Specific inhibition of biohydrogen-producing Clostridium sp after dilute-acid pretreatment of sunflower stalks

Florian Monlau, Quentin Aemig, Eric Trably, Jérôme J. Hamelin, Jean-Philippe Steyer, Hélène Carrère

Specific inhibition of biohydrogen-producing *Clostridium* sp. after dilute-acid pretreatment of sunflower stalks

MONLAU, Florian, AEMIG, Quentin, TRABLY*, Eric, HAMELIN, Jérôme, STEYER, Jean-Philippe, CARRERE, Hélène

INRA, UR050, Laboratoire de Biotechnologie de l'Environnement, Avenue des Etangs, 11100 Narbonne, France

* corresponding author : Eric Trably, INRA, UR050, Laboratoire de Biotechnologie de l'Environnement, Avenue des Etangs, Narbonne, F-11100, France.
Tel: +33 (0)4 68 42 51 72
Fax: +33 (0)4 68 42 51 60
E-mail: eric.trably@supagro.inra.fr

Abstract

Dilute-acid pretreatments are commonly used to solubilise holocelluloses of lignocellulosic materials and represent a promising route to enhance biohydrogen production by dark fermentation. Besides the soluble sugars released, furan derivatives, such as furfural and 5-HMF, as well as phenolic compounds can accumulate in dilute-acid hydrolyzates and that may affect fermentative microbial populations. In this study, biohydrogen production from glucose (5 g VS L^{-1}) in batch tests was investigated in presence of increasing volumes (0% - control, 3.75%, 7.5%, 15% and 35% (v/v)) of dilute acid hydrolyzate generated from sunflower stalks (170°C, 1h, 4 g HCl/ 100gTS). A sharp decrease of the hydrogen yield was observed from 2.04 mol H_2 $mol^{-1}_{\text{eq. hexose initial}}$ in the control to 0 mol H_2 $mol^{-1}_{\text{eq. hexose initial}}$ for volumes higher than 15% of added hydrolyzate. Although acetate and butyrate were the main end-products found in the control, ethanol and lactate accumulated accordingly with the increasing addition of hydrolyzate. A clear shift of dominant microbial populations from *Clostridium* sp. to *Sporolactobacillus* sp. was concomitantly observed, suggesting a specific inhibition of the biohydrogen-producing bacteria by adding increasing volumes of hydrolyzates.

Keywords

Agricultural residues; Biohydrogen; Dark fermentation; Furans; Microbial communities; Phenolic compounds.

1. Introduction

Dark fermentation of lignocellulosic residues by mixed cultures is a very promising biological process for producing biologically and efficiently hydrogen, so-called biohydrogen (bioH_2). Over the past ten years, many studies have focused on biohydrogen production from lignocellulosic materials which constitute realistic and sustainable sources of energy when considering both their high abundance and their low cost [1,2]. Among agricultural residues, sunflower stalks have currently few suitable end-uses since they are mostly burnt in fields which cause environmental pollution. Thus, they could be widely used for biohydrogen production. Similarly to bioethanol-producing bioprocesses, pretreatments of agricultural residues before dark fermentation are recommended to increase the total amount of fermentable sugars [3,4,5]. Dilute-acid pretreatments are particularly efficient to convert holocelluloses to sugars monomers that are further converted by H_2-producing bacteria [6, 7]. To date, using dilute-acid pretreatments of lignocellulosic residues prior to fermentative biohydrogen production has been investigated on rice straw [3], cornstalk [4], grass [8], poplar leaves [9] barley straw [10] and sugarcane bagasse [11]. As an illustration, Cui et *al.* (2010) observed a maximal hydrogen production of 33.45 mL H_2 $g^{-1}_{\text{dry poplar}}$ after dilute acid pretreatment of poplar leaves in 4 % HCl (w/v) boiling 30 min, which was 2.22 fold higher than from raw substrate [9]. Similarly, a maximum cumulative hydrogen yield of 72.21 ml H_2 $g^{-1}_{\text{dry grass}}$ was achieved by boiling grass (30 min) with 4% HCl (w/v), which was 16.45 fold higher than from untreated substrate [8]. In addition, a theoretical hydrogen yield of 163.2 mL H_2 $g^{-1}_{\text{dry matter}}$ was predicted, using surface response methodology, by applying dilute-acid pretreatment (4.8% HCl (w/v)) at 93°C for 23 min on *Laminaria japonica* [12]. However, a decrease of hydrogen production was shown under more drastic pretreatments conditions (12% HCl (w/v) at 160°C for 22.5 min) with only 9.5 mL H_2 $g^{-1}_{\text{dry matter}}$ and clearly an inverse relationship was found between the hydrogen potentials and the concentration of 5-Hydroxylmethylfurfural (5-HMF), a by-product formed during the pretreatment process [12].

Under extreme conditions, dilute-acid pretreatments are well known to generate on their hydrolyzates several unwanted by-products such as furan derivatives (furfural and 5-Hydroxylmethylfurfural) and phenolic compounds that can further reduce overall microbial activity [13]. The effect of lignocellulose-derived inhibitors has been extensively investigated in the case of ethanol production [14, 15] and at a

lower extent for methane production [16,17]. Delgenès et *al.* (1986) found that glucose-fermenting yeast *Saccharomyces cerevisiae* and xylose-fermenting yeasts *Candida shehatae* and *Pichia stipites*, were almost completely inhibited by furfural, HMF, syringaldehyde and vanillin at high concentrations (2–5 g/L) whereas glucose-fermenting yeast *Zymomonas mobilis* was found more resistant [15]. More recently, Barakat et *al.* (2011) showed that a separated addition of these by-products (furfural, 5-HMF) at a concentration of 1g L^{-1} did not affect methane production from xylose and surprisingly furfural and 5-HMF were also degraded into methane [16]. To date, only very few studies have investigated the effects of lignocellulose-derived inhibitors on biohydrogen production using pure cultures [18] or mixed cultures [19]. Cao et *al.* (2009) studied the effect of inhibitors generated by acid-pretreatment (121°C, 90 min, 2% (v/v) H_2SO_4) of corn stover on a pure culture of *Thermoanaerobacterium thermosaccharolyticum W16* [18]. The authors showed that hydrogen production decreased when inhibitor concentration was between 2 and 3 times higher than in original acid hydrolyzate. Biohydrogen production was totally inhibited when inhibitors were 4 times more concentrated [18]. Quéméneur et *al*. (2011) showed a negative impact on lag phase and hydrogen yield in mixed cultures operated with xylose as substrate when furans (furfural and 5-HMF) and phenolics compounds were added individually at a concentration of 1 g L^{-1} [19]. It was also observed that hydrogen yields were more specifically impacted by furan derivatives than phenolic compounds. Hydrogen yields of 0.4, 0.51 and 1.28 mol H_2 mol^{-1} xylose were observed for medium supplemented by 5-HMF, furfural and phenols, respectively, in comparison with a control operated with no addition of these products, *i.e.* 1.67 mol H_2 mol^{-1} xylose [19].

The aim of this work was to investigate the impact of added increasing volumes (3.75%, 7.5%, 15% and 35% (v/v)) of dilute-acid hydrolyzate (170°C, 1h, 4 g HCl/ 100gTS) of sunflower stalks on biohydrogen production using glucose (5 g L^{-1}) as carbon source regarding performances, metabolites pathways and microbial communities changes.

2. Materials and Methods

2.1 Preparation of the sunflower stalks hydrolyzate:

Sunflower stalks (Serin variety, collected in Lacq, south of France in 2010) were used as raw substrate. The stalks were crushed and milled to particle size of 2-3 mm using a SM-100 cutting mill. Sunflower stalks were then dried overnight at 45°C and stored at room temperature. Dilute-acid pretreatment of sunflower stalks was performed in a Zipperclave autoclave series 02-0378-1 (Autoclave ®, France). This stainless autoclave had a capacity of 1L with a maximal temperature of 250°C and maximal pressure of 79 bars. The substrate with a solid loading of 35 g L^{-1} was heated with a ceramic furnace and mixed with pales and two propellers at a mixing speed of 300 rpm. Dilute-acid pretreatment was performed at 170°C for 1 h with an acid concentration of 4g HCl / 100 gTS. After pretreatment, the hydrolyzate was separated from the solid fraction by filtration through a sieve of 0.25 mm pore size. The hydrolyzate was kept at 4°C for further characterisation of soluble sugars, metabolites and byproducts generated but also for biohydrogen potentials.

2.2 Chemical composition

Total solids (TS) and volatile solids (VS) were determined according to the APHA standard methods [20]. Carbohydrates (glucose, xylose and arabinose) as well as uronic acids, i.e. galacturonic and glucuronic acids were quantified in triplicates using a strong acid hydrolysis protocol adapted from Effland (1977) [21]. For this, 200 mg of solid samples were hydrolyzed with 12 M H_2SO_4 acid for 2 h at room temperature, and then diluted to reach a final acid concentration of 1.5 M and kept at 100°C for 3 h. Acid-insoluble lignin was measured by weighing the residues after drying at 105°C overnight. Then, carbohydrates and uronic acids were quantified by HPLC analysis coupled to refractometric detection (Waters R410). The components were separated in an Aminex HPX-87H column (Biorad). The elution phase corresponded to a solution of 0.005 M H_2SO_4 under a flow rate of 0.3 mL min^{-1}. The column temperature was maintained at 50°C. Since cellulose is a polymer of glucose and hemicelluloses with branched chains of shorter complex sugars composed of pentose units, such as xylose and arabinose, cellulose and hemicellulose contents were consequently determined as follows:

Cellulose (% VS) = Glucose (%VS) / cF (Equation 1)

Hemicelluloses (% TS) = [Xylose (%VS) + Arabinose (%VS)] / cF (Equation 2)

where the conversion factor (cF) was equal to 1.11 for the conversion of glucose-based polymers to monomeric units of glucose, and 1.13 for the conversion of xylose-based polymers to monomeric units of arabinose and xylose, according to Petersson et *al.* (2007) [22]. The main characteristics and composition of the substrates used in the experiments are presented in Table 1

[TABLE 1]

2.3 Quantification of sugar monomers, by-products and metabolic products

Volatile fatty acid (VFA) composition of the liquid phase, *i.e*. acetic (C2), propionic (C3), butyric and iso-butyric (C4 and iC4), valeric and iso-valeric (C5 and iC5) and caproic (C6) acids, was determined using a gas chromatograph (GC-3900, Varian) equipped with a flame ionization detector (FID). Concentrations of non-VFA metabolic end-products (lactate and ethanol), residual sugar monomers (glucose and xylose) and other hydrolyzate by-products (furfural and 5-hydroxylmethylfurfural) were measured by High Performance Liquid Chromatography (HPLC) coupled to refractometric detection (Waters R410). The components were separated with an Aminex HPX-87H column (Biorad). The eluting solution corresponded to 0.005 M H_2SO_4, and the flow rate was 0.4 mL min^{-1}. The column temperature was maintained at 35°C. Total phenols in the liquid fraction of the pretreated samples were determined using microtube tests (Spectroquant®, Merck) followed by a colorimetric measurement method at 500 nm.

2.4 Biohydrogen production in batch tests

Hydrogen production experiments were operated in batch mode over 30 days and were carried out in 500 mL plasma bottles with a working volume of 200 mL containing 100 mM of 2-(N-morpholino)ethanesulfonic acid (MES) buffer. An anaerobically digested sludge was used as inoculum and heat shock treated (90°C, 15 min) to inhibit the hydrogen consumers. One millilitre of inoculum (50 gVS L^{-1}) was added to the culture medium (ratio of substrate (g VS L^{-1}) / Inoculum (g VS L^{-1}) around 20). In each flask, 5 g VS L^{-1} of glucose were supplemented with increasing volumes (0%, 3.75%, 7.5%, 15% and 35% (v/v)) of hydrolyzate generated by dilute-acid pretreatment of the sunflower stalks (170°C, 1h, 4 g HCl/ 100gTS). The initial pH was adjusted to 5.5 using NaOH (1 M) and before

neutralization the pH ranged from 4 to 5 for all the samples due to the addition of the MES buffer. Duplicate bottles were incubated at 35°C. At start of the experiment, strict anaerobic conditions were reached by degasifying the headspace with nitrogen gas. The bottles were then sealed with a septum-type butyl rubber stopper. Once the maximum cumulative hydrogen production was reached, the liquid phase (2 mL) was sampled and stored at 4°C for metabolite analysis. Biogas volume was periodically measured using an acidified water displacement method. Biogas composition (CH_4, CO_2, H_2 and N_2) was analysed using a gas chromatograph (Clarus 280, Perkin Elmer) equipped with a column HayeSep Q, and a molecular sieve (5 Å), coupled to a thermal conductivity detector (TCD) The operating conditions were as follows: the carrier gas was argon under a pressure of 102 kPa and a flow rate of 4.5 mL min^{-1}; temperatures of the injector and the detector were both fixed at 150°C.

To assess accurately the hydrogen production kinetic parameters, the cumulative H_2 production (H) data was fitted to the following modified Gompertz equation (Equation 3):

—— (Equation 3)

where P is the maximum cumulative H_2 production (mol H_2 $mol^{-1}_{\text{eq. hexose initial}}$), Rm is the maximum H_2 production rate (mol H_2 $mol^{-1}_{\text{eq. hexose initial}}$ day^{-1}), λ is the lag-phase time (days), t is the incubation time (days) and e is exp (1). The cumulative H_2 production was expressed in mol H_2 $mol^{-1}_{\text{eq. hexose consumed}}$ by considering the variations of the working volume due to gas and liquid samplings. The values of P, Rm and λ were estimated using a non-linear regression algorithm developed with Matlab software (version 6.5, MathWorks).

2.5 DNA extraction, PCR amplification and CE-SSCP fingerprinting

Two milliliters of culture were collected at the end of the batch experiments. Microbial cells were collected by centrifugation (12,100 g - 15 min). Genomic DNA was extracted and purified using the Wizard Genomic DNA Purification kit (Promega) according to manufacturer recommendations. DNA quantity and purity in the extracts were measured by spectrophotometry (Infinite NanoQuant M200, Tecan). The 16S rRNA genes were amplified as detailed elsewhere by Quéméneur et *al*. (2011) for further fingerprinting analysis by CE-SSCP [23]. Normalization and comparison of the fingerprinting

profiles were performed using the StatFingerprint library in R software (R Development Core Team 2009) [24].

2.6 Characterization of microbial communities by pyrosequencing

One genomic DNA, representative of the CE-SSCP fingerprinting profiles, was selected for each experimental condition. The identification of the dominant bacterial populations was performed by pyrosequencing of the V4-V5 regions of the 16S rRNA gene (Molecular Research Laboratory, TX, USA). An average of 3841 sequences per sample was obtained, and sequence data derived from the sequencing process was processed using a proprietary analytical pipeline (Molecular Research Laboratory, TX, USA) [25]. Sequences were clustered at 3% of divergence and then taxonomically classified using BLASTn using a curated GreenGenes database.

3. Results and Discussion

3.1 Effect of dilute-acid pretreatment (170°C, 1h, 4 g HCl/ 100gTS) on sugar solubilisation and generation of secondary by-products.

In a preliminary batch test operated with untreated sunflower stalks, a low hydrogen yield of 2.3 ± 0.9 mL H_2 gVS^{-1} was obtained (data not shown). Similar low hydrogen yields from raw lignocellulosic materials were already reported in the literature, *e.g.* 3.16 ml H_2 gVS^{-1} for cornstalk [26] and 1 ml H_2 gVS^{-1} for wheat straw [27]. Surprisingly, by applying dilute-acid pretreatment (170°C, 1h, 4 g HCl/ 100gTS) on sunflower stalks, no hydrogen production was observed after ten days of fermentation (data not shown) whereas such conditions was found effective in increasing the methane potentials in an earlier study [28]. These results suggest that probably inhibitory compounds were released in the hydrolyzate after dilute-acid pretreatment of the sunflower stalks and affected the biohydrogen performances and biohydrogen producing bacteria.

Consequently, to understand this brutal inhibition of hydrogen production after dilute-acid pretreatment of sunflower stalks, the composition (ie soluble sugars and main by-products degradation) in the slurry was determined in a first time (Table 2). First, the dilute-acid pretreatment condition (170°C, 1h, 4 g HCl/ 100gTS) was highly efficient to hydrolyze hemicellulosic material, since about 3.14 g L^{-1} of xylose was

detected in the slurry. In contrast, low amounts of glucose were found, with only 0.28 g L^{-1}, confirming that the dilute acid pretreatments were not efficient to hydrolyse the cellulosic components. These observations are in agreement with Monlau et *al.* (2012) who reported a high hemicellulose solubilisation of 94.4% on sunflower stalks pretreated with the same dilute acid pretreatment condition (170°C, 1h, 4 g HCl/ 100gTS) [28]. Besides the high amount of xylose released in the hydrolyzate, other secondary by-products were generated (Table 2), *i.e.* carboxylic acids such as formate (0.6 g L^{-1}) and acetate (0.81 g L^{-1}), furans derivatives such as furfural (1.15 g L^{-1}) and 5-HMF (0.13 g L^{-1}), and phenolic compounds (20.2 mg L^{-1}).

[TABLE 2]

Basically, furfurals and 5-HMF are formed during pentose and hexose thermal degradation, respectively, whereas phenolic compounds are generated by thermal partial breakdown of lignin [22]. When furfural and 5-HMF are further broken down, formic acid can be produced, whereas acetic acid is generated by hydrolysis of the acetyl groups of hemicellulosic compounds [7, 13, 14]. At a concentration about 1 g L^{-1}, carboxylic acids such as acetate have not been reported to inhibit significantly the growth of clostridial species, which are the main hydrogen-producing bacteria found in dark fermentation processes. In contrast, furan derivatives and phenolic compounds can affect significantly the hydrogen-producing pathways [19, 20]. The release of these by-products was previously reported in similar studies using dilute-acid pretreatments [10, 28]. The nature and concentration of the by-products (*i.e.* furans, phenolic compounds...) released in the hydrolyzate varied from one study to another due to the nature of the lignocellulosic residues, but also the kind of pretreatments and the operational conditions (*i.e.* residence time, temperature, pressure, chemical concentrations) [29, 30]. A ratio of conversion efficiency adapted from Fangkum and Reungsang (2011) was calculated taking into account the sum of soluble sugars (ie glucose and xylose) concentrations divided by furfural concentration. Furfural has been considered because it is the main by-product released during dilute-acid pretreatment of lignocellulosic residues and it is also the most investigated in literature data [11]. Table 3 shows the values of conversion efficiency obtained in this study and makes it possible to compare it with some other relevant literature data applying thermal or thermal dilute-acid pretreatments. Interestingly, our value of 2.97 was lower than other ratios generally observed in the literature suggesting that in our case a large part of pentose sugars released was converted into furfural.

However, such a ratio has to be considered only as a rough indicator of conversion efficiency as many other by-products can be released in the slurry additionally to furfural. Indeed, Du et *al.* (2009) reported forty potentials by-products degradation of lignocellulosic substrates [30].

[TABLE 3]

3.2 Effect of dilute-acid hydrolyzate concentration on hydrogen performances

The impact of a gradual increase of added volumes of dilute acid hydrolyzate (0%, 3.75%, 7.5%, 15% and 35% (v/v)) was evaluated with glucose as carbon source (5g VS L^{-1}) to determine the level volume of hydrolyzate that inhibited biohydrogen production. Throughout all the batch experiments, no CH_4 was detected in the gas phase, indicating that methanogenic activity was efficiently suppressed after heat-shock treatment of the microbial inoculum. The results of cumulative hydrogen production are presented in Figure 1 and expressed in mL H_2 g^{-1} $_{eq.\ hexose\ consumed}$. At start of the experiment, the concentration of equivalent hexose was assessed by considering not only the glucose added initially but also the amount of equivalent hexose present in the hydrolyzate.

[FIGURE 1]

The kinetic parameters of the cumulative H_2 production curves were determined by fitting the data to a modified Gompertz model (Table 4). All correlation coefficient (R^2) values were higher than 0.95, indicating that the fitted curves matched well with the experimental data. In the control, *i.e.* with no hydrolyzate added, a maximal H_2 production of 2.04 (±0.14) mol H_2 mol^{-1} $_{eq.hexose\ initial}$ and a maximum hydrogen production rate of 0.32 (± 0.22) mol H_2 mol^{-1} $_{eq.\ hexose\ initial}$ day^{-1} were observed. These results are consistent with hydrogen yields usually reported in the literature, when measured with mesophilic fermentative mixed cultures as inoculum and glucose as carbon source [19]. When 3.75% (v/v) of hydrolyzate was added to the culture medium, neither the hydrogen production yield, the lag phase nor the maximum H_2 production rate were significantly impacted. Only the time to reach the maximal cumulated hydrogen production was slightly longer with 24 days instead of 16 days. By increasing the added volume of hydrolyzate (7.5% v/v) and, consequently, the concentration of secondary by-products,

the hydrogen fermentation performances decreased substantially with a lowering of the H_2 yields to 0.24 (± 0.05) mol H_2 mol^{-1} $_{eq.hexose\ initial}$. In this assay, the corresponding by-product concentrations were only 61 mg L^{-1} of acetate, 45 mg L^{-1} of formate, 86.2 mg L^{-1} of furfural, 9.5 mg L^{-1} of 5-HMF and 1.5 mg L^{-1} of phenolic compounds. After addition of more than 15% (v/v) of hydrolyzate, no more hydrogen production was observed even after 30 days of fermentation. It was concluded that the hydrogen production pathways were highly sensitive to a slight increase of dilute acid hydrolyzate volume in the bacth reactors.

[TABLE 4]

Kongjan et *al.* (2009) noticed similar results on thermophilic mixed cultures by investigating the biohydrogen production from wheat straw hydrolyzate after hydrothermal pretreatment [34]. The hydrogen yields decreased from 317 to 148 mL H_2 g^{-1} sugars when the hydrolyzate concentration increased from 5% to 25% (v/v) and no hydrogen was produced in presence of hydrolyzate concentration of 30% (v/v). Even if their results were highly similar to ours, it is complicated to compare them because the microbial communities involved in thermophilic conditions are not the same as in mesophilic conditions.

However, our results are in disagreement with some other literature data that investigated the impact of dilute acid pretreatment on biohydrogen production using mixed cultures and did not report inhibition [8, 9, 32]. Unfortunately, such studies did not report the concentration of the main by-products released during pretreatments process and thus it is difficult to compare to our results.

In addition, Fangkum and Reungsang (2011) have shown that dilute-acid pretreatment of sugarcane bagasse with H_2SO_4 concentration from 0.25% to 3% (v/v), led to an increased production of biohydrogen using mixed cultures of elephant dung in mesophilic conditions, despite the release of furfural during pretreatment [11]. However, the cumulative biohydrogen production decreased with an increase of the H_2SO_4 concentration from 4% to 5% (v/v). Table 5 shows the comparison of our results with some other literature data that reported both the concentration of by-products released during thermal and dilute-acid pretreatment and the impact on biohydrogen production using mesophilic mixed cultures. Fangkum and Reungsang (2011) have shown a similar trend of reduction of biohydrogen potential with similar concentrations of furfural inside the batch hydrogen fermentation reactor. Moreover, under similar

conditions as our study, Quéméneur et *al*. (2012) showed that furfural (1g L^{-1}) added alone to a control of xylose (5g VS L^{-1}) had a negative impact on hydrogen production using similar anaerobic mixed cultures but did not cause total inhibition [19]. In our study, total hydrogen inhibition was noticed for a concentration of furfural of 172 mg L^{-1} which was significantly lower than the value of 1g L^{-1} tested by Quéméneur et *al*. (2012), suggesting therefore a possible synergistic effect between furfural and other by-products released in the hydrolyzate [19]. Such synergistic effect of by-products (furfural, 5-HMF, phenolic compounds) was previously observed on bioethanol production and methane production from lignocellulosic hydrolyzates [17, 29].

If the content of by-products and their nature seems to be important parameters on biohydrogen fermentation, other factors should be considered in future works as the nature of inoculum used, the ratio of inoculum/by-products but also the possible synergistic impact of the by-products released.

[TABLE 5]

3.3 Effect of dilute-acid hydrolyzate concentration on metabolites routes

In order to understand the decrease of biohydrogen performances by adding gradual volumes of hydrolyzate, the metabolite patterns of each different conditions were determined. A mass balance in COD (Chemical Oxygen Demand) equivalents was calculated to check whether all metabolites were identified (Table 3). According to mass balance assumptions, the sum of the organic matter added at start of the experiment should be equal to the organic matter at the end. Carbon dioxide is not considered by the COD mass balance as it is already in the most oxidized form and hence is inorganic (COD value of CO_2 is null). Thus, this mass balance was carried out by converting the substrate such as added sugars and the products such as remaining sugars, metabolites and hydrogen gas, in terms of equivalent COD. The COD mass balance was completed at more or less 15% of the initial COD, without considering the biomass growth, which confirmed that all main microbial metabolites were identified and quantified.

Considering this, Figure 2 shows the distribution of soluble metabolites expressed in mol $mol^{-1}_{\text{eq. hexose consumed}}$ when the cumulative H_2 production (H) was maximal. In the control batch test operated with glucose as sole carbon source (5 g L^{-1}) and no hydrolyzate, high levels of butyrate and acetate were observed with 0.68 mol $_{\text{butyrate}}$ $mol^{-1}_{\text{eq. hexose consumed}}$ and 0.54 mol $_{\text{acetate}}$ $mol^{-1}_{\text{eq. hexose consumed}}$. This result suggested that hydrogen was

mainly produced from the acetate-butyrate fermentation pathways. In the culture supplemented with 3.75 % (v/v) of hydrolyzate, the main metabolites detected were butyrate, acetate as well as ethanol with 0.49 mol $_{butyrate}$ mol^{-1} $_{eq.\ hexose\ consumed}$, 0.32 mol $_{acetate}$ mol^{-1} $_{eq.\ hexose\ consumed}$ and 0.30 mol $_{ethanol}$ mol^{-1} $_{eq.\ hexose\ consumed}$, respectively. In the culture supplemented with 7.5 % (v/v) of hydrolyzate, the decrease of 83% of the hydrogen yield was consistent with lower contents in butyrate and acetate, and higher concentrations in ethanol (0.67 mol $_{ethanol}$ mol^{-1} $_{eq.\ hexose\ consumed}$) and lactate (0.78 mol $_{lactate}$ mol^{-1} $_{eq.\ hexose\ consumed}$). For volumes of hydrolyzate higher than 15 % (v/v), only ethanol and lactate were produced with a predominance of ethanol production with 1.53 and 1.72 mol $_{ethanol}$ mol^{-1} $_{eq.\ hexose\ consumed}$ for 15 % (v/v) and 35 % (v/v) of hydrolyzate added, respectively. Interestingly, the decrease of H_2 production occurring for volumes of hydrolyzate higher than 7.5 % (v/v) was therefore concomitant with the accumulation of lactate and ethanol. Ethanol and lactate are known as metabolites generated in zero-hydrogen balance pathways which is consistent with the absence of hydrogen production in these cases. Similarly, Kongjan et *al*. (2009) observed an increase of the proportion of lactate and ethanol produced during thermophilic dark fermentation by increasing the volume of hydrolyzate after thermal pretreatment of wheat straw [34].

[FIGURE 2]

3.4 Effect of dilute-acid hydrolyzate concentration on microbial community changes

Biohydrogen inhibition and metabolic shift observed could have been due to either (i) the development of H_2 consumers or metabolic competitors such as lactic acid bacteria and enterobacteria, or (ii) a metabolic shift resulting from stressful conditions for hydrogen-producing bacteria. In order to decipher what population shifts occurred; microbial communities present at the end of fermentation were identified and are presented in Table 6.

First, in the control experiment supplemented with glucose as sole carbon source and no hydrolyzate, the bacterial community was dominated by the *Clostridium* genus with the highest proportion of 90% and especially by *Clostridium tyrobutyricum* species that accounted for 70% of the sequences retrieved. This observation is in accordance with the literature where clostridia are the main dominant hydrogen-producing bacteria using rich carbohydrates substrates [19, 35, 36]. Rafrafi et *al*. (2013) reported that the abundance of bacteria belonging to the *Clostridium* genus ranged from 79 to 97% during the fermentation of glucose using

various kinds of mixed cultures as seed inoculum, *i.e.* anaerobic sludge, cassava, caecotrophs [36]. Similar results were observed by Fang et *al*. (2002) who showed that 64.4% of the clones were affiliated to clostridial species in granular sludge reactor fed with glucose as substrate, and with 43.8% of the clones being related to *Clostridium cellulosi*, 12.5% to *Clostridium acetobutylicum* and 8.3% to *Clostridium tyrobutyricum* [35].

For the culture supplemented with 3.75% of hydrolyzate, the *Clostridium* genus still remain the most dominant genus (79%) but dominant species shifted to *Clostridium saccharobutylicum* and *Clostridium pasteurianum* with 38% and 20% of the total abundance, respectively. The *Sporolactobacillus* genus was also observed at a proportion of 14%. When the culture was supplemented by 7.5% a decrease of the abundance of *Clostridium* genus was shown and a significant development of lactic acid bacteria of *Sporolactobacillus* genus was observed with a proportion of 55% and 40% of total abundance, respectively. Main species observed in this case were *Clostridium saccharobutylicum* and *Sporolactobacillus racemilacticus*. Such observations are in agreement with the decrease of hydrogen production concomitant to lactate production when the culture was supplemented with 7.5% of hydrolyzate (Figure 2). In addition, Noike et *al*. (2002) reported the specific reduction of hydrogen production in presence of lactic acid bacteria. They observed the replacement of hydrogen by lactic acid production when two hydrogen-producing strains *Clostridium acetobutylicum* and *Clostridium butyricum* where cultivated with two hydrogen consuming bacteria, ie *Lactobacillus paracasei* and *Enterococcus durans.* Such observations were explained by the secretion of bacteriocins by lactic acid bacteria that further inhibit biohydrogen production [37].

Furthermore, the culture supplemented with 15% and 35% of hydrolyzate were only dominated by *Sporolactobacillus* genus with a proportion of 75% and 69% of the sequences retrieved, respectively. Interestingly, the richness estimation ranging from 88 to 99 [38] remains stable along the different assays despite the wide gradient of hydrolyzate applied (Table 6). Thus, the shift in bacterial species that occurred could not be attributed to a loss of microbial diversity. Moreover, this bacterial shift from *Clostridium* to *Sporolactobacillus* species was concomitant to the strong inhibition of hydrogen production and the metabolic shift form acetate/butyrate to lactate/ethanol. However, in contrast with the predominance of *Sporolactobacillus* genus, the main metabolite was ethanol and not lactate. One explanation of the high production of ethanol for cultures supplemented by 15% and 35% can be the presence of solventogenesis of

the *Clostridium* genus when bacteriocins are generated by *Sporolactobacillus* genus. Indeed, other lactic acid bacteria of *Lactobacillus* spp. can decrease H_2 production by secreting bacteriocins in the culture media which can lead to stressfull conditions for clostridial cells, with a subsequent switch in their metabolic pathway from H_2 production to solvent production such as ethanol [37, 39].

Even though no H_2 was produced after addition of high amounts of hydrolyzate, microbial activity did not stopped and glucose was still consumed and microbial metabolites (ie lactate and ethanol) were generated. As dark fermentation is an intermediate stage of the anaerobic digestion process, these metabolites can be further used to produce methane whatever their distribution. This is in agreement with Barakat et *al*., (2011) who did not observe any inhibition of methane production from xylose in presence of similar by-products (furans derivatives and phenolics compounds) [16]. This suggests that alternative pathways to acetate or butyrate could be utilized by anaerobic bacteria to convert lignocellulosic residues in anaerobic digestion in presence of such specific hydrogen-producing pathways inhibitors.

[TABLE 6]

4. Conclusions:

After dilute acid pretreatment of lignocellulosic residues (170°C, 1h, 4 g HCl/ 100gTS), and besides the expected release of soluble sugars like glucose (0.28 g L^{-1}) and xylose (3.14 g L^{-1}), secondary by-products such as acetate (0.6 g L^{-1}), formate (0.81 g L^{-1}), furfural (1.15 g L^{-1}), 5-HMF (0.13 g L^{-1}) and phenolic compounds (20.2 mg L^{-1}) were generated. In this study, a strong and significant inhibition of biohydrogen fermentation was observed by adding increasing volume of dilute-acid hydrolyzate. For a low concentration of hydrolyzate added of 7.5% (v/v), a substantial decrease of hydrogen production was observed, suggesting a specific effect of the lignocellulosic degradation by-products released in the slurry on H_2-producing bacteria. Ethanol and lactate which are involved in zero-hydrogen balance pathways were mainly produced and were resulting from a population shift of *Clostridium* genus to *Sporolactobacillus* genus. The results suggested the generation of stress-full conditions for the remaining *Clostridium* hydrogen-producing bacteria that shift in solventogenesis in presence of lactic acid bacteria.

Acknowledgements

The authors are grateful to ADEME, the French Environment and Energy Management Agency, for financial support in the form of F. Monlau's PhD grant. The authors wish to thank Dr Diana Garcia-Bernet for her technical support.

References

[1] Guo, X.M., Trably, E., Latrille, E., Carrère, H., Steyer, J.P. Hydrogen production from agricultural waste by dark fermentation: A review. International Journal of Hydrogen Energy 2010, 35: 10660-10673.

[2] Cheng, C.L., Lo, Y.C., Lee, K.S., Lee, D.J., Lin, C.Y., Chang, J.S. Biohydrogen production from lignocellulosic feedstock. Bioresource Technology 2011, 102: 8514-8523.

[3] Chang, A.C.C., Tu, Y.H., Huang, M.H., Lay, C.H., Lin, C.Y. Hydrogen production by the anaerobic fermentation from acid hydrolyzed rice straw hydrolyzate. International Journal of Hydrogen Energy 2006, 36: 14280-14288.

[4] Pan, C.M., Ma, H.C., Fan, Y.T., Hou, H.W. Bioaugmented cellulosic hydrogen production from cornstalk by integrating dilute acid-enzyme hydrolysis and dark fermentation. International Journal of Hydrogen Energy 2011, 36: 4852-4862.

[5] Monlau, F., Sambusiti, C., Barakat, A., Guo, X.M., Latrille, E., Trably, E., Steyer, J.P., Carrere, H. Predictive models of biohydrogen and biomethane production based on the compositional and structural features of lignocellulosic materials. Environmental Science and Technology 2012, 46: 12217-12225.

[6] 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. Critical Reviews in Environmental Science and Technology 2013, 43(3): 260-322.

[7] Panagiotopoulos, I.A., Bakker, R.R., de Vrije, T., Koukios, E.G. Effect of pretreatment severity on the conversion of barley straw to fermentable substrates and the release of inhibitory compounds. Bioresource Technology 2011, 102: 11204-11211.

[8] Cui, M.J., Shen, J.Q. Effects of acid and alkaline pretreatments on the biohydrogen production from grass by anaerobic dark fermentation. International Journal of Hydrogen Energy 2011, 37: 1120-1124.

[9] Cui, M., Yuan, Z., Zhi, X., Wei, L.,Shen, J. Biohydrogen production from poplar leaves pretreated by different methods using anaerobic mixed bacteria. International Journal of Hydrogen Energy 2010, 35: 4041-4047.

[10] Panagiotopoulos, I.A., Bakker, R.R., Budde, M.A.W., de Vrije, T., Claassen, P.A.M., Koukios, E.G. Fermentative hydrogen production from pretreated biomass: A comparative study. Bioresource Technology 2009, 100: 6331-6338.

[11] Fangkum, A., Reungsang, A. Biohydrogen production from sugarcane bagasse hydrolysate by elephant dung: Effects of initial pH and substrate concentration. International Journal of Hydrogen Energy 2011, 36: 8687-8696.

[12] Jung, K.W., Kim, D.H., Kim, H.W., Shin, H.S. Optimization of combined (acid plus thermal) pretreatment for fermentative hydrogen production from Laminaria japonica using response surface methodology (RSM). International Journal of Hydrogen Energy 2011, 36: 9626-9631.

[13] Palmqvist, E., Hahn-Hagerdal, B. Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. Bioresource Technology 2000, 74: 25-33.

[14] Larsson, S., Palmqvist, E., Hahn-Hagerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., Nilvebrant, N.O. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme and Microbial Technology 1999, 24: 151-159.

[15] Delgenes, J.P., Moletta, R., Navarro, J.M. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichiastipitis, and Candida shehatae. Enzyme and Microbial Technology 1996, 19: 220-225.

[16] Barakat, A., Monlau, F., Steyer, J.P., Carrère, H. Effect of lignin-derived and furan compounds found in lignocellulosic hydrolysates on biomethane production. Bioresource Technology 2012, 104: 90-99.

[17] Badshah, M. Evaluation of process parameters and treatments of different raw materials for biogas production. Thesis, University of Lunds, 2012.

[18] Cao, G.L., Ren, N.Q., Wang, A.J., Guo, W.Q., Xu, J.F., Liu, B.F. Effect of lignocellulose-derived inhibitors on growth and hydrogen production by Thermoanaerobacterium thermosaccharolyticum W16. International Journal of Hydrogen Energy 2009, 35: 13475-13480.

[19] Quéméneur, M., Hamelin, J., Barakat, A., Steyer, J.-P., Carrere, H., Trably, E. Inhibition of fermentative hydrogen production by lignocellulose-derived compounds in mixed cultures. International Journal of Hydrogen Energy 2012, 37: 3150-3159.

[20] A.P.H.A. Standard Methods for the Examination of Water and Wastewater, 20th ed. 1998.

[21] Effland, M.J. Modified Procedure to Determine Acid-Insoluble Lignin in Wood and Pulp. Tappi 1977, 60: 143-144.

[22] Petersson, A., Thomsen, M.H., Hauggaard-Nielsen, H.,Thomsen, A.B. Potential bioethanol and biogas production using lignocellulosic biomass from winter rye, oilseed rape and faba bean. Biomass & Bioenergy 2007, 31: 812-819.

[23] Quéméneur, M., Hamelin, J., Latrille, E., Steyer, J.P., Trably, E. Functional versus phylogenetic fingerprint analyses for monitoring hydrogen-producing bacterial populations in dark fermentation cultures. International Journal of Hydrogen Energy 2011, 36: 3870-3879.

[24] Michelland, R.J., Dejean, S., Combes, S., Fortun-Lamothe, L., Cauquil, L. StatFingerprints: a friendly graphical interface program for processing and analysis of microbial fingerprint profiles. Molecular Ecology Resources 2009, 9(5):1359-1363.

[25] R Development Core Team 2009. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

[26] Zhang, M.L., Fan, Y.T., Xing, Y., Pan, C.M., Zhang, G.S., Lay, J.J. Enhanced biohydrogen production from cornstalk wastes with acidification pretreatment by mixed anaerobic cultures. Biomass & Bioenergy 2007, 31: 250-254.

[27] Fan, Y.T., Zhang, Y.H., Zhang, S.F., Hou, H.W.,Ren, B.Z. Efficient conversion of wheat straw wastes into biohydrogen gas by cow dung compost. Bioresource Technology 2005, 97: 500-505.

[28] Monlau, F., Barakat, A., Steyer, J.P., Carrère, H. Comparison of seven types of thermo-chemical pretreatments on the structural features and anaerobic digestion of sunflower stalks. Bioresource Technology 2012, 120: 241-247.

[29] Mussatto, S.I., Roberto, I.C. Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. Bioresource Technology 2004, 93(1): 1-10. .

[30] Du, B., Sharma, L.N., Becker, C., Chen, S.F., Mowery, R.A., Van Walsum, G.P., Chambliss, C.K. Effect of Varying Feedstock–Pretreatment Chemistry Combinations on the Formation and Accumulation of Potentially Inhibitory Degradation Products in Biomass Hydrolysates. Biotechnology and Bioengineering 2010, 107(3).

[31] Panagiotopoulos, I., Bakker, R., de Vrije, T., Van Niel, E., Koukios, E., Claassen, P.A.M. Exploring critical factors for fermentative hydogen production from various types of lignocellulosic biomass. Journal of the Japan institute of energy 2011, 90: 363-368.

[32] Kaparaju, P., Serrano, M., Thomsen, A.B., Kongjan, P. and Angelidaki, I. Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresource Technology 2009, 100(9): 2562-2568.

[33] Chong, P.S., Jahim, J.M., Harun, S., Lin, S.S., Mutalib, S.A., Hasson, O., Mohd Nor, M.T. Enhancement of batch biohydrogen production from prehydrolysate of acid treated oil palm empty fruit bunch. International Journal of Hydrogen Energy 2013, In press.

[34] Ruiz, E., Cara, C., Manzanares, P., Ballesteros, M., Castro, E. Evaluation of steam explosion pre-treatment for enzymatic hydrolysis of sunflower stalks. Enzyme and microbial Technology 2008, 42: 160-166.

[34] Kongjan, P., O-Thong, S., Kotay, M., Min, B., Angelidaki, I. Biohydrogen production from wheat straw hydrolyzate by dark fermentation using extreme thermophilic mixed culture. Biotechnology and Bioengineering 2009, 105: 899-908.

[35] Fang H.H.P., Zhang T., Liu H. Microbial diversity of a mesophilic hydrogen-producing sludge. Applied Microbiology and Biotechnology 2002, 58: 112–118.

[36] Rafrafi Y., Trably E., Hamelin J., Latrille E., Meynial-Salles E., Benomar S., Guidici-Orticoni, M.T., Steyer, J.P. Sub-dominant bacteria as keystone species in microbial communities producing biohydrogen. International Journal of Hydrogen Energy 2013, In press.

[37] Noike T., Takabatake H., Mizuno O., Ohba M. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. International Journal of Hydrogen Energy 2002, 27(11-12): 1367–1371.

[38] Chao, A. Nonparametric estimation of the number of classes in a population. Scandinavian Journal of Statistics 1984, 11: 265-270.

[39] Hawkes F.R., Dinsdale R., Hawkes D.L., Hussy I. Sustainable fermentative hydrogen production: challenges for process optimisation. International Journal of Hydrogen Energy 2002, 27(11-12): 1339

Table and Figure captions

Table 1. Composition of sunflower stalks (average values of triplicates and standard deviation errors).

Table 2. Composition in monomeric sugars and inhibitory by-products of the hydrolyzate generated after dilute-acid pretreatment (170°C, 1h, 4% HCl) of sunflower stalks

Table 3. Comparison of conversion efficiency ratio after different thermal and/or dilute-acid pretreatments on lignocellulosic residues.

Table 4. Performances of mixed-culture fermentative H_2 production in batch tests after increasing addition of hydrolyzate. Values correspond to means of two replicates of independent values ± confidence intervals (error bars).

Table 5. Comparison of mesophilic hydrogen production obtained from hydrolyzates after different thermal and/or dilute-acid pretreatments on lignocellulosic residues: impact of by-products concentration and inoculum to furfural concentrations ratio (I/F).

Table 6. Repartition of the bacterial population in % of genus and species during dark fermentation on the glucose control alone and supplemented by increasing volumes of hydrolyzate (3.75%, 7.5%, 15% and 35% (v/v))

Figure 1. Cumulative hydrogen curves by increasing addition volumes (3.75%, 7.5%, 15% and 35% (v/v)) of hydrolyzate (170°C, 4% HCl) on glucose (5 g L^{-1}). Values correspond to means of two replicates of independent values ± standard deviation (error bars).

Figure 2. Metabolite patterns after addition of increasing volumes of hydrolyzate (3.75%, 7.5%, 15% and 35% (v/v)) in fermentative mixed cultures. Values correspond to means of two replicates of independent values ± confidence intervals (error bars) determined at steady state (Hmax).

Table

Sunflower stalks characteristics	Mean±SD
TS (% wet weight)	96.4 ±0.3
VS (%wet weight)	89.4 ± 0.6
Cellulose (%VS)	25.1 ± 1.7
Hemicelluloses (%VS)	11,6 ± 1.2
Klason Lignin (%VS)	32.5 ± 0.6
Uronic acids (%VS)	2.2 ± 0.3

Table

Compounds	g L^{-1}
Glucose	0.28
Xylose	3.14
∑ soluble sugars [a]	3.42
Formate	0.60
Acetate	0.81
Furfural	1.15
5-HMF	0.13
Total phenols	0.02
∑ By-products [b]	1.3
E[c]	2.97

[a] Sum of glucose and xylose content.

[b] Sum of furfural, 5-HMF and total phenols.

[c] Efficiency coefficient is calculated by the sum of soluble sugars content divided by furfural content.

Table

Substrates	Conditions of pretreatment	Conversion efficiency coefficient	Ref.
Wheat straw	Sequential thermal pretreatments (first step: 80°C, 6 min; second step: 180°C, 15 min; third step: 190°C, 3 min)	11	[31]
Sugarcane bagasse	121°C, 60 min, 4 % H_2SO_4 (v/v)	55.5	[11]
Sugarcane bagasse	121°C, 60 min, 5 % H_2SO_4 (v/v)	38.5	[11]
Oil palm empty fruit bunch	120°C, 15 min, 6 % H_2SO_4 (w/v)	27.7	[32]
Oil palm empty fruit bunch	120°C, 15 min, 8 % H_2SO_4 (w/v)	9.9	[32]
Sunflower stalks	Steam explosion, 200°C, 5 min	75	[33]
Sunflower stalks	Steam explosion, 230°C, 5 min	6.25	[33]
Sunflower stalks	170°C, 1h, 4 g HCl/ 100gTS	2.97	Our study

Table

Glucose (5gVS L^{-1}) + added hydrolyzate (%(v/v))	Modified Gompertz equation parameter values				Glucose consumed (%)	Hydrogen yield (mol H_2 $mol^{-1}_{eq.\ hexose\ consumed}$)	COD mass balance (%)	Final pH
	P (mol H_2 $mol^{-1}_{eq.\ hexose\ initial}$)	Rm (mol H_2 $mol^{-1}_{eq.\ hexose\ initial}$ day^{-1})	λ (day)	R^2				
Glucose	2.04 (± 0.14)	0.32 (± 0.22)	3.8 (± 0.39)	0.99 (± 0.00)	100	2.04 (± 0.14)	85	3.42 (± 0.11)
Glucose + 3.75 %	1.83 (± 0.08)	0.21 (± 0.16)	2.24 (± 1.47)	0.97 (± 0.04)	100	1.83 (± 0.08)	85	3.60 (± 0.09)
Glucose + 7.5 %	0.24 (± 0.05)	0.08 (± 0.00)	5.82 (± 0.02)	0.95 (± 0.04)	63	0.45 (± 0.06)	103	3.23 (± 0.01)
Glucose + 15 %	0	0	> 30	-	100	0	102	3.67 (± 0.05)
Glucose + 35 %	0	0	> 30	-	59	0	94	4.83 (± 0.03)

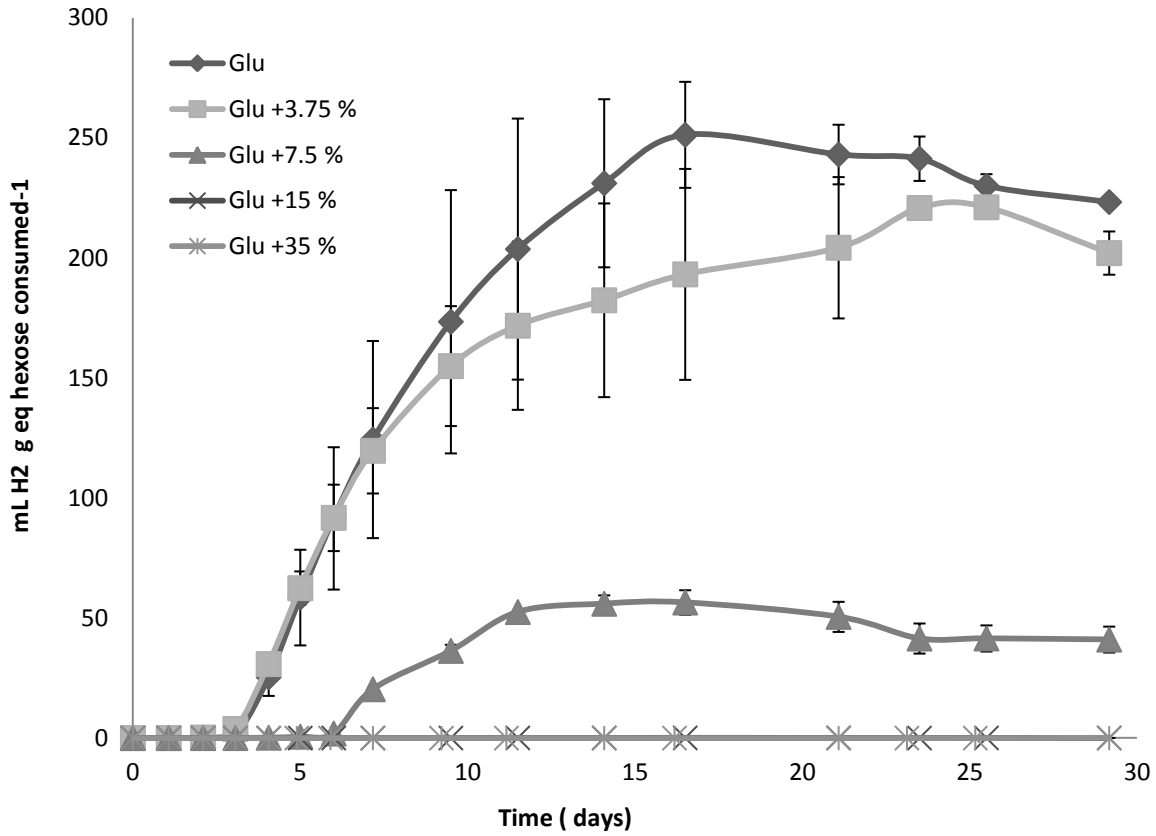
Table

Substrates	Conditions of pretretament	Batch dark fermentation conditions	Composition of soluble sugars and by-products identified in hydrogen batch fermenter	I/F ratio	Hydrogen potential (mL H_2 / g soluble sugars)	Ref
Xylose	no pretreatment	37°C, pH=5,5, anaerobic digested sludge pretreated at 90°C for 10 min	Soluble sugars: 5g/L Furfural: 1000 mg/L no other by-products	0.25	71	[19]
Sugarcane bagasse	Dilute-acid pretreatment (121°C, 60 min, 0.5 % H_2SO_4 (v/v); partial removal of phenolic compounds by $Ca(OH)_2$	37°C, pH=5.5, elephant dung pretreated at 100°C for 2h (857 mg VS added L^{-1})	Soluble sugars: 1.56g/L Furfural: 8 mg/L Acetic acid: 331 mg/L and probably other by-products not identified.	107	306	[11]
	Dilute-acid pretreatment (121°C, 60 min, 3 % H_2SO_4 (v/v); partial removal of phenolic compounds by $Ca(OH)_2$		Soluble sugars: 3.9g/L Furfural: 4 mg/L Acetic acid: 570 mg/L and probably other by-products not identified.	214	124	
	Dilute-acid pretreatment (121°C, 60 min, 4 % H_2SO_4 (v/v); partial removal of phenolic compounds by $Ca(OH)_2$		Soluble sugars: 5.1g/L Furfural: 90mg/L Acetic acid: 731 mg/L and probably other by-products not identified.	9.53	34	
	Dilute-acid pretreatment (121°C, 60 min, 5 % H_2SO_4 (v/v); partial removal of phenolic compounds by $Ca(OH)_2$		Soluble sugars: 5.2g/L Furfural: 135 mg/L Acetic acid: 735 mg/L and probably other by-products not identified.	6.3	31	
Sunflower stalks	Dilute-acid pretreatment (170°C, 1h, 4g HCl / 100 gTS)	35°C, pH=5.5, anaerobic digested sludge pretreated at 90°C for 15 min	Soluble sugars: 5.1g/L Furfural: 43 mg/L 5-HMF: 5 mg/L Acetic acid: 30 mg/L Total phenols: 1mg/L Probably other by-products not identified.	5.8	228	Our study
			Soluble sugars: 5.25g/L Furfural: 86 mg/L 5-HMF: 9 mg/L Acetic acid: 61 mg/L Total phenols: 2 mg/L Probably other by-products not identified.	2.9	29	
			Soluble sugars: 5.5g/L Furfural: 172 mg/L 5-HMF: 19 mg/L Acetic acid: 122 mg/L Total phenols: 3 mg/L Probably other by-products not identified.	1.56	0	

Table

	Conditions				
	Glu	**Glu + 3.75%**	**Glu + 7.5%**	**Glu + 15%**	**Glu + 35%**
Richness diversity	99(10)	96(10)	88(7)	92(9)	93(10)
	% **GENUS** / *Genus species*[a]				
CLOSTRIDIUM	***90***	***79***	***55***	***19***	***23***
Clostridium tyrobutyricum	70	4	0	1	1
Clostridium saccharobutylicum	0	38	30	1	0
Clostridium thiosulfatireducens	9	8	11	8	12
Clostridium pasteurianum	6	20	0	0	0
Clostridium bifermentans	3	2	4	4	3
Clostridium subterminale	1	1	3	3	3
Clostridium thermopalmarium	1	1	2	1	1
Clostridium tertium	0	0	1	1	1
SPOROLACTOBACILLUS	***0***	***14***	***40***	***75***	***69***
Sporolactobacillus racemilacticus	0	6	34	64	59
Sporolactobacillus terrae	0	1	3	6	4
Sporolactobacillus laevolacticus	0	1	2	4	5
Sporolactobacillus sp.	0	7	0	0	1
BACILLUS	***2***	***1***	***2***	***2***	***3***
Bacillus badius	2	0	0	1	1
Bacillus coagulans	0	0	0	1	1
PSEUDOMONAS	***2***	***1***	***1***	***1***	***1***
Pseudomonas plecoglossicida	1	1	1	0	1
OTHERS	***6***	***5***	***3***	***2***	***4***

Monlau_et_al_FIGURE1.pdf



Monlau_et_al_FIGURE2.pdf