Waste Biomass Valor (2016) 7:1017–1029
DOI 10.1007/s12649-016-9494-4

ORIGINAL PAPER

Biohydrogen Production from Liquid and Solid Fractions of Sugarcane Bagasse After Optimized Pretreatment with Hydrochloric Acid

Patrícia Lorencini[1] · Marcos Rechi Siqueira[1] · Bianca Chieregato Maniglia[1] · Delia Rita Tapia[1] · Sandra Imaculada Maintinguer[2] · Valeria Reginatto[1]

Received: 10 April 2015 / Accepted: 10 February 2016 / Published online: 17 February 2016

Abstract This work determined the optimal conditions to pretreat sucargane bagasse with HCl by using the liquid and the solid fractions resulting from the bagasse pretreament as substrate for fermentative hydrogen production by a mixed culture. A 2^3 full factorial central composite design (star configuration) helped to determine how temperature, time, and acid concentration affected the total monosaccharides (TM), total reducing sugars (TRS), and total inhibitors (TI) concentrations in the liquid fraction. Temperature, time, and acid concentration impacted the TRS and TM concentrations, but these variables did not influence the TI concentration significantly. The optimal pretreatment conditions were HCl at 7.36 % (v/v), 96.8 °C, and 441.6 min, which afforded the highest TRS concentration in the liquid hydrolysates. The liquid fraction obtained from the bagasse pretreated with acid under the optimal conditions (designated liq) was not suitable for H_2 production by the mixed culture before treatment of the fraction with activated carbon. The solid residual bagasse (designated sol) alone afforded 6.0 mL of H_2/g of bagasse. Liq treated with 10 % (m/v) activated carbon, to give liq + C, and sol added with the enzyme Celluclast® 10 U g^{-1}, to afford sol + E, yielded 45.3 and 7.8 mL of H_2/g of bagasse respectively, which amounted to 53.1 mL of H_2/g of bagasse. The volumetric productivities—1450 and 1423 mL of H_2 L^{-1} d^{-1} for liq + C and sol + E, respectively—are the highest ever reported in the literature for H_2 production from sugarcane bagasse by a microbial consortium.

Keywords Sugarcane bagasse · Hydrochloric acid · Pretreatment · Fermentation · Biohydrogen

Introduction

The annual Brazilian sugarcane production is ca. 716 million tons [1]. This amount corresponds to ca. 43 % of the global output, which makes Brazil the major sugarcane world producer. Every ton of sugarcane used to obtain sucrose and ethanol generates about 0.3 ton of bagasse, and burning of this residue produces electricity. Unfortunately, bagasse burning releases high quantities of carbon dioxide and particulate material into the atmosphere [2], which makes environmentally friendly strategies to treat sugarcane bagasse highly desirable.

Hydrogen gas constitutes an attractive sustainable fuel because its combustion produces water only. Besides, H_2 has high energy density (about 144 MJ kg^{-1}) and is an excellent energy carrier. Although H_2 production currently relies on fossil fuel-based methods, this fuel can also originate from fermentation of carbohydrate-rich materials. In this context, H_2 production from low-cost waste such as lignocellulosic materials like sugarcane bagasse could become a sustainable and economically viable process [3–8].

Sugarcane bagasse is a carbohydrate-rich waste that consists of ca. 40 % cellulose and 35 % hemicellulose; it also contains 15 % lignin, a non-carbohydrate constituent [9]. This composition has motivated the recent use of sugarcane bagasse as a lignocellulosic substrate for

✉ Valeria Reginatto
valeriars@ffclrp.usp.br

[1] Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, 14040-901 Ribeirão Preto, SP, Brazil

[2] Institute of Chemistry, Center for Monitoring and Research of the Quality of Fuels, Biofuels, Crude Oils and Derivatives – CEMPEQC, UNESP - São Paulo State University, Prof. Francisco Degni Street 55, 14800-900 Araraquara, SP, Brazil

fermentative H_2 production [3–7]. The successful application of this waste requires a pretreatment process that solubilizes fermenting sugars and makes the polysaccharides more accessible for later fermentation [9–11].

The literature contains description of acid, alkaline, enzymatic, and combined (acid or alkaline plus enzymatic) pretreatments to hydrolyze sugarcane bagasse for subsequent use as substrate in fermentative H_2 production [3, 6, 7, 12]. Chairattanamanokorn et al. [4] hydrolyzed sugarcane bagasse with 20 U g^{-1} of bagasse of commercial cellulase. These authors applied the hydrolysate in H_2 production by a thermally preheated sludge, to obtain 1.4 mmol of H_2/g of TVS. Pretreatment of sugarcane with NaOH 0.1 mol L^{-1} at 100 °C for 2 h before enzymatic hydrolysis promoted a 13-fold increase in H_2 yield (13.4 mmol g^{-1} of TVS).

The majority of investigations on the use of sugarcane bagasse as substrate for fermentative H_2 production have applied acid pretreatment with sulfuric acid at high temperatures to solubilize sugars. For example, Pattra et al. [3] pretreated sugarcane bagasse with 0.5 % H_2SO_4 at 121 °C for 60 min and used it as substrate for H_2 production by *Clostridium butyricum*, to obtain 1.73 mol of H_2 mol^{-1} of sugar. Saripan and Reungsang (2013) pretreated sugarcane bagasse with H_2SO_4 at 1 and 121 °C for 60 min, to achieve 1.12 and 0.84 mol of H_2 mol^{-1} of sugar in the presence of *Thermoanaerobacterim thermosaccharolyticum* at 55 °C and of a mixed culture at 37 °C, respectively [12]. However, acid pretreatment associated with high temperatures can produce sugar degradation compounds such as acetic acid (from hemicellulose), furfural (from pentose dehydration), and 5-hydroxymethylfurfural (HMF, from hexose dehydration), all of which inhibit fermentation [11, 13, 14].

In turn, pretreatment of lignocellulosic materials with sulfuric acid introduces sulfate into the hydrolysate, which could diminish the H_2 yield due to H_2 consumption by sulfate-reducing microorganisms in a mixed culture [15]. The literature brings no records of studies with hydrochloric acid to pretreat bagasse for biohydrogen production purposes. Therefore, this work reports on the development of a sugarcane bagasse pretreatment method involving the use of HCl under moderate temperature conditions (up to 100 °C, to avoid fermentation inhibitors generation) to explore sugarcane bagasse as a substrate for fermentative H_2 production by a mixed culture. To assess the whole potential of sugarcane bagasse for H_2 production, two fractions of acid-pretreated bagasse were evaluated as substrate: (1) the liquid hydrolysate (liq), which contained soluble sugars, and (2) the solid bagasse (sol) that remained after acid pretreatment.

Materials and Methods

Substrate

The sugarcane bagasse was obtained from a sugar and ethanol mill located near the city of Ribeirão Preto, state of São Paulo, Brazil. The sugarcane bagasse was washed with tap water to remove residual sugar (sucrose). Next, the sugarcane bagasse was dried at 60 °C until constant weight, milled in a knife mill (SL 31), sieved at 35 mesh or 0.417 mm, and homogenized in a single lot. The bagasse was then kept in desiccators until the pretreatment or fermentation tests. Hereafter, this bagasse will be referred to as *in natura* or dried bagasse.

Acid Pretreatment

The dried bagasse was pretreated at a 1:15 ratio for all the studied conditions. To this end, 3.33 g of dried bagasse and 50 mL of HCl solution were mixed in a 250-mL beaker; the temperature was controlled by a water bath. Table 1 describes the pretreatment conditions tested according to a 2^3 full factorial central composite design (star configuration). The temperature ranged from 63.2 to 96.8 °C; the treatment time ranged from 38.4 to 441.6 min. The acid concentration lay between 0.6 and 7.36 % (v/v), which corresponded to 3.9 g of HCl/100 g of TS and 47.6 g of HCl/100 g of TS, respectively, after correction of the HCl concentration (36.5 %) and density (1.18 g cm^{-3}). All the assays were carried out in triplicate.

Acid treatment was interrupted by cooling the mixture in an ice bath, which was followed by filtration through Whatmann filter paper under vacuum. The residual solid sugarcane bagasse retained in the filter paper was washed with distilled water; the solution was collected in the same volumetric flask as the hydrolysate. This procedure helped to standardize the final volume of the liquid hydrolysate (liq) for the chemical analyses (total residual sugars (TRS), total monosaccharides (TM), and total inhibitors (TI)), and fermentation assays. The filtrate was collected in a 100-mL volumetric flask. Before the flask volume was reached, the pH was adjusted to neutral with the aid of solid calcium hydroxide.

The solid sugarcane bagasse obtained after acid pretreatment was washed and dried at 60 °C until constant weight. The solid fraction (sol) was later used as substrate in fermentative assays for hydrogen production.

Table 1 Central composite design matrix showing the code and real values of the variables temperature (T), time (t), and acid concentration (A) as well as the responses for total monosaccharides (TM), total reducing sugars (TRS), and total inhibitors (TI) concentrations in g L^{-1} in the hydrolysates obtained from the pretreatment of sugarcane bagasse under the different conditions of the experimental design

Run	T (°C)	t (min)	A (%)	Glu	Xyl	Ara	TM	TRS	Ac	F	HMF	TI
1	70.0 (−1)	120.0 (−1)	2.0 (−1)	0.02	0.04	0.46	0.52	0.90	0.63	–	–	0.63
2	70.0 (−1)	120.0 (−1)	6.0 (+1)	0.05	2.62	0.51	3.18	5.81	0.89	–	–	0.89
3	70.0 (−1)	360.0 (+1)	2.0 (−1)	0.04	1.31	0.57	1.92	2.98	0.91	–	–	0.91
4	70.0 (−1)	360.0 (+1)	6.0 (+1)	0.20	4.93	0.72	5.85	9.57	1.00	0.008	–	1.01
5	90.0 (+1)	120.0 (−1)	2.0 (−1)	0.14	5.01	0.48	5.63	8.89	1.19	0.004	–	1.19
6	90.0 (+1)	120.0 (−1)	6.0 (+1)	0.65	5.55	0.66	6.86	9.76	0.94	0.006	–	0.95
7	90.0 (+1)	360.0 (+1)	2.0 (−1)	0.59	5.72	0.70	7.01	12.09	0.98	0.04	–	1.02
8	90.0 (+1)	360.0 (+1)	6.0 (+1)	1.22	6.07	0.71	8.00	13.88	0.97	0.26	0.0004	1.23
9	63.2 (−1.68)	240.0 (0)	4.0 (0)	0.04	0.73	0.56	1.33	8.22	0.90	–	–	0.90
10	96.8 (+1.68)	240.0 (0)	4.0 (0)	0.90	6.05	0.71	7.66	13.52	1.01	0.21	0.02	1.24
11	80.0 (0)	38.4 (−1,68)	4.0 (0)	0.04	0.72	0.84	1.60	7.29	0.81	–	–	0.81
12	80.0 (0)	441.6 (+1.68)	4.0 (0)	0.57	5.37	0.72	6.66	10.15	1.02	0.04	–	1.06
13	80.0 (0)	240.0 (0)	0.64 (−1.68)	0.03	0.43	0.55	1.01	5.46	0.78	–	–	0.78
14	80.0 (0)	240.0 (0)	7.36 (+1.68)	0.73	5.76	0.68	7.17	13.65	0.99	0.07	0.05	1.11
15	80.0 (0)	240.0 (0)	4.0 (0)	0.35	5.37	0.74	6.46	9.57	1.05	0.01	–	1.06
16	80.0 (0)	240.0 (0)	4.0 (0)	0.43	5.35	0.74	6.52	9.83	1.03	0.02	–	1.05
17	80.0 (0)	240.0 (0)	4.0 (0)	0.37	5.23	0.71	6.31	9.34	1.02	0.02	–	1.04

Glu glucose, *Xyl* xylose, *Ara* arabinose, *TM* Glu + Xyl + Ara concentrations, *Ac* acetic acid, *F* furfural, *HMF* hydroxymethylfurfural, *TI* Ac + F + HMF concentrations

Optimization of Sugarcane Bagasse Acid Pretreatment

Surface-response methodology (SRM) and multi-response analysis [16] helped to optimize the experimental conditions (temperature, time, and acid concentration) that led to the highest total reducing sugars (TRS) concentration possible. A 2^3 full factorial central composite design (star configuration) aided in this purpose (Table 1). SRM also enabled determination of the effect of temperature (T), time, and acid concentration on TRS.

The analysis of variance (ANOVA), the multiple comparison test, and all the statistical analyses were performed with the aid of the software Statistica 6.0. The data were fit to a second-order equation (Eq. 1) as a function of the independent variables.

$$Y_i = \beta o + \sum_{j=1}^{3} \beta_j X_j + \sum_{j=1}^{3} \beta_{jj} X_j^2 + \sum \sum_{j<k=2}^{3} \beta_{jk} X_j X_k$$

$$Y_i = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where β_n corresponds to constant regression coefficients; Y_i refers to the dependent variables (TRS, TM, and TI concentrations); and X_1, X_2, and X_3 are the coded independent variables temperature, time, and acid concentration, respectively.

After attainment of the surface-response results, multi-response analysis helped to optimize the process conditions [16]. This method transformed response variables (Yi) into an individual function of dimensionless desirability (gi) (Eq. 2) that ranged from 0 (undesirable response) to 1 (desired response). The geometric means of individual desires furnished the overall desirability function (G) (Eq. 3). The software Mathematic 5.0 helped to maximize G.

$$g_i = \frac{Y_i - Y_{\min}}{Y_{\max} - Y_{\min}}, \quad (2)$$

$$G = (g_1^{n_1} + g_1^{n_2} + \ldots\ldots + g_1^{n_k})^{1/K}, \quad (3)$$

where Y_{min} and Y_{max} are the response minimum and maximum values, respectively; k is the number of considered responses; and n is the weight of each response.

Finally, sugarcane bagasse liquid hydrolysates prepared in the optimal conditions helped to validate the optimized process conditions obtained by multi-response analysis. The validation experiments were performed in triplicate, and the resulting hydrolysates were characterized with respect to TRS, TM, and TI (the sum of acetic acid, furfural, and HMF concentrations) concentrations.

Fermentative Assays for Hydrogen Production

Inoculum and Culture Medium

The inoculum consisted of a mixed culture (sludge) collected from an upflow anaerobic sludge blanket (UASB) reactor used to treat the effluent from a sugar and ethanol mill near the city of Ribeirão Preto-SP, Brazil. The sludge was maintained in our laboratory by feeding with glucose (10 g L^{-1}, as carbon source) and nutrient solution. The latter solution, adapted from Gonzalez-Gil et al. [17], consisted of NH_4Cl (0.11 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.1 g L^{-1}), KH_2PO_4 (0.136 g L^{-1}), and Na_2HPO_4 (0.148 g L^{-1}) as macronutrients plus 1 mL L^{-1} of the trace elements $FeCl_2.4H_2O$ (2.0 mg L^{-1}), H_3BO_3 (50.0 mg L^{-1}), $ZnCl_2$ (50.0 mg L^{-1}), $CuCl_2.2H_2O$ (38.0 mg L^{-1}), $MnCl_2{\cdot}4H_2O$ (500.0 mg L^{-1}) $(NH_4)_6$-$Mo_7O_24{\cdot}4H_2O$ (50.0 mg L^{-1}), $AlCl_3{\cdot}6H_2O$ (90.0 mg L^{-1}), $CoCl_2{\cdot}6H_2O$ (2.0 mg L^{-1}), $NiCl_2{\cdot}6H_2O$ (142.0 mg L^{-1}), $Na_2SeO{\cdot}5H_2O$ (164.0 mg L^{-1}), EDTA (1.0 mg L^{-1}), and HCl 36 % (1.0 mg L^{-1}). All the chemicals were analytical grade.

Drying of the sludge at 105 °C for 12 h according to a procedure adapted from Buitrón and Carvajal [18] ensured its enrichment with H_2-producing bacteria. The volatile solids (VS) content of the dry sludge was analyzed before its use in fermentative assays.

Substrates

The following substrates were used for H_2 production during batch fermentative assays: 3.3 g of the bagasse *in natura* in 100 mL of distilled water (B); solution of glucose at the same concentration as the concentration of TRS in the optimal hydrolysate (ca. 20 g L^{-1}) (Glu); liquid hydrolysate (liq) from 3.3 g of sugarcane bagasse treated with acid in the optimal pretreatment conditions; 3.3 g of solid sugarcane bagasse that remained from the acid pretreatment in the optimal conditions (sol) (which corresponded to 4.62 g of dried sugarcane bagasse *in natura*) in 100 mL of distilled water; 3.3 g of sugarcane bagasse pretreated with acid in the optimal conditions (which corresponded to 4.62 g of dried bagasse without pretreatment) plus enzyme Celluclast ® 10 U g^{-1} in 100 mL of distilled water (sol + E); bagasse *in natura* plus the enzyme Celluclast ® 10 U g^{-1} in 100 mL of distilled water (B + E); and liquid hydrolysate from 3.3 g of sugarcane bagasse pretreated with acid in the optimal conditions detoxified with activated carbon (liq + C). All the experiments were supplemented with macronutrients plus 1 mL L^{-1} of the trace elements as described above in the culture medium composition.

The activated carbon 10 % (w/v) was added to the liquid hydrolysate (liq + C) under stirring at 200 rpm and room temperature. Stirring was conducted for 2 h, which was followed by filtration in Whatmann paper filter under vaccum to detoxify the liquid hydrolysate for use as substrate in the fermentative assays [19].

The Filter Paper (FP) activity of the enzyme was measured according to Ghose [20] before addition of the commercial enzyme Celluclast ® (Novozymes, USA) to the residual solid sugarcane bagasse pretreated with acid in the optimal conditions (sol). One enzyme unit (1 U) was defined as the amount of enzyme that released 1 μmol of TRS per min. The measured FP activity of Celluclast ® was 63.2 U mL^{-1}. Thus, 0.52 mL of the enzyme was added in the fermentation flasks containing 3.3 g of the substrate S, which gave 10 U of enzyme/g of acid-treated bagasse (sol).

Fermentative Batch Assays

H_2 production batch tests were carried out in triplicate, in 125-mL bioreactors containing 100 mL of one of the substrates (Glu, sol, sol + E, liq, or liq + C) and nutrient solution. The dried sludge (3.3 g) containing 45 % of VS was also added to the bioreactors. The initial pH of the assays was adjusted to 6.0 with NaOH 50 % (w/v), if necessary. Argon gas (0.1 m^3 min^{-1}) was bubbled through the bioreactor for 2 min, to ensure anaerobic conditions.

The bioreactors were operated at 35 °C and 100 rpm in a shaker (Dubnoff 304D, Nova Ética, Brasil). The shaker was coupled to pipes that led to a gas measurement system consisting of an inverted flask containing NaOH 5 % (w/v) solution and a flask. This arrangement helped to determine the volume that the produced gas displaced (Fig. 1). The volume and composition of the generated biogas were monitored during the tests. Gas chromatography revealed the composition of the gas, as will be described below.

Glucose, xylose, arabinose, acetic acid, furfural, HMF, and the soluble metabolites from fermentation (acetic acid, butyric acid, lactic acid, and ethanol) were quantified at the beginning and end of the fermentation assays.

Estimation of H_2 Production Kinetic Parameters

The modified Gompertz equation (Eq. 4) provided the kinetic data on biohydrogen production from the different substrates and control. The H_2 volume accumulated along the assay was introduced in the program Statistica 7 and modeled according to Eq. 4, to give the parameters R_m, H_{max}, and λ.

$$H = H_{\max}.\exp\left\{-\exp\left[\frac{R_m.e}{H_{\max}}(-t)+1\right]\right\} \quad (4)$$

where H = cumulative H_2 volume in tests; H_{max} = maximum potential from H_2 production (mL); R_m = Maximum H_2 production rate (mL h^{-1}); λ = lag phase or the time

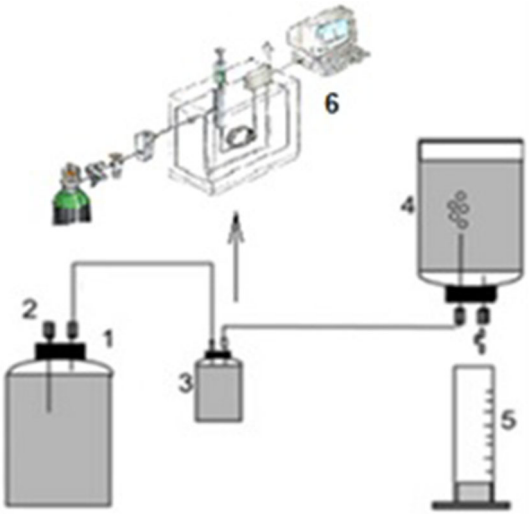

Fig. 1 Batch bioreactor for H_2 production and outline of the system employed for gas capture: *1* Bioreactor with tubing for the gas outlet, *2* Point of argon gas bubbling, *3* Security and gas sampling flask, *4* Inverted bottle containing 5 % NaOH, *5* Graduated bottle to collect the displaced NaOH volume, *6* Gas Chromatograph (adapted from Garcia-Morales et al. [21])

elapsed before H_2 production started (h), and t = duration of tests (h).

Analytical Methods

Analysis of cellulose, hemicellulose, and lignin contents in bagasse *in natura* (B) and in bagasse after acid pretreatment under optimized conditions (sol) was performed according to [22, 23] and to a modification from [24]. The hemicellulose content was obtained on the basis of the results achieved for holocellulose (cellulose + hemicellulose). All the analyses were accomplished in triplicate.

The VS concentration in the sludge was assayed according to the Standard Methods for Examination of Water and Wastewater [25].

With the aid of spectrophotometry, the TRS concentrations in the hydrolysate and at the beginning and at the end of fermentations were analyzed by the 3.5-dininitrosalycilyc acid (DNS) method described by Miller [26]. High performance liquid chromatography (HPLC) provided the concentration of glucose, xylose, and arabinose as well as the concentration of fermentation inhibitors such as acetic acid, furfural, and HMF in the hydrolysates. The concentration of the soluble metabolites acetic acid, butyric acid, lactic acid, and ethanol in the samples kept in the bioreactors were also analyzed by HPLC before the start and at the end of the kinetic assays. An Aminex HPX-87H column was used at 55 °C; the mobile phase was H_2SO_4 0.005 mol L^{-1} at a flow of 1.0 mL min^{-1}. All the samples were filtered through 0.45-μm acetate-cellulose membranes and analyzed according to the methodology described by Sá et al. [27]. The HPLC equipment was a Shimadzu (Japan) chromatograph. The Refraction Index Detector (RID) helped to quantify monosaccharides and ethanol. A Diode Array spectrophotometer operating at 280 nm helped to detect furfural, HMF, and the organic acids. The analytical column was Aminex HPX-87H; the mobile phase consisted of H_2SO_4 0.005 mol L^{-1} at a flow rate of 0.6 mL min^{-1} (84 Kgf cm^{-1}). All the reagents were purchased from Sigma-Aldrich, Germany. Data were acquired and processed with the aid of the software Class VP 6.1 (Shimadzu, Japan). The analyses were conducted in triplicate.

Gas chromatography (GC) allowed qualitative determination of the gases produced during fermentative assays; the detector temperature was 100 °C. Chromatographic analysis was carried out on a GC 35 gas chromatograph equipped with a thermal conductivity detector (TCD). The column consisted of 5-A molecular sieve measuring 2 m × 4.7 mm; the argon carrier gas flow was 30 mL min^{-1}. The temperatures of the injector, column, and detector were 80, 50, and 100 °C, respectively.

Results and Discussion

Optimization of Pretreatment

Table 1 lists the TRS, TM (sum of the concentrations of glucose, xylose, and arabinose), and TI (sum of the concentrations of acetic acid, HMF, and furfural) concentrations determined in the liquid hydrolysates obtained from the pretreatment of sugarcane bagasse under the different conditions of the experimental design.

Xylose and acetic acid were the main monosaccharide and inhibitor in the hydrolysates, respectively, which evidenced higher hemicellulose hydrolysis. Furfural, a product of pentose degradation [13, 28], emerged at low concentration, but it did not arise under the mildest experimental conditions (assays 1, 2, 3, 9, 11, and 13).

Glucose occurred at low concentrations (between 0.02 and 1.22 g L^{-1}) in the hydrolysates, which indicated poor cellulose hydrolysis in the experimental conditions. Because HMF originated from glucose dehydration, it also emerged at very low concentration. According to Hendriks and Zeeman [30], treatment of lignocellulosic materials with acid solubilizes hemicellulose, making cellulose more accessible for further enzymatic hydrolysis. Hence, the low concentration of glucose and its degradation derivative (HMF) in the hydrolysates does not mean that the cellulose structure remained intact.

According to the results displayed in Table 1, pretreatment with HCl at 6 %, at 90 °C, for 360 min (run 8) afforded the highest TRS concentration. Statistical data

analysis by SRM helped to fit these data to a second-order model. Table 2 depicts the analysis of variance (ANOVA) and the regression coefficients of the second-order polynomials for TM, TRS, and TI concentrations in the hydrolysates. ANOVA showed that only the models fit for TM and TRS were statistically significant ($p < 0.05$), because $F_{calculated}$ values > F_{listed}. These models were also predictive—their F ratio ($F_{calculated}/F_{listed}$) was higher than the corresponding F_{listed}. In these models, only the linear parameters of temperature, time, and acid concentration were statistically significant for TM and TRS ($p < 0.05$). The response-surface curves for TRS were generated for further fermentation (Fig. 2).

Figure 2a–c present the surface-response curves for TRS concentration as a function of temperature, time, and acid concentration. Temperature affected the TRS concentration in the hydrolysate more than time (Fig. 2a). The effect of time was only important at low temperatures, but it became less pronounced at intermediate temperatures. Temperatures and times ranging between 90 and 96.8 °C and between 320 and 441.6 min, respectively, gave a higher TRS.

Table 2 Regression coefficients and analysis of variance for TM, TRS, and TI concentrations in the hydrolysates obtained from the pretreatment of sugarcane bagasse under the different conditions of the experimental design

Coefficients	TM Y_1	TRS Y_2	TI Y_3
β_o	4.92*	8.88*	1.13*
Linear			
β_1	1.95*	2.51*	0.38*
β_2	1.10*	1.31*	0.095*
β_3	1.40*	2.04*	0.70*
Quadratic			
β_{11}	−0.51	0.07	0.095*
β_{22}	−0.64	−0.69	−0.30*
β_{33}	−0.66	−0.39	0.58*
Interactions			
β_{12}	−0.19	0.18	0.009
β_{13}	−0.55	−1.10	0.005
β_{23}	0.13	0.32	0.092*
R^2	0.85	0.81	0.55
$F^a_{calculated}$	25.40	18.15	1.57
F^b_{listed}	3.41	3.41	3.29
$F_{calculated}/F_{listed}$	7.44	5.32	0.48

* Significant at 5 % level

[a] Ratio between the mean square of regression and the mean square of residuals

[b] Tabulated for Fisher test using the significance level and degrees of freedom

According to Fig. 2b, temperatures between 63.2 and 75 °C and acid concentrations between 0.64 and 3 % afforded hydrolysates with the lowest TRS concentration. Higher temperatures and acid concentrations increased TRS in the hydrolysates. Temperatures between 90 and 96.8 °C and acid concentrations between 6 and 7.36 % produced hydrolysates with the highest TRS.

Both acid concentration and time (Fig. 2c) significantly influenced the TRS concentration. Acid concentrations ranging from 6 to 7.36 % and time from 360 to 441.6 min elicited the highest TRS.

The models calculated for TRS concentration (Table 2) furnished the desirability function (G). The minimum and maximum values of each response variable derived from the experimental results obtained in the experimental design (Table 1) enabled determination of the g_i function. The optimized conditions afforded hydrolysates with higher TRS. The process conditions that led to the maximum global desirability of the G function were 96.8 °C, 441.6 min, and acid at 7.36 %.

Table 3 compares the predicted and the experimental data obtained from triplicate assays that helped to validate the process conditions optimized by the multi-response analysis. The relative deviation values revealed that the predicted and experimental data correlated well.

In this work, the hydrolysate composition obtained under the optimized conditions was as follows: monosaccharides—xylose = 7.43 g L^{-1}, glucose = 3.75 g L^{-1}, and arabinose = 2.73 g L^{-1}; inhibitors—furfural = 0.08 g L^{-1}, HMF = 0.009 g L^{-1}, and acetic acid = 2.3 g L^{-1}. The TM concentration was similar to TM concentration values obtained for other bagasse hydrolysates submitted to treatment with H_2SO_4 at the same bagasse/acid solution ratio and final volume used herein. For example, Rai et al. [31] hydrolyzed sugarcane bagasse with H_2SO_4 for dark H_2 production integrated with H_2 production via photofermentation by pure cultures of *Enterobacter aerogenes* MTCC 2822 and *Rhodopseudomonas* BHU 01, respectively. Sugarcane bagasse pretreatment at 2 % H_2SO_4 (v/v) and at 121 °C, for 60 min, yielded a hydrolysate that contained glucose (3.41 g L^{-1}), xylose (8.36 g L^{-1}), and arabinose (0.55 g L^{-1}). However, authors observed lower inhibitors concentrations than ours—acetic acid and furfural concentrations were 1.49 and 0.19 g L^{-1}, respectively.

Table 4 presents the lignocelluloses composition of the bagasse before and after acid pretreatment under the optimized conditions.

Acid pretreatment diminished the hemicellulose content the most: from 26.92 to 1.31 %, which corresponded to total removal of 95 %. The cellulose content reduced by 22 %, from 46.7 to 36.35 %. The soluble lignin content decreased from 0.1 to 0.03 %, but the insoluble lignin

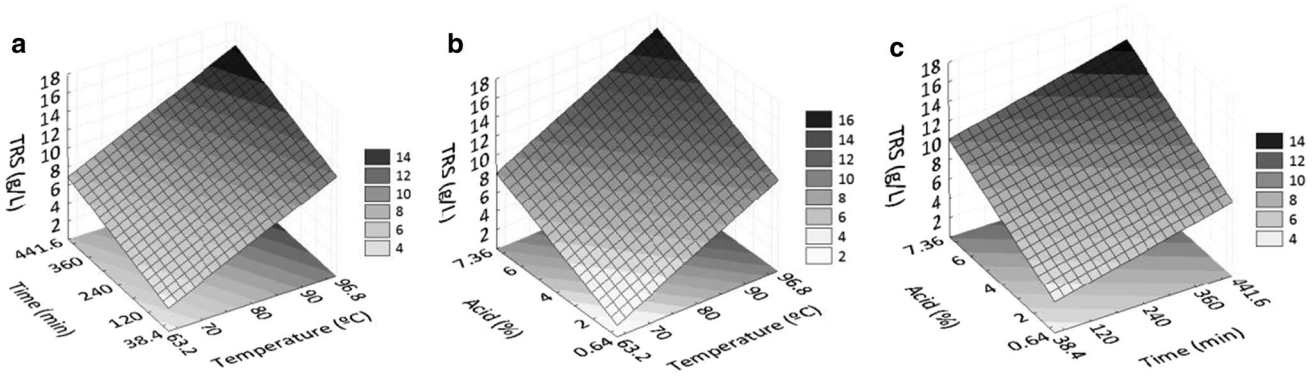

Fig. 2 Surface-response of TRS concentration as a function of **a** temperature versus time, **b** temperature versus acid concentration, and **c** time versus acid concentration

Table 3 Predicted and experimental responses of TRS and TM concentrations before and after acid pretreatment under the optimized conditions

Responses	Predicted	Experimental	RD (%)[b]
TRS	18.75	20.2	7.2
TM[a]	12.40	13.91	11.04

[a] TM = total monosaccharides content (glucose 3.75; xylose 7.43; arabinose 2.73 g L^{-1})

[b] Relative deviation (RD): [(experimental value—predicted value)/experimental value] × 100

content did not lower significantly. These results agreed with the data depicted in Table 1, which shows the highest concentration of xylose and arabinose in all hydrolysates. Glucose from cellulose appeared at much lower concentration than xylose (Table 1), and phenolic compounds did not arise in the hydrolysates, as corroborated by low lignin solubilization in the pretreatment conditions used here (Table 4). According to Monlau et al. [28], hemicelluloses are the most thermochemically sensitive of all the lignocellulosic components. During thermochemical pretreatment, the hemicelluloses side groups react first, followed by the hemicelluloses. Under neutral conditions, lignin normally starts to dissolve in water at around 180 °C. Therefore, dilute acid pretreatment appears to be a more promising process to hydrolyze hemicelluloses to sugars in high yields, and therefore to change the lignin structure and increase the cellulosic surface area. The changes promoted by acid pretreatment in the cellulose structure enhanced enzymatic cellulose hydrolysis and proved to be a very efficient method to saccharify lignocellulosic substrates [28].

H_2 Production Tests

All the literature studies on the acid hydrolysis and/or pretreatment of sugarcane bagasse for biohydrogen production have used sulfuric acid [3, 6, 7, 31]. However, when it comes to fermentative hydrogen production by a mixed culture, it is especially important to avoid introduction of sulfate and nitrate into the hydrolysates—sulfate- and nitrate-reducing bacteria can potentially consume H_2 from the medium, which could reduce the H_2 yield [15].

The next step after pretreatment of sugarcane bagasse with HCl under the optimized conditions (96.8 °C, 441.6 min, and acid at 7.36 %) was to conduct fermentative H_2 production assays using the sugarcane derived substrates (Fig. 3).

Bagasse *in natura* (B) and bagasse *in natura* added with Celluclast ® 10 U g^{-1} (B + E) did not produce H_2, probably because the H_2-producing microbial consortium, was not able to attack the lignocellulose of untreated even with

Table 4 Lignocellulosic content (cellulose, holocellulose, hemicellulose, and soluble and insoluble lignin) in bagasse *in natura* (B) and after acid pretreatment under optimized conditions (sol)

Bagasse	Cellulose (%)	Holocellulose (%)	Hemicellulose (%)	Soluble lignin (%)	Insoluble lignin (%)	Total lignin (%)
In natura (B)	46.70 ± 0.46[b]	73.62 ± 1.43[a]	26.92	0.10 ± 0.01[a]	20.65 ± 0.61[b]	20.75
After acid pretreatment (sol)	36.35 ± 1.15[a]	37.66 ± 0.29[b]	1.31	0.03 ± 0.002[b]	19.43 ± 0.47[b]	19.46

Holocellulose = Hemicellulose + Cellulose. The letters a and b in the same column represent a significant difference in the samples as calculated by Tukey Test ($p < 0.05$)

the aid of Celluclast®. All the researchers that have used Celluclast® to saccharify sugarcane bagasse employed a pretreatment method that made cellulose available; e.g., acid, alkaline, or steam explosion treatment [6, 7, 12, 32, 33]. For example, Martin et al. [32] achieved the highest convertibility of sugarcane bagasse, 74.9 %, when they used Celluclast® at 25 FPU/g of dry after pretreatment by wet oxidation at 195 C, for 15 min, in alkaline pH. Pretreatment was necessary even when an enzymatic cocktail containing components with different enzymatic activities was employed [33]. In the present work, addition of only Celluclast® at 10 U g^{-1} of bagasse was not enough to make bagasse *in natura* (B) fermentable.

Without treatment with activated carbon, the liquid hydrolysate (liq) was also not suitable for H_2 production, probably due to its inhibitors content.

Detoxification with activated carbon led the hydrolysate liq + C to produce a higher H_2 volume as compared with the control (Glu), which relied on glucose as substrate only. However, the time elapsed until the beginning of H_2 production was longer in the case of sol, sol + E, and liq + C as compared with glucose. For example, it took liq + C 73 h to begin H_2 production, whilst 23 h were necessary for glucose. This indicated that substrate adaptation by the microbial consortium was necessary (Fig. 3). In addition, the use of dried sludge enhanced the time elapsed until the beginning of H_2 production probably to allow the spores to return to their vegetative form.

Table 5 summarizes the modified Gompertz model kinetic parameters, yields, and productivities obtained on the basis of the results from the fermentative H_2 production assays. The control gave the highest maximum H_2 production rate (7.25 mL h^{-1}), followed by the detoxified hydrolysate liq + C (6.04 mL h^{-1}). However, liq + C afforded the largest H_2 volume (114.6 mL) and yield (0.72 mol of H_2/mol of consumed TRS) as compared with Glu (91.2 mL and 0.54 mol of H_2/mol of consumed TRS, respectively). The higher H_2 yield obtained with liq + C as compared with glucose (Table 5) was probably due to the sugar concentration. The TRS concentration in liq + C included disaccharides, which were probably used for H_2 production by the mixed culture, increasing H_2 yield.

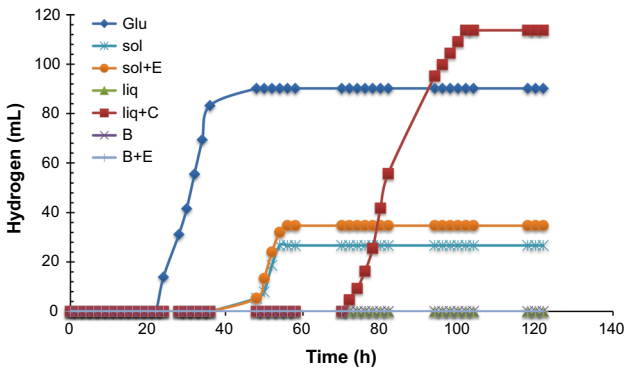

Fig. 3 Fermentative H_2 production from bagasse-derived substrates. Glu: glucose in the same initial TRS concentration as the optimal hydrolysate (20 g L^{-1}); liq: liquid hydrolysate obtained from pretreatment of the sugarcane bagasse in the optimized conditions; liq + C: liquid hydrolysate obtained from pretreatment of the sugarcane bagasse in the optimized conditions, after treatment with activated carbon; sol: solid bagasse that remained after pretreatment of the sugarcane bagasse in the optimized conditions; sol + E: solid bagasse that remained after pretreatment of the sugarcane bagasse in the optimized conditions plus the enzyme Celluclast ® 10 U g^{-1}. B: bagasse *in natura*; B + E: bagasse *in natura* + Celluclast ®

Detoxification of lignocellulosic hydrolysates with activated charcoal is a well-known cost-effective procedure that can absorb inhibitor compounds [34]. The hydrolysate that did not undergo treatment with activated carbon (liq) did not constitute a feasible substrate for H_2 production. Inhibitors emerged during acid pretreatment, even at the relative mild conditions applied in this work. Indeed, liquid hydrolysate (liq) obtained at the optimal conditions contained furfural, HMF, and acetic acid at 0.08, 0.009, and 2.3 g L^{-1}, respectively (Table 5). Quéméneur et al. [35] reported that addition of furfural at 1 g L^{-1} (a concentration that was much higher than the concentration of 0.08 g L^{-1} in the liquid hydrolysate) to xylose, as the main substrate, inhibited 76 % of maximum H_2 production by a mixed culture. As for acetic acid, it had lower inhibitory effect on H_2 production as compared with furfural. Wang et al. [36] verified that addition of acetic acid at 5 and 10 g L^{-1} (which are higher concentrations than the one verified herein, 2.3 g L^{-1}) inhibited maximum H_2 production by 29 and 64 %, respectively. In addition, lignocellulose degradation products, like phenolic compounds, are also known to be fermentation inhibitors [14], but they were not detected here.

The substrates sol and sol + E afforded similar H_2 production rates (5.6 and 5.9 mL of H_2/h, respectively), but addition of Celluclast ® to sol + E enhanced the volume of produced H_2 from 27.6 to 35.9 mL; i.e., a 23 % increase. The acid pretreatment of bagasse enhanced the ability of the enzyme to convert cellulose. Indeed, addition of Celluclast® to the untreated bagasse (B + E) had no effect on H_2 production. In other words, the enzyme helped to degrade the cellulose content of pretreated bagasse (36.35 %, Table 4) and to solubilize sugars, thereby raising the H_2 volume. Celluclast® acts mainly as endoglucanase, hydrolyzing the β-1,4 glycoside bonds of cellulose and releasing oligosaccharides, which are mostly non-fermentable. If a further enzyme like a β-glucosidase, which act mainly on cellobiose, had been used, the fermentable sugar concentration would enhance even more, and, probably, also H_2 volume. The combination of different enzymatic activities in enzyme blends or cocktails, with FPase, endoglucanase, xylanase,

Table 5 Modified Gompertz model parameters, yields, and productivity of fermentative H_2 production assays using glucose and sugarcane bagasse derivatives as substrates

Substrate	λ (h)	R_m (mL h^{-1})	H_{max} (mL)	$Y_{H2/GLU}$ (mol of H_2/mol of consumed TRS as glucose)	$Y_{H2/g}$ (mL of H_2/g of bagasse)	Productivity (mL of H_2 L^{-1} h^{-1}) or (mL of H_2 L^{-1} d^{-1})*
Glu	23.5 ± 0.27	7.25 ± 1.17	91.2 ± 7.88	0.54	–	72 or 1728*
Liq	0	0	0	0	–	
Liq + C	73.4 ± 0.14	6.04 ± 0.08	114.6 ± 2.6	0.72	45.3	60.4 or 1450*
Sol	48.4 ± 0.94	5.59 ± 0.58	27.6 ± 0.1	0.20	6.0	55.9 or 1342*
Sol + E	47.6 ± 0.26	5.93 ± 0.15	35.9 ± 2.4	0.33	7.83	59.3 or 1423*
(Liq + C) + (sol + E)				1.05	53.13	119.7 or 2873*

Glu: glucose in the same initial TRS concentration as the optimal hydrolysate (20 g L^{-1}); liq: liquid hydrolysate obtained from pretreatment of the sugarcane bagasse in the optimized conditions; liq + C: liquid hydrolysate obtained from pretreatment of the sugarcane bagasse in the optimized conditions treated with activated carbon; sol: solid bagasse that remained after pretreatment of the sugarcane bagasse in the optimized conditions; sol + E: solid bagasse that remained after pretreatment of the sugarcane bagasse in the optimized conditions plus enzyme Celluclast® 10 U g^{-1}. YH_2/Glu: mmol of H_2 produced per mmol of consumed TRS, as glucose equivalent (1 mmol = 180 mg). YH_2/g = mL of H_2 produced/g of bagasse (considering the amount of initial dried bagasse used in the pretreatment assays)

and β-glucosidase activities has been successfully employed to saccharify pretreated sugarcane bagasse for fermentations [33, 37].

Together, the substrates sol + E and liq + C afforded 53.13 mL of H_2/g of bagasse or 2.10 mmol of H_2/g of bagasse. The volumetric productivities found in the present work—1450 and 1423 mL of H_2 L^{-1} d^{-1} for liq + C and sol + E, respectively—are the highest ever reported in the literature for H_2 production from sugarcane bagasse by a microbial consortium.

Few literature works have studied sugarcane bagasse as substrate for H_2 production by microbial consortia. Chairattanamanokorn et al. [4] pretreated sugarcane bagasse with NaOH 0.1 mol L^{-1} at 100 °C, for 2 h, and added cellulolytic enzymes to improve hydrolysis and H_2 production from bagasse in the presence of a mixed culture. The addition of enzyme increased TRS from 0.25 to 0.79 g of reducing sugars/g of bagasse, which raised H_2 production from 1.4 to 13.39 mmol of H_2/g of Total Volatile Solids in the bagasse. Here, addition of Celluclast® to the solid substrate (sol) increased the volume of produced H_2 by ca. 25 % and raised the yield by 60 %, from 0.20 to 0.33 mol H_2/mol of consumed glucose.

Fangkum and Reunsang [6] worked on H_2 production from sugarcane bagasse pretreated with H_2SO_4, to achieve 0.84 mmol of H_2/mmol of consumed sugar when they used elephant dung as inoculum. Here, the yields were 0.20 and 0.72 mmol of H_2/mmol of consumed TRS as glucose for sol and liq + C, respectively (Table 5). Fangkum and Reunsang (2011) obtained 109.55 mL of H_2 L^{-1} d^{-1}, which was ca. 13 times lower as compared with our data for liq + C (1450 mL of H_2 L^{-1} d^{-1}, Table 5). This difference probably resulted from the type of acid applied to pretreat and detoxify the liquid hydrolysate (liq) with activated carbon. Overall, our data pointed out that the pretreatment of sugarcane bagasse with HCl enabled application of whole bagasse; that is, the liquid and the solid fractions, as substrate for H_2 production. It is noteworthy that the high yields obtained herein were only possible after detoxification of the liquid hydrolysate.

Authors who applied pure cultures to produce H_2 from bagasse reported similar or even higher volumetric rates than ours. For example, Pattra et al. [3] produced 1611 mL of H_2 L^{-1} d^{-1} when they used a pure culture of *Clostridium butyricum* as inoculum. Lai et al. [7] achieved the highest volumetric rates for H_2 production ever described in the literature—0.52 L of H_2 L^{-1} h^{-1}—when they employed *Thermoanaerobacterium aotearoense* SCUT27/Δldh as pure culture. However, for practical reasons, studies that apply mixed cultures are more realistic than those that use pure cultures. When it is desirable to produce a fuel such as H_2 continually and in huge amounts, the sterile conditions required by pure cultures would be impracticable.

Table 6 presents the concentrations of TRS, monosaccharides, inhibitors, and the main fermentation soluble metabolites including acetate, butyrate, lactate, and ethanol, at the beginning and at the end of the fermentative assays. According to Table 6, 56.8 % of the initial bagasse (3.3 g/100 mL) was converted to TRS, as detected in the liquid fraction (18.75 g L^{-1}), and 20.33 % remained in the solid fraction (6.71 g L^{-1}). The enzymatic treatment of the solid fraction (sol) helped to solubilize ca. 5 % more TRS (from 6.71 to 7.70 g L^{-1}).

In terms of total mL of H_2/g of bagasse (Table 6) obtained from the liquid and solid fractions of bagasse, ca. 85 % of the H_2 volume originated from liq + C and 15 % from sol + E. In addition, 2.42 and 1.0 mL of H_2/g of initial TRS concentration originated from liq + C and sol + E, respectively.

During the fermentation assays conducted with the bagasse-derived substrates, the mixed culture consumed the main monosaccharides detected in the bagasse hydrolysate (glucose, xylose, and arabinose). The fermentation assays that used liquid hydrolysate (liq) as substrate consumed TRS and monosaccharides, but this uptake did not result in H_2 formation (Fig. 2; Table 6) or in the metabolites acetate and butyrate. TRS were converted mainly into lactate (Table 6). The liquid hydrolysate treated with activated carbon (liq + C) contained reduced TRS and monosaccharides concentrations, mainly xylose and arabinose, at the beginning of the assays as compared with the untreated liquid hydrolisate (liq). This fact could be due to the treatment of liq with activated carbon. Xylose from hemicellulose was the main monosaccharide detected in the solid fraction of bagasse (sol) as well as in sol + E. This was probably because the acid pretreatment acted mainly on the hemicellulose, and washing of the pretreated bagasse was not enough to remove all the solubilized xylose. The presence of a pentose degradation product in the sol fraction, acetic acid, corroborated this.

The furfural and HMF inhibitors emerged at the beginning of the assays in liq, sol, and sol + E. At the end of the assays, the concentrations of these inhibitors were recovered in liq. However, in sol and sol + E, the concentrations of the inhibitors were lower than the detection limit of the method (10^{-3} g L^{-1}). Treatment of liq with activated carbon removed the inhibitors (furfural and HMF), as verified by their initial concentration in the liq + C assay. This removal was probably the reason why it was possible to use liq + C as substrate (Fig. 2). Treatment of the liquid hydrolysate with activated carbon may also have removed other fermentation inhibitors that went undetected here.

Acetate was the metabolite from fermentative H_2 production that furnished the highest H_2 yield from glucose—consumption of 1 mol of glucose formed 2 mols of acetate, and 4 mols of hydrogen [15]. However, acetic acid could also be an inhibitor or even a substrate for H_2 production [36–39]. During hydrolysis of lignocellulosic materials, acetic acid originates from hydrolysis of acetyl groups in hemicellulose and, to some extent, lignin [28]. In the undissociated form, acetic acid can penetrate the cell membrane and inhibit product formation, disrupting the pH balance at high concentration, inhibiting cell growth, or even killing cells [29, 37]. However, some strains, mainly those belonging to *Clostridia* species, can use acetic acid and other organic acids as substrate to produce H_2 [38]. In all the assays that produced H_2 (Glu, liq + C, sol, and sol + E), the acetate concentration rose. Compared with the mmol of H_2 produced (Table 5) in the assays Glu, liq + C, sol, sol + E, the acetate concentration (Table 6) represented 12, 3, 8.5, and 7.8 % of the maximum theoretical H_2 yield based on the stoichiometric ratio (2 mols of acetate for 4 mols of H_2).

On the other hand, the initial acetate concentration in the liquid hydrolysate (liq) diminished, and no H_2 evolved from this substrate. Most likely, microorganisms present in the mixed culture were able to use acetate. In a literature review, Monlau et al. [39] described that inhibition of biohydrogen production in the presence of lignocellulosic byproducts does not imply that bacterial activity is inexistent, because carbohydrates can undergo degradation through non-hydrogen-producing pathways such as lactate, ethanol, and propionate pathways. In addition, the presence of inhibitors during dark fermentation could shift the metabolism from H_2-producing pathways (i.e., acetate and butyrate) to non-H_2-producing pathways (i.e., lactate, ethanol, and propionate). Indeed, an increase in lactate and ethanol occurred when liquid hydrolisate (liq) was the substrate (Table 6).

The presence of ethanol and lactate did not impact the H_2 balance, because these metabolites originated from consumption of an additional electron from NADH. Low amounts of ethanol emerged in all cases, except for liq + C.

Butyrate is also a metabolite related to the fermentative H_2 production, because the consumption of 1 mol of glucose furnishes 1 mol of butyrate and 2 mols of hydrogen [15]. Butyrate emerged in all assays with H_2 production, but this emergence was more pronounced in the assays that afforded the highest H_2 production (Glu and liq + C).

Compared with the mmol of H_2 produced (Table 5) in the assays Glu, liq + C, sol, sol + E, the butyrate concentration (Table 6) represented 63.5, 75.4, 72.1, and 75.6 %, respectively, of the maximum theoretical H_2 yield based on the stoichiometric ratio (1 mols of butyrate for 2 mols of H_2). These results showed that H_2 production by this mixed culture using either glucose or other substrates preferentially followed the butyrate pathway. Use of the liquid hydrolysate and the sugarcane derived substrates enhanced this effect. Fangkum and Reusang [6] studied the effects of sugarcane bagasse hydrolysate as substrate for H_2 production, and they also detected butyrate as the main soluble product. These authors did not mention the presence of lactic acid during fermentation, but they attributed the low H_2 yield to the existence of *Sporolactobacillus* sp., acid lactic bacteria, in the microbial community. Although in the present work lactate emerged as a metabolite, it is not possible to affirm that it originated from liquid hydrolysate fermentation, because it also appeared as the main metabolite in the assay that used glucose as substrate.

Conclusions

The surface-response methodology used to optimize temperature, time, and HCl concentration conditions to pretreat sugarcane bagasse revealed that temperature, time, and

Table 6 Concentration of TRS, monosaccharides, inhibitors, and soluble fermentation metabolites at the beginning and at the end of the fermentation assays that used the sugarcane bagasse derivatives as substrate

Concentration (g L^{-1})	Substrate in the fermentative assay									
	Glu		Liq		Liq + C		Sol		Sol + E	
	I	F	I	F	I	F	I	F	I	F
Saccharide										
TRS	20.81 ± 1.93	8.91 ± 0.66	18.75 ± 2.35	15.8 ± 1.96	16.82 ± 2.08	5.6 ± 0.87	6.71 ± 2.17	–	7.7 ± 2.51	–
Glucose	19.93 ± 1.94	8.87 ± 0.25	3.75 ± 0.8	0.78 ± 0.07	3.26 ± 0.03	0.67 ± 0.02	0.64 ± 0.09	0.02	0.57 ± 0.01	0.02
Xylose	–	–	7.43 ± 0.73	–	5.98 ± 0.41	0.64 ± 0.08	1.49 ± 0.03	–	1.185 ± 0.05	0.30 ± 0.04
Arabinose	–	–	2.73 ± 0.80	1.4 ± 0.70	0.88 ± 0.02	0.012 ± 0.01	–	–	–	–
Inhibitor										
5-HMF	–	–	0.009	0.009	–	–	0.002	–	0.002	–
Furfural	–	–	0.083 ± 0.006	0.067 ± 0.007	–	–	0.007	–	0.008	–
Metabolite										
Acetate	–	0.013 ± 0.01	1.072 ± 0.06	0.67 ± 0.03	0.032 ± 0.002	0.074 ± 0.004	0.003	0.006	0.003	0.073 ± 0.01
Butyrate	–	0.131 ± 0.22	–	–	–	0.151 ± 0.04	–	0.034 ± 0.03	–	0.062 ± 0.02
Lactate	–	0.002	0.011 ± 0.005	0.065 ± 0.004	–	0.092 ± 0.03	–	0.001	–	–
Ethanol	–	0.002	0.012 ± 0.006	0.045 ± 0.002	–	–	–	0.005	–	0.003

I: initial concentration, F: final concentration. Concentrations without value or deviation means that they are less than 10^{-3} (except for TRS analysis). Glu: glucose in the same initial TRS concentration as the optimal hydrolysate (20 g L^{-1}); liq: liquid hydrolysate obtained from pretreatment of the sugarcane bagasse in the optimized conditions; liq + C: liquid hydrolysate obtained from pretreatment of the sugarcane bagasse in the optimized conditions treated with activated carbon; sol: solid bagasse that remained after pretreatment of the sugarcane bagasse in the optimized conditions; sol + E: solid bagasse that remained after pretreatment of the sugarcane bagasse in the optimized conditions plus enzyme Celluclast® 10 U g^{-1}

acid concentration affected the TRS and total monosaccharides (TM) concentrations, but these variables did not impact the TI concentration significantly. The conditions 96.8 °C, 7.36 % HCl, and 441.6 min afforded the highest TRS in the hydrolysates. However, the hydrolysate only became a suitable substrate to produce H_2 after detoxification with activated carbon, and the H_2 yield was higher as compared with a control containing glucose only. Pretreatment with HCl also made the remaining solid bagasse suitable for H_2 production. Subsequent enzymatic hydrolysis of this substrate increased the volume of H_2 produced from the initial bagasse even further. The appropriate pretreatment of sugarcane bagasse with HCl can transform the whole bagasse into a renewable substrate for H_2 production.

Acknowledgments The authors wish to thank 'Programa de Aperfeiçoamento de Pessoal de Nível Superior' (CAPES) as well as "Fundação de Amaro à Pesquisa do Estado de São Paulo" (FAPESP) for financial support.

References

1. Instituto Brasileiro de Geografia e Estatística - IBGE. Anuário Estatístico do Brasil, v.73, ISSN 0100-1299 (2013)
2. Hofsetz, K., Silva, M.A.: Brazilian sugarcane bagasse: energy and non-energyconsumption. Biomass Bioenergy **46**, 564–573 (2012)
3. Pattra, S., Sangyoka, S., Boonmee, M., Reungsang, A.: Bio-hydrogen production from the fermentation of sugarcane bagasse hydrolysate by *Clostridium butyricum*. Int. J. Hydrog. Energy **33**, 5256–5265 (2008)
4. Chairattanamanokorn, P., Penthamkeerati, P., Reungsang, A., Lo, Y.-C., Lu, W.B., Chang, J.S.: Production of biohydrogen from hydrolyzed bagasse with thermally preheated sludge. Int. J. Hydrog. Energy **34**, 7612–7617 (2009)
5. Cheng, C.L., Chang, J.S.: Hydrolysis of lignocellulosic feedstock by novel cellulases originating from *Pseudomonas* sp. CL3 for fermentative hydrogen production. Bioresour. Technol. **102**, 8628–8634 (2011)
6. Fangkum, A., Reungsang, A.: Biohydrogen production from sugarcane bagasse hydrolysate by elephant dung: effects of initial pH and substrate concentration. Int. J. Hydrog. Energy **36**, 8687–8696 (2011)
7. Lai, Z., Zhu, M., Yang, X., Wang, J., Li, S.: Optimization of key factors affecting hydrogen production from sugarcane bagasse by a thermophilic anaerobic pure culture. Biotechnol. Biofuels **7**, 119–130 (2014)
8. Ntaikou, I., Antonopoulou, G., Lyberatos, G.: Biohydrogen production from biomass and wastes via dark fermentation: a review. Waste Biomass Valor. **1**, 21–39 (2010)
9. Rezende, C.A., de Lima, M.A., Maziero, P., de Azevedo, E.R., Garcia, W., Polikarpov, I.: Chemical and morphological characterization of sugarcane bagasse submitted to a delignification process for enhanced enzymatic digestibility. Biotechnol. Biofuels **4**, 1–18 (2011)
10. Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J.: Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour. Technol. **101**, 4851–4861 (2010)
11. Monlau, F., Barakat, A., Trably, E., Dumas, C., Steyer, J.-P., Carrere, H.: Lignocellulosic materials into biohydrogen and biomethane: impact of structural features and pretreatment. Crit. Rev. Environ. Sci. Technol. **43**, 260–322 (2013)
12. Saripan, A.F., Reungsang, A.: Biohydrogen production by *Thermoanaerobacterium thermosaccharolyticum* KKU-ED1: culture conditions optimization using xylan as the substrate. Int. J. Hydrog. Energy **38**, 6167–6173 (2013)
13. Lavarack, B.P., Griffin, G.J., Rodman, D.: The acid hydrolysis of sugarcane bagasse hemicellulose to produce xylose, arabinose, glucose and other products. Biomass Bioenergy **23**, 367–380 (2002)
14. Jonsson, L., Alriksson, B.: Nilvebrant N–O bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol. Biofuels **6**, 16–22 (2013)
15. Valdez-Vazquez, I., Poggi-Varaldo, H.M.: Hydrogen production by fermentative consortia. Renew. Sustain. Energy Rev. **13**, 1000–1013 (2009)
16. Derringer, G., Suich, R.: Simultaneous optimization of several response variables. J. Qual. Technol. **12**, 214–219 (1980)
17. Gonzalez-Gil, G., Kleerebezem, R., Lettinga, G.: Assessment of metabolic properties and kinetic parameters of methanogenic sludge by on-line methane production rate measurements. Appl. Microbiol. Biotechnol. **58**, 248–254 (2002)
18. Buitrón, G., Carvajal, C.: Biohydrogen production from *Tequila vinasses* in an anaerobic sequencing batch reactor: effect of initial substrate concentration, temperature and hydraulic retention time. Bioresour. Technol. **101**, 9071–9077 (2010)
19. Hargraeves, P.I., Barcelos, C.A., Costa, A.C.A., Pereira Jr, N.: Production of ethanol 3G from *Kappaphycus alvarezii*: evaluation of different process strategies. Bioresour. Technol. **134**, 257–263 (2013)
20. Ghose, T.K.: Measurement of cellulase activities. Pure Appl. Chem. **59**, 257–268 (1987)
21. García-Morales, J.L., Nebot, E., Romero, L.I., Sales, D.: Comparison between acidogenic and methanogenic inhibition caused by liner alkylbenzzene-sulfonate (LAS). Chem. Biochem. Eng. Q. **15**, 13–19 (2001)
22. Sun, J.: Isolation and characterization of cellulose from sugarcane bagasse. Polym. Degrad Stabil. **84**, 331–339 (2004)
23. TAPPI. TAPPI Standard. Method T19 om-54. TAPPI test methods (1991)
24. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. Determination of structural carbohydrates and lignin in biomass. Technical Report. NREL/TP-510-42618 (2012)
25. APHA, AWWA, WEF. Standard methods for the examination of water and wastewater. 19th. edn. *American Public Health Association*. Washington, DC (1995)
26. Miller, G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. **30**, 785–793 (1959)
27. Sá, L.R.V., de Oliveira, M.A.L., Cammarota, M.C., Matos, A., Ferreira-Leitão, V.S.: Simultaneous analysis of carboydrates and volatile fatty acids by HPLC for monitoring fermentative biohydrogen production. Int. J. Hydrog. Energy **36**, 7543–7549 (2011)
28. Monlau, F., Barakat, A., Trably, E., Dumas, C., Steyer, J.P., Carrère, H.: Lignocellulosic materials into biohydrogen and biomethane: impact of structural features and pretreatment. Crit. Rev. Environ. Sci. Technol. **43**, 260–322 (2013)
29. Klinke, H.B., Thomsen, A.B., Ahring, B.K.: Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl. Microbiol. Biotechnol. **66**, 10–26 (2004)
30. Hendriks, A.T.W.M., Zeeman, G.: Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. **100**, 10–18 (2009)

31. Rai, P.K., Singh, S.P., Asthana, R.K., Singh, S.: Biohydrogen production from sugarcane bagasse by integrating dark- and photo-fermentation. Bioresour. Technol. **152**, 140–146 (2014)
32. Martin, C., Klinke, H., Thomsen, A.B.: Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzyme Microbial. Technol. **40**, 426–432 (2007)
33. Visser, E.M., Leal, T.F., Almeida, M.N., Guimarães, V.M.: Increased enzymatic hydrolysis of sugarcane bagasse from enzyme recycling. Biotechnol. Biofuels **8**, 5 (2015)
34. Canilha, L., Carvalho, W., Felipe, M.G.A., Silva, J.B.A.: Xylitol production from wheat straw hemicellulosic hydrolysate: hydrolysate detoxification and carbon source used for inoculum preparation. Brazilian J. Microbiol. **39**, 333–336 (2008)
35. Quermeneur, M., Hamelin, J., Barakat, A., Steyer, J.P., Carrere, H., Trably, E.: Inhibition of fermentative hydrogen production by lignocellulose-derived compounds in mixed cultures. Int. J. Hydrog. Energy **37**, 3150–3159 (2012)
36. Wang, Y., Zhao, Q.B., Yang, M., Yu, H.Q., Harad, H., Li, Y.Y.: Biohydrogen production with mixed anaerobic cultures in the presence of high-concentration acetate. Int. J. Hydrog. Energy **33**, 1164–1171 (2008)
37. Hernández-Salas, J.M., Villa-Ramírez, M.S., Veloz-Rendón, J.S., Rivera-Hernández, K.N., González-César, R.A., Plascencia-Espinosa, M.A., Trejo-Estrada, S.R.: Comparative hydrolysis and fermentation of sugarcane and agave bagasse. Bioresour. Technol. **100**, 1238–1245 (2009)
38. Matsumoto, M., Nishimura, Y.: Hydrogen production by fermentation using acetic acid and lactic acid. J. Biosci. Bioeng. **103**, 236–241 (2007)
39. Monlau, F., Sambusiti, C., Barakat, A., Quéméneur, M., Trably, E., Steyer, J.-P., Carrère, H.: Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review. Biotechnol. Adv. **32**, 934–951 (2014)