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

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18 Declaration of interest: none

19

20 **Abstract**

21 Hydrogen (H<sub>2</sub>) 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 H<sub>2</sub> production is limited by the accumulation of H<sub>2</sub> in the  
24 fermentation broth. In continuous stirred-tank reactors (CSTR), such phenomenon is associated  
25 with poor mass transfer conditions. Nevertheless, this parameter has been scarcely considered to  
26 enhance H<sub>2</sub> production. In this research, the effect of the H<sub>2</sub> mass transfer conditions on the  
27 productivity and efficiency of H<sub>2</sub> production was evaluated using a series of CSTR operated at H<sub>2</sub>  
28 mass transfer coefficients (k<sub>L</sub>a) ranging from 1.04 to 4.23 1/h. The results showed that volumetric  
29 H<sub>2</sub> production rate (VHPR) and H<sub>2</sub> yield increased 74 and 78%, respectively, due to enhanced mass  
30 transfer conditions. This behavior was driven by 53% decrease of the dissolved H<sub>2</sub> concentration.  
31 More specifically, a maximum VHPR of 7.66 L/L-d with a H<sub>2</sub> yield of 1.1 mol H<sub>2</sub>/mol hexose was  
32 obtained at a k<sub>L</sub>a = 4.23 1/h. Furthermore, 16S-DGGE analysis and sequencing revealed that  
33 *Clostridium* and *Lactobacillus* were the dominant bacterial genera in continuous operation. In  
34 particular, *Clostridium* increased its occurrence at k<sub>L</sub>a of 2.72-4.23 1/h as a response to lower  
35 dissolved H<sub>2</sub> concentrations. The novelty of this work relies on the demonstration that mass transfer  
36 conditions controls H<sub>2</sub> accumulation and enhances the reactor performance for H<sub>2</sub> production.

37 **Key words:** biohydrogen; CSTR; dark fermentation; mass transfer

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## 42 **1. Introduction**

43 The diversification of energy sources is a critical keystone in the contemporaneous vision of  
44 sustainable development. In this regard, biofuels are expected to play a relevant role to fulfill future  
45 requirements of the transportation sector and to be a complementary source in the forthcoming  
46 electricity grid. In this context, molecular hydrogen ( $H_2$ ) has been underlined due to its high energy  
47 content (120 kJ/g) and the highly efficient conversion to electricity through the  $H_2$  fuel-cell  
48 technology. Moreover,  $H_2$  can be produced from a wide range of residual biomass through dark  
49 fermentation, which also makes it an attractive option in waste valorization scenarios.

50 The production of  $H_2$  through dark fermentation is achieved by anaerobic microorganisms that use  
51 two principal metabolic routes: the pyruvate formate lyase and the pyruvate ferredoxin  
52 oxydoreductase pathways [1,2]. These routes are associated with maximum theoretical metabolic  
53 yields of 2 and 4 mol  $H_2$ /mol hexose, respectively. However, most studies in literature report  $H_2$   
54 yields below these thresholds. The low  $H_2$  production depends on multiple factors, including the  
55 microbial community composition, the operational conditions and the efficiency of liquid-gas mass  
56 transfer. The importance of this later topic relies on the fact that dissolved  $H_2$  concentration can  
57 exert a thermodynamic control on  $H_2$ -associated metabolic pathways. For instance, as  $H_2$   
58 accumulates ( $P_{H_2} > 60$  Pa),  $H_2$  synthesis from ferredoxin becomes theoretically unfeasible. As a  
59 consequence, the maximum  $H_2$  yield decreases from 4 to 2 mol  $H_2$ /mol hexose [3]. Further  
60 accumulation of  $H_2$  in the liquid phase ( $P_{H_2} > 500$  Pa) leads to the occurrence of homoacetogenesis,  
61 which is the metabolism through which acetate is synthesized from  $H_2$  and  $CO_2$  [4–6].

62 In dark fermentative systems, different alternatives have been proposed to enhance  $H_2$  mass transfer  
63 [7–13]. Mechanical stirring can be highlighted due to its low-cost and ease of implementation.  
64 Using mechanical stirring, Beckers et al. [13] successfully showed that  $H_2$  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.

67 In continuous regime, it can be hypothesized that different steady states of performance could arise  
68 as a function of mass transfer conditions with concomitant changes in metabolic pathways and  
69 microbial community composition. In this regard, the aim of this work was to compare steady state  
70 performances of dark fermentative systems at different conditions of mass transfer (associated with  
71 different stirring velocities) with a special focus on the potential shifts in metabolic pathways and  
72 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  
78 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  
80 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]:  $\text{NH}_4\text{Cl}$ , 2110;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 100;  $\text{CuCl}_2 \cdot \text{H}_2\text{O}$ , 1.25;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 7;  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ ,  
83 19.1;  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 102.5. In addition, a phosphate buffer ( $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ , pH 5.9) was added to  
84 reach a final concentration of 100 mM.

### 85 2.2 Bioreactors set-up and operational conditions

86 A series of five CSTR (Applikon Biotechnologies, USA) with a working volume of 1 L (9.5 cm of  
87 internal diameter and 70.88 cm<sup>2</sup> liquid-gas transfer area) was set up as shown in Fig. S1. The  
88 bioreactors were equipped with a stirrer and two Rushton-type impellers symmetrically positioned  
89 along the depth of the reactor working-volume.

90 CSTR I was inoculated with the pretreated anaerobic sludge (section 2.1) at a concentration of 4.5 g  
91 TS/L and started-up in batch mode for 24 h with an initial substrate concentration of 15 g lactose /L.  
92 Stirring, temperature and pH were controlled at 250 rpm, 37 °C and 5.9, respectively. Thereafter,  
93 the reactor was switched to continuous mode with a fixed hydraulic retention time (HRT) of 6 h.  
94 The reactor was monitored for a minimum of 20 HRT equivalents, i.e. 5 days, and until a steady  
95 state was reached in terms of volumetric H<sub>2</sub> 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  
98 min at 4°C. The resulting pellets were re-suspended in mineral medium without substrate,  
99 characterized in terms of volatile suspended solids (VSS) and stored at -4 °C until their use as  
100 inoculum in four more reactors CSTR II to V. CSTR II - V were inoculated with the recovered  
101 biomass from CSTR I at a concentration of 0.45 g VSS/L. The start-up strategy and operational  
102 conditions were maintained identical to CSTR I, except for the stirring velocity, which was set at  
103 100, 200, 300 and 400 rpm in CSTR II, CSTR III, CSTR IV and CSTR V, respectively. CSTR I – V  
104 were also operated and monitored for a minimum of 20 HRT and until stable VHPR was observed.

### 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  
108 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

110 determined by the phenol sulfuric method [16]. VFA were quantified from filtered (22  $\mu\text{m}$ ) samples  
111 by capillary electrophoresis (1600A, Agilent Technologies, Waldbronn, Germany) as reported  
112 elsewhere [17]. All  $\text{H}_2$  and VFAs yields were calculated considering the amount of hexose  
113 consumed.

114 Gas production was measured through a liquid displacement device (SEV. Puebla, Mexico), and its  
115 composition ( $\text{H}_2$  and  $\text{CO}_2$ ) was determined through a gas chromatograph equipped with a thermal  
116 conductivity detector (6890N, Agilent Technologies, Waldbronn, Germany). All gas volumes were  
117 reported at 1 atm and 273.15 K.

#### 118 2.4 Determination of $k_{\text{L}}a$ and dissolved $\text{H}_2$ concentration

119 To determine  $\text{H}_2$  mass transfer coefficients ( $k_{\text{L}}a$ , 1/h) of the CSTR, a series of  $\text{O}_2$  desorption  
120 experiments were performed at 50, 100, 200, 300 and 400 rpm using the gas-out method described  
121 elsewhere [13]. In brief, the reactor vessel was filled with mineral medium without substrate and  
122 inoculum. Stirring, temperature and pH were set to 50-400 rpm, 37  $^\circ\text{C}$  and 5.9, respectively. The  
123 system was assembled with an electrode to measure and record dissolved oxygen (DO)  
124 concentrations. For each experiment, the system was first degassed with  $\text{N}_2$  and then flushed with  
125 pure oxygen until the dissolved  $\text{O}_2$  concentration reached 100%. Afterwards, the  $\text{O}_2$  sparging was  
126 ceased and the decrease of the dissolved gas concentration was recorded until equilibrium was  
127 reached. Obtained data were normalized and adjusted to the following desorption equation:

$$128 \quad [DO(t)] = [DO]_{t=0} * e^{-(k_{\text{L}}a)_{\text{O}_2} * t} \quad (1)$$

129 Where  $t$  (h) represents the elapsed time and  $(k_{\text{L}}a)_{\text{O}_2}$  is the volumetric mass transfer coefficient of  
130  $\text{O}_2$ . The resulting  $(k_{\text{L}}a)_{\text{O}_2}$  value altogether with the  $\text{O}_2$  and  $\text{H}_2$  diffusivities ( $D_{\text{O}_2}$  and  $D_{\text{H}_2}$ ,  
131 respectively) were used to compute the  $(k_{\text{L}}a)_{\text{H}_2}$  considering the following relationship [13]:

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

Where  $D_{H_2}$  and  $D_{O_2}$  were  $5.91 \times 10^{-5}$  and  $2.62 \times 10^{-5}$  cm<sup>2</sup>/s at 40 and 37 °C, respectively [18,19].

Furthermore, to calculate dissolved H<sub>2</sub> concentrations at the different mass transfer conditions, it was first considered that mass transfer of H<sub>2</sub> from the liquid to the gas phase ( $Q_{H_2}$ , in mol<sub>H<sub>2</sub></sub>/L-h) can be described as follows:

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

Where  $(k_L a)_{H_2}$  (1/h) is the volumetric mass transfer coefficient for H<sub>2</sub>,  $C_{H_2,liq}$  (mol/L) is the concentration of dissolved H<sub>2</sub>,  $p_{H_2,gas}$  (atm) is the H<sub>2</sub> partial pressure of the headspace, and  $H^{cp}$  (mol/L-atm) is the Henry's coefficient of H<sub>2</sub> ( $8.47 \times 10^{-4}$  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  $p_{H_2,gas}$ ).

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

## 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'-

152 CGCCCGCCGCGCGCGGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGC  
153 AGCAG-3') and 907R (5'-CCGTCAATTCMTTGTGAGTTT) to amplify the V3 - V5 regions. The  
154 PCR products were loaded in polyacrylamide gels (8%) with a denaturing gradient (urea-  
155 formamide) that ranged from 30 to 60 %. The DGGE electrophoresis conditions were 70 V for 20 h  
156 at 60 °C. After electrophoresis, the gel was fixed with acetic acid (10%), treated with a AgNO<sub>3</sub>  
157 solution (1 g/L) and revealed with a Na<sub>2</sub>CO<sub>3</sub> (23.3 g/L) solution. The gel bands were photographed  
158 under visible light with a digital camera. The DGGE images were analyzed with the BioNumeric  
159 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  
161 through a UPGMA dendrogram computed in the R environment [22]. Furthermore, selected DNA  
162 bands were cut, reamplified by PCR using 341F (without GC-clamp) and 907R primers, and  
163 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  
165 (Ibis Therapeutics, USA). Edited sequences were compared with the reference 16S-rRNA database  
166 of 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<sub>2</sub>, acetate and butyrate was performed as suggested elsewhere [23,24]:

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

171 Where VFA and H<sub>2</sub> are given in mol/d.

172 To confirm and characterize the H<sub>2</sub> consumption capacity of the microbial community, a series of  
173 H<sub>2</sub> consumption experiments was also carried out. These experiments were conducted in 120 mL  
174 serum bottles with a working volume of 80 mL, using biomass harvested from the CSTR III (200

175 rpm) as inoculum. For this purpose, enough volume of effluent was recovered from CSTR III and  
176 centrifuged at 3500 rpm for 10 min at 4°C. Resulting pellets were re-suspended in mineral medium  
177 (section 2.1), characterized in terms of VSS, and stored at -4 °C until their use. The experiments  
178 were prepared with an initial concentration of 2 g VSS/L using the mineral medium with the  
179 composition described in section 2.1 and supplemented with 560 mg/L of NaHCO<sub>3</sub>. No organic  
180 substrate was added. The serum bottles were hermetically sealed and the headspace displaced first  
181 with N<sub>2</sub> and then with pure H<sub>2</sub>. A second set of experiments was prepared identically, but H<sub>2</sub> was  
182 pressurized at 1.4 atm. Two additional bottles were prepared, one without biomass and the other  
183 without H<sub>2</sub>, to serve as physicochemical and endogenous controls, respectively. All experiments  
184 were incubated at 37 °C. The H<sub>2</sub> consumption was computed from the decrease of the system  
185 pressure and headspace composition.

186 The cumulative H<sub>2</sub> consumption was modelled utilizing the Gompertz model [25,26]:

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

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

## 191 2.7 Statistic analysis

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

### 198 3. Results and discussion

#### 199 3.1 $k_{La}$ determination in the dark fermentative system

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

#### 206 3.2 The $H_2$ mass transfer conditions as a mechanism controlling the productivity and the efficiency 207 of dark fermentation

208 CSTR I was operated for an equivalent time of 34 HRT (8.5 days) at an organic loading rate (OLR)  
209 of 60 g lactose/L-d with the main objective of producing seed-biomass for subsequent experiments  
210 (CSTR II - V). The performance of CSTR I was stable in terms of productivity and efficiency, with  
211 an average VHPR of  $7.1 \pm 1.0$  L/L-d and  $H_2$  yield of  $0.94 \pm 0.1$  mol  $H_2$ /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  $H_2$  yield  
213 of 1.2 mol  $H_2$ /mol hexose, which was similar to the results showed in the present work. The  
214 stability of  $H_2$  production and the similarity with previous reports were clear indications of a  
215 successful establishment of the dark fermentative  $H_2$  production. Thus, the biomass was then  
216 recovered to serve as inoculum in the following experiments.

217 CSTR II to V were independently operated at  $k_{La}$  values in the range of 1.04 to 4.23 1/h, associated  
218 with stirring velocities ranging from 100 to 400 rpm (Table S1). The results showed that the mass  
219 transfer coefficient strongly affected dark fermentation in terms of VHPR ( $F_{3, 50}=13.05$ ,  $p<0.05$ ) and  
220  $H_2$  yield ( $F_{3, 50}=13.04$ ,  $p<0.05$ ) (Fig. 1). At the lowest  $k_{La}$  tested (1.04 1/h), the VHPR was  $4.4 \pm 1.3$

221 L/L-d with an H<sub>2</sub> yield of  $0.6 \pm 0.15$  mol H<sub>2</sub>/mol hexose. In contrast, at the highest value of  $k_{La}$   
222 ( $4.23$  1/h), an average VHPR of  $7.66 \pm 1.42$  L/L-d was obtained with an H<sub>2</sub> yield of  $1.08 \pm 0.21$  mol  
223 H<sub>2</sub>/mol hexose. These results represent an increase of 74% in terms of VHPR and 78% in terms of  
224 H<sub>2</sub> yield.

225 The extent of improvement, in both VHPR and H<sub>2</sub> yield, achieved in this work was consistent with  
226 previous reports that focused on H<sub>2</sub> mass transfer (Table S2). For instance, Beckers et al. [13]  
227 reported an improvement of approximately 89 and 19 % in terms of H<sub>2</sub> production rate and H<sub>2</sub>  
228 yield, respectively, in an anaerobic stirred tank reactor after having increased the stirring conditions  
229 from 0 to 400 rpm. Using CO<sub>2</sub> sparging, Kim et al. [7] were able to increase the VHPR and H<sub>2</sub> yield  
230 in a CSTR by 56% and 118%, respectively. Nevertheless, such an approach resulted in H<sub>2</sub> 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<sub>2</sub> yield were reached,  
233 avoiding the utilization of additional gases and, therefore, the H<sub>2</sub> produced remains concentrated.

### 234 3.3 Enhancement of mass transfer conditions intensifies metabolic routes leading to H<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>  
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<sub>2</sub> production by dark fermentation.

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

### 253 3.4 Microbial community

254 The analysis of the 16S rRNA-DGGE (Fig. 3) and the sequencing results (Table 1) showed that the  
255 microbial community was mainly composed of *Clostridium* and *Lactobacillus* species. These two  
256 genera have been previously reported to play important roles in dark fermentative systems  
257 [14,29,30]. *Clostridium spp.* are mostly related to H<sub>2</sub>-producing bacteria and are widely found in  
258 dark fermentative systems associated with high efficiencies (e.g. [1,31]). In contrast, *Lactobacillus*  
259 *spp.* correspond to lactic acid bacteria that have been identified as substrate competitors of H<sub>2</sub>-  
260 producers during the fermentation of cheese whey [32]. Moreover, it has been shown that the  
261 abundance of lactic acid bacteria increased under relatively high organic loading rates (58.8 and  
262 88.2 g lactose/L-d), and it was likely associated with the accumulation of H<sub>2</sub> in the fermentative  
263 medium [29].

264 Interestingly, the DGGE analysis showed that *Lactobacillus* (band at DGGE relative distance ~ 68)  
265 was more abundant in the samples taken at the end of CSTRs operation (Fig. 3), suggesting that  
266 these microorganisms were enriched during the H<sub>2</sub> production process. DGGE profiles also  
267 revealed that bands associated with *Clostridium* (two bands at DGGE relative distances of 45 and  
268 50, approximately) were less intense in the samples taken at the end of CSTR II and CSTR III (1.04

269 and 1.64 1/h) than those taken in CSTR IV and CSTR V (2.72 and 4.23 1/h). Altogether, these  
270 findings indicate that H<sub>2</sub> transfer conditions did not only affect the productivity, efficiency and  
271 metabolism in dark fermentative systems, but they have also important implications on shaping  
272 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<sub>2</sub> through the homoacetogenesis pathway in response to high dissolved H<sub>2</sub>  
276 concentrations. To quantify this activity in mixed cultures, previous studies reported an approach  
277 based on balancing the acetate, butyrate and H<sub>2</sub> productivities [23,24]. In accordance with this  
278 method, it was found that homoacetogenic acetate production rates ranged from 31.1 to 68.6 mol  
279 acetate/L-d. In terms of percentage, the acetate produced by homoacetogenesis was 32-46 % of total  
280 acetate quantified in the systems (Table 2). Similar values were reported elsewhere by Luo et al.  
281 [24] at different conditions of pH, temperature and sludge pretreatment. Also, in UASB reactors,  
282 Carrillo-Reyes et al. [33] reported homoacetogenic productivities that represented about 50% of the  
283 total acetate observed. In regard to homoacetogenesis estimation, it is important to mention that its  
284 theoretical evaluation is subjected to uncertainties that are difficult to control: 1) homoacetogenesis  
285 is carried out through the Wood-Ljungdahl pathway, which could theoretically lead to other not  
286 considered metabolites, e.g. ethanol and propionate [34], and 2) the mass balance assumes that  
287 acetate is associated with a H<sub>2</sub> molar yield of 4 mol H<sub>2</sub>/mol hexose, which is not accurate in most of  
288 the cases.

289 To confirm the dual characteristics of dark fermentative microorganisms and propose a method to  
290 avoid uncertainties of homoacetogenesis estimation, biomass harvested from a H<sub>2</sub>-producing reactor  
291 (CSTR III) was used to perform additional experiments to study the consumption of H<sub>2</sub>. The results  
292 showed that the H<sub>2</sub> consumption profile was successfully modeled with the Gompertz equation

293 (Fig. 4). The corresponding kinetic parameters are presented in Table 3. Interestingly, the system  
294 with an initial pressure of 1.4 atm showed remarkably higher velocity of H<sub>2</sub> uptake in comparison  
295 with the system at 1 atm. This finding confirms that H<sub>2</sub> accumulation controls the metabolism  
296 leading to its own consumption. Furthermore, the capillary electrophoresis analysis revealed that  
297 acetate was the main metabolite, produced at final concentrations of  $94.8 \pm 39$  and  $175 \pm 46$  mg/L  
298 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 H<sub>2</sub> mass transfer conditions. It was found that the  
302 increase of the mass transfer coefficient,  $k_{L,a}$ , enhanced the VHPR from  $4.4 \pm 1.3$  L/L-d at a  $k_{L,a}$  of  
303 1.04 1/h (100 rpm) to  $7.6 \pm 1.4$  L/L-d at  $k_{L,a}$  of 4.23 1/h (400 rpm), which is equivalent to a 74 %  
304 increase. Similarly, the H<sub>2</sub> yield shifted from  $0.6 \pm 0.15$  mol H<sub>2</sub>/mol hexose to  $1.08 \pm 0.21$  mol  
305 H<sub>2</sub>/mol hexose, i.e. an increment of 78%. The improvement in mass transfer conditions produced  
306 lower concentrations of dissolved H<sub>2</sub>, which favored the dominance of *Clostridium sp.* over  
307 *Lactobacillus sp.*, which led to an enhancement of H<sub>2</sub> production through the acetate and butyrate  
308 pathways. The dual capability of the hydrogenogenic biomass was confirmed through microcosm  
309 studies that allowed to develop a first approach towards the characterization of H<sub>2</sub> consuming mixed  
310 cultures. Overall, it was demonstrated that the dark fermentation could be successfully controlled by  
311 mass transfer conditions.

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319 **Appendix A.** Supplementary material.

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- 426

427 Legends to figures

428

429 Fig. 1. Improvement of A) volumetric hydrogen production rate and B)  $H_2$  yield as results of the  
430 modification of the hydrogen mass transfer coefficient ( $k_{La}$ ) through stirring velocity. Boxes with  
431 same letters are not significant different at  $p < 0.05$  in accordance with the Tukey test.

432

433 Fig. 2. Effect of the hydrogen mass transfer coefficient ( $k_{La}$ ) on A) the fermentative byproducts, B)  
434 total volatile fatty acids concentration, and C) dissolved hydrogen concentrations.

435

436 Fig. 3. DGGE (Denaturing gradient gel electrophoresis) profiles from dark fermentation systems  
437 operated under different mass transfer conditions. Groups with a euclidean distance among its  
438 members  $< 1$  are colored to aid in the figure interpretation. Band numbers indicate sequenced  
439 samples (see Table 1).

440

441 Fig. 4. Hydrogen consumption profiles utilizing hydrogenogenic biomass harvested from the  
442 continuous stirred-tank reactor III. Experimental points ( $\square$ ), physicochemical control ( $-\Delta-$ ) and  
443 gompertz model ( $- - -$ ). A) Experiment performed at an initial  $P_{H_2}$  of 1 atm. B) Experiment  
444 performed at an initial  $P_{H_2}$  of 1.4 atm.

445

**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	<i>Clostridiales/Clostridiaceae/Clostridium/ C. butyricum</i>	549/551 (99%)	NR_113244.1
2	<i>Clostridiales/Clostridiaceae/Clostridium/ C. butyricum</i>	551/551 (100%)	NR_113244.1
3	<i>Clostridiales/Clostridiaceae/Clostridium/ C. butyricum</i>	438/441 (99%)	NR_113244.1
4	<i>Clostridiales/Ruminococcaceae/Caproiciproducens/ C. galactitolivorans</i>	470/478 (98%)	NR_145929.1
5	<i>Clostridiales/Ruminococcaceae/Caproiciproducens/ C. galactitolivorans</i>	336/352 (95%)	NR_145929.1
6	<i>Bacillales/Sporolactobacillaceae/ Sporolactobacillus/ S. nakayamae</i>	339/352 (96%)	NR_114001.1
7	<i>Clostridiales/Clostridiaceae/Clostridium/ C. jeddahense</i>	549/553 (99%)	NR_144697.1
8	<i>Bacillales/Sporolactobacillaceae/Sporolactobacillus/ S. terrae</i>	457/470 (97%)	NR_112772.1
9	<i>Clostridiales/Clostridiaceae/Clostridium/ C. butyricum</i>	555/562 (99%)	NR_113244.1
10	<i>Clostridiales/Clostridiaceae/Clostridium/ C. butyricum</i>	542/548 (99%)	NR_113244.1
11	<i>Clostridiales/Clostridiaceae/Clostridium/ C. butyricum</i>	551/551 (100%)	NR_113244.1
12	<i>Lactobacillales/Lactobacillaceae/ Lactobacillus/ L. paracasei</i>	543/543 (100%)	NR_113337.1
13	<i>Lactobacillales/Lactobacillaceae/ Lactobacillus/ L. paracasei</i>	546/547 (99%)	NR_113337.1
14	<i>Enterobacterales/Enterobacteriaceae/Klebsiella/ K. variicola</i>	528/529 (99%)	NR_025635.1

**Table 2.** Theoretical determinations of H<sub>2</sub> consumption by homoacetogenesis.

Stirring (rpm)	kLa (1/h)	VHPR (L/L-d)	H <sub>2</sub> yield (mol H <sub>2</sub> /mol hexose)	Homoacetogenesis			
				(mol acetate/L-d)	% of total acetate	(L H <sub>2</sub> -eq/L-d) <sup>a</sup>	% of theoretical H <sub>2</sub> <sup>b</sup>
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%

VHPR: Volumetric hydrogen production rate

<sup>a</sup>Accounts the amount of H<sub>2</sub> consumed in the synthesis of homoacetogenic acetate

<sup>b</sup>Estimates the percentage of H<sub>2</sub> that is lost by the homoacetogenic route

**Table 3.** Summary of H<sub>2</sub> consumption experiments. For both experimental conditions, two additional serum bottles were set-up to account for the inoculum activity (without the addition of H<sub>2</sub> gas) and the contribution of physicochemical phenomena (e.g. mass transfer).

Experiment	Initial pressure (atm)	Gompertz parameters			pH final	Acetate concentration (mg/L)
		H <sub>max</sub> (mmol <sub>H2</sub> )	R <sub>max</sub> (mmol H <sub>2</sub> /h)	λ (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

Figure 1  
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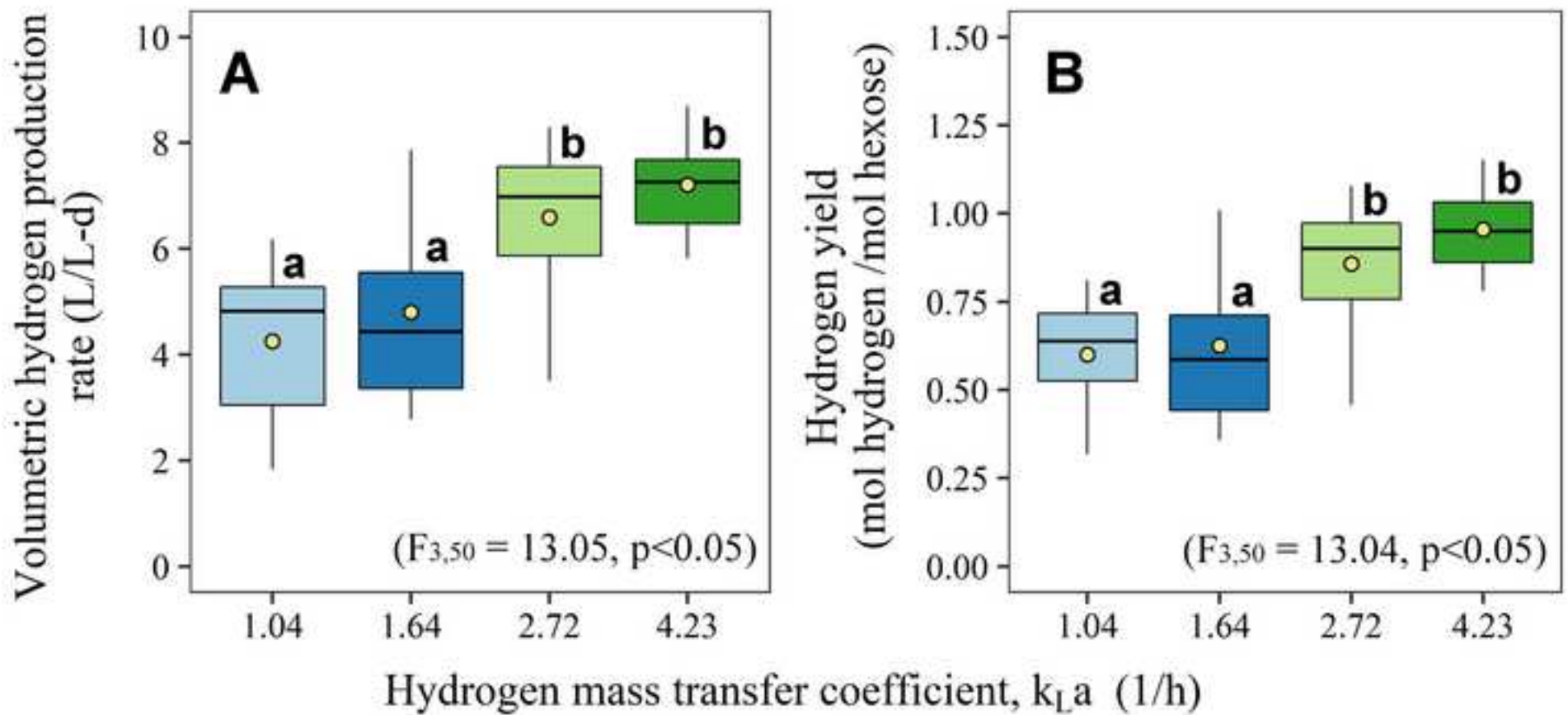


Figure 2  
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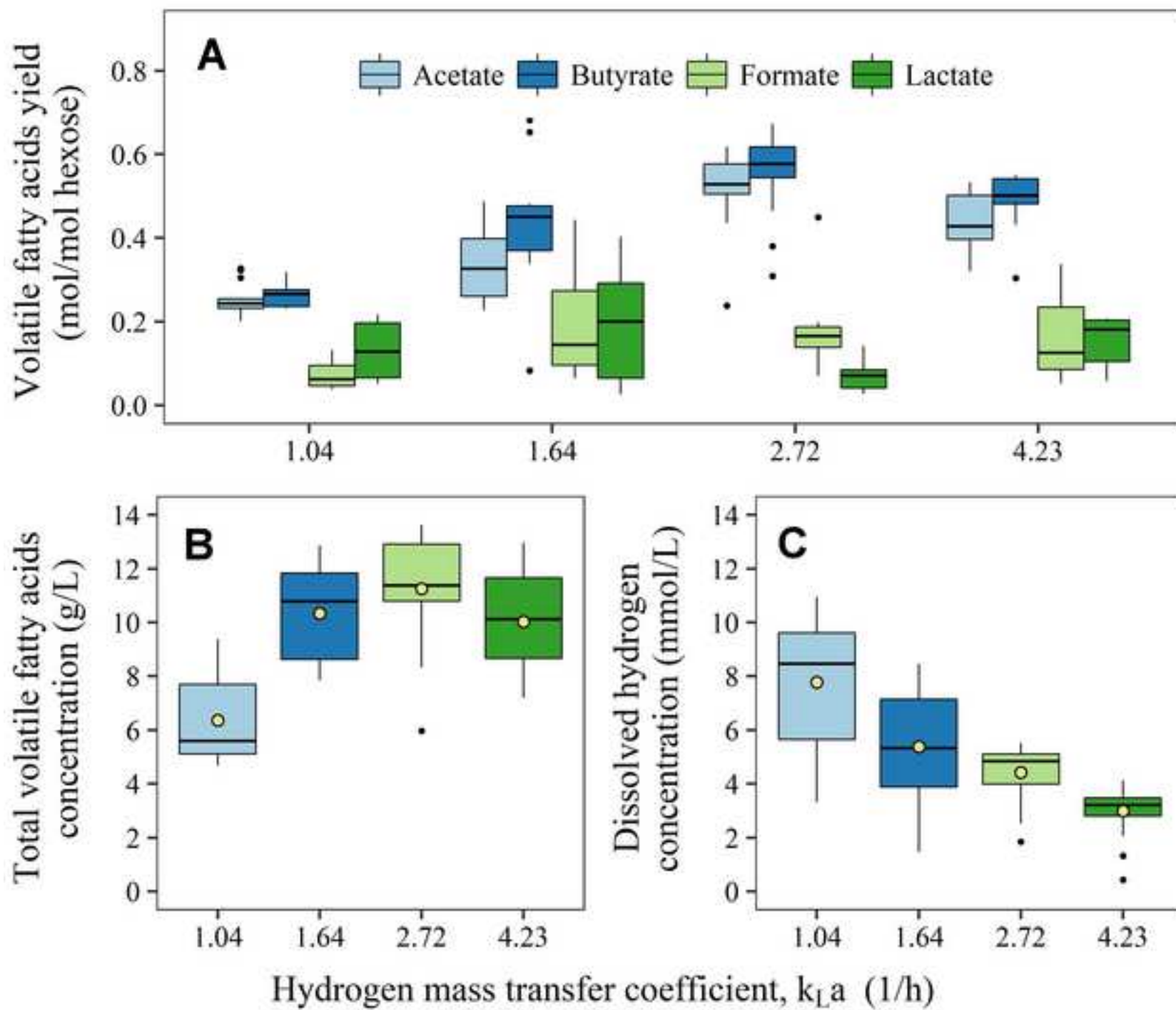


Figure 3  
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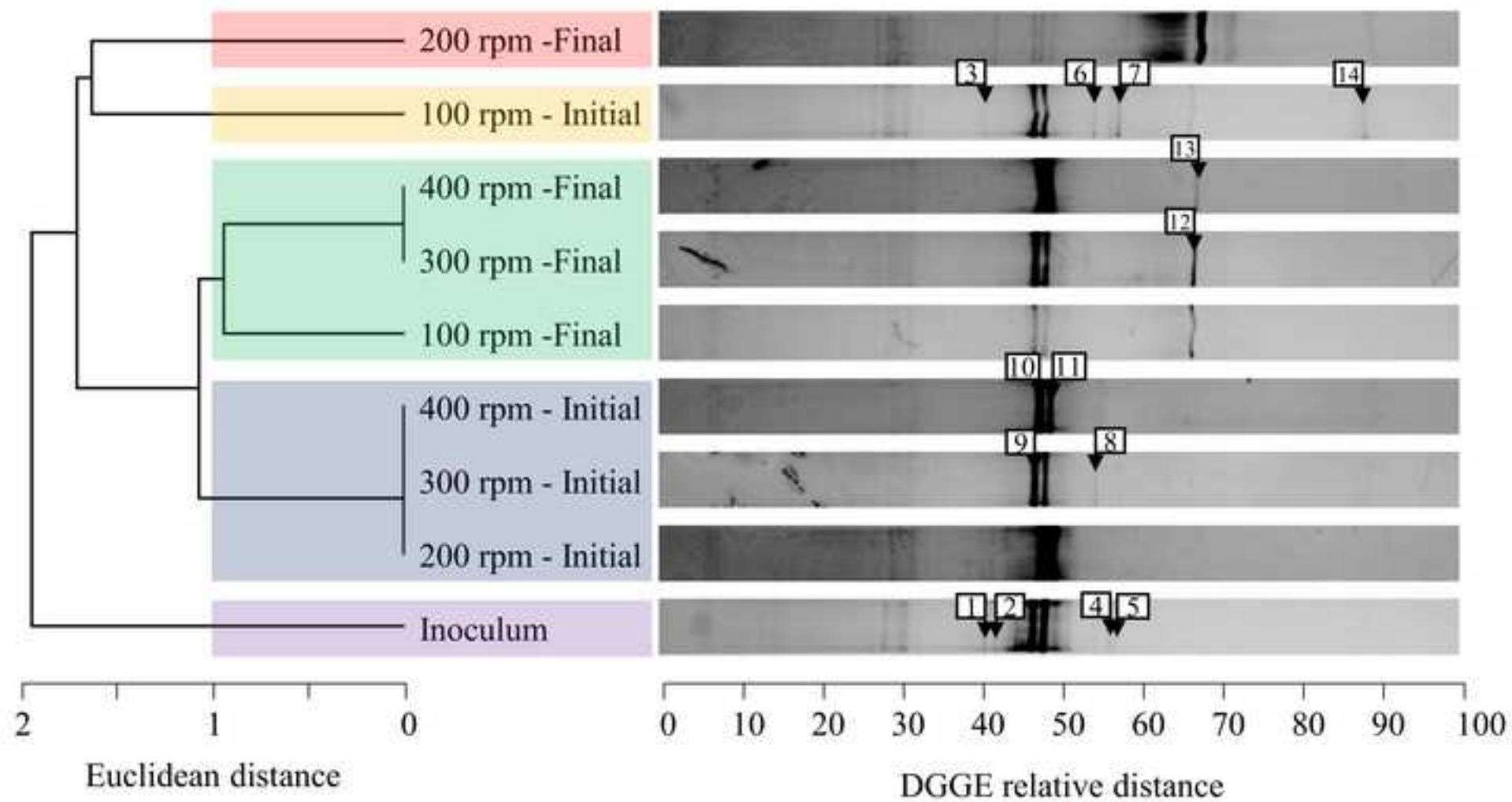


Figure 4  
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