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

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1 **REVISED VERSION**

2 A standardized biohydrogen potential protocol: an international round 3 robin test approach

4

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

46

47 **Abstract**

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

61

62 **Keywords**

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

64

65 ## 1. INTRODUCTION

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

75 bioenergy generation [2,3]. This shows an optimistic scenario for biohydrogen production,
76 as an added-value process to the conventional anaerobic digestion-based waste stream
77 treatment processes. Significant improvements have been made in hydrogen yields and
78 production rates in the last two decades [4].
79 Alongside the positive outlook for hydrogen production through dark fermentation, there is
80 an increasing demand for better understanding of the main factors impacting the system at a
81 process level [5]. Through the assessment of the biological hydrogen potential (BHP), the
82 information provided by former case studies has been valuable to evaluate several
83 parameters, like new potential substrates, e.g., crop residues, microalgal biomass, aquatic
84 plants [6–9]; the effect of different substrate compositions, carbohydrates content and its
85 complexity, or solids content [10–12]; and further optimizing of operational parameters like
86 substrate pretreatment [13]. However, observations are mostly depending on the type of
87 substrate, the source of inoculum and the operational conditions, wherein comparisons
88 between them become a challenging task.
89 Particularly, two major limitations have been identified when dealing with the comparison
90 of BHP results issued from the literature: one of them is the lack of fairness when
91 comparing these results with regard to the use of different protocols, and, on the other hand,
92 the diverse precision levels due to non-standardized practices (i.e., due to manual
93 operations), which causes a broad inconsistency as well as different reliability level of the
94 experimental data. In this context, the normalization of the testing guideline criterion
95 becomes a key factor in order to count with homogenized data on hydrogen production.
96 One way to attain a better comparison viability is through the establishment of a
97 comprehensive technique that can encompass the most suitable conditions reported for dark
98 fermentation, as they have been already identified in previous studies. In this sense, the
99 design and implementation of a standardized BHP protocol could allow to systematically
100 characterize and compare different process alternatives under the same technical baseline.
101 At the same time, it is important to establish quality control criteria on central parameters of
102 the process discerning whether the experimental data are valid. Nonetheless, Venkata
103 Mohan et al. [14] proposed a batch mode protocol to evaluate the BHP of wastewater. This
104 method was validated with two real substrates but only one source of inoculum and
105 pretreatment. To the best of our knowledge, no interlaboratory study to validate a batch

106 mode protocol giving an evaluation of biohydrogen potential (BHP) has been conducted so
107 far.
108 Among the most influencing parameters on hydrogen production stand the initial pH,
109 hydrogen partial pressure, temperature, as well as the acclimation of the microbial
110 communities in the inoculum for the selection of hydrogen-producing bacteria [15].
111 Specifically, Davila-Vazquez et al. [16] performed a thorough study on the effect of
112 different pH and initial substrate concentrations for different substrates on the hydrogen
113 molar yield (HMY) and the volumetric hydrogen production rate (VHPR) at a temperature
114 of 37ºC, confirming that the best set of conditions for higher HMY were initial pH 7.5 and
115 5 g L^{-1} when glucose was used as a model substrate.
116 On the other hand, there is groundwork in the effort of standardizing the method for the
117 acclimation of microbial community in the anaerobic mixed culture. This step is essential to
118 inactivate methanogens while selecting spore-forming hydrogenogenic microorganisms.
119 Some authors have converged in the higher suitability of applying thermal shock to
120 anaerobic mixed cultures at 103-106°C [17,18]. Also, due to the fact that a high partial
121 pressure will cause higher dissolved hydrogen concentration, with the consequential
122 reduction of oxidized ferredoxin and thus hindering the hydrogen production yield [3], the
123 selection of a system that allows for the continuous release of such pressure – such as an
124 automatic gas release monitoring unit – is strategic for maximizing hydrogen production
125 during BHP testing.
126 In addition, the modified Gompertz equation constitutes by far the most widely accepted
127 model to describe the kinetics of hydrogen production when using anaerobic mixed cultures
128 [19,20]. This consideration is fundamental in the establishment of a suitable BHP protocol.
129 The statement of important criteria for biochemical methane potential test has been
130 suggested [21], likewise quality control criteria such as repeatability and reproducibility of
131 results, to properly validate or reject them, and in this way, guarantee its reliability [22].
132 Such quality criteria could apply to BHP testing.
133 The aim of this study was to propose and validate a comprehensive standard protocol for
134 BHP testing in batch-mode considering all of the aforementioned conditions, in order to
135 assess the level of repeatability and reproducibility of the results, at an interlaboratory level.
136 For this purpose, the protocol was validated in several laboratories from different countries

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

143

144 2. METHODOLOGY

145 2.1 Substrate

146 Glucose was used as the only carbon source at 5 and 20 g L^{-1}. Two different buffer
147 solutions – modified from the medium proposed by Mizuno et al. [23] – were tested, one
148 based in phosphate salt and another using MES monohydrate (2-[N-Morpholino]
149 ethanesulfonic acid, 4-Morpholineethanesulfonic acid monohydrate), as suggested
150 previously [11]. The phosphate buffer composition in mg L^{-1} was: NH_4Cl, 2600; K_2HPO_4,
151 250; $MgCl_2 \cdot 6H_2O$, 125; $FeSO_4 \cdot 7H_2O$, 100; $CoCl_2 \cdot 6H_2O$, 2.5; $MnCl_2 \cdot 4H_2O$, 2.5; KI, 2.5;
152 $NiCl_2 \cdot 6H_2O$, 0.5; $ZnCl_2$, 0.5. In the MES-based buffer, the K_2HPO_4 was substituted by
153 MES at 1200 mg L^{-1}.

154

155 2.2 Sources of inoculum

156 Different sources of inoculum were tested among the laboratories: i) heat treated anaerobic
157 sludge from a thermophilic digester of sewage sludge (HTT); ii) heat treated anaerobic
158 sludge from a mesophilic full-scale UASB reactor treating brewery wastewater (HTM); iii)
159 biomass from an auto-fermented effluent rich in sucrose (AF) [24]; iv) aerobic sludge
160 pretreated by cell wash-out (WO); v) compost from kitchen wastes (C) [25]; vi) heat treated
161 anaerobic sludge from a pilot-scale digester treating the organic fraction of solid wastes
162 (HTS). The heat pretreatment procedure applied to HTT, HTM, and HTS was at 105°C for
163 24 hours to select spore-forming bacteria, suppressing potential hydrogen consumers such
164 as methanogens [17]; then the dried sludge was grounded and mesh-sieved through a #20-
165 mesh (particle size of 850 μm). During the WO pretreatment, the aerobic sludge was fed
166 into a completely stirred tank reactor with glucose at 10 g L^{-1} and at 8 h of hydraulic
167 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

199 produced was continuously released, passing through a CO_2-absorption unit (NaOH, 3N)
200 and measured online each 10 mL. Run stopped when the hydrogen production achieved the
201 stationary phase, according to the online graph shown by the software in the automatic
202 device. Gas samples from the headspace were taken at the end of the gas production for its
203 composition analysis. The automatic device reported the gas production at standard
204 conditions by using its internal temperature and pressure sensors.
205
206 **2.3 Interlaboratory tests**
207 The protocol was tested in 8 independent laboratories from 5 different countries (Brazil,
208 Chile, France, Mexico and Uruguay). Testing was divided into stages as specified in the
209 following subsections.
210
211 **2.3.1 Use of phosphate buffer in manual protocol**
212 At first instance, a set of interlaboratory experiments by 6 different independent
213 laboratories was performed under a manual procedure (2.2.1) with phosphate buffer and 20
214 g L^{-1} glucose as model substrate, using the same inoculum (HTT).
215
216 **2.3.2 Phosphate vs. MES buffer in manual protocol**
217 Manual procedure (2.2.1) was run among 4 laboratories using glucose at 5 g L^{-1}, two
218 different buffer solutions and the own inoculum from the laboratories, HTT, HTM, AF,
219 WO were evaluated as comparison.
220
221 **2.3.3 Use of MES buffer in automatic protocol**
222 Finally, the automatic procedure in AMPTS II (2.2.2) was tested using 5 g L^{-1} glucose with
223 the selected buffer solution (MES), comparing three inocula, HTT, HTM, and HTS, among
224 4 laboratories.
225
226 **2.4 Analytical methods**
227 The final soluble chemical oxygen demand (COD) was analyzed by standard methods [26],
228 and residual sugars were determined by the sulfuric acid-phenol method [27]. Soluble
229 metabolites (e.g. acetate, propionate, butyrate, ethanol) were determined by different

230 methodologies previously reported, gas chromatography with flame ionization detector
231 [17,24,25,28,29] and High Performance Liquid Chromatography (HPLC) [11,30], and
232 capillary electrophoresis [16]. H_2 and CO_2 content in the biogas were determined by gas
233 chromatography with thermal conductivity detector [11,16,17,24,25,28,29].
234
235 **2.5 Data analysis**
236 The experimental cumulative hydrogen production was fitted to the modified Gompertz
237 equation 1 [16] using the Solver add in Microsoft Excel (v 16.18, Microsoft, USA):
238 $$H(t) = H_{max} exp\left\{-exp\left[\frac{2.71828R_{max}}{H_{max}}(\lambda - t) + 1\right]\right\}$$
239 Eq.1
240 where H(t) (L) is the total volume of hydrogen produced at culture time t (d); H_{max} (L) is
241 the maximal amount of hydrogen produced; R_{max} (L d^{-1}) is the maximum hydrogen
242 production rate and λ (d) is the lag time before exponential hydrogen production. The
243 hydrogen potential (HP), the hydrogen production rate (HPR), and the hydrogen molar
244 yield (HMY), were defined as response variables. HP and HMY were calculated from H_{max}
245 and defined as L H_2 L^{-1} (per liter of working volume) and mol H_2 mol $glucose_{consumed}^{-1}$,
246 respectively; and the HPR was calculated from R_{max} and defined as L H_2 L-d^{-1}.
247 Average values and the corresponding standard deviations of response variables were used
248 to calculate the intralaboratory (within-lab) and interlaboratory (between-lab) coefficient of
249 variation (CV), or relative standard deviation, as repeatability and reproducibility
250 indicators, respectively. The statistical analysis of the data for estimate of the precision
251 statistics was a one-way and two-way analysis of variance (ANOVA), carried out for each
252 response variable, as recommended for interlaboratory studies [31]. Variables with
253 statistically significant effects were analyzed. The statistical analysis was run in the R
254 environment (v 3.1.2, RStudio, RStudio Inc.).
255
256 **3. RESULTS AND DISCUSSION**
257
258 **3.1 Effects on hydrogen potential, production rate and molar yield**

259 In the present work, BHP assays were performed by different laboratories to evaluate the
260 HP, HMY and HPR for each laboratory, using glucose as an individual model substrate
261 with mixed anaerobic consortia.
262 In experiments with 20 g L^{-1} glucose, using the manual protocol and phosphate buffer
263 (Figure 1), values for HP, HPR and HMY ranged among 0.65 – 2.03 L H_2 L^{-1}, 0.63 – 5.23
264 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
265 the same manual protocol and buffer conditions (Figure 2) the intervals were 0.14 – 0.78 L
266 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
267 studies have focused on the evaluation of BHP from different feedstocks using mixed
268 anaerobic consortia and manual protocols. Some of these studies have been intended for
269 evaluating different model substrates in individual mode [16,32], co-substrate mode [32],
270 industrial wastewater effluents [18], among others. In terms of the protocol conditions, the
271 selected for the present work was based on the best according to some previous studies on
272 batch protocols for hydrogen production [16], and using heat-treated inoculum to overcome
273 the activity of methanogenic archaea [17,33]. Several other authors have also addressed the
274 relevance of initial pH [5,15,20,34], temperature [5,15,20,34,35], and concentration of
275 inoculum and substrate [20,35]. From previous works and to maximize the production of
276 hydrogen, in this work the starting pH was at 7.5, the temperature of incubation at 37°C and
277 the substrate on inoculum ratio at 2.5 for all experiments. The suitability of the modified
278 Gompertz model to describe and to model a batch fermentative hydrogen production
279 process with mixed anaerobic cultures has been successfully proved [16,36], in this work
280 all hydrogen production kinetics were adjusted to this model.
281 The values of HP and HPR obtained for the manual mode with phosphate buffer and 20 g
282 L^{-1} glucose, were similar to the values reported in previous studies with the same substrate,
283 buffer, and a similar heat-treatment of inoculum. For instance, Davila-Vazquez et al. [16]
284 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]
285 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
286 might be due to the initial substrate/inoculum ratio. Such consistency in the range of results
287 as compared with previous works [16,32] reflects the robustness of using a batch protocol
288 and heat-treated sludge as inoculum.

289 To increase the repeatability of the proposed protocol, glucose concentration was decreased
290 to 5 g L^{-1} (as explained below, section 3.2), and two different buffers were tested. Despite
291 the substrate concentration decreased by 4-fold, the average HP and HPR only decreased by
292 50% and 62%, respectively, in comparison to using 20 g L^{-1}; and the average HMY
293 increased by 56% (Figure 1 and 2). In addition to the better HMY, lowering the glucose
294 concentration increased its consumption from 74 ± 29 to 98 ± 4 %, and increased the
295 hydrogen content in the biogas, from 43 ± 9 to 56 ± 11 %, comparing experiments with
296 initial values at 20 and 5 g L^{-1}, respectively. The average final pH using phosphate buffer
297 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}
298 could be affected by the ethanol concentration (2.6 g L^{-1}), where its accumulation at 1.14 g
299 L^{-1} has been proved to affect the metabolic pathways in dark fermentation [37]. In this
300 sense, the preferential yields of produced metabolites change from
301 ethanol>acetate>propionate to acetate>propionate>butyrate, comparing 20 to 5 g L^{-1} using
302 phosphate buffer, respectively (Figure 3, A and C).
303 When comparing phosphate with MES buffer in manual mode at 5 g L^{-1} glucose, using
304 MES improved HP, HMY and HPR by 2.20, 1.80 and 0.27-fold, respectively (Figure 2).
305 Although no other studies have addressed the use of MES buffer for H_2 production
306 protocols, this improvement could be attributed to the fact that these two buffers portray
307 clear differences in buffer capacity. In this sense, the buffer capacity for both solutions
308 from pH 7.5 to 6.5 is similar, 1.3 and 1.1 mM for MES and phosphate; respectively; but at
309 lower values from 6.5 to 4.5 the MES buffer capacity (4.1 mM) is higher than that for
310 phosphate (1 mM). This difference in the buffer capacity was reflected by the final average
311 pH comparing both experiments, being 4.4 ± 0.6 and 3.8 ± 0.5, for MES and phosphate,
312 respectively. The metabolic pathways were also driven by the buffer solution, with
313 preferential production of acetate>butyrate and acetate>propionate, using MES and
314 phosphate buffer, respectively (Figure 3, B and C). Buffer implications have also already
315 been discussed in previous works [38]. However, the buffer composition is not usually
316 adjusted to the substrate concentration, which is a relevant consideration working with
317 batch protocols without pH control.
318 Recently, the hydrogen potential of agroindustrial effluents has been tested in automatic
319 equipment [39,40]. To compare the selected parameters of this work, 5 g L^{-1} glucose and

320 MES buffer, different inocula were evaluated with the automatic protocol by different
321 laboratories.
322 Further experiments were performed at 5 g L^{-1} glucose in automatic equipment (AMPTS II)
323 with the selected buffer system (MES), achieving a level of 0.47 – 1.02 L H_2 L^{-1}, 1.60 –
324 2.79 L H_2 L-d^{-1}, and 0.78 – 1.65 mol H_2 $mol_{glucose}^{-1}$ (Figure 4). The general comparison
325 showed a similar metabolic pathway using the manual or the automatic protocol
326 (acetate>butyrate, with the same average value 0.29 and 0.28 mol/mol; Fig. 4 D to F).
327 Besides, values of glucose consumption and hydrogen content, 94 ± 6 and 53 ± 6 %
328 respectively, were similar to the achieved with the manual protocol. The closest comparison
329 of a manual and automatic protocol is the one reported by Wang et al. [41], who evaluated
330 anaerobic digestion for cellulose under both regimes, without a significant effect on
331 methane yield. In the present study for hydrogen production, using the automatic protocol
332 decrease 25% the HP and HMY compared to the manual one (Fig. 2 and 3). Only the HPR
333 was improved by the automatic protocol, becoming 70% higher than the manual one;
334 explained by the continuous gas released and its online recording. Several authors have
335 addressed the positive effects and implications of low hydrogen partial pressure (among
336 other different process conditions) on H_2 production [5,15]. Even though the slight
337 difference between HP, HPR and HMY in the manual and automatic protocol, considering
338 results with the same inoculum (HTT or HTM), the statistical analysis showed non-
339 significant difference considering p values higher than 0.01, but significant differences
340 considering p values higher than 0.05. In this sense, both methods resulted in comparable
341 results, regardless of operational differences like working volume and stirring conditions.
342 In terms of the different inoculum tested, average values were in narrow range for HP, HPR
343 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 $mol_{glucose}^{-1}$,
344 respectively. Those similar results are attributed to the same heat-treatment selecting spore-
345 forming bacteria [17], regardless of the different source of anaerobic sludge.
346 The COD measurements were stable at different evaluated conditions, with final average
347 concentrations at 19.4 ± 1.3, 5.1 ± 0.4, and 5.3 ± 0.9 g L^{-1}, for the manual protocol with 20
348 and 5 g L^{-1} glucose, and the automatic protocol, respectively; which correspond to COD
349 removals from 1-11%. Similar COD removals have been reported for dark fermentation,
350 where most of the COD is converted to acids; other strategies like coupling dark

351 fermentation to photo-fermentation are meant to increase the hydrogen productivity and
352 decrease the COD content in wastewaters [42].
353

354 **3.2 Repeatability and reproducibility evaluation**

355 The repeatability and the reproducibility of common process variables (HP, HPR and
356 HMY) were evaluated in the present international work, evaluating the effect of substrate
357 concentration and buffer solution on different inoculum (Figure 1 and 2), and the use of an
358 automatic device comparing different inoculum (Figure 4). The first interlaboratory
359 approach analyzing the same inoculum with the manual protocol and glucose at 20 g L^{-1}
360 resulted in a low reproducibility, were the variable "Laboratory" had a significant effect on
361 HPR and HMY (Table 1). This low reproducibility is explained by the intra-laboratory
362 variation (intra CV), where one-third of the laboratories had values higher than 20% for
363 HP, HPR and HMY; resulting in an inter-laboratory variation (inter CV) up to 72% (Figure
364 1). A concentration of 20 g L^{-1} was tested following the recommendation of Davila-
365 Vazquez et al. [16] between their highest HPR at 15 and 25 g L^{-1} of glucose, with intra CV
366 from 5 to 21 %, respectively. In the present study, using glucose at 20 g L^{-1} produced a
367 maximum volume of 120 mL of biogas every three hours during the exponential phase,
368 which could produce errors during the data register using the manual method, due to the
369 resolution of the graduated cylinder.
370 To increase the repeatability and reproducibility, a second approach in the present work
371 was evaluated using 5 g L^{-1} glucose, considering the lower CV (from 3 to 10%) found by
372 Davila-Vazquez et al. [16] at such concentration. Previously, several glucose
373 concentrations were tested between 5 and 25 g L^{-1}, where the lowest concentration
374 maximized the HMY [16]; in this sense, evaluating 5 g L^{-1} had the additional purpose to
375 increase the BHP. Indeed, different inoculum commonly used for each laboratory and
376 different buffer solution were evaluated. In average, the repeatability using 5 g L^{-1} glucose
377 reduced the intra CV to 18% (17 % and 20 %, for MES and phosphate buffer, respectively)
378 compared to the 22% at 20 g L^{-1}. Phosphate buffer produced significant differences in
379 terms of hydrogen potential and molar yield, whereas using MES buffer had no statistical
380 effect on hydrogen potential and molar yield, whatever the tested inoculum (Table 1). The
381 higher repeatability achieved using MES is attributed to a better buffer capacity than the

382 phosphate solution. In this sense, buffer and the microbial inoculum showed both
383 significant effects on HP and HMY (Table 2).
384 Using the automatic equipment, the repeatability improved in comparison to the manual
385 protocol, reducing the average intra CV to 9.3, 15 and 10.5% for HP, HPR and HMY
386 (Figure 4). However, such comparable reproducibility was only achieved using the HTT
387 inoculum, where inter CV of 15, 42 and 25%, were similar than the obtained using the
388 manual protocol, i.e. 12, 56 and 16%, for HP, HPR and HMY, respectively. The different
389 inoculum had a significant effect on the response variables (Table 2), resulting in higher
390 inter CV using HTS and HTM inoculum, 113 and 28% for HP and HMY, compared to the
391 obtained with HTT.
392

393 **3.3 Recommendations to run BHP batch tests**

394 To our knowledge, there is only one previous study focused on the design and
395 standardization of a methodology (protocol) to evaluate Biological Hydrogen Production
396 (BHP tests) [14]. As the authors proposed a workflow for a process optimization [14], their
397 aim was not to determine statistical parameters to evaluate the repeatability or
398 reproducibility of the protocol at interlaboratory level. The closest approaches to this work
399 are those that aim to standardize a biochemical batch protocol for methane production,
400 specially using organic solid wastes [22,43,44]; which is a common practice for the design
401 of industrial anaerobic digesters [22]. The first studies were focused on proposing general
402 parameters for methane production tests [45] and analyzing the method repeatability and
403 reproducibility [43]. Two approaches have focused on interlaboratory studies [44] and
404 general recommendations for the test validation [22]. In this sense, the results of the present
405 work are analyzed to made recommendations in terms of: i) parameters that influence the
406 repeatability and reproducibility of results, ii) criteria for results validation and acceptance,
407 iii) workload analysis of the different tested protocols.
408

409 **3.3.1 Repeatability and reproducibility**

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

413 (Table 3). In terms of reproducibility, the criteria and sequence were as follows: reducing
414 substrate concentration> increasing the buffer capacity; where using the manual or the
415 automatic protocol produced higher and lower inter CV for HP and HPR, respectively
416 (Table 3). A common strategy to optimize hydrogen producing processes is to look for the
417 maximum organic load supported [14]; however, awareness is needed on the fact that at
418 carbohydrate concentrations higher than 5 g L^{-1} repeatability decrease, and possible
419 comparison to literature will be limited due to the loss of reproducibility.
420

421 **3.3.2 Criteria for results validation and acceptance**

422 One common criterion for validation of results established for methane potential tests is the
423 methane recovery using a model substrate in comparison to the theoretical recovery [22].
424 The theoretical hydrogen yield is not a suitable criterion for accepting BHP results,
425 considering that the well-known maximum biochemical yield 4 mol H_2 $mol_{glucose}^{-1}$, depends
426 mainly on low H_2 partial pressure, the pH, and other parameters such as the nature of the
427 inoculum [5].
428 Based on the relative standard deviation proposed as acceptable by Holliger et al. [22] for
429 methane potential tests, the HP results obtained in the present work fitted to both criteria, 5-
430 10% for intralaboratory CV, and ≥ 14 % of interlaboratory CV using the manual protocol;
431 but only the intralaboratory CV criteria was fitted by the automatic protocol (Table 3).
432 However, the HPR results did not fit to any of both, having closer results if the automatic
433 protocol is used. Since BHP tests are normally carried out by only one laboratory,
434 acceptable intra CV for the HPR are 25 and 15 %, using the manual or automatic protocol,
435 respectively (average values, Table 3).
436

437 **3.3.3 Workload analysis**

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

444 mainly due to the time saved for chromatographic analysis; but the manual protocol has the
445 advantage that no specialized equipment is required. To reduce the workload analysis in the
446 manual protocol a CO_2 trap could be applied similarly to the automatic device, but the
447 proper experiment setup would need further validation.
448

449 4. CONCLUSIONS

450 In this work, a standardized protocol for biohydrogen production potential was evaluated in
451 interlaboratory mode. Evaluating the effect of operational parameters, such as substrate
452 concentration, buffer capacity and a manual or automatic gas measuring method, the
453 repeatability and reproducibility were increased. In this sense, the interlaboratory variation
454 of the HP and HPR were reduced from 32 to 12 %, and from 72 to 33 %, respectively,
455 showing the robustness of the proposed protocol. The intralaboratory variation among their
456 replicates was reduced up to 13 and 9% for HP, and up to 25 and 15 % for HPR, using the
457 manual and automatic protocol, respectively; such variations were stated as criteria for
458 acceptance of the results. The automatic protocol increases the reproducibility and
459 repeatability of the BHP test, while also reduces the workload in comparison with the
460 manual protocol, although the manual protocol also reaches acceptable variation values.
461 Hence, both protocols – manual and automatic – are suitable to get acceptable results. Due
462 to this protocol was standardized with a model substrate, further validation is envisioned
463 using real wastewater or effluents, following the same operational parameters S/X ratio,
464 soluble carbohydrate content, and buffer capacity. The present work sets a precedent for
465 further interlaboratory comparison of other worth studying parameters for hydrogen
466 production by dark fermentation.
467

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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.

505 [8] Varanasi JL, Kumari S, Das D. Improvement of energy recovery from water hyacinth
506 by using integrated system. Int J Hydrog Energy 2018;43:1303–18.
507 doi:10.1016/j.ijhydene.2017.11.110.
508 [9] Carrillo-Reyes J, Buitrón G. Biohydrogen and methane production via a two-step
509 process using an acid pretreated native microalgae consortium. Bioresour Technol
510 2016;221:324–30. doi:10.1016/j.biortech.2016.09.050.
511 [10] Guo XM, Trably E, Latrille E, Carrere H, Steyer J-P. Predictive and explicative
512 models of fermentative hydrogen production from solid organic waste: Role of
513 butyrate and lactate pathways. Int J Hydrog Energy 2014;39:7476–85.
514 doi:10.1016/j.ijhydene.2013.08.079.
515 [11] Chatellard L, Trably E, Carrère H. The type of carbohydrates specifically selects
516 microbial community structures and fermentation patterns. Bioresour Technol
517 2016;221:541–9. doi:10.1016/j.biortech.2016.09.084.
518 [12] Turhal S, Turanbaev M, Argun H. Hydrogen production from melon and watermelon
519 mixture by dark fermentation. Int J Hydrog Energy 2019;44:18811–7.
520 doi:10.1016/j.ijhydene.2018.10.011.
521 [13] Karaosmanoglu Gorgec F, Karapinar I. Biohydrogen production from hydrolyzed
522 waste wheat by dark fermentation in a continuously operated packed bed reactor: The
523 effect of hydraulic retention time. Int J Hydrog Energy 2019;44:136–43.
524 doi:10.1016/j.ijhydene.2018.08.155.
525 [14] Venkata Mohan S, Chiranjeevi P, Mohanakrishna G. A rapid and simple protocol for
526 evaluating biohydrogen production potential (BHP) of wastewater with simultaneous
527 process optimization. Int J Hydrog Energy 2012;37:3130–41.
528 doi:10.1016/j.ijhydene.2011.10.109.
529 [15] Guo XM, Trably E, Latrille E, Carrère H, Steyer J-P. Hydrogen production from
530 agricultural waste by dark fermentation: A review. Int J Hydrog Energy
531 2010;35:10660–73. doi:10.1016/j.ijhydene.2010.03.008.
532 [16] Davila-Vazquez G, Alatriste-Mondragón F, de León-Rodríguez A, Razo-Flores E.
533 Fermentative hydrogen production in batch experiments using lactose, cheese whey
534 and glucose: Influence of initial substrate concentration and pH. Int J Hydrog Energy
535 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.

566 [28] García-Depraect O, Gómez-Romero J, León-Becerril E, López-López A. A novel
567 biohydrogen production process: Co-digestion of vinasse and Nejayote as complex
568 raw substrates using a robust inoculum. Int J Hydrog Energy 2017;42:5820–31.
569 doi:10.1016/j.ijhydene.2016.11.204.
570 [29] Silva-Illanes F, Tapia-Venegas E, Schiappacasse MC, Trably E, Ruiz-Filippi G.
571 Impact of hydraulic retention time (HRT) and pH on dark fermentative hydrogen
572 production from glycerol. Energy 2017;141:358–67.
573 doi:10.1016/j.energy.2017.09.073.
574 [30] Arreola-Vargas J, Flores-Larios A, González-Álvarez V, Corona-González RI,
575 Méndez-Acosta HO. Single and two-stage anaerobic digestion for hydrogen and
576 methane production from acid and enzymatic hydrolysates of Agave tequilana
577 bagasse. Int J Hydrog Energy 2016;41:897–904. doi:10.1016/j.ijhydene.2015.11.016.
578 [31] ASTM E 691 - 99. Standard Practice for Conducting an Interlaboratory Study to
579 Determine the Precision of a Test Method. ASTM International 1999
580 [32] Gupta M, Velayutham P, Elbeshbishy E, Hafez H, Khafipour E, Derakhshani H, et al.
581 Co-fermentation of glucose, starch, and cellulose for mesophilic biohydrogen
582 production. Int J Hydrog Energy 2014;39:20958–67.
583 doi:10.1016/j.ijhydene.2014.10.079.
584 [33] Bundhoo MAZ, Mohee R, Hassan MA. Effects of pre-treatment technologies on dark
585 fermentative biohydrogen production: A review. J Environ Manage 2015;157:20–48.
586 doi:10.1016/j.jenvman.2015.04.006.
587 [34] Wang J, Wan W. Factors influencing fermentative hydrogen production: A review. Int
588 J Hydrog Energy 2009;34:799–811. doi:10.1016/j.ijhydene.2008.11.015.
589 [35] Davila-Vazquez G, Arriaga S, Alatriste-Mondragón F, de León-Rodríguez A, Rosales-
590 Colunga LM, Razo-Flores E. Fermentative biohydrogen production: trends and
591 perspectives. Rev Env Sci Biotechnol 2008;7:27–45.
592 [36] Wang JL, Wan W. Kinetic models for fermentative hydrogen production: A review.
593 Int J Hydrog Energy 2009;34:3313–23. doi:10.1016/j.ijhydene.2009.02.031.
594 [37] Wang B, Wan W, Wang JL. Inhibitory effect of ethanol, acetic acid, propionic acid
595 and butyric acid on fermentative hydrogen production. Int J Hydrog Energy
596 2008;33:7013–9. doi:10.1016/j.ijhydene.2008.09.027.

597 [38] Davila-Vazquez G, de León-Rodríguez A, Alatriste-Mondragón F, Razo-Flores E. The
598 buffer composition impacts the hydrogen production and the microbial community
599 composition in non-axenic cultures. Biomass Bioenergy 2011;35:3174–81.
600 doi:10.1016/j.biombioe.2011.04.046.
601 [39] Galindo-Hernández KL, Tapia-Rodríguez A, Alatriste-Mondragón F, Celis LB,
602 Arreola-Vargas J, Razo-Flores E. Enhancing saccharification of Agave tequilana
603 bagasse by oxidative delignification and enzymatic synergism for the production of
604 hydrogen and methane. Int J Hydrog Energy 2018;43:22116–25.
605 doi:10.1016/j.ijhydene.2018.10.071.
606 [40] Valdez-Guzmán BE, Rios-Del Toro EE, Cardenas-López RL, Méndez-Acosta HO,
607 González-Álvarez V, Arreola-Vargas J. Enhancing biohydrogen production from
608 Agave tequilana bagasse: Detoxified vs. Undetoxified acid hydrolysates. Bioresour
609 Technol 2019;276:74–80. doi:10.1016/j.biortech.2018.12.101.
610 [41] Wang B, Nges IA, Nistor M, Liu J. Determination of methane yield of cellulose using
611 different experimental setups. Water Sci Technol 2014;70:599–604.
612 doi:10.2166/wst.2014.275.
613 [42] Hitit ZY, Zampol Lazaro C, Hallenbeck PC. Increased hydrogen yield and COD
614 removal from starch/glucose based medium by sequential dark and photo-
615 fermentation using Clostridium butyricum and Rhodopseudomonas palustris. Int J
616 Hydrog Energy 2017;42:18832–43. doi:10.1016/j.ijhydene.2017.05.161.
617 [43] Hansen TL, Schmidt JE, Angelidaki I, Marca E, Jansen J la C, Mosbæk H, et al.
618 Method for determination of methane potentials of solid organic waste. Waste Manag
619 2004;24:393–400. doi:10.1016/j.wasman.2003.09.009.
620 [44] Raposo F, Fernández-Cegrí V, De la Rubia M a., Borja R, Béline F, Cavinato C, et al.
621 Biochemical methane potential (BMP) of solid organic substrates: evaluation of
622 anaerobic biodegradability using data from an international interlaboratory study. J
623 Chem Technol Biotechnol 2011;86:1088–98. doi:10.1002/jctb.2622.
624 [45] Owen WF, Stuckey DC, Healy Jr. JB, Young LY, McCarty PL. Bioassay for
625 monitoring biochemical methane potential and anaerobic toxicity. Water Res
626 1979;13:485–92. doi:10.1016/0043-1354(79)90043-5.
627
628

629 **Figure and table captions**
630
631 Figure 1. Evaluation of the manual protocol using glucose at 20 g L^{-1} and phosphate buffer
632 for 6 different laboratories: hydrogen potential (A), production rate (B), molar yield
633 achieved for consumed glucose (C), and the coefficient of variations (CV) obtained at each
634 laboratory (intra) and interlaboratories (inter).
635
636 Figure 2. Evaluation of the manual protocol using glucose at 5 g L^{-1} comparing the
637 phosphate and MES buffer, for 4 different laboratories using different inoculum: hydrogen
638 production potential (A), production rate (B), molar yield achieved for consumed glucose
639 (C), and the intralaboratory coefficients of variation (CV).
640
641 Figure 3. Evaluation of the automatic protocol using glucose at 5 g L^{-1} with MESS buffer,
642 comparing different inoculum among 4 different laboratories: hydrogen production
643 potential (A), production rate (B), molar yield achieved for consumed glucose (C), and the
644 coefficient of variations (CV) obtained at each laboratory (intra) and interlaboratories
645 (inter) for each parameter are shown.
646
647 Figure 4. Molar yield per mol of consumed glucose for the metabolites produced at the
648 different experiments: evaluation of the manual protocol using glucose at 20 g L^{-1} and
649 phosphate buffer (A); evaluation of the manual protocol using glucose at 5 g L^{-1} comparing
650 the MES (B) and phosphate buffer (C); evaluation of the automatic protocol with MES
651 buffer, comparing different inoculum HTT (D), HTM (E) and HTS (F).
652
653 Table 1. One-way ANOVA results for the evaluation of the manual protocol using the same
654 inoculum (20 g L^{-1} glucose with phosphate buffer), and the different inoculum (5 g L^{-1}
655 glucose with phosphate and MES buffer).
656
657 Table 2. Two-ways ANOVA results for the evaluation of the manual protocol using
658 different buffer (5 g L^{-1} glucose, phosphate and MES buffer) and the automatic protocol
659 using different inoculum (5 g L^{-1} glucose and MES buffer).
660

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

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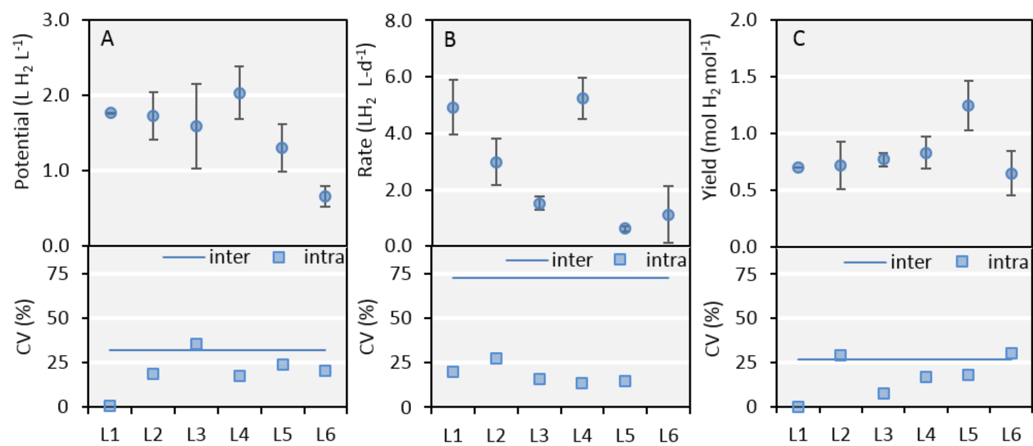
668

669 Figure 1



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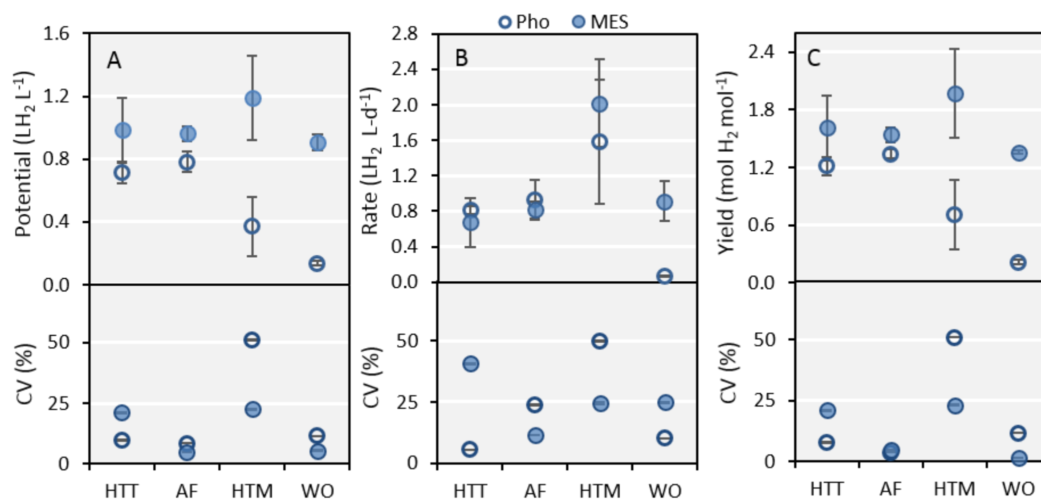
673 Figure 2



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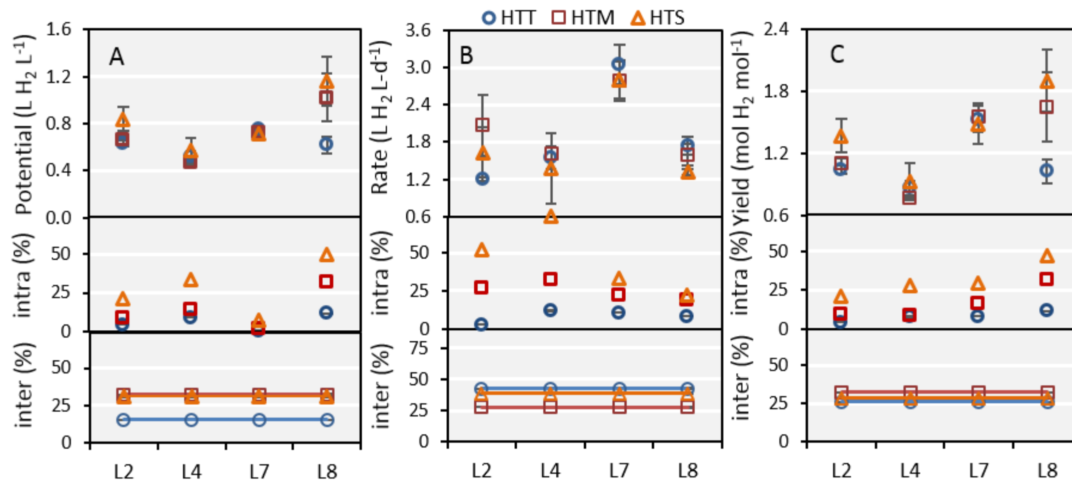
677 Figure 3



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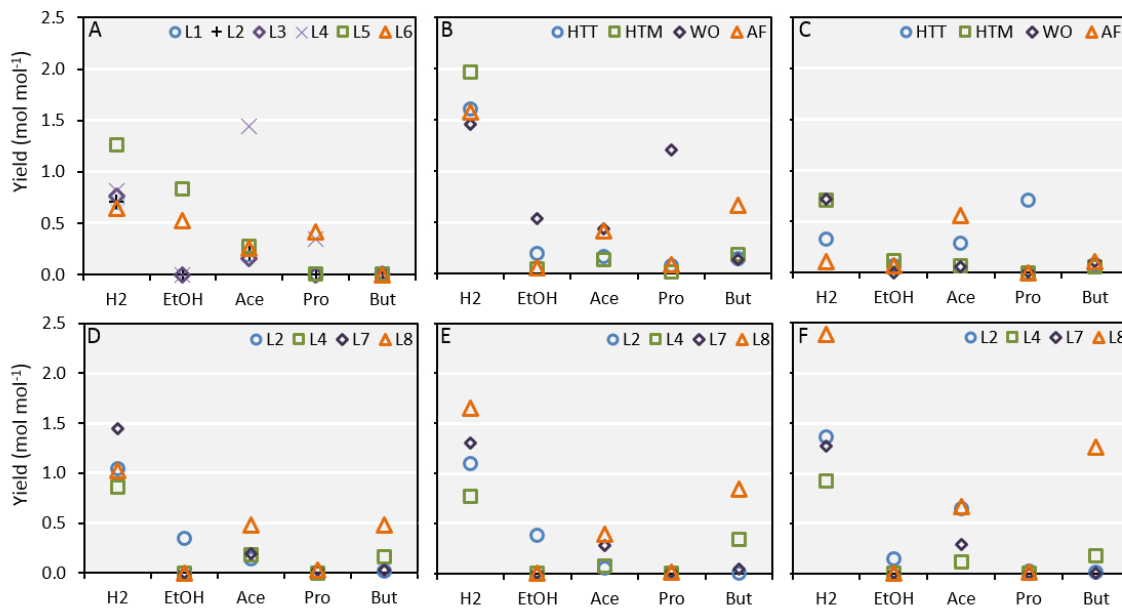
680 Figure 4



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*
Manual protocol			
Potential	Inoculum	**$1.5e^{-3}$**	a) HTM, AF b) HTT c) WO
Potential	Buffer	**$4.8e^{-7}$**	
Potential	Inoculum:Buffer	**$2.2e^{-3}$**	
Rate	Inoculum	**$1.0e^{-3}$**	a) AF, HTT, WO b) HTM
Rate	Buffer	0.205	
Rate	Inoculum:Buffer	0.217	
Yield	Inoculum	**$2.1e^{-3}$**	a) HTM, AF, HTT b) WO, HTT
Yield	Buffer	**$2.7e^{-7}$**	
Yield	Inoculum:Buffer	**0.015**	

Response variable	Evaluated variable	*p* value	Groups of variables *
Automatic protocol			
Potential	Inoculum	**$4.6e^{-6}$**	a) HTS b) HTT, HTM
Potential	Laboratory	**$1.9e^{-7}$**	a) L2, L7 b) L4 c) L8
Potential	Inoculum:Laboratory	**$3.1e^{-4}$**	
Rate	Inoculum	0.065 **$4.6e^{-9}$**	a) L2,L4,L8 b) L4,L8 c) L7
Rate	Laboratory		
Rate	Inoculum:Laboratory	0.052	
Yield	Inoculum	**$6.4e^{-5}$**	a) HTS 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