Hydrogen Production by Anaerobic Digestion from *Agave lechuguilla* Hydrolysates

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Hydrogen production from enzymatic hydrolysates of *Agave lechuguilla* pretreated by autohydrolysis was assessed in this work. The pretreatment was carried out in a high-pressure reactor using a solid/liquid ratio of 1:6 (w/v) at 190 °C for 30 min at 200 rpm. The pretreated solids were enzymatically hydrolyzed and then were digested with a treated mixed consortium under specified conditions with a Taguchi ($L_9(3^4)$) experimental array. The results showed that the xylan was 65.2% solubilized during pretreatment, and the glucan preserved was 77.5% hydrolyzed, obtaining a hydrolysate with 55 g/L of glucose. The production of hydrogen after anaerobic digestion of hydrolysates was significantly influenced mainly by the temperature (80.6%) and glucose concentration (15.1%). The best conditions were 40 ºC, glucose 20 g/L, inoculum 5% (v/v), and initial pH 7. Under optimal conditions, the hydrogen yield achieved was 3.48 mol H_2/mol glucose consumed at 120 h.

Keywords: Agave lechuguilla; Autohydrolysis pretreatment; Anaerobic digestion; Hydrogen

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INTRODUCTION

Energy plays a major role in world economic and social development; however, today's energy is produced mainly from non-renewable sources that are considered pollutants. Therefore, continuous diversification of energy sources is of crucial importance to every nation, and Mexico is no exception (Arreola-Vargas *et al.* 2015*)*. Hydrogen is one of the most promising energy carriers due to its high energy-yield efficiency and low generation of pollutants (Han *et al*. 2016). Natural gas reforming is a well-established technology used in many refineries and chemical industries in Mexico for large-scale H_2 production (Ortiz *et al*. 2016). However, the production of hydrogen *via* this process generates large quantities of carbon dioxide (CO_2), one of the main causes of global warming (Arriaga *et al*. 2011).

The biological process of anaerobic digestion is an environmental friendly process and can utilize a wide range of substrates (Sattar *et al*. 2016), including different lignocellulosic feedstocks such as forest and agricultural residues or crops not used for food or feed (Liu *et al*. 2014). However, due to the complex plant cell wall structures, lignocellulosic materials are not capable of undergoing fermentation without previous pretreatment and hydrolysis (Zhao *et a*l. 2013). In second-generation (2G) biofuel production process from lignocellulosic biomass, the pretreatment stage is of major importance; therefore, the selection of an adequate pretreatment method that can improve the hydrolysis of structural carbohydrates (cellulose and hemicellulose) and can also be ecofriendly and low cost is crucial. Autohydrolysis pretreatment is a method that does not

require the use or addition of chemicals and only uses water, making this method an attractive option to obtain a pretreated material with a high digestibility (Rios-González *et al*. 2017). To our knowledge, previous reports by Rios-González *et al*. (2017) and Ortíz-Méndez *et al*. (2017) are the only ones to mention the use of autohydrolysis to pretreat agave biomass (*Agave tequilana* and *Agave lechuguilla* respectively) to produce 2G ethanol.

There is abundant research on hydrogen production from lignocellulosic hydrolysates (Arriaga *et al*. 2011; Zhao *et al*. 2014; Arreola-Vargas *et al.* 2015; Baêta *et al*. 2016; Ding *et al*. 2016; Gonzales *et al*. 2016; Han *et al*. 2016; Rorke and Kana 2016; Sangyoka *et al*. 2016; Sattar *et al*. 2016; Kumar *et al*. 2017). However, only a few studies of hydrogen production from agave biomass are available in the current literature. These studies were carried out using *Agave tequilana* bagasse without pretreatment and only applying hydrolysis with diluted acid at high temperatures (Arreola-Vargas *et al*. 2015, 2016) and by enzymatic hydrolysis (Contreras *et al*. 2017).

Agave lechuguilla (lechuguilla) is a common plant of northern Mexico, occupying the largest range of all agaves with almost 20 million hectares of the arid and semiarid lands of Mexico (Castillo *et al*. 2011). *A. lechuguilla* traditionally has been exploited for extracting fibers used in the manufacture of metal polishing brushes, furniture and car seat fillings, carpets and cleaning brushes, as a construction material in combination with thermoplastic resins, and as a concrete reinforcement (Pando-Moreno *et al.* 2008). *A. lechuguilla* cogollos (the heart or pulpy central stem with attached leaf bases) can be harvested many times without sacrificing the whole plant. The annual productivity, with 427 mm of average rainfall, is 4 tons per hectare (Escamilla-Treviño 2012), making it an attractive energy crop. Moreover, *A. lechuguilla* has potential as a feedstock for ethanol production (Morales-Martínez *et al*. 2017; Ortíz-Mendez *et al*. 2017; Díaz-Blanco *et al.* 2018).

During anaerobic digestion, the sludge contains different microorganism groups. Hydrogen consumers such as methanogens are present and are strongly subjected to deactivation *via* different methods (high temperature, UV radiation, extremely low or high pH) to obtain an enriched mixed consortium of *Clostridium*-like microorganisms that are known to produce hydrogen (Sattar *et al*. 2016). Several factors affect hydrogen production in addition to the nature and treatment of the mixed consortia, such as temperature, pH, mineral medium formulation, type of inoculum, the profile of organic acids produced, and the type and concentration of substrate (Sangyoka *et al*. 2016).

The present work assesses the effects of temperature, substrate concentration, initial pH, and inoculum on hydrogen production from *A. lechuguilla* hydrolysates in batch reactors to optimize the process *via* a Taguchi ($L_9(3^4)$) experimental array.

EXPERIMENTAL

Materials and Methods

Feedstock

All cogollos (*A. lechuguilla*) were harvested from Ramos Arizpe, Coahuila, Mexico (latitude 25° 55' 47" North, longitude 101° 55' 47" West). For storage purposes, the cladodes of the cogollos were completely separated and dried for 24 to 30 h at 45 °C in a tray dehydrator (model KL10, Queretaro, Mexico). The dried material was milled to obtain a particle size of 2 mm in a cutting mill (Retsch SM100, Retsch, Haan, Germany). After

milling, the material was stored in plastic containers at room temperature until further use.

Determination of feedstock composition and pretreatment

Before chemical composition determination, the moisture content was determined with a moisture analyzer (Moisture Analyzer OHAUS; Parsippany, NJ). Cellulose (glucan), hemicellulose (xylan), and lignin were quantified according to Rios *et al*. (2017) using *A. lechuguilla* extractives free, which was removed and determined using the analytical method NREL/TP-510-42619 (Sluiter *et al*. 2005). Finally, ashes and proteins were determined using the analytical method NREL/TP-510-42622 (Sluiter *et al*. 2008) and Kjeldahl method, respectively.

According to a previous report by Ortíz-Méndez *et al*. (2017), the optimum conditions to pretreat *A. lechuguilla* biomass by autohydrolysis were at 190 °C for 30 min, corresponding to a severity factor (SF) of 4.127. Under these conditions, a greater quantity of xylan is hydrolyzed and glucan is largely preserved. Additionally, the enzymatic digestibility increases. Therefore, the autohydrolysis pretreatment of *A. lechuguilla* was carried out in a 5-gallon high-pressure stainless-steel reactor equipped with a Rushton impeller agitation system (Parr Instruments Company, Moline, IL, USA). The dried and milled material (2.192 kg of *A. lechuguilla*) was suspended in 13.15 L of distilled water (resulting in a 1:6 w/v solid/liquid ratio) at 190 °C and 200 rpm for 30 min, corresponding to a SF of 4.127. The heating up (190 °C) and cooling down (50 °C) time was 56 min and 45 min respectively. However, to calculate the SF according to Fan and Ragauskas (2012) this time is not taken in account,

$$\boldsymbol{SF = log\left\{t\, exp\left[\frac{T_H - T_R}{14.75}\right]\right\}} \quad (1)$$

where t is the reaction time in minutes, T_H is the reaction temperature in °C, and T_R is the reference temperature (100 °C). The value of 14.75 is an empirical parameter related to the activation energy and temperature.

The reactor was opened when the temperature had decreased to 50 °C. Then the pretreated material was washed with water, and the glucan, xylan, and lignin content were determined as described previously (without extractives removal). The enzymatic loading calculations were established according to the glucan content present in the pretreated material.

Enzymatic hydrolysis of the pretreated solids

The pretreated solid fraction was hydrolyzed using Cellic® CTec3 (kindly provided by Novozymes A/S, Kalundborg, Denmark), with a cellulase activity of 217 FPU/mL. FPU is the activity unit of cellulase when filter paper is used as the enzymatic hydrolysis substrate, according to Ghose (1987). According to previous studies carried out by Morales-Martínez *et al*. (2017), the enzymatic hydrolysis was carried out at a solids loading of 25% (w/w) in the presence of 0.05 M sodium citrate buffer at a pH of 4.8, using an enzyme loading of 15 FPU per gram of glucan in a 15 L glass reactor with mechanical stirring and controlled with an eZ Applikon® module (Schiedam, Netherlands). The reactor was maintained at 50 °C and 200 rpm for 72 h. The slurry obtained was withdrawn from the reactor and centrifuged at 10000 × *g* for 15 min. The liquid fraction (hydrolysate) was filtered and analyzed by HPLC to determine the glucose concentration.

The enzymatic hydrolysis yield was expressed as the relationship between the amount of glucose released during saccharification and the initial amount of glucan present in the pretreated material. The enzymatic hydrolysis yield was calculated as described by Fang *et al*. (2010).

Inoculum and treatment

The anaerobic granular sludge used in the present work was obtained from a large-scale up-flow anaerobic sludge blanket (UASB) reactor provided by the Modelo S. De R. L. De C. V. brewery located in Torreon, Coahuila, Mexico. To eliminate hydrogen-consuming microorganisms (methanogens) and to favor hydrogen spore-formers mainly *Clostridium* species, the sludge was heated in a boiling water bath at 105 °C for 30 min, then cooled down and followed by an acid treatment that involved decreasing the pH of the sludge to 3.0 using 0.1 N HCl solution for 24 h. After this period, the pH was adjusted to 7.0 with a 0.1 N NaOH solution (Hu and Chen 2007). The sludge obtained was separated by filtration and used as an inoculum in the hydrogen production assays.

Optimization of hydrogen production from enzymatic hydrolysate

An orthogonal experimental array ($L_9(3^4)$) was applied to optimize the hydrogen production from enzymatic hydrolysates of *A. lechuguilla* biomass. Nine experiments were carried out at the temperatures 30, 40, and 50 °C; glucose concentrations 20, 37.5, and 55 g/L; inoculum of 5, 10, and 15% (v/v); and initial pH values 5, 6, and 7. The hydrogen fermentation conditions and the orthogonal experiment array are shown in the Tables 1 and 2, respectively.

Table 1. Factors and Levels in the Orthogonal Experiments

Factors	Levels		
	1	2	3
A, Temperature (°C)	30(A1)	40(A2)	50(A3)
B, Glucose concentration (g/L)	20(B1)	37.5(B2)	55(B3)
C, Inoculum (v/v, %)	5(C1)	10(C2)	15(C3)
D, Initial pH	5(D1)	6(D2)	7(D3)

Table 2. Orthogonal Experiment Array

Experiment No.	Factors/Levels			
	A, Temperature (°C)	B, Glucose Concentration (g/L)	C, Inoculum (v/v, %)	D, Initial pH
1	A1	B1	C1	D1
2	A1	B2	C2	D2
3	A1	B3	C3	D3
4	A2	B1	C2	D3
5	A2	B2	C3	D1
6	A2	B3	C1	D2
7	A3	B1	C3	D2
8	A3	B2	C1	D3
9	A3	B3	C2	D1

Each experiment was carried out in a 25 mL glass bottle that contained 10 mL of hydrolysate. The hydrolysate was previously supplemented with a mineral medium described by Contreras-Dávila *et al*. (2017), in g/L: $NH_4H_2PO_4$, 4.5; Na_2HPO_4, 0.635;

K_2HPO_4, 0.125; $MgCl_2 \cdot 6H_2O$, 0.1; $ZnCl_2$, 0.075; $FeSO_4 \cdot 7H_2O$, 0.025; $MnSO_4 \cdot H_2O$, 0.009; and $CuSO_4 \cdot 5H_2O$, 0.005. The glucose concentration and pH of the hydrolysates were adjusted as described in the experimental design. The amount of anaerobic treated sludge (inoculum) added for each experiment is described in Table 2.

After adding the inoculum, the reactors were sealed with butyl rubber stoppers and aluminum caps to avoid gas leakage and flushed with N_2 (100%) gas for 15 min to promote an anaerobic environment. Hydrogen and methane production were determined by gas chromatography and measured at 20, 44, 68, and 92 h. After every measurement, the reactors were flushed with N_2 (100%) as described above. The initial and final glucose concentration were determined by High Performance Liquid Chromatography (HPLC). The hydrogen production yield (mol H_2/mol of consumed glucose) was considered the dependent variable. The experimental data was analyzed statistically by the ANOVA method using Qualitek-4® software (Nutek, Inc., Bloomfield Hills, MI, USA). To validate the results, a set of experiments were further performed using the obtained optimized conditions with a 50.5 mL of hydrolysates.

Analytical Methods

The hydrogen and methane produced were measured by gas chromatography (Varian 3400, Palo Alto, CA, USA) equipped with a TCD detector at 200 °C and a Molecular Sieve 5A packed column at 30 °C, using argon as the carrier gas with a flow rate of 6 mL/min. The sugars (glucose, xylose, cellobiose, and arabinose) were determined by HPLC (Agilent 1260 Infinity, CA, USA) equipped with a refractive index detector at 45 °C, using an Agilent Hi-Plex H column at 35 °C (7.7 x 300 mm, CA, USA) and 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.5 mL/min. All experiments were carried out in triplicate, and the average values are reported.

RESULTS AND DISCUSSION

Composition of *A. lechuguilla* and Autohydrolysis Pretreatment

The extractives were the main component in *A. lechuguilla* cogollos in dry base w/w (29.8%). The glucan, xylan, and lignin contents were 18.2%, 7.7%, and 21.7%, respectively. The ashes and protein contents were 8% and 5.5%, respectively and a 9.07% corresponded to non-detected components (Table 3).

The solids composition of *A. lechuguilla* pretreated by autohydrolysis is summarized in Table 3. The solids recovered after pretreatment were 55.8% from the raw material, mainly due to the solubilization of extractives and xylan during the process. The glucan content was increased after pretreatment compared with untreated biomass (increasing from 18.2% to 28.2%). From the initial glucan content present in the untreated material, 86.4% remained in the solid phase. However, the glucan content present in the pretreated material (28.19%) does not coincide with previous results reported by Ortíz-Méndez *et al*. (2017) and Morales-Martínez *et al*. (2017) in which the same SF factor was applied (4.127). These differences can be attributed to the different location at which the raw material was collected. The hydrolysis of xylan is one of the main effects of the autohydrolysis process, and its degradation products are dissolved in the liquid phase during pretreatment. As expected, autohydrolysis mainly affected the hemicellulosic components, and under these conditions 65.2% of the original xylan content was solubilized.

The glucan and most of the insoluble lignin were retained completely in the solid phase (Amiri and Karimi 2015; Zhuang *et al*. 2016). The lignin was not solubilized during the pretreatment process, recovering 99.2%. These results are similar to previous reports (Ortíz-Méndez *et al*. 2017; Morales-Martínez *et al*. 2017; Rios-González *et al*. 2017) and with other materials pretreated with the same method (Moniz *et al*. 2013; Buruiana *et al*. 2014).

Delignification is not the only factor to decrease lignocellulose recalcitrance; in a previous study on ethanol production from *Agave tequilana* bagasse pretreated by autohydrolysis (Rios-González *et al*. 2017), the hemicellulose (xylan) removal improved the enzymatic hydrolysis (obtaining an 81.5% hydrolysis yield).

Table 3. *A. lechuguilla* Composition after Autohydrolysis Pretreatment

Component	Untreated solid (%)	Pretreated Solid (%)	Solids recovered (%)	Components remained (%)
Glucan	18.21 ± 0.35	28.19 ± 0.94	15.74 ± 0.37	86.44 ± 1.1
Xylan	7.71 ± 0.16	4.81 ± 0.36	2.68 ± 0.61	34.76 ± 0.94
Lignin	21.67 ± 0.41	38.50 ± 0.72	21.49 ± 0.72	99.17 ± 1.6
Others	52.41 ± 0.30	28.5 ± 0.67	15.91 ± 0.56	30.4 ± 0.63
Total	100 ± 0.30	100 ± 0.67	55.82 ± 0.56	-

Enzymatic Hydrolysis of the Pretreated Solids

Table 4 shows the enzymatic hydrolysis of pretreated *A. lechuguilla*, reaching a glucose concentration of 55 g/L at 72 h, corresponding to a hydrolysis yield of 77.5%. However, no significant increase in the glucose concentration was observed after 24 h of enzymatic hydrolysis, and it is likely that in this period, the cellulases started to present inhibition. This result can be attributed to an increase in the diffusional limitation of cellulases by a high solid loading, or to lignin's effect on the action of enzymes *via* the blocking of access to the cellulose (López-Linares *et al*. 2014). Comparing the results obtained in the present study with the results reported previously by Ortíz-Méndez *et al*. (2017), the hydrolysis yield was similar (60.8%) at a solid and enzyme loadings of 20% (w/w) and 25 FPU per gram of glucan, respectively, with the difference that the enzyme complex used in this previous report was Acellerase 1500 (Genencor®, USA). Meanwhile, the glucose released and the hydrolysis yield were lower than those reported (previous results) by Morales-Martínez *et al*. (2017) using the same enzyme complex (Cellic® CTec3, Novozymes) used in the present study.

The sugar (glucose) concentrations used (55 g/L, 37.5 g/L, and 20 g/L) are similar to other reports of H_2 production using hydrolysates obtained from *A. tequilana* bagasse. Arreola-Vargas *et al*. (2015) assessed the H_2 production using hydrolysates, with total sugar concentrations of 27.9 and 18.9 g/L from cooked and uncooked bagasse, respectively, obtained by acid hydrolysis with HCl. Using the same raw material, Arreola-Vargas *et al*. (2016) assessed H_2 production using acid and enzymatic hydrolysates, with total sugar concentrations of 17.3 g/L and 8.9 g/L, respectively. Contreras-Dávila *et al*. (2017) assessed this same process (H_2 production) using two enzymatic hydrolysates with a total sugar concentration that ranged from 11 to 12.5 g/L.

Table 4. Performance of Enzymatic Hydrolysis of Pretreated Solids

Time (h)	Glucose (g/L)	Hydrolysis Yield (%)
0	0 ± 0	0 ± 0
24	49.2 ± 0.24	69.34 ± 0.24
48	53.6 ± 0.46	75.54 ± 0.46
72	55.0 ± 0.51	77.52 ± 0.51

Optimization of Hydrogen Production from Enzymatic Hydrolysate

Table 5 shows that maximum H_2 production was obtained at 40 °C (3.58 to 4.09 × 10^{-3} mol), with a significant decrease at 50 °C (0.733 to 0.936 × 10^{-3} mol). The optimal temperature for H_2 production varies widely based on the nature of the biocatalyst and the type of substrate used. For mixed consortia, diverse optimum temperatures have been reported (Bharathiraja *et al*. 2016). Glucose consumption was observed in all cases; however, higher hydrogen yields were observed in the cases where the initial glucose concentration was lower (20 g/L), while the maximum hydrogen yield (3.3 mol H_2/mol glucose consumed) was observed in the case of an experiment carried out at 30 °C, 10% (v/v) of inoculum and initial pH of 7.0. The decrease in the hydrogen yield can be attributed to an inhibition caused by high initial substrate concentration or to the presence of a higher concentration of inhibitory by-products present in the hydrolysate, such as furfural and HMF (Gonzales *et al*. 2016). In addition, a high substrate concentration can cause a buildup of cell concentration and volatile fatty acids (VFAs) in the system, leading to a decline of pH in the reactor that could inhibit hydrogen production (Fan *et al*. 2004).

Table 5. Hydrogen Production and Glucose Consumption from *A. lechuguilla* Hydrolysate at 92 h of Fermentation

Experiment No.	H_2 (mol x 10^{-3})	Glucose Consumed (mol x 10^{-3})	Final pH	mol H_2/mol Glucose Consumed
1	2.4 ± 0.12	1.1 ± 0.05	5.1 ± 0.01	2.2 ± 0.08
2	1.6 ± 0.08	1.6 ± 0.14	4.9 ± 0.03	1.0 ± 0.11
3	3.37 ± 0.13	2.3 ± 0.12	5.0 ± 0.01	1.4 ± 0.12
4	3.58 ± 0.07	1.1 ± 0.08	4.8 ± 0.04	3.3 ± 0.07
5	4.53 ± 0.28	1.7 ± 0.11	5.2 ± 0.08	2.7 ± 0.19
6	4.09 ± 0.43	1.7 ± 0.19	5.0 ± 0.03	2.4 ± 0.31
7	0.799 ± 0.09	0.8 ± 0.06	5.1 ± 0.15	0.9 ± 0.07
8	0.936 ± 0.08	1.4 ± 0.15	5.0 ± 0.09	0.7 ± 0.11
9	0.733 ± 0.05	1.9 ± 0.11	5.2 ± 0.18	0.4 ± 0.08
*Methane not detected in all cases.				

As mentioned above, an inhibitory effect of high substrate concentration generally occurs in anaerobic digestion processes, depending on the type of substrates and microorganisms. The initial substrate concentration plays an important role on the yield and production rate of hydrogen (Fabiano and Perego 2002). Sangyoka *et al*. (2016) observed a decrease in H_2 production at a sugar concentration of 30 g/L present in the hydrolysate of sugar cane bagasse that was previously subjected to acidic hydrolysis. Chen *et al*. (2005) reported that H_2 production from sucrose by *Clostridium butyricum* CGS5

was higher at an initial sucrose concentration of 20 g-COD (Chemical Oxygen Demand)/L, while the fermentation process was inhibited at an initial sucrose concentration of 30 g-COD/L. Similar behavior was mentioned by Oh *et al*. (2003), who reported the H_2 production by *Citrobacter* sp. Y19 with glucose as a carbon source and mentioned that hydrogen yield gradually decreased with the increase of glucose concentration at levels higher than 20 g/L.

Table 5 shows that pH values were similar for all cases (4.8 to 5.2). The pH, as mentioned before, is an important factor in anaerobic biological processes, due to its effects on Fe-hydrogenase activity, metabolic pathways, and the duration of the lag phase (Lay 2000). Carbohydrate-rich substrates have a greater potential to acidify the media during anaerobic fermentation, and as a result, in some situations system stability is hard to maintain. In hydrogen fermentation, reactors tend to acidify readily, and a reduction in the pH may take place. Low initial pH values below 5.0 inhibit hydrogen production. On the other hand, high initial pH values such as 9.0 decrease the lag phase time but tend to produce less hydrogen (Sangyoka *et al*. 2016).

Table 6 shows the average effect of the factors at the assigned levels on the hydrogen yield. Both the lowest (0.4) and the highest (3.3) hydrogen yield were attributed to Factor A (Temperature) at level 2 (40 ºC) and level 3 (50 ºC), respectively. In the present study, three levels for each factor were selected, and the R values (Range extreme difference) were calculated based on the difference between the highest and lowest hydrogen yield. The highest R value was considered the most influential factor. As seen in the Table 5, the influence of these four factors on hydrogen yield was in the order A, B, D, C, based on R values.

Table 6. Main Effects of Selected Factors

Factor	Level 1	Level 2	Level 3	R
(A) Temperature (ºC)	1.60	2.78	0.67	2.12
(B) Glucose (g/L)	2.22	1.42	1.42	0.80
(C) Inoculum (% v/v)	1.75	1.63	1.67	0.12
(D) pH	1.78	1.45	1.82	0.37

Figure 1 shows the influence of each individual factor. The increase in the glucose concentration resulted in a decrease in hydrogen yield. For the temperature, the hydrogen yield was higher in level 2; increasing the temperature over 40 °C led to a reduction in hydrogen yield. The pH and inoculum were the factors with less influence over hydrogen yield, obtaining similar results for all levels. The pH did not present a significant effect, which was attributed to the fact that the factor was only controlled at the beginning of the experiment and a rapid decrease in the initial pH (after 20 h) was observed neutralizing the effect of the factor during the rest of the experiment. Taking into account the optimization process for the four factors at the levels assessed, the optimum conditions were A2 (40 °C); B1 (20 g/L glucose); C1 (5 % v/v inoculum); D3 (initial pH 7).

Table 7 shows the ANOVA for hydrogen yield. According to the Fisher test (F), the temperature is a more significant factor than the hydrogen yield. After the temperature, the glucose concentration was more significant than hydrogen yield, and pH and inoculum were the factors with the least significance.

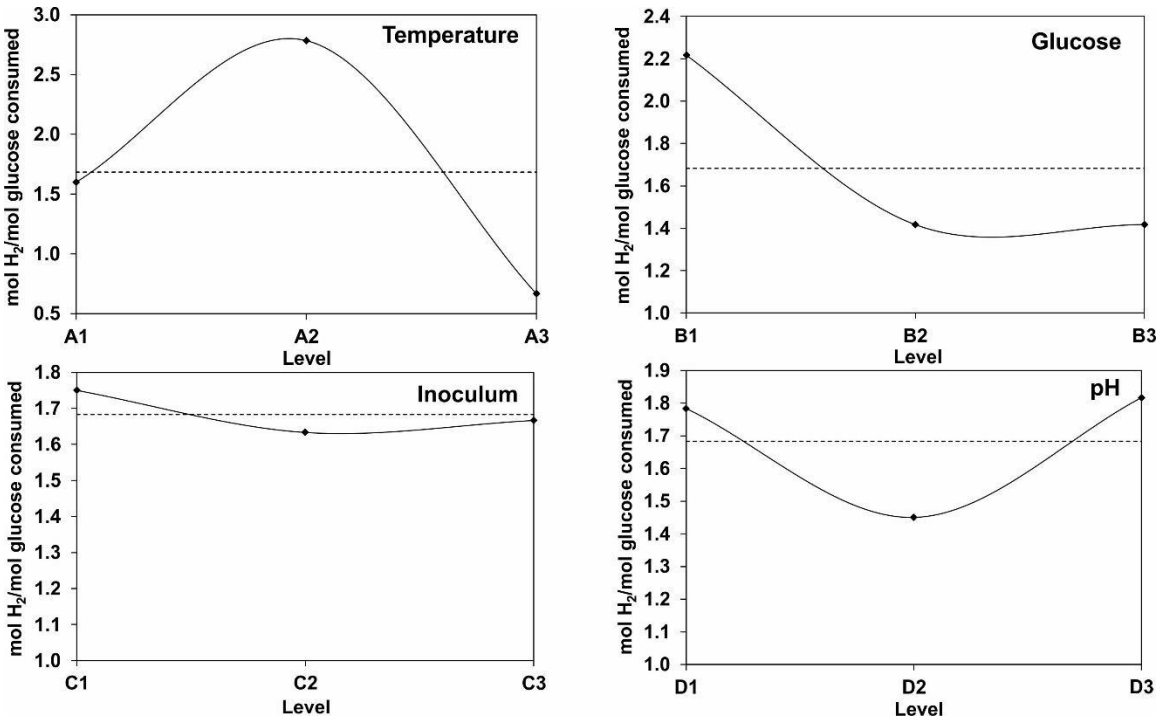

Fig. 1. Individual factors performance at different levels

Table 7. Analysis of Variance (ANOVA)

Factors	DOF	Sums of Squares	Variance	F-Ratio	Pure Sum	Percentage
Temperature	2	13.50	6.75	578.72	13.48	80.6
Glucose	2	2.56	1.28	109.71	2.54	15.185
Inoculum	2	0.04	0.02	1.86	0.02	0.119
pH	2	0.49	0.25	21.14	0.47	2.813
Other/Error	9	0.10	0.01			1.189
Total	17	16.71				

The temperature showed the highest impact on hydrogen yield (80.6%), followed by the glucose concentration (15.18%), the initial pH (2.81%), and finally the inoculum (0.11%). Controlling each factor individually or as a whole can lead to a major increase in hydrogen yield. By studying the main effects of each factor, the general trends of the influence of the factors towards the process can be characterized. The characteristics can be controlled such that a lower or a higher value in a particular influencing factor can produce the preferred result. Therefore, the levels of factors to produce the best results can be predicted, so that the higher levels of hydrogen yield can be achieved with optimized conditions obtained: temperature of 40 ºC, glucose of 20 g/L, inoculum of 5% (v/v), and pH of 7. The expected result under optimum conditions was 3.515 mol H_2/mol glucose consumed.

The final experimental stage consisted of applying the optimum conditions obtained to confirm or validate the results of the previous stage. Figure 2 shows the experimental results using optimum conditions predicted by the Taguchi L9 orthogonal array, from which it can be seen that hydrogen yield is greatly improved at the selected levels, resulting in a value of 3.48 mol H_2/mol glucose consumed at 120 h, very similar to

the previously mentioned expected value. The hydrogen yield in the present work was greater than that reported by Arreola-Vargas *et al*. (2014) and Contreras-Dávila *et al*. (2017) in which they used an enzymatic hydrolysate from *Agave tequilana* bagasse, obtaining a hydrogen yield of 3.4 mol H_2/mol hexose (in batch mode) and 1.53 mol H_2/mol substrate (in continuous mode), respectively.

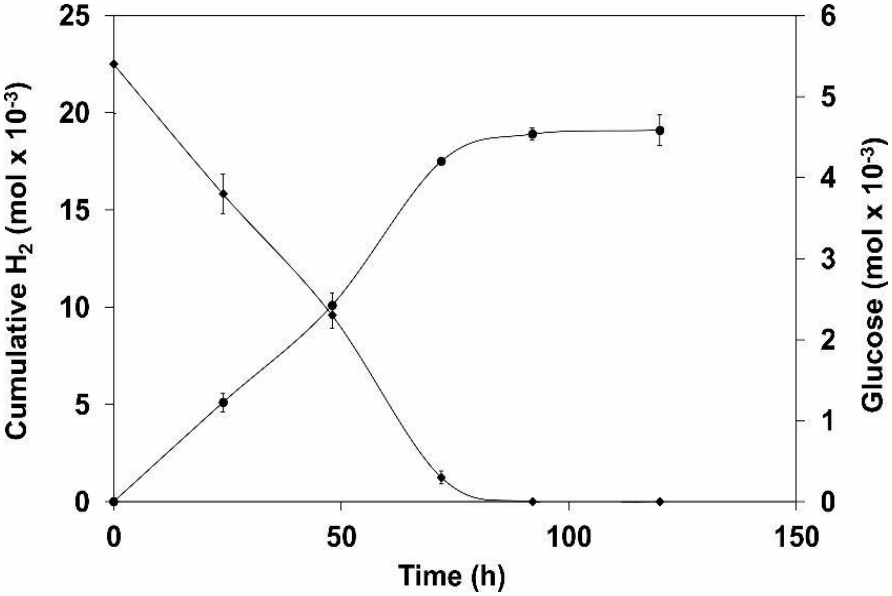

Fig. 2. Hydrogen production (circle mark) and glucose consumption (diamond shape mark) from *A. lechuguilla* hydrolysate during validation of results under optimum conditions

CONCLUSIONS

1. The results demonstrated the potential of hydrogen production from the enzymatic hydrolysates of *A. lechuguilla* pretreated by autohydrolysis.
2. The hydrogen production was significantly influenced by the operational conditions, mainly by the temperature and the initial glucose concentration. The hydrogen yield achieved (3.48 mol H_2/mol glucose consumed) was greater compared to early reports using hydrolysates of agaves.
3. Future research will be focused on assessing the production of hydrogen during continuous mode operation at different organic loading rates to study the economic feasibility of this process on a large-scale.

ACKNOWLEDGMENTS

The authors are grateful to Innovation Promotion Program (Programa de Estímulos a la Innovación PEI-2016) from the National Council of Science and Technology of Mexico (CONACyT) for financial support.

REFERENCES CITED

Amiri, H., and Karimi, K. (2015). "A promising pretreatment for the improvement of acetone, butanol, and ethanol production from woody materials," *Chem. Eng. Sci*. 137, 722-729. DOI: 10.1016/j.ces.2015.07.020

Arreola-Vargas, J., Alatriste-Mondragón, F., Celis, L. B., Razo-Flores, J., López-López, A., and Méndez-Acosta, H. O. (2014). "Continuous hydrogen production in a trickling bed reactor by using triticale silage as inoculum: Effect of simple and complex substrates," *J. Chem. Technol. Biotechnol*. 90, 1062-1069. DOI: 10.1002/jctb.4410

Arreola-Vargas, J., Ojeda-Castillo, V., Snell-Castro, R., Corona-González, R. I., Alatriste-Mondragón, F., and Méndez-Acosta, H. O. (2015). "Methane production from acid hydrolysates of *Agave tequilana* bagasse: Evaluation of hydrolysis conditions and methane yield," *Bioresource Technol*. 181, 191-199. DOI: 10.1016/j.biortech.2015.01.036

Arreola-Vargas, J., Flores-Larios, A., González-Álvarez, V., Corona-González, R. I., and Méndez-Acosta, H. O. (2016). "Single and two-stage anaerobic digestion for hydrogen and methane production from acid and enzymatic hydrolysates of *Agave tequilana* bagasse," *Int. J. Hydrogen Energ*. 41, 897-904. DOI: 10.1016/j.ijhydene.2015.11.016

Arriaga, S., Rosas, I., Alatriste-Mondragón, F., and Razo-Flores, E. (2011). "Continuous production of hydrogen from oat straw hydrolysate in a biotrickling filter," *Int. J. Hydrogen Energy* 36, 3442-3449. DOI: 10.1016/j.ijhydene.2010.12.019

Baêta, B. E. L., Lima, D. R. S., Filho, J. G. B., Adarme, O. F. H., Gurgel, L. V. A., and de Aquino, S. F. (2016). "Evaluation of hydrogen and methane production from sugarcane bagasse hemicellulose hydrolysates by two-stage anaerobic digestion process," *Bioresource Technol*. 218, 436-446. DOI: 10.1016/j.biortech.2016.06.113

Bharathiraja, B., Sudharsanaa, T., Bharghavi, A., Jayamuthunagai, J., and Praveenkumar, R. (2016). "Biohydrogen and biogas – An overview on feedstocks and enhancement process," *Fuel* 185, 810-828. DOI: 10.1016/j.fuel.2016.08.030

Buruiana, C. T., Vizireanua, C., Garrote, G., and Parajó, J. C. (2014). "Optimization of corn stover biorefinery for coproduction of oligomers and second generation bioethanol using non-isothermal autohydrolysis," *Ind. Crop. and Prod*. 54, 32-39. DOI: 10.1016/j.indcrop.2014.01.003

Castillo, Q. D., Mares, A. O., and Villavicencio, G. E. E. (2011). "Lechuguilla (*Agave lechuguilla* Torr.) planta suculenta de importancia económica y social de las zonas áridas y semiáridas de México," *Boletín de la Sociedad Latinoamericana y del Caribe de Cactáceas y otras Suculentas* 8, 6-9.

Chen, W. M., Tseng, Z. J., Lee, K. S., and Chang, J. S. (2005). "Fermentative hydrogen production with *Clostridium butyricum* CGS5 isolated from anaerobic sewage sludge," *Int. J. Hydrogen Energ*. 30, 1063-1070. DOI: 10.1016/j.ijhydene.2004.09.008

Contreras-Dávila, C. A., Méndez-Acosta, H. O., Arellano-García, L., Alatriste-Mondragón, F., and Razo-Flores, E. (2017). "Continuous hydrogen production from enzymatic hydrolysate of *Agave tequilana* bagasse: Effect of the organic loading rate and reactor configuration," *Chem. Eng. J.* 313, 671-679. DOI: 10.1016/j.cej.2016.12.084

Díaz-Blanco, D. I., de La Cruz, J. R., López-Linares, J. C., Morales-Martínez, T. K.,

Ruiz, E., Rios-González, L. J., Romero, I., and Castro, E. (2018). "Optimization of dilute acid pretreatment of *Agave lechuguilla* and ethanol production by co-fermentation with *Escherichia coli* MM16," *Ind. Crop. Prod*. 114, 154-163. DOI: 10.1016/j.indcrop.2018.01.074

Ding, L., Cheng, J., Yue, L., Liu, J., Zhang, L., Zhou, J., and Cen, K. (2016). "Fermentative hydrogen and methane co-production from pretreated *Spartina anglica* biomass with optimal saccharification effect under acid/alkali-assisted steam/ microwave heating and enzymolysis," *Energ. Convers. Manage*. 127, 554-560. DOI: 10.1016/j.enconman.2016.09.045

Escamilla-Treviño, L. L. (2012). "Potential of plants from the genus agave as bioenergy crops," *Bioenerg. Res*. 5, 1-9. DOI: 10.1007/s12155-011-9159-x

Fabiano, B., and Perego, P. (2002). "Thermodynamic study and optimization of hydrogen production by *Enterobacter aerogenes*," *Int. J. Hydrogen Energ*. 27, 149-156. DOI: 10.1016/S0360-3199(01)00102-1

Fang, H., Zhao, C., and Song, X.-Y. (2010). "Optimization of enzymatic hydrolysis of steam-exploded corn stover by two approaches: Response surface methodology or using cellulase from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* NL02," *Bioresource Technol*. 101, 4111-4119. DOI: 10.1016/j.biortech.2010.01.078

Fan, H., and Ragauskas, A. (2012). "Pretreatment and lignocellulosic chemistry," *Bioenergy Resources* 5, 1043-1066. DOI: 10.1007/s12155-012-9208-0

Fan, Y. T., Li, C. L., Lay, J. J., Hou, H. W., and Zhang, G. S. (2004). "Optimization of initial substrate and pH levels for germination of sporing hydrogen-producing anaerobes in cow dung compost," *Bioresource Technol*. 91, 189-193. DOI: 10.1016/s0960-8524(03)00175

Ghose, T. K. (1987). "Measurement of cellulase activities," *Pure Appl. Chem*. 59, 257-268. DOI: 10.1351/pac198759020257

Gonzales, R. R., Sivagurunathan, P., Parthiban, A., and Kim, S.-H. (2016). "Optimization of substrate concentration of dilute acid hydrolyzate of lignocellulosic biomass in batch hydrogen production," *Int. Biodeterior. Biodegradation*. 113, 22-27. DOI: 10.1016/j.ibiod.2016.04.016

Han, W., Hu, Y., Li, S., Li, F., and Tang, J. (2016). "Biohydrogen production in the suspended and attached microbial growth systems from waste pastry hydrolysate," *Bioresource Technol*. 218, 589-594. DOI: 10.1016/j.biortech.2016.07.009

Hu, B., and Chen, S. (2007). "Pretreatment of methanogenic granules for immobilized hydrogen fermentation," *Int. J. Hydrogen Energ*. 32, 3266-3273. DOI: 10.1016/j.ijhydene.2007.03.005

Kumar, K., Sivagurunathan, P., Sen, B., Kim, S.-H., and Lin, C.-Y. (2017). "Mesophilic continuous fermentative hydrogen production from acid pretreated de-oiled jatropha waste hydrolysate using immobilized microorganisms," *Bioresource Technol*. DOI: 10.1016/j.biortech.2017.03.059

Lay, J. J. (2000). "Modeling and optimization of anaerobic digested sludge converting starch to hydrogen," *Biotechnol. Bioeng*. 68, 269-278. DOI: 10.1002/(SICI)1097-0290(20000505) 68:3< 269:: AID-BIT5>3.0.co;2-T

Liu, C.-M., Wu, S.-Y., Chu, C.-Y., and Chou, Y.-P. (2014). "Biohydrogen production from rice straw hydrolyzate in a continuously external circulating bioreactor," *Int. J. Hydrogen Energ*. 39, 19317-19322. DOI: 10.1016/j.ijhydene.2014.05.175

López-Linares, J. C., Romero, I., Cara, C., Ruiz, E., Moya, M., and Castro, E. (2014).

"Bioethanol production from rapeseed straw at high solids loading with different process configurations," *Fuel* 122, 112-118. DOI: 10.1016/j.fuel.2014.01.024

Moniz, P., Pereira, H., Quilhó, T., and Carvalheiro, F. (2013). "Characterization and hydrothermal processing of corn straw towards the selective fraction of hemicelluloses," *Ind. Crop. Prod*. 50, 145-153. DOI: 10.1016/j.indcrop.2013.06.037

Morales-Martínez, T. K., Díaz-Blanco, D. I., Rodríguez-de la Garza, J. A., Morlett-Chávez, J., Castro-Montoya, A. J., Quintero, J., Aroca, G., and Rios-González, L. J. (2017). "Assessment of different saccharification and fermentation configurations for ethanol production from *Agave lechuguilla*," *BioResources* 12, 8093-8105. DOI: 10.15376/biores.12.4.8093-8015

Oh, Y. K., Seol, E. H., Kim, J. R., and Park, S. (2003). "Fermentative biohydrogen production by a new chemoheterotrophic bacterium *Citrobacter* sp. Y19," *Int. J. Hydrogen Energ*. 28, 1353-1359. DOI: 10.1016/S0360-3199(03)00024-7

Ortiz, A. L., Zaragoza, M. J. M., and Collins-Martínez, V. (2016). "Hydrogen production research in Mexico: A review," *Int. J. Hydrogen Energ*. 41, 23363-23379. DOI: 10.1016/j.ijhydene.2016.07.004

Ortíz-Méndez, O. H., Morales-Martínez, T. K., Rios-González, L. J., Rodríguez-de la Garza, J. A., Quintero, J., and Aroca, G. (2017). "Bioethanol production from *Agave lechuguilla* biomass pretreated by autohydrolysis," *Rev. Mex. Ing. Quim*. 16, 467-476.

Pando-Moreno, M., Pulido, R., Castillo, D., Jurado, E., and Jiménez, J. (2008). "Estimating fiber for lechuguilla (*Agave lechuguilla* Torr., Agavaceae), a traditional non-timber forest product in Mexico," *Forest. Ecol. Manag*. 255, 3686-3690. DOI: 10.1016/j.foreco.2008.02.053

Rios-González, L. J., Morales Martínez, T. K., Rodríguez Flores, M. F., Rodríguez de la Garza, J. A., Castillo Quiroz, D., Castro Montoya, A. J., and Martínez, A. (2017). "Autohydrolysis pretreatment assessment in ethanol production from agave bagasse," *Bioresource Technol*. 242, 184-190. DOI: 10.1016/j.biortech.2017.03.039

Rorke, D., and Kana, E. B.G. (2016). "Biohydrogen process development on waste sorghum (*Sorghum bicolor*) leaves: Optimization of saccharification, hydrogen production and preliminary scale up," *Int. J. Hydrogen Energy* 12941-12952. DOI: 10.1016/j.ijhydene.2016.06.112

Sangyoka, S., Reungsang, A., and Lin, C.-Y. (2016). "Optimization of biohydrogen production from sugarcane bagasse by mixed cultures using a statistical method," *Sustainable Environment Research* 26, 235-242. DOI: 10.1016/j.serj.2016.05.001

Sattar, A., Arslan, C., Ji, C., Sattar, S., Umair, M., Sattar, S., and Bakht, M. Z. (2016). "Quantification of temperature effect on batch production of bio-hydrogen from rice crop wastes in an anaerobic bio reactor," *Int. J. Hydrogen Energ*. 41, 11050-11061. DOI: 10.1016/j.ijhydene.2016.04.087

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., and Templeton, D. (2008). *Determination of Ash in Biomass* (Report No. TP-510-42622), National Renewable Energy Laboratory, Golden, CO.

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., and Templeton, D. (2005). *Determination of Extractives in Biomass* (Report No. TP-510-42619), National Renewable Energy Laboratory, Golden, CO.

Zhao, L., Cao, G.-L., Wang, A.-J., Ren, H.-Y., and Ren, N.-Q. (2013). "Evaluation of continuous biohydrogen production from enzymatically treated cornstalk hydrolysate," *Int. J. Hydrogen Energ*. 38, 15100-15104. DOI: 10.1016/j.ijhydene.2013.09.100

Zhao, L., Cao, G.-L., Wang, A.-J., Ren, H.-Y., and Ren, N.-Q. (2014). "An anaerobic sequential batch reactor for enhanced continuous hydrogen production from fungal pretreated cornstalk hydrolysate," *Int. J. Hydrogen Energ*. 39, 19311-19316. DOI: 10.1016/j.ijhydene.2014.05.167

Zhuang, X., Wang, W., Xu, Q., Qi, W., Wan, Q., Tan, X., Zhou, G., and Yuan, Z. (2016). "Liquid hot water pretreatment of lignocellulosic biomass for bioethanol production accompanying with high valuable products," *Bioresource Technol*. 199, 68-75. DOI: 10.1016/j.biortech.2015.08.051

Article submitted: May 31, 2018; Peer review completed: August 10, 2018; Revised version received: August 21, 2018; Accepted: August 22, 2018; Published: August 27, 2018.
DOI: 10.15376/biores.13.4.7766-7779