

Enhancement of mass transfer conditions to increase the productivity and efficiency of dark fermentation in continuous reactors

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- 1 Enhancement of mass transfer conditions to increase the productivity and efficiency
- 2 of dark fermentation
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- 19

20 Abstract

21 Hydrogen (H₂) produced by dark fermentation is an alternative to fulfill the requirements of the 22 transportation sector and to be a complementary source in the forthcoming electricity grid. 23 However, the dark fermentative H₂ production is limited by the accumulation of H₂ in the fermentation broth. In continuous stirred-tank reactors (CSTR), such phenomenon is associated 24 25 with poor mass transfer conditions. Nevertheless, this parameter has been scarcely considered to enhance H₂ production. In this research, the effect of the H₂ mass transfer conditions on the 26 productivity and efficiency of H_2 production was evaluated using a series of CSTR operated at H_2 27 mass transfer coefficients (k_La) ranging from 1.04 to 4.23 1/h. The results showed that volumetric 28 H₂ production rate (VHPR) and H₂ yield increased 74 and 78%, respectively, due to enhanced mass 29 30 transfer conditions. This behavior was driven by 53% decrease of the dissolved H₂ concentration. 31 More specifically, a maximum VHPR of 7.66 L/L-d with a H₂ yield of 1.1 mol H₂/mol hexose was obtained at a $k_La = 4.23$ 1/h. Furthermore, 16S-DGGE analysis and sequencing revealed that 32 33 *Clostridium* and *Lactobacillus* were the dominant bacterial genera in continuous operation. In 34 particular, *Clostridium* increased its occurrence at k_La of 2.72-4.23 1/h as a response to lower dissolved H₂ concentrations. The novelty of this work relies on the demonstration that mass transfer 35 conditions controls H₂ accumulation and enhances the reactor performance for H₂ production. 36

- 37 Key words: biohydrogen; CSTR; dark fermentation; mass transfer
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- 39
- 40

42 1. Introduction

43

sustainable development. In this regard, biofuels are expected to play a relevant role to fulfill future 44 45 requirements of the transportation sector and to be a complementary source in the forthcoming electricity grid. In this context, molecular hydrogen (H₂) has been underlined due to its high energy 46 47 content (120 kJ/g) and the highly efficient conversion to electricity through the H₂ fuel-cell technology. Moreover, H₂ can be produced from a wide range of residual biomass through dark 48 fermentation, which also makes it an attractive option in waste valorization scenarios. 49 50 The production of H₂ through dark fermentation is achieved by anaerobic microorganisms that use two principal metabolic routes: the pyruvate formate lyase and the pyruvate ferredoxin 51 oxydoreductase pathways [1,2]. These routes are associated with maximum theoretical metabolic 52 yields of 2 and 4 mol H₂/mol hexose, respectively. However, most studies in literature report H₂ 53 yields below these thresholds. The low H₂ production depends on multiple factors, including the 54 microbial community composition, the operational conditions and the efficiency of liquid-gas mass 55 transfer. The importance of this later topic relies on the fact that dissolved H₂ concentration can 56 exert a thermodynamic control on H2-associated metabolic pathways. For instance, as H2 57 accumulates ($P_{H2} > 60$ Pa), H_2 synthesis from ferredoxin becomes theoretically unfeasible. As a 58 consequence, the maximum H₂ yield decreases from 4 to 2 mol H₂/mol hexose [3]. Further 59 accumulation of H_2 in the liquid phase ($P_{H2} > 500$ Pa) leads to the occurrence of homoacetogenesis, 60 61 which is the metabolism through which acetate is synthesized from H_2 and CO_2 [4–6]. In dark fermentative systems, different alternatives have been proposed to enhance H₂ mass transfer 62 63 [7–13]. Mechanical stirring can be highlighted due to its low-cost and ease of implementation.

The diversification of energy sources is a critical keystone in the contemporaneous vision of

64 Using mechanical stirring, Beckers et al. [13] successfully showed that H₂ yield was positively

65 linked to the increase of the volumetric mass transfer coefficient (k_La), which was controlled in turn 66 by the stirring velocity and gas sparging.

In continuous regime, it can be hypothesized that different steady states of performance could arise as a function of mass transfer conditions with concomitant changes in metabolic pathways and microbial community composition. In this regard, the aim of this work was to compare steady state performances of dark fermentative systems at different conditions of mass transfer (associated with different stirring velocities) with a special focus on the potential shifts in metabolic pathways and microbial communities.

73 2. Material and methods

74 2.1 Inoculum and fermentation medium

75 The seed sludge was obtained from a full-scale UASB reactor treating wastewater from a tequila

⁷⁶ factory (Casa Herradura, Jalisco, Mexico). Before its use, the sludge was heat treated at 105 °C for

77 24 h, pulverized in a mortar, and sieved through 0.5 mm mesh. The resulting powder was added to

the reactor at a total solids (TS) concentration of 4.5 g TS/L for the startup of CSTR I (Section 2.2).

79 In all the fermentation experiments, cheese whey powder (CWP) (Darigold, USA) with a lactose

so content of 75.5% was used as substrate at a fixed inlet concentration of 15 g lactose/L. The

81 fermentation medium was supplemented with the following components as described previously

82 (mg/L) [14]: NH₄Cl, 2110; MgCl₂·6H₂O, 100; CuCl₂·H₂O, 1.25; MnCl₂·4H₂O, 7; FeCl₂·4H₂O,

19.1; NiCl₂·6H₂O, 102.5. In addition, a phosphate buffer (KH₂PO₄-Na₂HPO₄, pH 5.9) was added to

reach a final concentration of 100 mM.

85 2.2 Bioreactors set-up and operational conditions

A series of five CSTR (Applikon Biotechnologies, USA) with a working volume of 1 L (9.5 cm of internal diameter and 70.88 cm² liquid-gas transfer area) was set up as shown in Fig. S1. The bioreactors were equipped with a stirrer and two Rushton-type impellers symmetrically positioned along the depth of the reactor working-volume.

CSTR I was inoculated with the pretreated anaerobic sludge (section 2.1) at a concentration of 4.5 g 90 TS/L and started-up in batch mode for 24 h with an initial substrate concentration of 15 g lactose /L. 91 Stirring, temperature and pH were controlled at 250 rpm, 37 °C and 5.9, respectively. Thereafter, 92 the reactor was switched to continuous mode with a fixed hydraulic retention time (HRT) of 6 h. 93 The reactor was monitored for a minimum of 20 HRT equivalents, i.e. 5 days, and until a steady 94 95 state was reached in terms of volumetric H₂ production rate (VHPR). The steady state was defined 96 as the phase where the variation of three consecutive measurements was less than 10% of the 97 VHPR. In such state, enough volume of effluent was recovered and centrifuged at 3500 rpm for 10 min at 4°C. The resulting pellets were re-suspended in mineral medium without substrate, 98 99 characterized in terms of volatile suspended solids (VSS) and stored at -4 °C until their use as inoculum in four more reactors CSTR II to V. CSTR II - V were inoculated with the recovered 100 biomass from CSTR I at a concentration of 0.45 g VSS/L. The start-up strategy and operational 101 conditions were maintained identical to CSTR I, except for the stirring velocity, which was set at 102 103 100, 200, 300 and 400 rpm in CSTR II, CSTR III, CSTR IV and CSTR V, respectively. CSTR I – V were also operated and monitored for a minimum of 20 HRT and until stable VHPR was observed. 104

105 2.3 Analytical methods

106 Liquid samples were collected on a regular basis and used to determine the concentrations of

107 biomass, soluble chemical oxygen demand (COD), total carbohydrates and short-chain volatile fatty

acids (VFA). Biomass concentration (as volatile suspended solids, VSS) and soluble COD were

109 quantified as described in standard methods [15]. The concentration of total carbohydrates was

determined by the phenol sulfuric method [16]. VFA were quantified from filtered (22 µm) samples
by capillary electrophoresis (1600A, Agilent Technologies, Waldbronn, Germany) as reported
elsewhere [17]. All H₂ and VFAs yields were calculated considering the amount of hexose
consumed.

Gas production was measured through a liquid displacement device (SEV. Puebla, Mexico), and its composition (H_2 and CO_2) was determined through a gas chromatograph equipped with a thermal conductivity detector (6890N, Agilent Technologies, Waldbronn, Germany). All gas volumes were reported at 1 atm and 273.15 K.

118 2.4 Determination of k_La and dissolved H_2 concentration

To determine H₂ mass transfer coefficients (k_La, 1/h) of the CSTR, a series of O₂ desorption 119 120 experiments were performed at 50, 100, 200, 300 and 400 rpm using the gas-out method described elsewhere [13]. In brief, the reactor vessel was filled with mineral medium without substrate and 121 inoculum. Stirring, temperature and pH were set to 50-400 rpm, 37 °C and 5.9, respectively. The 122 system was assembled with an electrode to measure and record dissolved oxygen (DO) 123 concentrations. For each experiment, the system was first degassed with N2 and then flushed with 124 pure oxygen until the dissolved O₂ concentration reached 100%. Afterwards, the O₂ sparging was 125 ceased and the decrease of the dissolved gas concentration was recorded until equilibrium was 126 reached. Obtained data were normalized and adjusted to the following desorption equation: 127

128
$$[DO(t)] = [DO]_{t=0} * e^{-(k_L a)_{O2} * t}$$
 (1)

Where *t* (h) represents the elapsed time and $(k_L a)_{O_2}$ is the volumetric mass transfer coefficient of O₂. The resulting $(k_L a)_{O_2}$ value altogether with the O₂ and H₂ diffusivities $(D_{O_2} \text{ and } D_{H_2},$

131 respectively) were used to compute the $(k_L a)_{H_2}$ considering the following relationship [13]:

132
$$(k_L a)_{H_2} = (k_L a)_{O_2} * \left(\frac{D_{H_2}}{D_{O_2}} \right)^{1/2}$$
 (2)

133 Where D_{H_2} and D_{O_2} were 5.91 x 10⁻⁵ and 2.62 x 10⁻⁵ cm²/s at 40 and 37 °C, respectively [18,19].

Furthermore, to calculate dissolved H₂ concentrations at the different mass transfer conditions, it was first considered that mass transfer of H₂ from the liquid to the gas phase (Q_{H_2} , in mol_{H2}/L-h) can be described as follows:

137
$$Q_{H_2} = (k_L a)_{H_2} * (C_{H_2, liq} - p_{H_2, gas} * H^{cp})$$
(3)

Where $(k_L a)_{H_2}$ (1/h) is the volumetric mass transfer coefficient for H₂, $C_{H_2,liq}$ (mol/L) is the concentration of dissolved H₂, $p_{H_2,gas}$ (atm) is the H₂ partial pressure of the headspace, and H^{cp} (mol/L-atm) is the Henry's coefficient of H₂ (8.47 x10⁻⁴ mol/L-atm at 37°C, Sander, 2015). Solving the equation for $C_{H_2,liq}$ (Eq. 4), we obtain an expression with two known constants (i.e. $k_L a$ and H^{cp}) and two variables that can be derived from bioreactors operation (i.e. Q_{H_2} and $p_{H_2,gas}$).

143
$$C_{H_2,liq} = \frac{Q_{H_2}}{\{k_L a\}_{H_2}} + p_{H_2,gas} * H^{cp}$$
 (4)

144 2.5 Microbial community analysis

To investigate the microbial community structure and identify potential changes in response to
differences of mass transfer conditions, a PCR-DGGE approach was followed as described
previously [21]. DNA was extracted from biomass recovered after 24h of batch cultivation (start-up
phase) and at the end of each stage using a DNA extraction kit (Zymo-Research, USA). The 16S
rRNA gene was amplified by PCR using the 27F (5'-AGAGTTTGATCCTGGCCAG) and 1492R
(5'-GGTTACCTTGTTACGACTT) universal primers for bacteria. A nested PCR was conducted
with amplicons from the previous stage and primers 357F-GC (5'-

AGCAG-3') and 907R (5'-CCGTCAATTCMTTTGAGTTT) to amplify the V3 - V5 regions. The

154 PCR products were loaded in polyacrylamide gels (8%) with a denaturing gradient (urea-

formamide) that ranged from 30 to 60 %. The DGGE electrophoresis conditions were 70 V for 20 h

at 60 °C. After electrophoresis, the gel was fixed with acetic acid (10%), treated with a AgNO₃

solution (1 g/L) and revealed with a Na₂CO₃ (23.3 g/L) solution. The gel bands were photographed

under visible light with a digital camera. The DGGE images were analyzed with the BioNumeric

bioinformatics software (Applied Maths, Belgium) to create a presence-absence matrix from which

160 Euclidean distances were calculated. The distances between the DGGE profiles were visualized

through a UPGMA dendrogram computed in the R environment [22]. Furthermore, selected DNA

bands were cut, reamplified by PCR using 341F (without GC-clamp) and 907R primers, and

sequenced by the dideoxynucleotides method in a 3130 Genetic Analyzer (Applied Biosystems,

164 USA). DNA sequences were edited to remove low quality nucleotides with the BioEdit software

(Ibis Therapeutics, USA). Edited sequences were compared with the reference 16S-rRNA databaseof NCBI to find the closest relatives.

167 2.6 Hydrogen consumption

168 To evaluate the homoacetogenic activity under the different mass transfer conditions tested, the 169 following mass balance on H_2 , acetate and butyrate was performed as suggested elsewhere [23,24]:

170 Homoacetogenic acetate =
$$\frac{(2 \cdot Acetate + 2 \cdot Butyrate - Propionate - H_2)}{6}$$
 (5)

171 Where VFA and H_2 are given in mol/d.

To confirm and characterize the H_2 consumption capacity of the microbial community, a series of H₂ consumption experiments was also carried out. These experiments were conducted in 120 mL serum bottles with a working volume of 80 mL, using biomass harvested from the CSTR III (200

rpm) as inoculum. For this purpose, enough volume of effluent was recovered from CSTR III and 175 176 centrifuged at 3500 rpm for 10 min at 4°C. Resulting pellets were re-suspended in mineral medium (section 2.1), characterized in terms of VSS, and stored at -4 °C until their use. The experiments 177 were prepared with an initial concentration of 2 g VSS/L using the mineral medium with the 178 composition described in section 2.1 and supplemented with 560 mg/L of NaHCO₃. No organic 179 substrate was added. The serum bottles were hermetically sealed and the headspace displaced first 180 181 with N₂ and then with pure H₂. A second set of experiments was prepared identically, but H₂ was pressurized at 1.4 atm. Two additional bottles were prepared, one without biomass and the other 182 without H₂, to serve as physicochemical and endogenous controls, respectively. All experiments 183 184 were incubated at 37 °C. The H₂ consumption was computed from the decrease of the system 185 pressure and headspace composition.

186 The cumulative H_2 consumption was modelled utilizing the Gompertz model [25,26]:

187
$$H_{2_{cummulative}}(t) = H_{max} \cdot exp\left\{-exp\left[\frac{2.71828 \cdot R_{max}}{H_{max}}(\lambda - t) + 1\right]\right\}$$
(6)

188 Where $H_{2_{cumulative}}(t)$ (mmol) is the cumulative H₂ consumed at time t, H_{max} (mmol) is the 189 maximum amount of H₂ consumed in the experiment, R_{max} (mmol/h) is the maximum rate of H₂ 190 consumption, and λ (h) is the lag time before H₂ consumption.

191 2.7 Statistic analysis

To evaluate the effects of mass transfer conditions on the different response variables of this study, an analysis of variance was conducted. The effect was considered to be significant at a p value lower than 0.05. The response variable was verified to be normally distributed through graphical inspection (q-q plot). The heteroscedasticity was also verified with residual plots. Variables not normally distributed were transformed previously to the analysis. Alternatively, the non-parametric test of Kruskal-Wallis was used. All statistical analysis were conducted with R software [22].

198 **3. Results and discussion**

199 $3.1 \text{ k}_{\text{L}}a$ determination in the dark fermentative system

The $(k_L a)_{H_2}$ coefficients, named here " $k_L a$ " for simplicity, were determined in accordance with previously reported methodology [13]. The $k_L a$ values were in the range of 0.58 - 4.23 1/h and function of the stirring velocity (Supplementary Information Table S1). Such values are specific for the configuration, geometry and specific transfer area of the reactor. Nevertheless, these results are consistent to similar systems as reported elsewhere [13,27]. The subsequent biological experiments were conducted under rpm ≥ 100 rpm to avoid possible stagnation at lower speeds.

- 3.2 The H₂ mass transfer conditions as a mechanism controlling the productivity and the efficiency
 of dark fermentation
- CSTR I was operated for an equivalent time of 34 HRT (8.5 days) at an organic loading rate (OLR) 208 209 of 60 g lactose/L-d with the main objective of producing seed-biomass for subsequent experiments (CSTR II - V). The performance of CSTR I was stable in terms of productivity and efficiency, with 210 211 an average VHPR of 7.1 \pm 1.0 L/L-d and H₂ yield of 0.94 \pm 0.1 mol H₂/mol hexose. Under similar 212 OLR (55.4 g lactose/L-d), Davila-Vazquez et al. [28] reported a VHPR of 8.8 L/L-d and an H₂ yield of 1.2 mol H₂/mol hexose, which was similar to the results showed in the present work. The 213 214 stability of H₂ production and the similarity with previous reports were clear indications of a successful establishment of the dark fermentative H₂ production. Thus, the biomass was then 215 recovered to serve as inoculum in the following experiments. 216
- CSTR II to V were independently operated at k_La values in the range of 1.04 to 4.23 1/h, associated with stirring velocities ranging from 100 to 400 rpm (Table S1). The results showed that the mass transfer coefficient strongly affected dark fermentation in terms of VHPR ($F_{3, 50}=13.05$, p<0.05) and H_2 yield ($F_{3, 50}=13.04$, p<0.05) (Fig. 1). At the lowest k_La tested (1.04 1/h), the VHPR was 4.4 ± 1.3

221 L/L-d with an H₂ yield of 0.6 ± 0.15 mol H₂/mol hexose. In contrast, at the highest value of k_La 222 (4.23 1/h), an average VHPR of 7.66 ± 1.42 L/L-d was obtained with an H₂ yield of 1.08 ± 0.21 mol 223 H₂/mol hexose. These results represent an increase of 74% in terms of VHPR and 78% in terms of 224 H₂ yield.

225	The extent of improvement, in both VHPR and H ₂ yield, achieved in this work was consistent with
226	previous reports that focused on H_2 mass transfer (Table S2). For instance, Beckers et al. [13]
227	reported an improvement of approximately 89 and 19 % in terms of H_2 production rate and H_2
228	yield, respectively, in an anaerobic stirred tank reactor after having increased the stirring conditions
229	from 0 to 400 rpm. Using CO ₂ sparging, Kim et al. [7] were able to increase the VHPR and H ₂ yield
230	in a CSTR by 56% and 118%, respectively. Nevertheless, such an approach resulted in H_2 dilution,
231	which is not convenient for practical applications. In comparison, with optimized mixed conditions
232	reported in the present work, remarkable increases in the VHPR and H ₂ yield were reached,
233	avoiding the utilization of additional gases and, therefore, the H ₂ produced remains concentrated.
234	3.3 Enhancement of mass transfer conditions intensifies metabolic routes leading to H_2 production
235	To investigate the influence of mass transfer conditions on metabolic pathways, main VFA (i.e.
236	formate, acetate, butyrate and lactate) concentrations were determined at the different conditions of
237	stirring velocities. Results revealed that the enhancement of H ₂ mass transfer performance was
238	accompanied by an increase of the VFA molar yield. In particular, significant differences in acetate
239	and butyrate production yields (Fig. 1 A and B) were observed due to the enhancement of H_2
240	transfer conditions. In CSTR II, with k_La of 1.04 1/h, the production of acetate and butyrate was
241	0.29 and 0.31 mol/mol hexose, respectively. Meanwhile, in CSTR V, with k_La of 4.23 1/h, the
242	acetate and butyrate yields increased up to 0.44 and 0.5 mol/mol hexose, respectively. This finding
243	is consistent with the fact that the acetate and butyrate pathways are the most efficient routes in
244	terms of H ₂ production by dark fermentation.

On the other hand, H₂ concentrations in the fermentation broth (Fig. 2 C) clearly indicated that the 245 246 increase of acetate and butyrate yields were associated with the change of mass transfer conditions and the subsequent decrease of dissolved H₂. The theoretical H₂ concentrations in the fermentation 247 liquid were in the range of 7.5 to 3.4 mmol H_2/L , with the lowest value at the k_La of 4.23 1/h. 248 Nevertheless, considering the results of VFA and dissolved H₂ concentration, as well as the VHPR 249 250 and H₂ yield (Fig. 1), it seemed that mass-transfer did not affect H₂ metabolism beyond a k_La of 2.6 251 1/h, i.e. 300 rpm. Thus, the operation at 300 rpm was considered as the most suitable stirring velocity for this type of reactor. 252

253 3.4 Microbial community

The analysis of the 16S rRNA-DGGE (Fig. 3) and the sequencing results (Table 1) showed that the 254 microbial community was mainly composed of *Clostridium* and *Lactobacillus* species. These two 255 genera have been previously reported to play important roles in dark fermentative systems 256 [14,29,30]. *Clostridium spp.* are mostly related to H₂-producing bacteria and are widely found in 257 dark fermentative systems associated with high efficiencies (e.g. [1,31]). In contrast, Lactobacillus 258 spp. correspond to lactic acid bacteria that have been identified as substrate competitors of H₂-259 producers during the fermentation of cheese whey [32]. Moreover, it has been shown that the 260 261 abundance of lactic acid bacteria increased under relatively high organic loading rates (58.8 and 88.2 g lactose/L-d), and it was likely associated with the accumulation of H₂ in the fermentative 262 medium [29]. 263

Interestingly, the DGGE analysis showed that *Lactobacillus* (band at DGGE relative distance ~ 68) was more abundant in the samples taken at the end of CSTRs operation (Fig. 3), suggesting that these microorganisms were enriched during the H₂ production process. DGGE profiles also revealed that bands associated with *Clostridium* (two bands at DGGE relative distances of 45 and 50, approximately) were less intense in the samples taken at the end of CSTR II and CSTR III (1.04 and 1.64 1/h) than those taken in CSTR IV and CSTR V (2.72 and 4.23 1/h). Altogether, these findings indicate that H_2 transfer conditions did not only affect the productivity, efficiency and metabolism in dark fermentative systems, but they have also important implications on shaping microbial communities of dark fermentation.

273 3.5 The dual capacity of hydrogenogenic microorganisms

274 As largely reported, several dark fermentative species issued from the *Clostridium* genus have the 275 capacity to consume H_2 through the homoacetogenesis pathway in response to high dissolved H_2 276 concentrations. To quantify this activity in mixed cultures, previous studies reported an approach 277 based on balancing the acetate, butyrate and H₂ productivities [23,24]. In accordance with this method, it was found that homoacetogenic acetate production rates ranged from 31.1 to 68.6 mol 278 acetate/L-d. In terms of percentage, the acetate produced by homoacetogenesis was 32-46 % of total 279 280 acetate quantified in the systems (Table 2). Similar values were reported elsewhere by Luo et al. [24] at different conditions of pH, temperature and sludge pretreatment. Also, in UASB reactors, 281 Carrillo-Reyes et al. [33] reported homoacetogenic productivities that represented about 50% of the 282 total acetate observed. In regard to homoacetogenesis estimation, it is important to mention that its 283 theoretical evaluation is subjected to uncertainties that are difficult to control: 1) homoacetogenesis 284 is carried out through the Wood-Ljundahl pathway, which could theoretically lead to other not 285 considered metabolites, e.g. ethanol and propionate [34], and 2) the mass balance assumes that 286 acetate is associated with a H₂ molar yield of 4 mol H₂/mol hexose, which is not accurate in most of 287 the cases. 288

To confirm the dual characteristics of dark fermentative microorganisms and propose a method to avoid uncertainties of homoacetogenesis estimation, biomass harvested from a H_2 -producing reactor (CSTR III) was used to perform additional experiments to study the consumption of H_2 . The results showed that the H_2 consumption profile was successfully modeled with the Gompertz equation

(Fig. 4). The corresponding kinetic parameters are presented in Table 3. Interestingly, the system with an initial pressure of 1.4 atm showed remarkably higher velocity of H₂ uptake in comparison with the system at 1 atm. This finding confirms that H₂ accumulation controls the metabolism leading to its own consumption. Furthermore, the capillary electrophoresis analysis revealed that acetate was the main metabolite, produced at final concentrations of 94.8 \pm 39 and 175 \pm 46 mg/L in the system with an initial pressure of 1 and 1.4 atm, respectively.

299 **4.** Conclusions

300 This research demonstrates that the dark fermentation pathways and the related microbial 301 communities can be controlled by improving H₂ mass transfer conditions. It was found that the increase of the mass transfer coefficient, k_La , enhanced the VHPR from 4.4 \pm 1.3 L/L-d at a k_La of 302 1.04 1/h (100 rpm) to 7.6 \pm 1.4 L/L-d at k_La of 4.23 1/h (400 rpm), which is equivalent to a 74 % 303 304 increase. Similarly, the H₂ yield shifted from 0.6 ± 0.15 mol H₂/mol hexose to 1.08 ± 0.21 mol H₂/mol hexose, i.e. an increment of 78%. The improvement in mass transfer conditions produced 305 lower concentrations of dissolved H₂, which favored the dominance of *Clostridium sp.* over 306 Lactobacillus sp., which led to an enhancement of H₂ production through the acetate and butyrate 307 pathways. The dual capability of the hydrogenogenic biomass was confirmed through microcosm 308 studies that allowed to develop a first approach towards the characterization of H₂ consuming mixed 309 cultures. Overall, it was demonstrated that the dark fermentation could be successfully controlled by 310 mass transfer conditions. 311

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- 319 Appendix A. Supplementary material.
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427 Legends to figures

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Fig. 1. Improvement of A) volumetric hydrogen production rate and B) H₂ yield as results of the 429 modification of the hydrogen mass transfer coefficient (k_La) through stirring velocity. Boxes with 430 same letters are not significant different at p<0.05 in accordance with the Tukey test. 431 432 Fig. 2. Effect of the hydrogen mass transfer coefficient (k_La) on A) the fermentative byproducts, B) 433 total volatile fatty acids concentration, and C) dissolved hydrogen concentrations. 434 435 Fig. 3. DGGE (Denaturing gradient gel electrophoresis) profiles from dark fermentation systems 436 operated under different mass transfer conditions. Groups with a euclidean distance among its 437 members <1 are colored to aid in the figure interpretation. Band numbers indicate sequenced 438 samples (see Table 1). 439 440 Fig. 4. Hydrogen consumption profiles utilizing hydrogenogenic biomass harvested from the 441 442 continuous stirred-tank reactor III. Experimental points (\Box), physicochemical control (- Δ -) and 443 gompertz model (- - -). A) Experiment performed at an initial P_{H2} of 1 atm. B) Experiment performed at an initial P_{H2} of 1.4 atm. 444

Table 1. Affiliation of DGGE (denaturing gradient gel electrophoresis) bands from samples taken under different mass transfer conditions.

Band	Closest relative (order/family/genus/species)	Identity	Accession No.
1	Clostridiales/Clostridiaceae/Clostridium/ C. butyricum	549/551 (99%)	NR_113244.1
2	Clostridiales/Clostridiaceae/Clostridium/ C. butyricum	551/551 (100%)	NR_113244.1
3	Clostridiales/Clostridiaceae/Clostridium/ C. butyricum	438/441 (99%)	NR_113244.1
4	Clostridiales/Ruminococcaceae/Caproiciproducens/ C. galactitolivorans	470/478 (98%)	NR_145929.1
5	Clostridiales/Ruminococcaceae/Caproiciproducens/ C. galactitolivorans	336/352 (95%)	NR_145929.1
6	Bacillales/Sporolactobacillaceae/ Sporolactobacillus/ S. nakayamae	339/352 (96%)	NR_114001.1
7	Clostridiales/Clostridiaceae/Clostridium/ C. jeddahense	549/553 (99%)	NR_144697.1
8	Bacillales/Sporolactobacillaceae/Sporolactobacillus/ S. terrae	457/470 (97%)	NR_112772.1
9	Clostridiales/Clostridiaceae/Clostridium/ C. butyricum	555/562 (99%)	NR_113244.1
10	Clostridiales/Clostridiaceae/Clostridium/ C. butyricum	542/548 (99%)	NR_113244.1
11	Clostridiales/Clostridiaceae/Clostridium/ C. butyricum	551/551 (100%)	NR_113244.1
12	Lactobacillales/Lactobacillaceae/ Lactobacillus/ L. paracasei	543/543 (100%)	NR_113337.1
13	Lactobacillales/Lactobacillaceae/ Lactobacillus/ L. paracasei	546/547 (99%)	NR_113337.1
14	Enterobacterales/Enterobacteriaceae/Klebsiella/ K. variicola	528/529 (99%)	NR_025635.1

Stirri	ng kLa	VHPR	H ₂ yield	Homoacetoge			
(rpm)	(1/h)	(L/L-d)	$(mol H_2/mol$	(mol	% of total	(L H ₂ -eq/L-d) ^a	% of
(-p)	(1/1)		hexose)	acetate/L-d)	acetate		theoretical H ₂ ^b
100	1.04	4.40	0.61	31.1	32	2.79	39%
200	1.64	5.60	0.73	61.1	46	5.47	49%
300	2.72	7.67	1.00	68.6	38	6.15	44%
400	4.23	7.66	1.08	45.8	33	4.10	35%

Table 2. Theoretical determinations of H₂ consumption by homoacetogenesis.

VHPR: Volumetric hydrogen production rate

 $^{a}\mbox{Accounts}$ the amount of H_{2} consumed in the synthesis of homoacetogenic acetate

 $^{b}\mbox{Estimates}$ the percentage of \mbox{H}_{2} that is lost by the homoacetogenic route

	Initial	Goi	npertz paramet		Acetate	
Experiment	pressure	H _{max}	R _{max} (mmol	λ (h)	[–] pH final	concentration (mg/L)
	(atm)	(mmol _{H2})	H ₂ /h)			
A (n=3)	1	12.64	0.75	9.49	5.47 ± 0.02	94.9 ± 39
B (n=3)	1.4	17.87	1.24	12.52	5.44 ± 0.19	175 ± 46

Table 3. Summary of H_2 consumption experiments. For both experimental conditions, two additional serum bottles were set-up to account for the inoculum activity (without the addition of H_2 gas) and the contribution of physicochemical phenomena (e.g. mass transfer).



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