A standardized biohydrogen potential protocol: An international round robin test approach

Julian Carrillo-Reyes, Aída Tapia-Rodríguez, German Buitron, Iván Moreno-Andrade, Rodolfo Palomo-Briones, Elías Razo-Flores, Oscar Aguilar Juárez, Jorge Arreola-Vargas, Nicolas Bernet, Adriana Ferreira Maluf Braga, et al.

REVISED VERSION

A standardized biohydrogen potential protocol: an international round robin test approach

Julián Carrillo-Reyes[a]*, Aida Cecilia Tapia-Rodríguez[b], Germán Buitrón[a], Iván Moreno-Andrade[a], Rodolfo Palomo-Briones[b], Elías Razo-Flores[b], Oscar Aguilar Juárez[c], Jorge Arreola-Vargas[d], Nicolas Bernet[e], Adriana Ferreira Maluf Braga[f], Lucia Braga[g], Elena Castelló[g], Lucile Chatellard[e], Claudia Etchebehere[h], Laura Fuentes[h], Elizabeth León-Becerril[c], Hugo Oscar Méndez-Acosta[i], Gonzalo Ruiz-Filippi[j], Estela Tapia Venegas[j], Eric Trably[e], Jorge Wenzel[h], Marcelo Zaiat[f]

[a] Laboratory for Research on Advanced Processes for Water Treatment, Instituto de Ingeniería, Unidad Académica Juriquilla, Universidad Nacional Autónoma de México, Blvd. Juriquilla 3001, Queretaro 76230, Mexico.

[b] División de Ciencias Ambientales, Instituto Potosino de Investigación Científica y Tecnológica A.C., Camino a la Presa San José No. 2055, Col. Lomas 4a Sección, C.P. 78216, San Luis Potosí, SLP, México

[c] Department of Environmental Technology, Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C., Av. Normalistas 800, Col. Colinas de la Normal, C.P. 44270 Guadalajara, Jalisco, Mexico

[d] División de Procesos Industriales, Universidad Tecnológica de Jalisco, Luis J. Jiménez No. 577, 1o de Mayo, C.P. 44979, Guadalajara, Jalisco, México

[e] LBE, Univ Montpellier, INRA, Narbonne, France

[f] Biological Process Laboratory, São Carlos School of Engineering, University of São Paulo (LPB/EESC/USP), Av. João Dagnone 1100, São Carlos, São Paulo 13563-120, Brazil

[g] Laboratorio BioProA, Facultad de Ingeniería, Universidad de la República de Uruguay, Av. Julio Herrera y Reissig 565, Montevideo, Uruguay.

[h] Laboratorio de Ecología Microbiana, Departamento de Bioquímica y Genómica Microbiana. Instituto de Investigaciones Biológicas Clemente Estable. Av. Italia 3318, Montevideo, Uruguay.

[i] Departamento de Ingeniería Química, CUCEI-Universidad de Guadalajara, Blvd. M. García Barragan 1451, C.P. 44430, Guadalajara, Jalisco, Mexico

[j] Escuela de Ingeniería Bioquímica, Facultad de Ingeniería, Pontificia Universidad Católica de Valparaíso, Av. Brasil 2085, Valparaíso, Chile

[*] **Corresponding author (J. Carrillo-Reyes)**: JCarrilloR@iingen.unam.mx

Instituto de Ingeniería, Unidad Académica Juriquilla, Universidad Nacional Autónoma de México, Blvd. Juriquilla 3001, Queretaro 76230, Mexico.

Tel: +52 442 1926174

Fax: +52 442 1926185

A standardized biohydrogen potential protocol: an international round robin test approach

Abstract

Hydrogen production by dark fermentation is an emerging technology of increasing interest due to its renewable feature. Recent scientific advances have well investigated the operational conditions to produce hydrogen through the valorization of several wastes or wastewaters. However, the development of standardized protocols to accurately assess the biohydrogen potential (BHP) is of crucial importance. This work is the first interlaboratory and international effort to validate a protocol estimating hydrogen potential using batch tests, using glucose as individual model substrate. The repeatability of the hydrogen potential (HP) increased with variations of the proposed protocol: reducing substrate concentration, increasing the buffer capacity, and using an automatic device. The interlaboratory variation of the HP was reduced from 32 to 12 %, demonstrating the reproducibility and robustness of the proposed protocol. Recommendations to run BHP tests were formulated in terms of i) repeatability and reproducibility of results, ii) criteria for results validation and acceptance, iii) workload of the proposed protocols.

Keywords

Biohydrogen; batch protocol; dark fermentation; glucose; heat-treated inoculum

1. INTRODUCTION

Hydrogen constitutes a valuable energy carrier due to its highest energy density among fuels, producing only water as by-product. Dark fermentation is a suitable strategy to produce renewable hydrogen, wherein fermentative bacteria are able to produce it mainly from fermentable sugars from wastewaters or wastes with a high organic content as substrates, or other molecules like proteins and glycerol [1,2]. According to the Web of Science data-base, more than 1600 scientific papers dealing with ‘hydrogen production’ by ‘dark fermentation’ have been published since 2000, with an average of 175 published papers per year over the past four years. The high flexibility of dark fermentation to process a wide variety of renewable organic wastes sets a remarkable gap of opportunity for

bioenergy generation [2,3]. This shows an optimistic scenario for biohydrogen production, as an added-value process to the conventional anaerobic digestion-based waste stream treatment processes. Significant improvements have been made in hydrogen yields and production rates in the last two decades [4].

Alongside the positive outlook for hydrogen production through dark fermentation, there is an increasing demand for better understanding of the main factors impacting the system at a process level [5]. Through the assessment of the biological hydrogen potential (BHP), the information provided by former case studies has been valuable to evaluate several parameters, like new potential substrates, e.g., crop residues, microalgal biomass, aquatic plants [6–9]; the effect of different substrate compositions, carbohydrates content and its complexity, or solids content [10–12]; and further optimizing of operational parameters like substrate pretreatment [13]. However, observations are mostly depending on the type of substrate, the source of inoculum and the operational conditions, wherein comparisons between them become a challenging task.

Particularly, two major limitations have been identified when dealing with the comparison of BHP results issued from the literature: one of them is the lack of fairness when comparing these results with regard to the use of different protocols, and, on the other hand, the diverse precision levels due to non-standardized practices (i.e., due to manual operations), which causes a broad inconsistency as well as different reliability level of the experimental data. In this context, the normalization of the testing guideline criterion becomes a key factor in order to count with homogenized data on hydrogen production.

One way to attain a better comparison viability is through the establishment of a comprehensive technique that can encompass the most suitable conditions reported for dark fermentation, as they have been already identified in previous studies. In this sense, the design and implementation of a standardized BHP protocol could allow to systematically characterize and compare different process alternatives under the same technical baseline. At the same time, it is important to establish quality control criteria on central parameters of the process discerning whether the experimental data are valid. Nonetheless, Venkata Mohan et al. [14] proposed a batch mode protocol to evaluate the BHP of wastewater. This method was validated with two real substrates but only one source of inoculum and pretreatment. To the best of our knowledge, no interlaboratory study to validate a batch

mode protocol giving an evaluation of biohydrogen potential (BHP) has been conducted so far.

Among the most influencing parameters on hydrogen production stand the initial pH, hydrogen partial pressure, temperature, as well as the acclimation of the microbial communities in the inoculum for the selection of hydrogen-producing bacteria [15]. Specifically, Davila-Vazquez et al. [16] performed a thorough study on the effect of different pH and initial substrate concentrations for different substrates on the hydrogen molar yield (HMY) and the volumetric hydrogen production rate (VHPR) at a temperature of 37ºC, confirming that the best set of conditions for higher HMY were initial pH 7.5 and 5 g L^{-1} when glucose was used as a model substrate.

On the other hand, there is groundwork in the effort of standardizing the method for the acclimation of microbial community in the anaerobic mixed culture. This step is essential to inactivate methanogens while selecting spore-forming hydrogenogenic microorganisms. Some authors have converged in the higher suitability of applying thermal shock to anaerobic mixed cultures at 103-106°C [17,18]. Also, due to the fact that a high partial pressure will cause higher dissolved hydrogen concentration, with the consequential reduction of oxidized ferredoxin and thus hindering the hydrogen production yield [3], the selection of a system that allows for the continuous release of such pressure – such as an automatic gas release monitoring unit – is strategic for maximizing hydrogen production during BHP testing.

In addition, the modified Gompertz equation constitutes by far the most widely accepted model to describe the kinetics of hydrogen production when using anaerobic mixed cultures [19,20]. This consideration is fundamental in the establishment of a suitable BHP protocol.

The statement of important criteria for biochemical methane potential test has been suggested [21], likewise quality control criteria such as repeatability and reproducibility of results, to properly validate or reject them, and in this way, guarantee its reliability [22]. Such quality criteria could apply to BHP testing.

The aim of this study was to propose and validate a comprehensive standard protocol for BHP testing in batch-mode considering all of the aforementioned conditions, in order to assess the level of repeatability and reproducibility of the results, at an interlaboratory level. For this purpose, the protocol was validated in several laboratories from different countries

using the same inoculum at first instance, and then using several sources of fermentative bacteria, all performed with a model substrate. The collected data were compared in terms of the main kinetic parameters for hydrogen production, with the purpose of providing insight on the level of interlaboratory (between-lab) and intralaboratory (within-lab) replication, as well as the identification of the main key factors and specific challenges of this tool for performing further hydrogen production studies.

2. METHODOLOGY

2.1 Substrate

Glucose was used as the only carbon source at 5 and 20 g L^{-1}. Two different buffer solutions – modified from the medium proposed by Mizuno et al. [23] – were tested, one based in phosphate salt and another using MES monohydrate (2-[N-Morpholino] ethanesulfonic acid, 4-Morpholineethanesulfonic acid monohydrate), as suggested previously [11]. The phosphate buffer composition in mg L^{-1} was: NH_4Cl, 2600; K_2HPO_4, 250; $MgCl_2 \cdot 6H_2O$, 125; $FeSO_4 \cdot 7H_2O$, 100; $CoCl_2 \cdot 6H_2O$, 2.5; $MnCl_2 \cdot 4H_2O$, 2.5; KI, 2.5; $NiCl_2 \cdot 6H_2O$, 0.5; $ZnCl_2$, 0.5. In the MES-based buffer, the K_2HPO_4 was substituted by MES at 1200 mg L^{-1}.

2.2 Sources of inoculum

Different sources of inoculum were tested among the laboratories: i) heat treated anaerobic sludge from a thermophilic digester of sewage sludge (HTT); ii) heat treated anaerobic sludge from a mesophilic full-scale UASB reactor treating brewery wastewater (HTM); iii) biomass from an auto-fermented effluent rich in sucrose (AF) [24]; iv) aerobic sludge pretreated by cell wash-out (WO); v) compost from kitchen wastes (C) [25]; vi) heat treated anaerobic sludge from a pilot-scale digester treating the organic fraction of solid wastes (HTS). The heat pretreatment procedure applied to HTT, HTM, and HTS was at 105°C for 24 hours to select spore-forming bacteria, suppressing potential hydrogen consumers such as methanogens [17]; then the dried sludge was grounded and mesh-sieved through a #20-mesh (particle size of 850 µm). During the WO pretreatment, the aerobic sludge was fed into a completely stirred tank reactor with glucose at 10 g L^{-1} and at 8 h of hydraulic retention time (HRT) to select fermentative bacteria and wash-out those not capable to

grow at such HRT [1]. For all tests, the inoculum was added at a substrate/inoculum ratio of 2.7 (g glucose/g volatile solids).

2.2 Biohydrogen production batch mode protocol

Two different gas measuring strategies were evaluated: i) manual procedure for periodic biogas release, and ii) automatic device with a continuous biogas release. Common conditions among manual and automatic procedures were the following: initial pH was adjusted at 7.5 with HCl or NaOH 5N solutions; bottles were closed and sealed, exchanging the headspace with N_2 by 1 min, and incubated at 37°C. All evaluated conditions were run in triplicates with their respective endogenous controls without substrate. When tests came to a halt, liquid phase samples were collected to determine soluble metabolites and residual glucose as indicated in the analytical methods (section 2.4).

2.2.1 Manual protocol

The manual test procedure was proposed by Davila-Vazquez et al. [16]; in brief, experiments were performed in serological bottles of 120 mL, with 80 mL of working volume, and horizontal shaking at 150 rpm. Gas produced was released every three hours and measured by liquid displacement using an acidic solution (pH < 2), to avoid CO_2 absorption, in an inverted graduated cylinder. Gas samples were taken for its composition analysis by chromatography with a thermal conductivity detector. Run stopped when the hydrogen production reached the stationary phase and the coefficient of variation between the last three measurements was lower than 5 %, as suggested previously for biochemical methane potential tests [21]. The temperature during the gas production records and the atmospheric pressure of each laboratory was considered to express the gas production results at standard conditions (273.15 K, 101.325 kPa).

2.2.2 Automatic protocol

The automatic tests procedure was run in the Automatic Methane Potential Test System (AMPTS II, Bioprocess Control AB, Sweden). Tests were run in glass bottles of 600 mL, with 360 mL of working volume, accordingly to manufacturer recommendations. Bottles were incubated with intermittent shaking, 60 s on/180 s off at 60 % (approx. 120 rpm). Gas

produced was continuously released, passing through a CO_2-absorption unit (NaOH, 3N) and measured online each 10 mL. Run stopped when the hydrogen production achieved the stationary phase, according to the online graph shown by the software in the automatic device. Gas samples from the headspace were taken at the end of the gas production for its composition analysis. The automatic device reported the gas production at standard conditions by using its internal temperature and pressure sensors.

2.3 Interlaboratory tests

The protocol was tested in 8 independent laboratories from 5 different countries (Brazil, Chile, France, Mexico and Uruguay). Testing was divided into stages as specified in the following subsections.

2.3.1 Use of phosphate buffer in manual protocol

At first instance, a set of interlaboratory experiments by 6 different independent laboratories was performed under a manual procedure (2.2.1) with phosphate buffer and 20 g L^{-1} glucose as model substrate, using the same inoculum (HTT).

2.3.2 Phosphate vs. MES buffer in manual protocol

Manual procedure (2.2.1) was run among 4 laboratories using glucose at 5 g L^{-1}, two different buffer solutions and the own inoculum from the laboratories, HTT, HTM, AF, WO were evaluated as comparison.

2.3.3 Use of MES buffer in automatic protocol

Finally, the automatic procedure in AMPTS II (2.2.2) was tested using 5 g L^{-1} glucose with the selected buffer solution (MES), comparing three inocula, HTT, HTM, and HTS, among 4 laboratories.

2.4 Analytical methods

The final soluble chemical oxygen demand (COD) was analyzed by standard methods [26], and residual sugars were determined by the sulfuric acid-phenol method [27]. Soluble metabolites (e.g. acetate, propionate, butyrate, ethanol) were determined by different

methodologies previously reported, gas chromatography with flame ionization detector [17,24,25,28,29] and High Performance Liquid Chromatography (HPLC) [11,30], and capillary electrophoresis [16]. H_2 and CO_2 content in the biogas were determined by gas chromatography with thermal conductivity detector [11,16,17,24,25,28,29].

2.5 Data analysis

The experimental cumulative hydrogen production was fitted to the modified Gompertz equation 1 [16] using the Solver add in Microsoft Excel (v 16.18, Microsoft, USA):

$$H(t) = H_{max} exp\left\{-exp\left[\frac{2.71828 R_{max}}{H_{max}}(\lambda - t) + 1\right]\right\} \quad \text{Eq.1}$$

where H(t) (L) is the total volume of hydrogen produced at culture time t (d); H_{max} (L) is the maximal amount of hydrogen produced; R_{max} (L d^{-1}) is the maximum hydrogen production rate and λ (d) is the lag time before exponential hydrogen production. The hydrogen potential (HP), the hydrogen production rate (HPR), and the hydrogen molar yield (HMY), were defined as response variables. HP and HMY were calculated from H_{max} and defined as L H_2 L^{-1} (per liter of working volume) and mol H_2 mol $glucose_{consumed}^{-1}$, respectively; and the HPR was calculated from R_{max} and defined as L H_2 L-d^{-1}.

Average values and the corresponding standard deviations of response variables were used to calculate the intralaboratory (within-lab) and interlaboratory (between-lab) coefficient of variation (CV), or relative standard deviation, as repeatability and reproducibility indicators, respectively. The statistical analysis of the data for estimate of the precision statistics was a one-way and two-way analysis of variance (ANOVA), carried out for each response variable, as recommended for interlaboratory studies [31]. Variables with statistically significant effects were analyzed. The statistical analysis was run in the R environment (v 3.1.2, RStudio, RStudio Inc.).

3. RESULTS AND DISCUSSION

3.1 Effects on hydrogen potential, production rate and molar yield

In the present work, BHP assays were performed by different laboratories to evaluate the HP, HMY and HPR for each laboratory, using glucose as an individual model substrate with mixed anaerobic consortia.

In experiments with 20 g L^{-1} glucose, using the manual protocol and phosphate buffer (Figure 1), values for HP, HPR and HMY ranged among 0.65 – 2.03 L H_2 L^{-1}, 0.63 – 5.23 L H_2 L-d^{-1}, and 0.65 – 1.25 mol H_2 $mol_{glucose}^{-1}$, respectively; whereas for 5 g L^{-1} glucose at the same manual protocol and buffer conditions (Figure 2) the intervals were 0.14 – 0.78 L H_2 L^{-1}, 0.06 – 1.58 L H_2 L-d^{-1}, and 0.21 – 1.34 mol H_2 $mol_{glucose}^{-1}$, respectively. Various studies have focused on the evaluation of BHP from different feedstocks using mixed anaerobic consortia and manual protocols. Some of these studies have been intended for evaluating different model substrates in individual mode [16,32], co-substrate mode [32], industrial wastewater effluents [18], among others. In terms of the protocol conditions, the selected for the present work was based on the best according to some previous studies on batch protocols for hydrogen production [16], and using heat-treated inoculum to overcome the activity of methanogenic archaea [17,33]. Several other authors have also addressed the relevance of initial pH [5,15,20,34], temperature [5,15,20,34,35], and concentration of inoculum and substrate [20,35]. From previous works and to maximize the production of hydrogen, in this work the starting pH was at 7.5, the temperature of incubation at 37°C and the substrate on inoculum ratio at 2.5 for all experiments. The suitability of the modified Gompertz model to describe and to model a batch fermentative hydrogen production process with mixed anaerobic cultures has been successfully proved [16,36], in this work all hydrogen production kinetics were adjusted to this model.

The values of HP and HPR obtained for the manual mode with phosphate buffer and 20 g L^{-1} glucose, were similar to the values reported in previous studies with the same substrate, buffer, and a similar heat-treatment of inoculum. For instance, Davila-Vazquez et al. [16] produced 2.2 L H_2 L^{-1} and 6.36 L H_2 L-d^{-1}, with 25 g L^{-1} glucose; and Gupta et al. [32] produced 2.1 L H_2 L^{-1} and 3.12 L H_2 L-d^{-1} with 11.5 g L^{-1} glucose. These small differences might be due to the initial substrate/inoculum ratio. Such consistency in the range of results as compared with previous works [16,32] reflects the robustness of using a batch protocol and heat-treated sludge as inoculum.

To increase the repeatability of the proposed protocol, glucose concentration was decreased to 5 g L^{-1} (as explained below, section 3.2), and two different buffers were tested. Despite the substrate concentration decreased by 4-fold, the average HP and HPR only decreased by 50% and 62%, respectively, in comparison to using 20 g L^{-1}; and the average HMY increased by 56% (Figure 1 and 2). In addition to the better HMY, lowering the glucose concentration increased its consumption from 74 ± 29 to 98 ± 4 %, and increased the hydrogen content in the biogas, from 43 ± 9 to 56 ± 11 %, comparing experiments with initial values at 20 and 5 g L^{-1}, respectively. The average final pH using phosphate buffer were 4.6 ± 0.3 and 3.8 ± 0.5 at 20 g L^{-1} and 5 g L^{-1}, respectively. The lower yield at 20 g L^{-1} could be affected by the ethanol concentration (2.6 g L^{-1}), where its accumulation at 1.14 g L^{-1} has been proved to affect the metabolic pathways in dark fermentation [37]. In this sense, the preferential yields of produced metabolites change from ethanol>acetate>propionate to acetate>propionate>butyrate, comparing 20 to 5 g L^{-1} using phosphate buffer, respectively (Figure 3, A and C).

When comparing phosphate with MES buffer in manual mode at 5 g L^{-1} glucose, using MES improved HP, HMY and HPR by 2.20, 1.80 and 0.27-fold, respectively (Figure 2). Although no other studies have addressed the use of MES buffer for H_2 production protocols, this improvement could be attributed to the fact that these two buffers portray clear differences in buffer capacity. In this sense, the buffer capacity for both solutions from pH 7.5 to 6.5 is similar, 1.3 and 1.1 mM for MES and phosphate; respectively; but at lower values from 6.5 to 4.5 the MES buffer capacity (4.1 mM) is higher than that for phosphate (1 mM). This difference in the buffer capacity was reflected by the final average pH comparing both experiments, being 4.4 ± 0.6 and 3.8 ± 0.5, for MES and phosphate, respectively. The metabolic pathways were also driven by the buffer solution, with preferential production of acetate>butyrate and acetate>propionate, using MES and phosphate buffer, respectively (Figure 3, B and C). Buffer implications have also already been discussed in previous works [38]. However, the buffer composition is not usually adjusted to the substrate concentration, which is a relevant consideration working with batch protocols without pH control.

Recently, the hydrogen potential of agroindustrial effluents has been tested in automatic equipment [39,40]. To compare the selected parameters of this work, 5 g L^{-1} glucose and

MES buffer, different inocula were evaluated with the automatic protocol by different laboratories.

Further experiments were performed at 5 g L^{-1} glucose in automatic equipment (AMPTS II) with the selected buffer system (MES), achieving a level of 0.47 – 1.02 L H_2 L^{-1}, 1.60 – 2.79 L H_2 L-d^{-1}, and 0.78 – 1.65 mol H_2 $mol_{glucose}^{-1}$ (Figure 4). The general comparison showed a similar metabolic pathway using the manual or the automatic protocol (acetate>butyrate, with the same average value 0.29 and 0.28 mol/mol; Fig. 4 D to F). Besides, values of glucose consumption and hydrogen content, 94 ± 6 and 53 ± 6 % respectively, were similar to the achieved with the manual protocol. The closest comparison of a manual and automatic protocol is the one reported by Wang et al. [41], who evaluated anaerobic digestion for cellulose under both regimes, without a significant effect on methane yield. In the present study for hydrogen production, using the automatic protocol decrease 25% the HP and HMY compared to the manual one (Fig. 2 and 3). Only the HPR was improved by the automatic protocol, becoming 70% higher than the manual one; explained by the continuous gas released and its online recording. Several authors have addressed the positive effects and implications of low hydrogen partial pressure (among other different process conditions) on H_2 production [5,15]. Even though the slight difference between HP, HPR and HMY in the manual and automatic protocol, considering results with the same inoculum (HTT or HTM), the statistical analysis showed non-significant difference considering p values higher than 0.01, but significant differences considering p values higher than 0.05. In this sense, both methods resulted in comparable results, regardless of operational differences like working volume and stirring conditions.

In terms of the different inoculum tested, average values were in narrow range for HP, HPR and HMY at 0.6 – 0.8 L H_2 L^{-1}, 1.8 – 2.0 L H_2 L-d^{-1} and 1.1 – 1.4 mol H_2 $mol_{glucose}^{-1}$, respectively. Those similar results are attributed to the same heat-treatment selecting spore-forming bacteria [17], regardless of the different source of anaerobic sludge.

The COD measurements were stable at different evaluated conditions, with final average concentrations at 19.4 ± 1.3, 5.1 ± 0.4, and 5.3 ± 0.9 g L^{-1}, for the manual protocol with 20 and 5 g L^{-1} glucose, and the automatic protocol, respectively; which correspond to COD removals from 1-11%. Similar COD removals have been reported for dark fermentation, where most of the COD is converted to acids; other strategies like coupling dark

fermentation to photo-fermentation are meant to increase the hydrogen productivity and decrease the COD content in wastewaters [42].

3.2 Repeatability and reproducibility evaluation

The repeatability and the reproducibility of common process variables (HP, HPR and HMY) were evaluated in the present international work, evaluating the effect of substrate concentration and buffer solution on different inoculum (Figure 1 and 2), and the use of an automatic device comparing different inoculum (Figure 4). The first interlaboratory approach analyzing the same inoculum with the manual protocol and glucose at 20 g L^{-1} resulted in a low reproducibility, were the variable "Laboratory" had a significant effect on HPR and HMY (Table 1). This low reproducibility is explained by the intra-laboratory variation (intra CV), where one-third of the laboratories had values higher than 20% for HP, HPR and HMY; resulting in an inter-laboratory variation (inter CV) up to 72% (Figure 1). A concentration of 20 g L^{-1} was tested following the recommendation of Davila-Vazquez et al. [16] between their highest HPR at 15 and 25 g L^{-1} of glucose, with intra CV from 5 to 21 %, respectively. In the present study, using glucose at 20 g L^{-1} produced a maximum volume of 120 mL of biogas every three hours during the exponential phase, which could produce errors during the data register using the manual method, due to the resolution of the graduated cylinder.

To increase the repeatability and reproducibility, a second approach in the present work was evaluated using 5 g L^{-1} glucose, considering the lower CV (from 3 to 10%) found by Davila-Vazquez et al. [16] at such concentration. Previously, several glucose concentrations were tested between 5 and 25 g L^{-1}, where the lowest concentration maximized the HMY [16]; in this sense, evaluating 5 g L^{-1} had the additional purpose to increase the BHP. Indeed, different inoculum commonly used for each laboratory and different buffer solution were evaluated. In average, the repeatability using 5 g L^{-1} glucose reduced the intra CV to 18% (17 % and 20 %, for MES and phosphate buffer, respectively) compared to the 22% at 20 g L^{-1}. Phosphate buffer produced significant differences in terms of hydrogen potential and molar yield, whereas using MES buffer had no statistical effect on hydrogen potential and molar yield, whatever the tested inoculum (Table 1). The higher repeatability achieved using MES is attributed to a better buffer capacity than the

phosphate solution. In this sense, buffer and the microbial inoculum showed both significant effects on HP and HMY (Table 2).

Using the automatic equipment, the repeatability improved in comparison to the manual protocol, reducing the average intra CV to 9.3, 15 and 10.5% for HP, HPR and HMY (Figure 4). However, such comparable reproducibility was only achieved using the HTT inoculum, where inter CV of 15, 42 and 25%, were similar than the obtained using the manual protocol, i.e. 12, 56 and 16%, for HP, HPR and HMY, respectively. The different inoculum had a significant effect on the response variables (Table 2), resulting in higher inter CV using HTS and HTM inoculum, 113 and 28% for HP and HMY, compared to the obtained with HTT.

3.3 Recommendations to run BHP batch tests

To our knowledge, there is only one previous study focused on the design and standardization of a methodology (protocol) to evaluate Biological Hydrogen Production (BHP tests) [14]. As the authors proposed a workflow for a process optimization [14], their aim was not to determine statistical parameters to evaluate the repeatability or reproducibility of the protocol at interlaboratory level. The closest approaches to this work are those that aim to standardize a biochemical batch protocol for methane production, specially using organic solid wastes [22,43,44]; which is a common practice for the design of industrial anaerobic digesters [22]. The first studies were focused on proposing general parameters for methane production tests [45] and analyzing the method repeatability and reproducibility [43]. Two approaches have focused on interlaboratory studies [44] and general recommendations for the test validation [22]. In this sense, the results of the present work are analyzed to made recommendations in terms of: i) parameters that influence the repeatability and reproducibility of results, ii) criteria for results validation and acceptance, iii) workload analysis of the different tested protocols.

3.3.1 Repeatability and reproducibility

Considering HP and HPR, the repeatability of the proposed protocol was significantly improved (reduction of intralaboratory variation), with the following criteria and sequence: reducing substrate concentration>increasing the buffer capacity> using an automatic device

(Table 3). In terms of reproducibility, the criteria and sequence were as follows: reducing substrate concentration> increasing the buffer capacity; where using the manual or the automatic protocol produced higher and lower inter CV for HP and HPR, respectively (Table 3). A common strategy to optimize hydrogen producing processes is to look for the maximum organic load supported [14]; however, awareness is needed on the fact that at carbohydrate concentrations higher than 5 g L^{-1} repeatability decrease, and possible comparison to literature will be limited due to the loss of reproducibility.

3.3.2 Criteria for results validation and acceptance

One common criterion for validation of results established for methane potential tests is the methane recovery using a model substrate in comparison to the theoretical recovery [22]. The theoretical hydrogen yield is not a suitable criterion for accepting BHP results, considering that the well-known maximum biochemical yield 4 mol H_2 $mol_{glucose}^{-1}$, depends mainly on low H_2 partial pressure, the pH, and other parameters such as the nature of the inoculum [5].

Based on the relative standard deviation proposed as acceptable by Holliger et al. [22] for methane potential tests, the HP results obtained in the present work fitted to both criteria, 5-10% for intralaboratory CV, and ≥ 14 % of interlaboratory CV using the manual protocol; but only the intralaboratory CV criteria was fitted by the automatic protocol (Table 3). However, the HPR results did not fit to any of both, having closer results if the automatic protocol is used. Since BHP tests are normally carried out by only one laboratory, acceptable intra CV for the HPR are 25 and 15 %, using the manual or automatic protocol, respectively (average values, Table 3).

3.3.3 Workload analysis

A helpful criterion to choose a particular protocol is the available time for performing the test, where a time-saving experimental set-up is pursued [43,44]. The workload for particular operations in the protocols include the preparation of the inoculum and mineral solution, set-up of batch reactors, monitoring gas production and composition, as well as data management and evaluation (Table 4). For one or five samples sets, the automatic protocol reduces the workload time from 32 to 65 % compared to the manual protocol,

mainly due to the time saved for chromatographic analysis; but the manual protocol has the advantage that no specialized equipment is required. To reduce the workload analysis in the manual protocol a CO_2 trap could be applied similarly to the automatic device, but the proper experiment setup would need further validation.

4. CONCLUSIONS

In this work, a standardized protocol for biohydrogen production potential was evaluated in interlaboratory mode. Evaluating the effect of operational parameters, such as substrate concentration, buffer capacity and a manual or automatic gas measuring method, the repeatability and reproducibility were increased. In this sense, the interlaboratory variation of the HP and HPR were reduced from 32 to 12 %, and from 72 to 33 %, respectively, showing the robustness of the proposed protocol. The intralaboratory variation among their replicates was reduced up to 13 and 9% for HP, and up to 25 and 15 % for HPR, using the manual and automatic protocol, respectively; such variations were stated as criteria for acceptance of the results. The automatic protocol increases the reproducibility and repeatability of the BHP test, while also reduces the workload in comparison with the manual protocol, although the manual protocol also reaches acceptable variation values. Hence, both protocols – manual and automatic – are suitable to get acceptable results. Due to this protocol was standardized with a model substrate, further validation is envisioned using real wastewater or effluents, following the same operational parameters S/X ratio, soluble carbohydrate content, and buffer capacity. The present work sets a precedent for further interlaboratory comparison of other worth studying parameters for hydrogen production by dark fermentation.

Acknowledgements

This work was performed in the frame of the Biohydrogen Latin America network. The authors thanks the funding of the following projects: Fondo de Sustentabilidad Energética SENER-CONACYT, Clúster Biocombustibles Gaseosos, 247006 and CONACYT 240087; PAPIIT IA100518 (UNAM); Fondecyt 3160219; Program ECOS-CONICYT project N° C12E06, GRAIL 613667 (FP7-KBBE-2013); ANII-FSE 102488 (Uruguay); FAPESP (2015/06246-7) and CNPq processes 406751/2013-7, 150641/2015-0, 150475/2016-0

(Brazil); and program EU Waste2bioHy project (FP7-MC-IEF - 326974). The technical assistance of Gloria Moreno Rodríguez, Jaime Pérez Trevilla, Ángel A. Hernández Huerta, Guillermo Vidriales-Escobar and Roberto E. Bolaños Rosales is also acknowledged.

Bibliography

[1] Tapia-Venegas E, Ramirez-Morales JE, Silva-Illanes F, Toledo-Alarcón J, Paillet F, Escudie R, et al. Biohydrogen production by dark fermentation: scaling-up and technologies integration for a sustainable system. Rev Environ Sci Biotechnol 2015:1–25. doi:10.1007/s11157-015-9383-5.

[2] Rezania S, Din MFM, Taib SM, Sohaili J, Chelliapan S, Kamyab H, et al. Review on fermentative biohydrogen production from water hyacinth, wheat straw and rice straw with focus on recent perspectives. Int J Hydrog Energy 2017;42:20955–69. doi:10.1016/j.ijhydene.2017.07.007.

[3] Chong M-L, Sabaratnam V, Shirai Y, Hassan MA. Biohydrogen production from biomass and industrial wastes by dark fermentation. Int J Hydrog Energy 2009;34:3277–87. doi:10.1016/j.ijhydene.2009.02.010.

[4] Singh A, Rathore D. Biohydrogen: Next Generation Fuel. Biohydrogen Prod. Sustain. Curr. Technol. Future Perspect., Springer, New Delhi; 2017, p. 1–10. doi:10.1007/978-81-322-3577-4_1.

[5] Khanna N, Das D. Biohydrogen production by dark fermentation: Biohydrogen production by dark fermentation. Wiley Interdiscip Rev Energy Environ 2013;2:401–21. doi:10.1002/wene.15.

[6] Arreola-Vargas J, Razo-Flores E, Celis LB, Alatriste-Mondragón F. Sequential hydrolysis of oat straw and hydrogen production from hydrolysates: Role of hydrolysates constituents. Int J Hydrog Energy 2015;40:10756–65. doi:10.1016/j.ijhydene.2015.05.200.

[7] Chong PS, Jahim JMd, Harun S, Lim SS, Mutalib SAbd, Hassan O, et al. Enhancement of batch biohydrogen production from prehydrolysate of acid treated oil palm empty fruit bunch. Int J Hydrog Energy 2013;38:9592–9. doi:10.1016/j.ijhydene.2013.01.154.

[8] Varanasi JL, Kumari S, Das D. Improvement of energy recovery from water hyacinth by using integrated system. Int J Hydrog Energy 2018;43:1303–18. doi:10.1016/j.ijhydene.2017.11.110.

[9] Carrillo-Reyes J, Buitrón G. Biohydrogen and methane production via a two-step process using an acid pretreated native microalgae consortium. Bioresour Technol 2016;221:324–30. doi:10.1016/j.biortech.2016.09.050.

[10] Guo XM, Trably E, Latrille E, Carrere H, Steyer J-P. Predictive and explicative models of fermentative hydrogen production from solid organic waste: Role of butyrate and lactate pathways. Int J Hydrog Energy 2014;39:7476–85. doi:10.1016/j.ijhydene.2013.08.079.

[11] Chatellard L, Trably E, Carrère H. The type of carbohydrates specifically selects microbial community structures and fermentation patterns. Bioresour Technol 2016;221:541–9. doi:10.1016/j.biortech.2016.09.084.

[12] Turhal S, Turanbaev M, Argun H. Hydrogen production from melon and watermelon mixture by dark fermentation. Int J Hydrog Energy 2019;44:18811–7. doi:10.1016/j.ijhydene.2018.10.011.

[13] Karaosmanoglu Gorgec F, Karapinar I. Biohydrogen production from hydrolyzed waste wheat by dark fermentation in a continuously operated packed bed reactor: The effect of hydraulic retention time. Int J Hydrog Energy 2019;44:136–43. doi:10.1016/j.ijhydene.2018.08.155.

[14] Venkata Mohan S, Chiranjeevi P, Mohanakrishna G. A rapid and simple protocol for evaluating biohydrogen production potential (BHP) of wastewater with simultaneous process optimization. Int J Hydrog Energy 2012;37:3130–41. doi:10.1016/j.ijhydene.2011.10.109.

[15] Guo XM, Trably E, Latrille E, Carrère H, Steyer J-P. Hydrogen production from agricultural waste by dark fermentation: A review. Int J Hydrog Energy 2010;35:10660–73. doi:10.1016/j.ijhydene.2010.03.008.

[16] Davila-Vazquez G, Alatriste-Mondragón F, de León-Rodríguez A, Razo-Flores E. Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: Influence of initial substrate concentration and pH. Int J Hydrog Energy 2008;33:4989 – 4997.

536 [17] Buitrón G, Carvajal C. Biohydrogen production from Tequila vinasses in an anaerobic
537 sequencing batch reactor: Effect of initial substrate concentration, temperature and
538 hydraulic retention time. Bioresour Technol 2010;101:9071–7.
539 doi:10.1016/j.biortech.2010.06.127.
540 [18] Moreno-Andrade I, Moreno G, Kumar G, Buitrón G. Biohydrogen production from
541 industrial wastewaters. Water Sci Technol 2015;71:105–10.
542 doi:10.2166/wst.2014.471.
543 [19] Zwietering MH, Jongenburger I, Rombouts FM, van 't Riet K. Modeling of the
544 Bacterial Growth Curve. Appl Environ Microbiol 1990;56:1875–81.
545 [20] Mu Y, Yu H-Q, Wang G. A kinetic approach to anaerobic hydrogen-producing
546 process. Water Res 2007;41:1152–60. doi:10.1016/j.watres.2006.11.047.
547 [21] Moreno-Andrade I, Buitrón G. Influence of the origin of the inoculum on the
548 anaerobic biodegradability test. Water Sci Technol 2004;49:53–9.
549 [22] Holliger C, Alves M, Andrade D, Angelidaki I, Astals S, Baier U, et al. Towards a
550 standardization of biomethane potential tests. Water Sci Technol 2016; 74: 2515-22.
551 doi:10.2166/wst.2016.336.
552 [23] Mizuno O, Dinsdale R, Hawkes FR, Hawkes DL, Noike T. Enhancement of hydrogen
553 production from glucose by nitrogen gas sparging. Bioresour Technol 2000;73:59–65.
554 doi:10.1016/S0960-8524(99)00130-3.
555 [24] Fontes Lima DM, Zaiat M. The influence of the degree of back-mixing on hydrogen
556 production in an anaerobic fixed-bed reactor. Int J Hydrog Energy 2012;37:9630–5.
557 doi:10.1016/j.ijhydene.2012.03.097.
558 [25] Castelló E, Perna V, Wenzel J, Borzacconi L, Etchebehere C. Microbial community
559 composition and reactor performance during hydrogen production in a UASB reactor
560 fed with raw cheese whey inoculated with compost. Water Sci Technol
561 2011;64:2265–73. doi:10.2166/wst.2011.706.
562 [26] APHA/AWWA/WFE. Standard methods for the examination of water and wastewater.
563 21th edn, Washington DC, USA 2005.
564 [27] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric Method for
565 Determination of Sugars and Related Substances. Anal Chem 1956;28:350–6.

[28] García-Depraect O, Gómez-Romero J, León-Becerril E, López-López A. A novel biohydrogen production process: Co-digestion of vinasse and Nejayote as complex raw substrates using a robust inoculum. Int J Hydrog Energy 2017;42:5820–31. doi:10.1016/j.ijhydene.2016.11.204.

[29] Silva-Illanes F, Tapia-Venegas E, Schiappacasse MC, Trably E, Ruiz-Filippi G. Impact of hydraulic retention time (HRT) and pH on dark fermentative hydrogen production from glycerol. Energy 2017;141:358–67. doi:10.1016/j.energy.2017.09.073.

[30] Arreola-Vargas J, Flores-Larios A, González-Álvarez V, Corona-González RI, Méndez-Acosta HO. Single and two-stage anaerobic digestion for hydrogen and methane production from acid and enzymatic hydrolysates of Agave tequilana bagasse. Int J Hydrog Energy 2016;41:897–904. doi:10.1016/j.ijhydene.2015.11.016.

[31] ASTM E 691 - 99. Standard Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method. ASTM International 1999

[32] Gupta M, Velayutham P, Elbeshbishy E, Hafez H, Khafipour E, Derakhshani H, et al. Co-fermentation of glucose, starch, and cellulose for mesophilic biohydrogen production. Int J Hydrog Energy 2014;39:20958–67. doi:10.1016/j.ijhydene.2014.10.079.

[33] Bundhoo MAZ, Mohee R, Hassan MA. Effects of pre-treatment technologies on dark fermentative biohydrogen production: A review. J Environ Manage 2015;157:20–48. doi:10.1016/j.jenvman.2015.04.006.

[34] Wang J, Wan W. Factors influencing fermentative hydrogen production: A review. Int J Hydrog Energy 2009;34:799–811. doi:10.1016/j.ijhydene.2008.11.015.

[35] Davila-Vazquez G, Arriaga S, Alatriste-Mondragón F, de León-Rodríguez A, Rosales-Colunga LM, Razo-Flores E. Fermentative biohydrogen production: trends and perspectives. Rev Env Sci Biotechnol 2008;7:27–45.

[36] Wang JL, Wan W. Kinetic models for fermentative hydrogen production: A review. Int J Hydrog Energy 2009;34:3313–23. doi:10.1016/j.ijhydene.2009.02.031.

[37] Wang B, Wan W, Wang JL. Inhibitory effect of ethanol, acetic acid, propionic acid and butyric acid on fermentative hydrogen production. Int J Hydrog Energy 2008;33:7013–9. doi:10.1016/j.ijhydene.2008.09.027.

[38] Davila-Vazquez G, de León-Rodríguez A, Alatriste-Mondragón F, Razo-Flores E. The buffer composition impacts the hydrogen production and the microbial community composition in non-axenic cultures. Biomass Bioenergy 2011;35:3174–81. doi:10.1016/j.biombioe.2011.04.046.

[39] Galindo-Hernández KL, Tapia-Rodríguez A, Alatriste-Mondragón F, Celis LB, Arreola-Vargas J, Razo-Flores E. Enhancing saccharification of Agave tequilana bagasse by oxidative delignification and enzymatic synergism for the production of hydrogen and methane. Int J Hydrog Energy 2018;43:22116–25. doi:10.1016/j.ijhydene.2018.10.071.

[40] Valdez-Guzmán BE, Rios-Del Toro EE, Cardenas-López RL, Méndez-Acosta HO, González-Álvarez V, Arreola-Vargas J. Enhancing biohydrogen production from Agave tequilana bagasse: Detoxified vs. Undetoxified acid hydrolysates. Bioresour Technol 2019;276:74–80. doi:10.1016/j.biortech.2018.12.101.

[41] Wang B, Nges IA, Nistor M, Liu J. Determination of methane yield of cellulose using different experimental setups. Water Sci Technol 2014;70:599–604. doi:10.2166/wst.2014.275.

[42] Hitit ZY, Zampol Lazaro C, Hallenbeck PC. Increased hydrogen yield and COD removal from starch/glucose based medium by sequential dark and photo-fermentation using Clostridium butyricum and Rhodopseudomonas palustris. Int J Hydrog Energy 2017;42:18832–43. doi:10.1016/j.ijhydene.2017.05.161.

[43] Hansen TL, Schmidt JE, Angelidaki I, Marca E, Jansen J la C, Mosbæk H, et al. Method for determination of methane potentials of solid organic waste. Waste Manag 2004;24:393–400. doi:10.1016/j.wasman.2003.09.009.

[44] Raposo F, Fernández-Cegrí V, De la Rubia M a., Borja R, Béline F, Cavinato C, et al. Biochemical methane potential (BMP) of solid organic substrates: evaluation of anaerobic biodegradability using data from an international interlaboratory study. J Chem Technol Biotechnol 2011;86:1088–98. doi:10.1002/jctb.2622.

[45] Owen WF, Stuckey DC, Healy Jr. JB, Young LY, McCarty PL. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Res 1979;13:485–92. doi:10.1016/0043-1354(79)90043-5.

Figure and table captions

Figure 1. Evaluation of the manual protocol using glucose at 20 g L^{-1} and phosphate buffer for 6 different laboratories: hydrogen potential (A), production rate (B), molar yield achieved for consumed glucose (C), and the coefficient of variations (CV) obtained at each laboratory (intra) and interlaboratories (inter).

Figure 2. Evaluation of the manual protocol using glucose at 5 g L^{-1} comparing the phosphate and MES buffer, for 4 different laboratories using different inoculum: hydrogen production potential (A), production rate (B), molar yield achieved for consumed glucose (C), and the intralaboratory coefficients of variation (CV).

Figure 3. Evaluation of the automatic protocol using glucose at 5 g L^{-1} with MESS buffer, comparing different inoculum among 4 different laboratories: hydrogen production potential (A), production rate (B), molar yield achieved for consumed glucose (C), and the coefficient of variations (CV) obtained at each laboratory (intra) and interlaboratories (inter) for each parameter are shown.

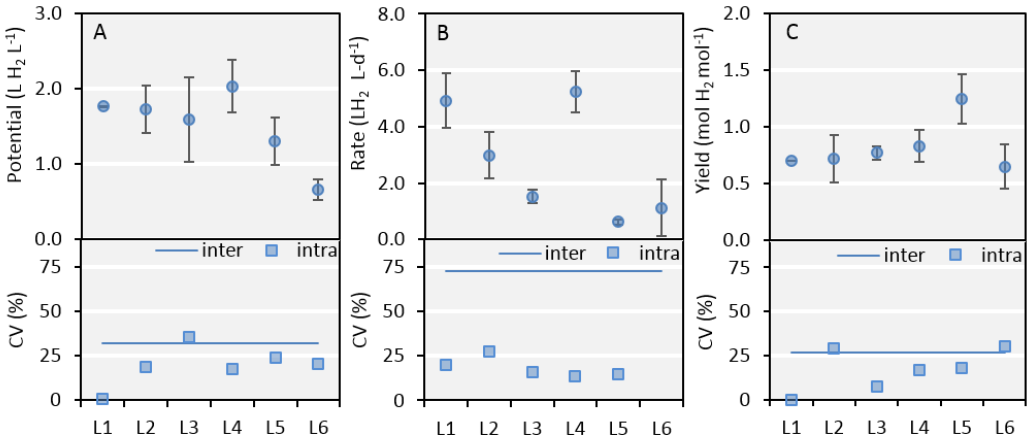
Figure 4. Molar yield per mol of consumed glucose for the metabolites produced at the different experiments: evaluation of the manual protocol using glucose at 20 g L^{-1} and phosphate buffer (A); evaluation of the manual protocol using glucose at 5 g L^{-1} comparing the MES (B) and phosphate buffer (C); evaluation of the automatic protocol with MES buffer, comparing different inoculum HTT (D), HTM (E) and HTS (F).

Table 1. One-way ANOVA results for the evaluation of the manual protocol using the same inoculum (20 g L^{-1} glucose with phosphate buffer), and the different inoculum (5 g L^{-1} glucose with phosphate and MES buffer).

Table 2. Two-ways ANOVA results for the evaluation of the manual protocol using different buffer (5 g L^{-1} glucose, phosphate and MES buffer) and the automatic protocol using different inoculum (5 g L^{-1} glucose and MES buffer).

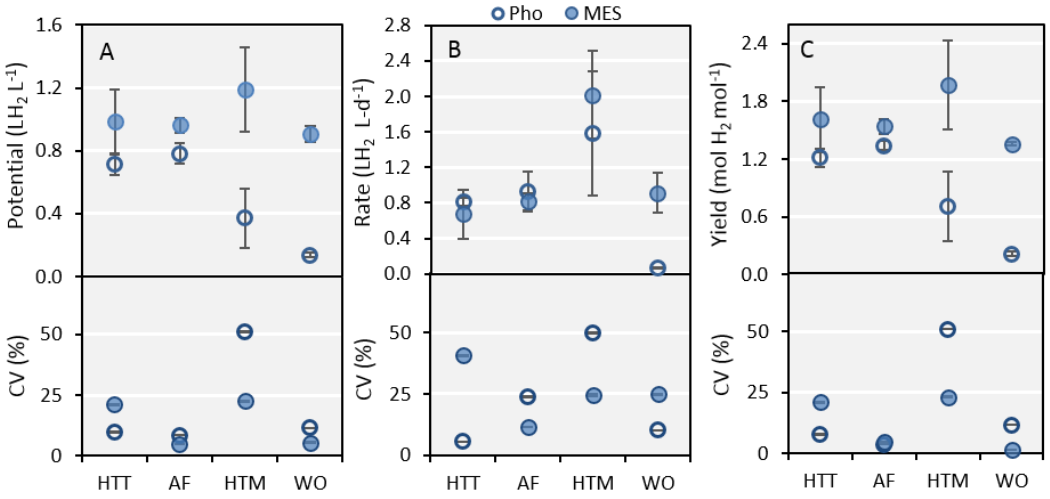
661 Table 3. Summary of the average coefficient of variations obtained at the tested parameters
662 for HP and HPR.
663
664 Table 4. Workload of BHP test by different experimental setups, considering one and five
665 samples by triplicate.
666

667
668
669 Figure 1



670
671
672

673 Figure 2

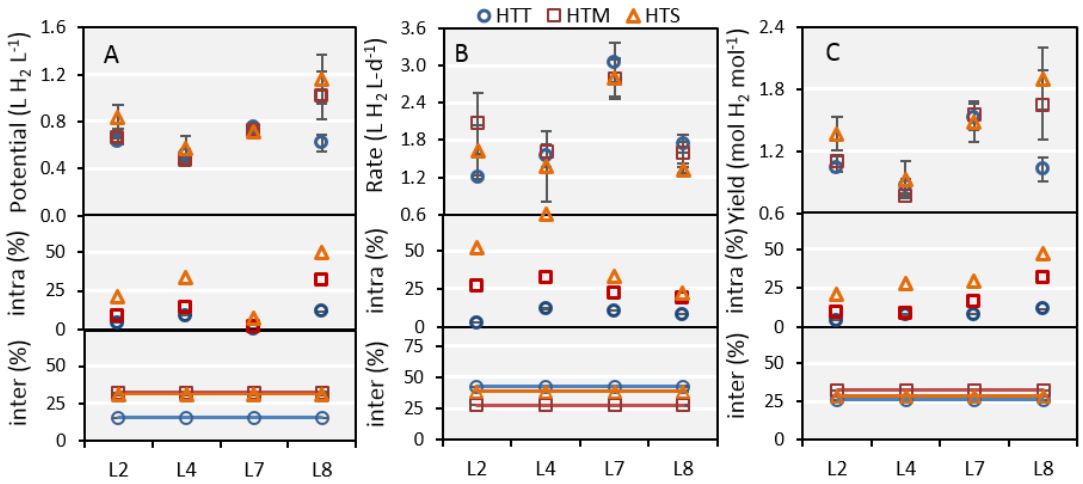


674

675

676

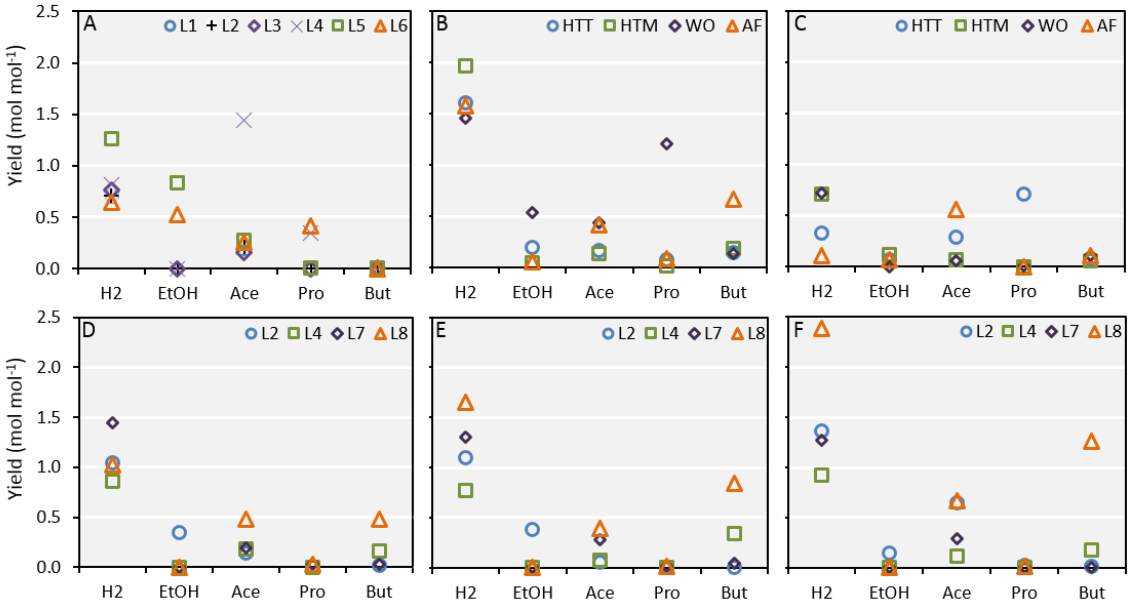
677 Figure 3



678

679

680 Figure 4



681

Table 1

Response variable	Evaluated variable	*p* value	Groups of variables*
20 g L^{-1} and phosphate buffer			
Potential	Laboratory	0.075	
Rate	Laboratory	**1.1e^{-3}**	a) L1, L2, L4
			b) L2, L3, L4
			c) L6
			d) L3, L5, L6
Yield	Laboratory	**0.022**	a) L1, L2, L3
			b) L4, L6
			c) L4, L5
5 g L^{-1} and phosphate buffer			
Potential	Laboratory	**2.7e^{-3}**	a) L1
			b) L2,L5
Rate	Laboratory	0.110	
Yield	Laboratory	**2.5e^{-4}**	a) L1, L2
			b) L5
5 g L^{-1} and MES buffer			
Potential	Laboratory	0.385	
Rate	Laboratory	0.429	
Yield	Laboratory	0.332	

* Groups without significant difference determined by a Tukey test; Bold letters stands for significant effects.

Table 2

Response variable	Evaluated variable	*p* value	Groups of variables*	Response variable	Evaluated variable	*p* value	Groups of variables *
Manual protocol				Automatic protocol			
Potential	Inoculum	**$1.5e^{-3}$**	a) HTM, AF	Potential	Inoculum	**$4.6e^{-6}$**	a) HTS
			b) HTT				b) HTT, HTM
			c) WO	Potential	Laboratory	**$1.9e^{-7}$**	a) L2, L7
Potential	Buffer	**$4.8e^{-7}$**					b) L4
Potential	Inoculum:Buffer	**$2.2e^{-3}$**					c) L8
Rate	Inoculum	**$1.0e^{-3}$**	a) AF, HTT, WO	Potential	Inoculum:Laboratory	**$3.1e^{-4}$**	
			b) HTM	Rate	Inoculum	0.065	a) L2,L4,L8
Rate	Buffer	0.205				**$4.6e^{-9}$**	b) L4,L8
Rate	Inoculum:Buffer	0.217					c) L7
Yield	Inoculum	**$2.1e^{-3}$**	a) HTM, AF, HTT	Rate	Laboratory		
			b) WO, HTT	Rate	Inoculum:Laboratory	0.052	
Yield	Buffer	**$2.7e^{-7}$**		Yield	Inoculum	**$6.4e^{-5}$**	a) HTS
Yield	Inoculum:Buffer	**0.015**					b) HTM, HTT
				Yield	Laboratory	**$1.7e^{-8}$**	a) L2, L7
							b) L4
							c) L8
				Yield	Inoculum:Laboratory	**$4.2e^{-6}$**	

* Groups without significant difference determined by a Tukey test; Bold letters stands for significant effects.

Table 3.

Method	Substrate concentration ($g\ L^{-1}$)	Buffer	HP			HPR		
			Coefficient of variation (%)*					
			intra		Inter	intra		Inter
			Ave	Min		Ave	Min	
Manual	20	Phosphate	19	1	32	30	14	72
Manual	5	Phosphate	20	9	60	22	6	73
Manual	5	MES	13	5	12	25	12	55
Automatic	5	MES	9	0.5	28	15	3	33

*Intra, intralaboratory; Inter, interlaboratory; Ave, average value; Min, minimal value.

Table 4.

Experimental set-up	A (h)	B (min)	C (min)	D (min)	E (min)	F[a] (min)	G[a,b] (min)	H[b] (min)	I (min)	Total (h)	Total/sample (h)
Set of one sample per triplicate											
Manual	5	30	30	10	-	10x7x3	10x7x3	-	15x3	13.9	4.6
Automatic	5	30	30	60	60	-	-	10x3	15x3	9.3	3.1
Set of five samples per triplicate											
Manual	5	30	150	10	-	10x7x15	10x7x15	-	15x15	46.9	3.1
Automatic	5	30	150	60	60	-	-	10x15	15x15	16.3	1.

Workload times for: A; preparation of inoculum and buffer solution; B, preparation of substrate solution; C, set up of reactors (inoculum weight, addition of substrate and buffer); D, set-up of device for gas measuring; E, connecting batch reactors to gas measuring; F, monitoring gas production during whole incubation; G, monitoring gas composition during whole incubation; H, monitoring final gas composition; I, data management (using an existing MS Excel-sheet) and interpretation. Total workload: A+B+ C+D+E+F+G+H. [a] Considering 7 gas production records during the incubation time around 75 h; [b] Considering in average 10 min for chromatographic gas analysis