and obtaining average H₂ productivity of 2.2 L L⁻¹d⁻¹ after 20 days of operation (Table 1). Although the xylose concentration used in the former study was two-fold higher than in this study, the applied OLR was 1.4-fold lower (40 g COD L⁻¹d⁻¹ vs. 55.6 g COD L⁻¹d⁻¹) because of the different HRT applied. Those authors reported that the H₂ production was negatively affected by a high concentration of xylose (>20 g COD L⁻¹) and no H₂ production at a pH of 5.

It was observed that the granules' structure was more affected with the use of xylose compared to the structure of the granules obtained with glucose as substrate (Figure 2(a) and 2(b)). The granules' disaggregation could be a combination of effects, including the type of substrate and the high OLR used. It has been observed that the change of substrate can cause a structural rearrangement of the microorganisms in the granule, which in some cases is related to its rupture (Liu *et al.* 2003). Therefore, new anaerobic granules were added (1/3 of the initial volume), and the substrate was changed to a mixture of xylose-glucose.

The xylose-glucose mixture was composed of 88% xylose and 12% glucose, simulating the average proportion observed in the agave bagasse hydrolysates experimentally obtained. Statistical analysis (Student's t-test) for the obtained results for the xylose-glucose mixture indicates that results were significantly different from the values obtained when the reactor was operated only with xylose. The EGSB was operated at a discontinuous mode with a substrate concentration of 10 g L⁻¹, reaching a H₂ productivity of 1,915 mL H₂ L⁻¹d⁻¹ and a H₂ yield of 1.33 mol H₂ per mol glucose; then it was operated in continuous mode. After six days in continuous operation, the H₂ productivity was $2,889 \pm 502$ mL H₂ L⁻¹d⁻¹ with a yield of 1.02 ± 0.59 mol H₂ per mol glucose. Hydrogen percentage in the gas was $54 \pm 3\%$ and the removal of carbohydrates was $31 \pm 8\%$, which was a similar performance to that observed when the reactor was operated with glucose as the only substrate. These results supported the assumption that the inoculum could not metabolize xylose as the only substrate at HRT of 5.5 h. The presence of a more suitable substrate such as glucose facilitates xylose fermentation (Prakasham *et al.* 2009b). On the other hand, the carbohydrate consumption (37%) was 1.9-fold lower than that obtained from the continuous operation with glucose. The low carbohydrate intake may be due to differences in glucose and xylose metabolism; before entering the Embden–Meyerhof–Parnas (EMP) pathway, unlike glucose, xylose undergoes a series of steps (Temudo *et al.* 2009; Reginatto & Antônio 2015). Besides, the microorganisms need the necessary time of contact to consume the substrate, and 5.5 h HRT resulted in a limitation for the microorganisms to use it efficiently. The H₂ productivity from a mixture of xylose-glucose (88–12%) was 1.8-fold higher than that reported by Arreola-Vargas *et al.* (2014), who used a mixture 1:1 in a trickling bed reactor (Table 1), mainly because they used a lower OLR because of the different HRT and substrate concentration used (5.5 h vs. 12 h, and 12 g L⁻¹ vs. 5 g COD L⁻¹).

Regarding the soluble metabolites produced (Figure 3), butyrate was the primary acid detected during the fermentation; this reveals that the principal reaction was a butyric fermentation type. The complete disruption of some granules was observed at the end of the operation (Figure 2(c)). That indicates that the organic load of 50 g xylose L⁻¹ negatively affected the stability, compared to the mono-fermentation with xylose. For that reason, bioaugmentation of acclimated granular sludge was done by adding 1/3 of the original volume.

Hydrogen production using agave bagasse acidic hydrolysates

Once steady H₂ production was reached from the mixture of xylose-glucose (88–12%), the evaluation of the H₂ production potential using 100% agave bagasse acidic hydrolysate was tested using 10 g L⁻¹. As a result, the H₂ productivity decreased from 34 mL H₂ L⁻¹d⁻¹ to 7 mL H₂ L⁻¹d⁻¹; the same trend was observed in the carbohydrates removal and the H₂ yield (Figure 4). Then, glucose was added to the hydrolysates (12%), maintaining the same initial carbohydrate concentration, as an attempt to increase the H₂ production, but there was no improvement in the performance. The low hydrogen production with the acidic hydrolysates could be associated with inhibitors' presence (Valdez-Guzmán *et al.* 2019); therefore,

Table 2 | H₂ productivity and yields from xylose, xylose-glucose, and acidic hydrolysates using mixed cultures

Substrate	Substrate concentration	Inoculum	Reactor	Operation mode	T (°C)	pH	HRT (h)	Productivity (L L ⁻¹ d ⁻¹)	Yield (mol H ₂ per mol sugar)	References
Xylose-glucose (1:1)	5 g COD L ⁻¹	TT ^a Anaerobic granular sludge	AnSBR	Sequencing batch	35	4.5	8*	6.7	2.0 ^b	Arreola-Vargas <i>et al.</i> (2013)
Xylose-glucose (25–75%)	10 g _{carbohydrate} L ⁻¹	Anaerobic granular sludge	EGSB	Sequencing batch	35	4.5	NR	1.4	1.33 ^b	This study
Xylose-glucose (88–12%)	10 g _{carbohydrate} L ⁻¹	Anaerobic granular sludge	EGSB	Sequencing batch	35	4.5	NR	1.9	1.2 ^b	This study
Xylose-glucose (1:1)	5 g COD L ⁻¹	TT ^a triticale silage from a dairy farm	Trickling bed reactor	Continuous	35	5.0	12	1.6	1.6 ^b	Arreola-Vargas <i>et al.</i> (2014)
Xylose-glucose (88–12%)	10 g _{carbohydrate} L ⁻¹	Anaerobic granular sludge	EGSB	Continuous	35	4.5	5.5	2.9	1.02 ^b	This study
Xylose	2 g L ⁻¹	Compost	CSTR	Sequencing batch	55	5.0	22	1.4	1.7	Calli <i>et al.</i> (2008)
Xylose	20 g L ⁻¹	Sewage sludge	Chemostat	Continuous	35	7.1	12	2.2	0.7	Lin & Cheng (2006)
Xylose	12 g _{carbohydrate} L ⁻¹	Anaerobic granular sludge	EGSB	Continuous	35	4.5	5.5	0.06	0.08 ^b	This study
Acidic agave bagasse hydrolysates	3.3 g _{carbohydrate} L ⁻¹	Anaerobic granular sludge	EGSB	Sequencing batch	35	4.5	NR	0.1	1.16 ^b	This study
Acidic oat straw hydrolysates	10 g COD L ⁻¹	TT ^a triticale silage from a dairy farm	Trickling bed reactor	Continuous	35	5.0	12	0	0	Arreola-Vargas <i>et al.</i> (2014)
Acidic oat straw hydrolysate + xylose/ glucose (1:1)	10 g COD L ⁻¹	TT ^a triticale silage from a dairy farm	Trickling bed reactor	Continuous	35	5.0	12	1.3	0.7 ^b	Arreola-Vargas <i>et al.</i> (2014)
Acidic agave bagasse hydrolysates	3.3 g _{carbohydrate} L ⁻¹	Anaerobic granular sludge	EGSB	Continuous	35	4.5	11	0.37	NR	Muñoz-Páez <i>et al.</i> (2020b)

AnSBR, anaerobic sequencing batch reactor; CSTR, continuous stirred tank reactor; EGSB, expanded granular sludge reactor; NR, not reported.

^aThermally treated.

^bConsumed sugar.

*Equivalent HRT.

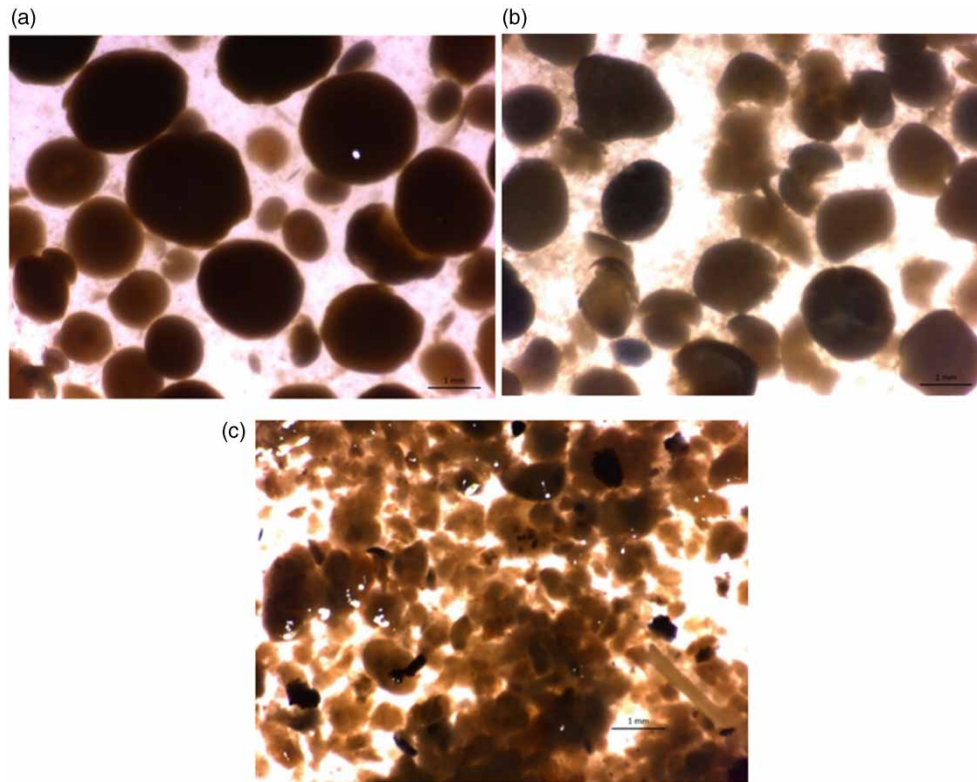


Figure 2 | Granular sludge at the final of the H_2 production: (a) Glucose, $35 \text{ g L}^{-1}\text{d}^{-1}$; (b) Xylose-glucose, sequencing batch operation (10 g L^{-1}); (c) Xylose-glucose 88–12%, continuous operation (52 g L^{-1}).

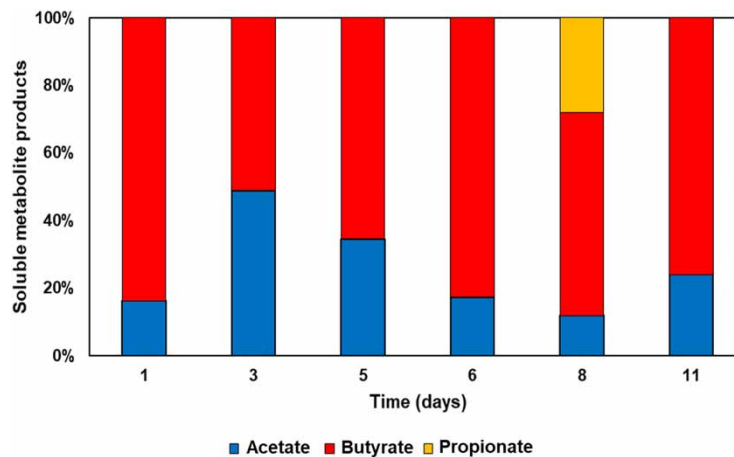


Figure 3 | Soluble metabolite products during the continuous fermentation of xylose-glucose (88–12%).

the hydrolysates were diluted to a carbohydrate concentration of 3.3 g L^{-1} in order to reduce the potential compounds that caused the inhibition of the microbial communities. As a result, the H_2 productivity and yield increased to $101 \text{ mL H}_2 \text{ L}^{-1}\text{d}^{-1}$ and 1.16 mol H_2 per mol carbohydrates.

The kinetic of H_2 production during this condition (3.3 g L^{-1}) was followed (Figure 5), observing a biphasic production. The biphasic or diauxic behavior has been described in the presence of a mixture of sugars, probably caused by sequential use of the substrates, utilizing first the preferential substrate followed by a short latency phase after which the microorganisms could use the second one (Temudo *et al.* 2009; Solopova *et al.* 2014). The latency period after the consumption of each

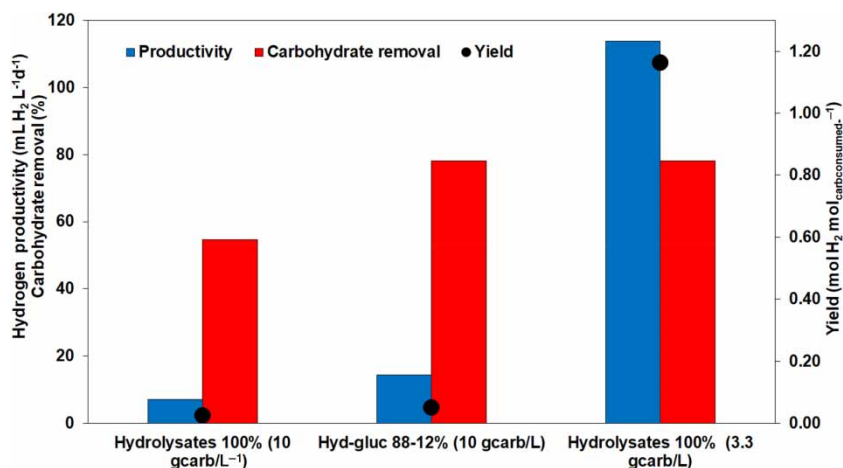


Figure 4 | Performance of the sequencing batch operation with acidic hydrolysates.

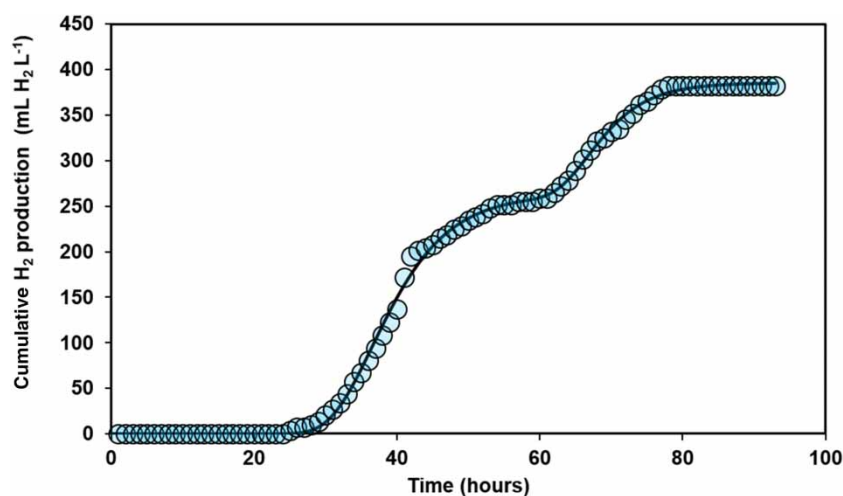


Figure 5 | Cumulative H₂ production per liter of the reactor from 100% hydrolysates at 3.3 g L⁻¹. The black line represents the modified Gompertz model fitted to the experimental data.

substrate has been related to two processes: (1) the time in which the cells made enzymatic adaptations to be able to switch to the consumption of the second sugar and (2) the time in which the global regulatory system of catabolic carbon repression is activated (Solopova *et al.* 2014). The main difference between the two options is that the first one mentioned a period of enzymatic adaptation in the entire population, whereas the second describes a heterogeneous response at the cellular level. Solopova *et al.* (2014) reported that the latency period was caused by how the global regulatory system of catabolic carbon repression is activated, meaning that the cells with the capacity to perform the necessary changes to use the second sugar before the activation of the astringent system remain active, while cells without this capability enter a non-growing state until they find another carbon source easier to use.

The hydrogen production was adjusted to the modified Gompertz equation (Kim *et al.* 2003), obtaining a good fit ($R^2 = 0.999$; Figure 5). In the first phase, after a latency period (λ_1) of 30 h, a maximum velocity (R1) of 16 mL H₂ L⁻¹ h⁻¹ was obtained, reaching the maximum hydrogen production (P1) of 263 mL H₂ L⁻¹ in the first 51 h. During the second phase, the latency period (λ_2) was 9 h, and the maximum velocity (R2) was 10 mL H₂ L⁻¹ h⁻¹, reaching a maximum production (P2) of 122 mL H₂ L⁻¹ in the next 20 h, obtaining a total hydrogen production of 385 mL H₂ L⁻¹ in 80 h. The diauxic behavior has already been reported during the methane production from agro-industrial-wastes (Buitrón *et al.* 2019). The authors evaluated the biochemical potential of methane of the solid fractions of hydrothermal treatment of rice straw, wheat straw, sugar cane and agave bagasse. They observed the diauxic behavior in all the solid fractions studied, whereas only in the

leachates with high COD concentration, indicating that compounds more difficult to digest were produced during the hydrothermal treatment. Valdez-Guzmán *et al.* (2019) found similar results during the hydrogen production from non-detoxified and detoxified agave bagasse acidic hydrolysates and attributed them to the sugars xylose, mannose and galactose present in the hydrolysates. Although the diauxic behavior is generally attributed to the sugars in the substrate, in the case of hydrolysates, other products such as oligosaccharides are released within the acidic hydrolysis of lignocellulosic biomass, which is more difficult to degrade than monosaccharide sugars and could contribute to biphasic production of biogas (Arreola-Vargas *et al.* 2014; Vasconcelos *et al.* 2020).

The decrease of the carbohydrates removal observed in the presence of xylose as principal substrate and the diauxic behavior observed during the sequential batch production from hydrolysates suggest that the operation of the EGSB at HRT of 5.5 h could result in a limitation on time necessary for the hydrogen production from hydrolysates. Therefore, to allow the carbohydrates consumption in the continuous operation with acidic hydrolysates of agave bagasse, the TRH was changed to 11 h. Continuous operation results have been reported before; the comparison of its performance with a suspended cell anaerobic bioreactor can be found in Muñoz-Páez *et al.* (2020b).

During the continuous operation with acidic hydrolyzed agave bagasse, the carbohydrate removal was 1.8 higher than that observed in the continuous production with the xylose-glucose model substrate mixture (68% vs. 37%), showing that the change of the HRT to 11 allowed the microbial community to consume the carbohydrates in the acidic hydrolysates. When comparing both productivities, particular caution is necessary because the carbohydrate concentration used was also different, almost three-fold lower with hydrolysates than with xylose-glucose. Therefore, the OLR with acidic hydrolysates was 7.4 g carbohydrates L⁻¹d⁻¹, almost 6.7-fold lower than that applied with the model substrate. The H₂ productivity obtained in hydrolysates' continuous fermentation was 361 ± 130 mL H₂ L⁻¹d⁻¹, almost 5.4-fold lower than that reported with the model substrate. It seems that the lower H₂ productivity is not only related to the decrease of the OLR, suggesting that the microbial activity was not completely inhibited by the toxic compounds present in the hydrolysates. However, the transport of carbon was diverted towards other pathways different than the hydrogen generation. Valdez-Guzmán *et al.* (2019) reported the deviation of the hydrogen production pathway due to inhibitory compounds in the dark fermentation. The soluble product metabolites obtained in this study were dominated by acetate and butyrate acids (Muñoz-Páez *et al.* 2020b), the preferential routes in hydrogen production. In other studies of dark fermentation with acidic hydrolysates, lactate has been reported, which was not measured in the present work, so carbon consumption could also be destined for this route (Liu *et al.* 2014; Valdez-Guzmán *et al.* 2019).

The H₂ productivity obtained with the acidic hydrolysates of agave bagasse was higher than that observed in the batch experiment under the same initial concentration of carbohydrates (3.3 g L⁻¹). One reason for these results could be that the continuous operation allowed the dilution of the metabolites generated in the fermentation and the inhibitory compounds from the acidic hydrolysates; therefore, these toxic compounds' contact time with the inoculum decreased, allowing higher productivity. In this context, a maximum hydrogen productivity was observed for the mixture of xylose 88% and glucose 12% in continuous mode. A similar result was obtained when the acidic hydrolysates of agave bagasse were employed in continuous mode. Finally, the continuous fermentation stopped because of the rupture of the granules on day 19.

The H₂ productivity obtained in the sequential batch experiments was 13-fold lower than that reported by Arreola-Vargas *et al.* (2014) (Table 1). However, they use a mixture of acidic hydrolysates with a mixture of xylose-glucose, which in their case, could improve the hydrogen production in the AnSBR. Regarding the continuous operation, H₂ production was not observed during the operation with hydrolysates, only with hydrolysates and a mixture of xylose-glucose in a trickling bed reactor at a similar HRT and pH to those in our work (12 h and 5.0, respectively; Arreola-Vargas *et al.* 2014). Finally, considering the results shown in this study, the H₂ production from agro-industrial hydrolysates is challenging not only because the compounds in the medium could be inhibitors of the fermentation but by the types and proportions of the sugars released in the hydrolysates that could negatively affect the performance of the dark fermentation. Therefore, a more in-depth characterization of the acidic hydrolysates should be done, and the selection of the inocula should consider the main sugars in the hydrolysates to use a microbial community with the capacity to use these sugars.

CONCLUSIONS

The anaerobic granular sludge present in the EGSB reactor did not produce H₂ when xylose was the only substrate and using an OLR of 52 g/L-d and 5.5 h of HRT. The reactor's performance with the mixture xylose-glucose (88–12%) was similar to

that of only glucose. The granules' structure was affected negatively by the operation with xylose, xylose-glucose, and acidic agave bagasse hydrolysates. Finally, hydrolysate concentrations higher than 3.3 g/L negatively affected the H₂ production in sequential batch fermentation.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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