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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
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 H₂ production. In this research, the effect of the H₂ mass transfer conditions on the
27 productivity and efficiency of H₂ production was evaluated using a series of CSTR operated at H₂
28 mass transfer coefficients (k_La) ranging from 1.04 to 4.23 1/h. The results showed that volumetric
29 H₂ production rate (VHPR) and H₂ yield increased 74 and 78%, respectively, due to enhanced mass
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
32 obtained at a k_La = 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_La of 2.72-4.23 1/h as a response to lower
35 dissolved H₂ concentrations. The novelty of this work relies on the demonstration that mass transfer
36 conditions controls H₂ accumulation and enhances the reactor performance for H₂ 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]: NH_4Cl , 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 cm² 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 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
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 μm) samples
111 by capillary electrophoresis (1600A, Agilent Technologies, Waldbronn, Germany) as reported
112 elsewhere [17]. All 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 (H_2 and 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 H_2 concentration

119 To determine H_2 mass transfer coefficients ($k_{\text{L}}a$, 1/h) of the CSTR, a series of 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 N_2 and then flushed with
125 pure oxygen until the dissolved O_2 concentration reached 100%. Afterwards, the 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 O_2 . The resulting $(k_{\text{L}}a)_{\text{O}_2}$ value altogether with the O_2 and H_2 diffusivities (D_{O_2} and D_{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 5.91×10^{-5} and 2.62×10^{-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:

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

Where $(k_L a)_{H_2}$ (1/h) is the volumetric mass transfer coefficient for 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 (8.47×10^{-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'-CCGTCAATTCMTTTGAGTTT) 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 AgNO₃
157 solution (1 g/L) and revealed with a Na₂CO₃ (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 H₂, 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 H₂ are given in mol/d.

172 To confirm and characterize the H₂ consumption capacity of the microbial community, a series of
173 H₂ 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 NaHCO₃. No organic
180 substrate was added. The serum bottles were hermetically sealed and the headspace displaced first
181 with N₂ and then with pure H₂. A second set of experiments was prepared identically, but H₂ was
182 pressurized at 1.4 atm. Two additional bottles were prepared, one without biomass and the other
183 without H₂, to serve as physicochemical and endogenous controls, respectively. All experiments
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 H₂ 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 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

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 ± 1.0 L/L-d and H_2 yield of 0.94 ± 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 ± 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 H₂ yield, achieved in this work was consistent with
226 previous reports that focused on H₂ mass transfer (Table S2). For instance, Beckers et al. [13]
227 reported an improvement of approximately 89 and 19 % in terms of H₂ production rate and H₂
228 yield, respectively, in an anaerobic stirred tank reactor after having increased the stirring conditions
229 from 0 to 400 rpm. Using CO₂ sparging, Kim et al. [7] were able to increase the VHPR and H₂ yield
230 in a CSTR by 56% and 118%, respectively. Nevertheless, such an approach resulted in H₂ 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 H₂ yield were reached,
233 avoiding the utilization of additional gases and, therefore, the H₂ produced remains concentrated.

234 3.3 Enhancement of mass transfer conditions intensifies metabolic routes leading to H₂ 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 H₂ 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 H₂
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 H₂ production by dark fermentation.

245 On the other hand, H₂ 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 H₂. The theoretical H₂ concentrations in the fermentation
248 liquid were in the range of 7.5 to 3.4 mmol H₂/L, with the lowest value at the k_La of 4.23 1/h.
249 Nevertheless, considering the results of VFA and dissolved H₂ concentration, as well as the VHPR
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
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 H₂-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 H₂-
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 H₂ 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 H₂ 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 H₂ 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 H₂ through the homoacetogenesis pathway in response to high dissolved H₂
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
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 H₂ molar yield of 4 mol H₂/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 H₂-producing reactor
291 (CSTR III) was used to perform additional experiments to study the consumption of H₂. The results
292 showed that the H₂ 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 H₂ uptake in comparison
295 with the system at 1 atm. This finding confirms that H₂ 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 ± 39 and 175 ± 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 H₂ mass transfer conditions. It was found that the
302 increase of the mass transfer coefficient, $k_{L,a}$, enhanced the VHPR from 4.4 ± 1.3 L/L-d at a $k_{L,a}$ of
303 1.04 1/h (100 rpm) to 7.6 ± 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 H₂ yield shifted from 0.6 ± 0.15 mol H₂/mol hexose to 1.08 ± 0.21 mol
305 H₂/mol hexose, i.e. an increment of 78%. The improvement in mass transfer conditions produced
306 lower concentrations of dissolved H₂, which favored the dominance of *Clostridium sp.* over
307 *Lactobacillus sp.*, which led to an enhancement of H₂ 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 H₂ 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.

320 **References**

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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 ($k_L a$) 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 ($k_L a$) 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 H₂ consumption by homoacetogenesis.

Stirring (rpm)	kLa (1/h)	VHPR (L/L-d)	H ₂ yield (mol H ₂ /mol hexose)	Homoacetogenesis			
				(mol acetate/L-d)	% of total acetate	(L H ₂ -eq/L-d) ^a	% of 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%

VHPR: Volumetric hydrogen production rate

^aAccounts the amount of H₂ consumed in the synthesis of homoacetogenic acetate

^bEstimates the percentage of H₂ that is lost by the homoacetogenic route

Table 3. Summary of H₂ consumption experiments. For both experimental conditions, two additional serum bottles were set-up to account for the inoculum activity (without the addition of H₂ gas) and the contribution of physicochemical phenomena (e.g. mass transfer).

Experiment	Initial pressure (atm)	Gompertz parameters			pH final	Acetate concentration (mg/L)
		H _{max} (mmol _{H2})	R _{max} (mmol H ₂ /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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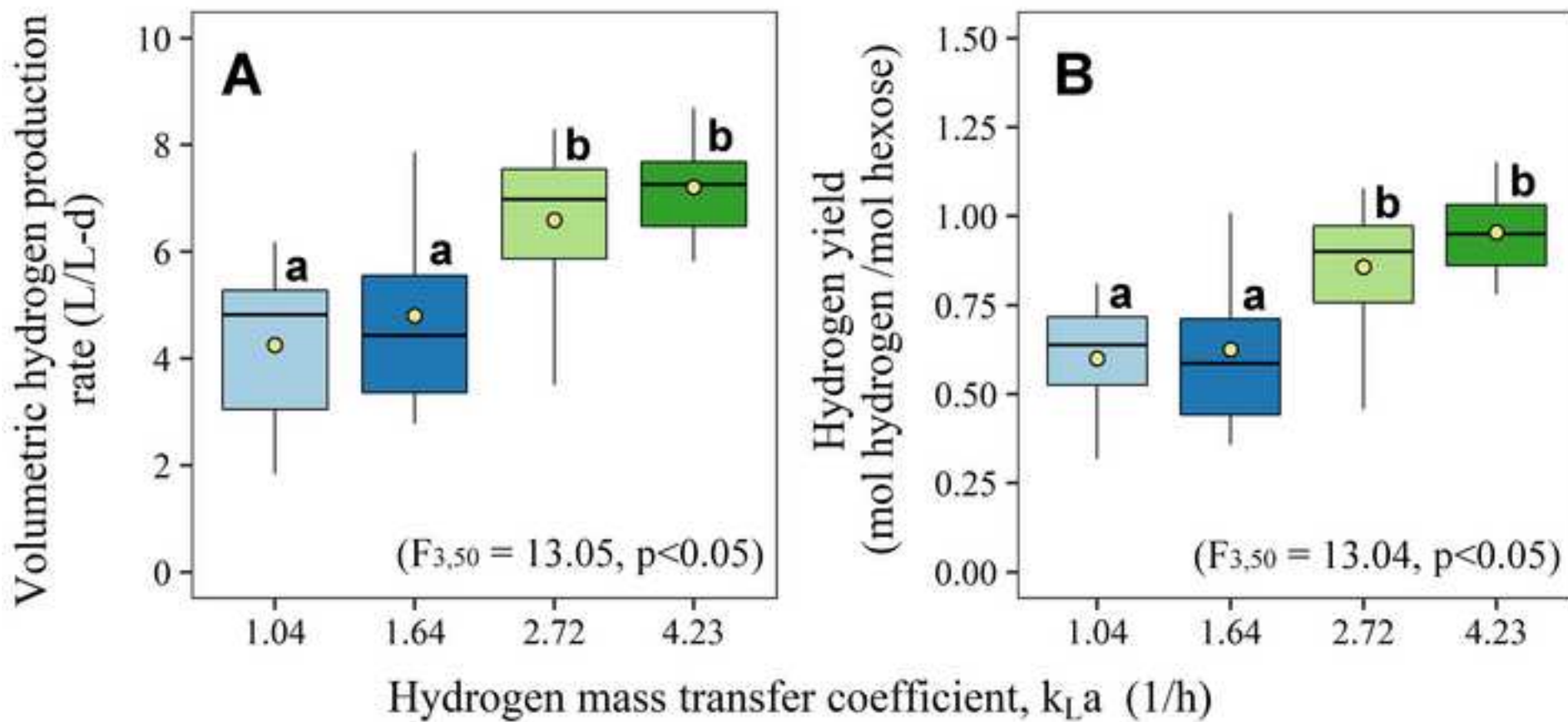


Figure 2
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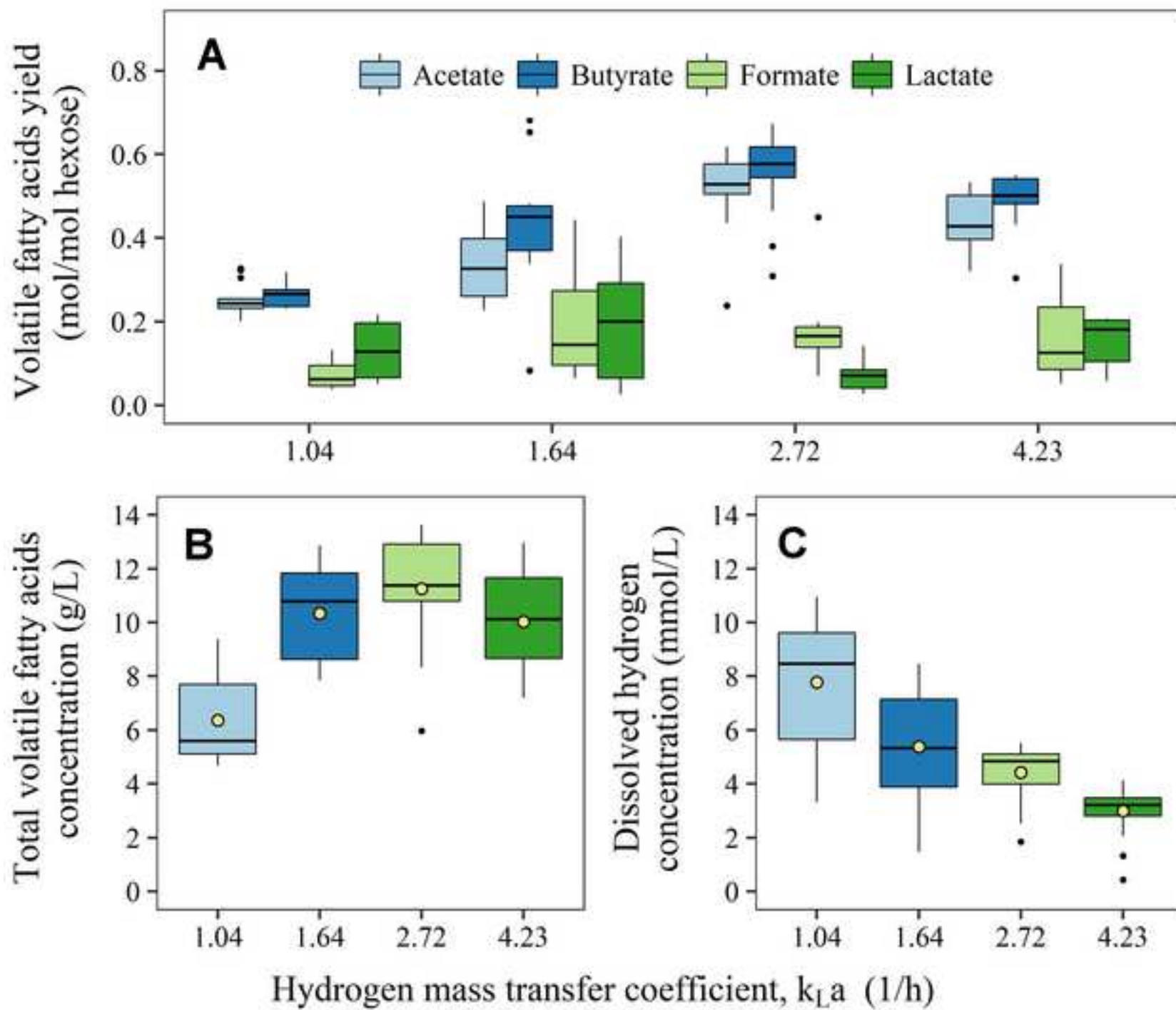


Figure 3
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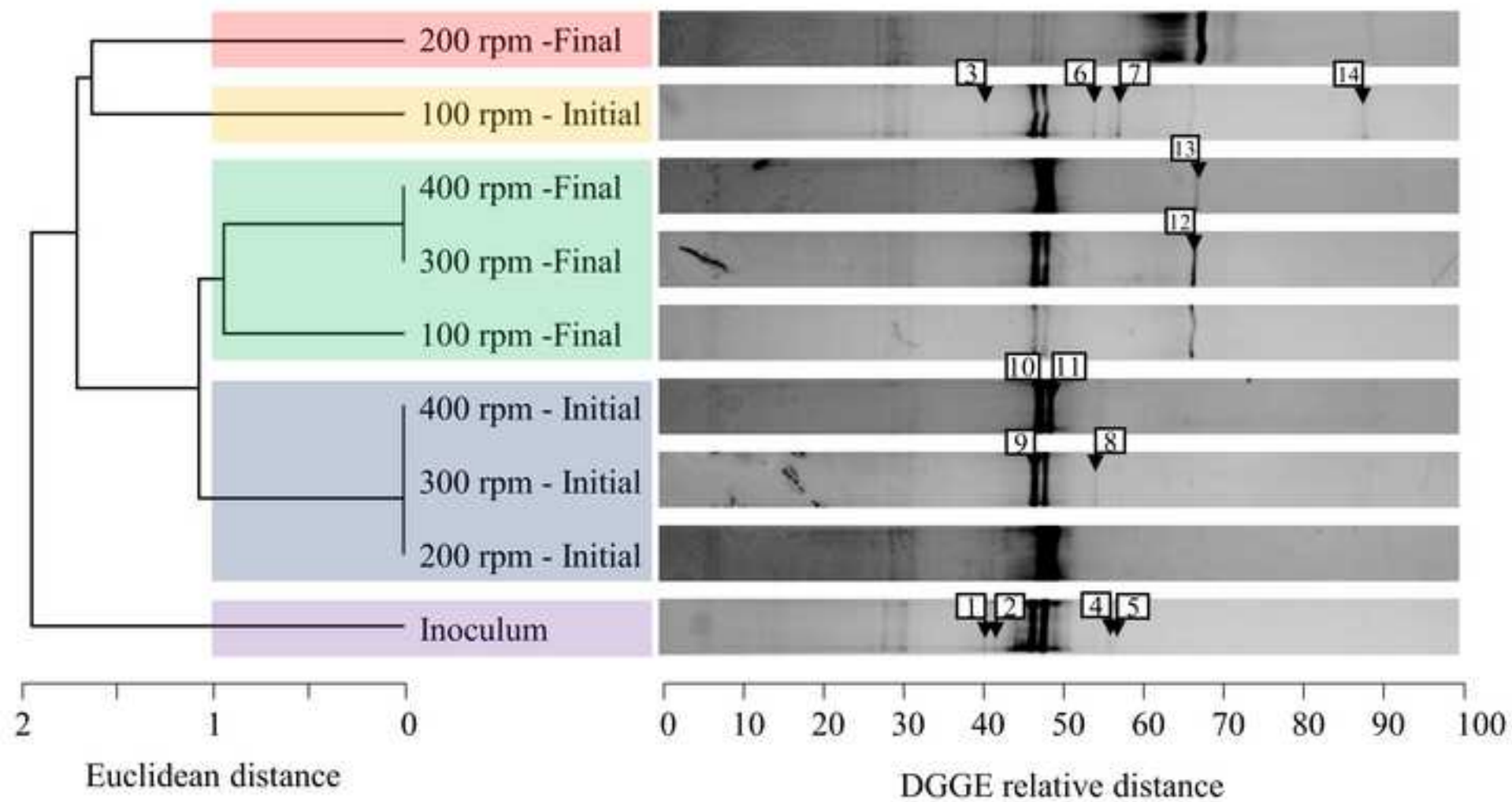


Figure 4
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