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## Functional versus phylogenetic fingerprint analyses for monitoring hydrogen-producing bacterial populations in dark fermentation cultures

Marianne Quéméneur, Jérôme Hamelin, Eric Latrille, Jean-Philippe Steyer,  
Eric Trably

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1 **Functional versus phylogenetic fingerprint analyses for monitoring**  
2 **hydrogen-producing bacterial populations in dark fermentation cultures**

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7 Marianne Quéméneur, Jérôme Hamelin, Eric Latrille, Jean-Philippe Steyer, and Eric Trably\*

8

9

10 Author address:

11 INRA, UR050, Laboratoire de Biotechnologie de l'Environnement, avenue des Etangs,

12 Narbonne, F-11100, France

13

14

15 \* Corresponding author:

16

17 Tel.: +33(0)468425151 ; Fax: +33(0)468425160 ; E-mail address: [trably@supagro.inra.fr](mailto:trably@supagro.inra.fr);

18

## 19 **Abstract**

20 The fermentative hydrogen production during the anaerobic digestion of organic matter is a  
21 promising technology for the renewable energy search. However, the mixed culture  
22 fermentation performances vary considerably depending on environmental conditions such as  
23 pH. We investigated the ability of a molecular CE-SSCP (capillary electrophoresis-single  
24 strand conformation polymorphism) fingerprinting method based on the *hydA* functional  
25 genes to describe better the bacterial community dynamics, as compared to the standard 16S  
26 rDNA-based method. A series of batch experiments was performed from sucrose at different  
27 initial pH from 4 to 6. As expected, higher H<sub>2</sub> production potentials (H<sub>max</sub>) and rates (R<sub>max</sub>)  
28 were obtained at the highest pH. Changes in batch performances were associated with shifts  
29 in the *hydA* diversity and structure. In contrast, 16S rDNA-based fingerprints were less  
30 sensitive to changes in H<sub>2</sub> production performances. The H<sub>max</sub> was related to lower *hydA*  
31 diversity, with *Clostridium sporogenes* as the major H<sub>2</sub> producer. Communities harboring  
32 larger *hydA* diversities were found in experiments with the higher R<sub>max</sub>, suggesting that  
33 species coexistence may have positive effects on H<sub>2</sub> productivity.

34

## 35 **Keywords**

36 Biohydrogen; 16S rDNA fingerprint; *hydA* functional genes; anaerobic digestion; mixed  
37 cultures; microbial diversity.

## 38 **1. Introduction**

39

40 Dark fermentation using mixed anaerobic microbial communities is an attractive way to  
41 produce hydrogen (H<sub>2</sub>), one of the most promising clean and sustainable energy carriers [1,  
42 2]. In recent years, the mixed microbial cultures have been investigated for producing H<sub>2</sub> at  
43 low cost from renewable organic wastes [2, 3, 4]. With regard to pure cultures, mixed cultures  
44 are more suitable for degrading a wide range of substrates, due to their better adaptation to  
45 diverse media and their high resistance and resilience against environmental changes or  
46 organic matter loads.

47 H<sub>2</sub> is a key metabolic intermediate used for electron carrier for interspecies energy transfer  
48 exchanges in anoxic systems. Therefore, when environmental conditions change, the H<sub>2</sub>  
49 production performances observed in mixed cultures are not only affected by the metabolic  
50 shifts, but also by the changes in population structures [5-7]. Indeed, the presence and activity  
51 of efficient H<sub>2</sub>-producing bacteria (HPB), in mixed cultures, can be strongly affected by the  
52 metabolic and phylogenetic interactions between the different community members.  
53 Consequently, a better understanding of the bacterial population dynamics is needed for  
54 improving the stability and the performances of H<sub>2</sub> dark fermentation bioprocesses. These  
55 bacterial population dynamics can be analyzed using different levels of resolution:  
56 phylogenetic and/or functional. To this end, accurate molecular tools and descriptors have to  
57 be used to gain insights into the links between diversity/structure of the bacterial community  
58 and H<sub>2</sub> evolution.

59 For the past decade, the fingerprinting techniques based on 16S rDNA sequences have been  
60 widely used to characterize the bacterial communities in H<sub>2</sub> dark fermentation processes [3, 5,  
61 8-12]. These fingerprinting techniques, including denaturing gradient gel electrophoresis  
62 (DGGE) [13], terminal restriction fragment length polymorphism analysis (T-RFLP) [14] or

63 capillary electrophoresis-single strand conformation polymorphism analysis (CE-SSCP) [15]  
64 provide simultaneously: (i) a rapid analysis of the relative abundance of the different  
65 community members; and (ii) a comparative analysis of the microbial population structures in  
66 relation to the changes in environmental conditions. These fingerprinting techniques were  
67 previously applied to identify the prominent species in HPB mixed cultures and to correlate  
68 their presence to the H<sub>2</sub> production performances and the environmental conditions. In this  
69 way, the highest H<sub>2</sub> production yields were usually observed when mixed cultures were  
70 predominantly composed of *Clostridium* species [2, 4, 7]. The fingerprinting techniques are  
71 well adapted for analyzing the microbial communities and investigating the inter-species  
72 interactions in dark fermentation bioreactors, because H<sub>2</sub> production often relies on a  
73 relatively small number of highly specialized microbial species. However, relatively little is  
74 known about the relationship existing between the microbial community structure and the H<sub>2</sub>  
75 production performances. Methods based on 16S rDNA provide information about the  
76 phylogeny of bacterial species present in the community, but not direct physiological  
77 information and fail occasionally to distinguish closely related H<sub>2</sub>-producing *Clostridium*  
78 species [6, 16].

79 Regarding the overall 16S rDNA fingerprint methods, very few studies have specifically  
80 characterized the HPB populations using functional fingerprinting methods [6, 11]. In  
81 particular, the fingerprinting methods based on *hydA* genes, encoding the [Fe-Fe]-  
82 hydrogenases (H<sub>2</sub>ases) are very meaningful, as they specifically target the bacterial  
83 populations directly involved in H<sub>2</sub> evolution, and provide valuable information about their  
84 structure and diversity. Several methods are now available for evaluating and comparing the  
85 fingerprint patterns [17-19]. Moreover, the diversity and dynamics of bacterial communities  
86 can be quantified from CE-SSCP profiles, using molecular descriptors such as Simpson's  
87 diversity index or Euclidean distances [20]. At last, if combined with multivariate analyses,

88 they can be used to find statistical correlations between the microbial community structures  
89 and the environmental parameters [20, 21]. Many environmental parameters can change the  
90 performance of fermentative H<sub>2</sub> production by modifying the bacterial metabolic pathways  
91 and community structure [2, 4, 7]. Among them, the pH appears to be one of the most  
92 important factor influencing H<sub>2</sub> performances [4, 22, 23]. The pH also influences the  
93 microbial community structure because it is a key parameter affecting carbon metabolism and  
94 growth rates of microorganisms [12].

95 In this paper, both *hydA*- and 16S rRNA gene-based fingerprinting methods were compared  
96 for monitoring bacterial population dynamics in response to pH changes. A series of batch  
97 tests were operated at different initial pH to induce a wide range of H<sub>2</sub> production  
98 performances and different bacterial composition. The fingerprint characterization followed  
99 by identification of H<sub>2</sub> producers were also used to assess link between H<sub>2</sub> production  
100 performances and bacterial community structures and diversities.

101

## 102 **2. Materials and methods**

103

### 104 **2.1. Hydrogen production in batch tests**

105

106 The H<sub>2</sub> production experiments were carried out in 500 mL glass bottles under batch  
107 conditions. The inoculum corresponded to an anaerobically digested sludge pretreated by  
108 heat/shock treatment (90°C, 10 min). Two milliliters of the pretreated inoculum (final  
109 concentration of 225 mg-COD/L) were inoculated in the following culture medium: 40 mM  
110 MES, 10 g sucrose, 1L deionized water. The total working volume was 200 mL. The initial  
111 pH was adjusted to 4, 4.5, 5, 5.5 and 6. All batch tests were performed in triplicates. After  
112 inoculation, each bottle was flushed with nitrogen for 5 min to maintain anaerobic conditions.

113 The bottles were then capped with a rubber stopper and incubated at 37°C for 24 days. Four  
114 milliliters of the mixed cultures were collected daily during the first 10 days of the  
115 experiments, and every 2<sup>nd</sup>-3<sup>rd</sup> day during the last 14 days of the experiments. The samples  
116 were centrifuged (20,000 g, 10 min) and the supernatants and the pellets were stored at -20°C  
117 for further chemical analyses and DNA extraction, respectively.

118

## 119 **2.2. Chemical analysis (biogas, metabolic byproducts)**

120

121 The volume of biogas was daily measured using an acidified water displacement method. The  
122 biogas composition (CH<sub>4</sub>, CO<sub>2</sub>, H<sub>2</sub> and N<sub>2</sub>) was determined by gas chromatography (GC-14A,  
123 Shimadzu) as previously described by Aceves-Lara *et al.* [23].

124 The volatile fatty acids (VFA) composition in the liquid phase, *i.e.* acetic acid (C<sub>2</sub>), propionic  
125 acid (C<sub>3</sub>), butyric and iso-butyric acids (C<sub>4</sub> and iC<sub>4</sub>) and valeric and iso-valeric acids (C<sub>5</sub> and  
126 iC<sub>5</sub>), were determined by gas chromatography coupled to catharometric detection (GC 8000,  
127 Fisons Instruments). The concentrations of sugars and non-VFAs metabolic byproducts, such  
128 as organic acids (lactate), ethanol or acetone were assessed by High Performance Liquid  
129 Chromatography analysis coupled to a refractometer (Waters Assoc.). The substrate  
130 degradation efficiency was estimated by dividing the amount of sucrose consumed by the  
131 amount of initial sucrose. The H<sub>2</sub> production performances were characterized with the  
132 following parameters, as described by Quéméneur *et al.* [6]: H<sub>2</sub> production potential (mL/L or  
133 mmol-H<sub>2</sub>/L), H<sub>2</sub> production rates (mmol-H<sub>2</sub>/L/d) and H<sub>2</sub> production yield (mol-H<sub>2</sub>/mol-  
134 hexose-consumed). The H<sub>2</sub> production potential (H) was the cumulative H<sub>2</sub> production. The  
135 H<sub>2</sub> production rate (R) represented the daily H<sub>2</sub> production increase. The H<sub>2</sub> yield was  
136 calculated by dividing the maximum H<sub>2</sub> production potential by the sucrose consumed.

137

### 138 **2.3. DNA extraction and PCR amplification**

139

140 For each experiment, molecular analyses of the bacterial communities were performed on the  
141 triplicate samples at the start of the experiments (initial time), at the maximum H<sub>2</sub> production  
142 rate (R<sub>max</sub>), at the maximum H<sub>2</sub> production potential (H<sub>max</sub>) and at the end of the experiments  
143 (after 24-day incubation).

144 Genomic DNA was extracted and purified from cell pellets using the Wizard Genomic DNA  
145 Purification kit (Promega). The purity and amount of DNA in the extracts were measured by  
146 spectrophotometry (Infinite NanoQuant M200, Tecan).

147 The *hydA* genes were amplified using two non-degenerated primers, *hydAClosF* (5' –  
148 ACCGGTGGAGTTATGGAAGC – 3', *C. pasteurianum* position F1258) and 5'-fluorescein  
149 phosphoramidite-labeled *hydAClosR* (5' – CATCCACCTGGACATGCCAT – 3', *C.*  
150 *pasteurianum* position R1508), designed from two consensus regions of an alignment of 31  
151 *hydA Clostridium* reference sequences [6]. Each PCR mixture (50 µL) contained 1X *Pfu*  
152 Turbo DNA polymerase buffer, 200 µM of each dNTP, 0.5 µM of each primer, 0.5 U of *Pfu*  
153 Turbo DNA polymerase (Stratagene) and 10 ng of genomic DNA. Reactions were performed  
154 in a Mastercycler thermal cycler (Eppendorf). The *hydA* genes were amplified as follows:  
155 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s,  
156 with a final extension at 72 °C for 10 min. The presence and size of about 200 bp PCR  
157 products were determined by ethidium bromide-stained (2%) gel electrophoresis.

158 Meanwhile, the 16S rRNA genes were amplified using *Pfu* Turbo DNA polymerase  
159 (Stratagene) and universal primers W49 (5'-ACGGTCCAGACTCCTACGGG-3', *Escherichia*  
160 *coli* position F331) [24] and 5'-fluorescein phosphoramidite-labeled W104 (5'-  
161 TTACCGCGGCTGCTGGCAC-3', *E. coli* position R533) [24], targeting about 200 bp of the  
162 V3 region of the 16S rRNA gene. The PCR reactions were performed as described above,



163 except that 25 cycles and primer hybridization at 61 °C were applied and 130 ng of each  
164 primer were used. The *Clostridium* genus was specifically analyzed by nested PCR. First, the  
165 16S rRNA genes of the *Clostridium* genus were amplified using AccuPrime *Taq* DNA  
166 polymerase (Invitrogen), a bacterial domain forward primer W18 (5'-  
167 GAGTTTGATCMTGGCTCAG -3', *E. coli* position F9) [15] and a *Clostridium*-specific  
168 reverse primer W109 (5'- CCCTTTACACCCAGTAA -3', *E. coli* position R561) [25], as  
169 described by Peu *et al.* [26]. Then, the diluted PCR products was used as a template for a  
170 universal 16S rDNA PCR, carried out using the primer set W49/W104.

171

#### 172 **2.4. CE-SSCP electrophoresis and statistical analyses**

173

174 One microliter of 5- to 2000-fold diluted PCR product was mixed with 18.8 µL of formamide  
175 and 0.2 µL of internal standard GeneScan ROX (Applied Biosystems). Samples were heat-  
176 denatured at 95°C for 5 min and immediately cooled in ice. CE-SSCP electrophoresis was  
177 performed in an ABI Prism 3130 genetic analyser (Applied Biosystems) with 50 cm-long  
178 capillary tubes filled with a non-denaturing 5.6% conformation analysis polymer (Applied  
179 Biosystems). Samples were eluted at 12 kV and 32°C for 30 min (for 16S rRNA gene) or 64  
180 min (for *hydA* gene). Raw CE-SSCP data were analyzed using GeneScan software (Applied  
181 Biosystems).

182 The CE-SSCP profiles were aligned with the internal standard ROX to correct any change in  
183 the electrophoretic motility between runs. The sum of the peak areas were normalized to unit  
184 before statistical analysis. The StatFingerprints library from R was used (R Development  
185 Core Team 2009) [27]. Euclidean Distances (ED) between each pair of CE-SSCP profiles  
186 were calculated for matrix generation and used as measurements of dissimilarity between  
187 bacterial communities.

188 An analysis of similarity (ANOSIM) was also used to investigate the effects of the initial pH,  
189 considered as a qualitative variable, on the structure of bacterial communities [28]. ANOSIM  
190 gives a statistic, ANOSIM R, which indicates the magnitude of dissimilarities among groups  
191 of samples. An ANOSIM R = 1 indicates that the groups of communities with different initial  
192 pH are dissimilar, and at the opposite, ANOSIM R = 0 indicates that the groups cannot be  
193 distinguished statistically. The statistical significance of ANOSIM R was tested by Monte  
194 Carlo randomization.

195 ED were also used to compare, over time, the effect of the initial pH on the divergence of the  
196 bacterial communities at functional (*hydA* genes) and phylogenetic levels (16S rRNA genes).  
197 For each batch tests, the ED was normalized by dividing the values obtained at each time by  
198 the largest distance calculated for the time course, giving values between 0 and 1 during the  
199 24-day incubations. A normalized ED = 1 means that the community is the most dissimilar to  
200 that on day 0 and a normalized ED = 0 means that the community is identical to that on day 0.  
201 The complexity of the bacterial community was estimated using Simpson's diversity index  
202 (D) from a CE-SSCP profile, by considering the number of species (number of peaks) as well  
203 as their relative abundance (area under each peak) [29].

204

## 205 **2.5. Assignment of the partial *hydA* sequences in mixed cultures**

206

207 The PCR products obtained from the H<sub>2</sub>-producing mixed cultures were purified using a PCR  
208 Purification Kit (Qiagen). The *hydA* clone libraries were constructed using the TA Cloning kit  
209 (Invitrogen). The PCR-CE-SSCP profile of each PCR product, amplified using plasmid-  
210 targeted primers T7 and P13, was compared with the HPB culture profiles for peak  
211 assignment. The cloned inserts were sent for sequencing (MilleGen company, Toulouse,  
212 France). The protein sequences, deduced from the nucleotide *hydA* sequences, were aligned

213 with reference sequences retrieved from the Genbank database using the CLUSTALW  
214 program [30], and further refined manually using the BioEdit program [31]. Distances were  
215 estimated by the Kimura method [32], and the neighbor-joining method was used for inferring  
216 the tree topologies [33]. Tree topology confidence was determined by bootstrap analysis on  
217 1000 replicates [34]. All phylogenetic programs were implemented with SeaView software  
218 [35]. The *hydA* gene sequences were deposited in the Genbank database under the accession  
219 numbers HQ241275 to HQ241278.

220

### 221 **3. Results and discussion**

222

#### 223 **3.1. Hydrogen production in batch tests**

224

225 The biogas produced by mixed cultures contained only H<sub>2</sub> and CO<sub>2</sub>, without detectable CH<sub>4</sub>,  
226 suggesting that the heat shock pretreatment was effective for inhibiting the methanogenic  
227 bacteria issued from the anaerobic sludge.

228 Figure 1 shows the accumulated H<sub>2</sub> production in mixed cultures at different initial pH over  
229 the 24 days of incubation. Table 1 summarizes the results of pH experiments. In comparison  
230 with other reports [12, 36, 38], time to reach the higher cumulated H<sub>2</sub> is large (ranging  
231 between 9.7 and 24 days) because the nutrients (except carbon source) were just provided by  
232 the inoculum. The maximal H<sub>2</sub> production rates ( $R_{\max}$ ) increased gradually from 16.4 to 38.3  
233 mmol-H<sub>2</sub>/L/d with the increase in pH from pH 4 to 6 ( $p < 0.05$ ). Initial pH also affected H<sub>2</sub>  
234 production with the highest maximum H<sub>2</sub> production ( $H_{\max}$ ) of 102.7 mmol-H<sub>2</sub>/L attained at  
235 pH 5 ( $p < 0.05$ ). Indeed, the  $H_{\max}$  increased with the rise in pH from 4 to 5, but reached a level  
236 of equilibrium in the pH range from 5 to 6 (Table 1). It is noteworthy that the  $H_{\max}$  obtained at  
237 pH 5.5 and pH 6 were reached earlier than pH 5, indicating that higher pH improves H<sub>2</sub>

238 production reaction. Interestingly, at pH 5.5 and pH 6, the H<sub>2</sub> production phase was followed  
239 by H<sub>2</sub> consumption phase (Figure 1). The H<sub>2</sub> content in the gas phase increased until the H<sub>max</sub>  
240 was reached (44.5% and 43.3%, for pH 5.5 and pH 6 respectively), and then declined at the  
241 end of experiments (28.5% and 27.4%, respectively) (Table 1). Similar H<sub>2</sub> consumption  
242 phases have previously been reported during fermentative H<sub>2</sub> production batch tests [36, 37].  
243 The present results showed that the optimal pH for H<sub>2</sub> production was in the range of 5 to 6.  
244 They were in agreement with comparable studies (*i.e.* batch cultures, without pH control,  
245 using 10 g-sucrose/L and heat-shocked mixed microflora) [38, 39]. For instance, Van Ginkel  
246 *et al.* [39] studied H<sub>2</sub> production in a pH range 4.5-6.5, using compost, potato soil or soybean  
247 soil as seed microflora, and found that pH 5-6 were the most suitable for H<sub>2</sub> production.  
248 Khanal *et al.* [38] studied H<sub>2</sub> production in a pH range 4.5-6.5 using compost, and showed  
249 that higher R<sub>max</sub> were obtained at higher pH. Shorter duration of the H<sub>2</sub> production was also  
250 found at higher pH and final pH increased with the increase in initial pH (Table 1). However,  
251 our results were different from this latter study [38], because these authors found the highest  
252 H<sub>max</sub> at pH 4.5. A low pH 4.5 was also found optimal for H<sub>2</sub> production in a pH range 4-7  
253 using anaerobic digester sludge [12]. In our study, no significant difference was observed  
254 between the H<sub>2</sub> yields ranging between 1.7 and 2 mol-H<sub>2</sub>/mol-hexose (Table 1). This finding  
255 can be explained by the variation of the substrate degradation efficiency increasing from  
256 53.4% to 100% with the increase in pH (Table 1). H<sub>2</sub> yield of 2 mol-H<sub>2</sub>/mol-hexose  
257 associated with optimal pH 5.5-6 were previously obtained using mixed cultures [40] or pure  
258 cultures [41].

259 As expected, the cumulated H<sub>2</sub> production was strongly correlated to intermediate metabolite  
260 productions (Table 1). The gradual increase of R<sub>max</sub> in the pH range from 4 to 6 matched with  
261 the increase in intermediate metabolites. The productions of total VFAs and overall soluble  
262 microbial products (SMPs) also increased with the increase of H<sub>max</sub> in the pH range from 4 to

263 5, but achieved a level of equilibrium within pH range 5 to 6 (Table 1). When  $H_{\max}$  was  
264 reached, butyrate (Bu) and acetate (Ac) contents in the SMPs ranged from 63.0% to 72.3%  
265 and 25.3% to 27.7%, respectively, while ethanol constituted only 5.6 to 11.5%. The  
266 production of Bu and Ac, as the major fermentation products, suggested that *Clostridium*  
267 species were the dominant bacteria in the mixed cultures. No significant difference of Bu/Ac  
268 ratios, ranging between 2.5 and 2.7, were detected according to pH (Table 1), suggesting that  
269 no metabolic switches occurred when  $H_{\max}$  was reached. At the end of the experiment, after  
270 the  $H_2$  consumption phase, at pH 5.5 and pH 6, caproate accumulated and represented 4.0%  
271 and 6.1% of the SMPs, respectively. Moreover, at pH 5.5 and pH 6, Bu/Ac ratios decreased  
272 significantly from 2.7 and 2.5 to 1.8 and 2.1, respectively. The  $H_2$  consumption phase can  
273 result from two metabolic pathways, either homoacetogenesis, producing only acetate from  
274  $H_2$  and  $CO_2$ , and caproate formation, produced from  $H_2$  and equimolar acetate and butyrate.  
275 Such changes in Bu/Ac ratios can be explained by metabolic alterations or switches due to  
276 environmental changes such as pH [38], but also can result from microbial community  
277 structure shifts during the fermentative  $H_2$  production.

278

### 279 **3.2. Functional and phylogenetic dynamics of the bacterial community**

280

281 CE-SSCP fingerprinting was used to compare the bacterial community structures at various  
282 pHs. Whatever the initial pH, highly similar bacterial- and *Clostridium*-specific 16S rDNA  
283 CE-SSCP fingerprinting patterns were obtained from all the  $H_2$ -producing cultures (data not  
284 shown). This result indicated that the *Clostridium* genus was largely dominant and played a  
285 key role in these  $H_2$ -producing experiments. This finding suggested that the use of a heat-  
286 shock pretreated sludge as inoculum led to select a large majority of *Clostridium* species as  
287 already mentioned in previous studies [4-7].

288 Figure 2 represents the relative divergence of the bacterial communities over time in response  
289 to the pH changes. Whatever the gene tested, the highest changes in CE-SSCP profiles were  
290 observed during the first days of the experiments when  $R_{\max}$  was reached. The divergence  
291 gradually increased with decreasing pH. Moreover, the highest increase of the community  
292 divergence was obtained at pH 4 between day 3 and 6 and the lowest was at pH 6. It is  
293 noteworthy to say that  $R_{\max}$  was the lowest at pH 4 and the highest at pH 6 (Table 1). For all  
294 pH, the maximal relative divergences were observed after 10 days of experiment. At pH 4-5,  
295 the relative divergence was stable until the end of the experiments. In contrast, at pH 5.5 and  
296 6, a decrease in the relative divergence was shown at day 24, when  $H_2$  consumption was  
297 observed (Figure 1). Thus, the shift of the CE-SSCP profiles over time was correlated to the  
298 pH changes. Fang *et al.* [12] also showed that total bacterial community structure changed  
299 with respect to pH changes. However, our study showed that the *hydA* structure was globally  
300 more affected by the pH than the total bacteria population structure (*hydA* ANOSIM  $R = 0.29$ ,  
301  $p < 0.001$ ; 16S rDNA ANOSIM  $R = 0.16$ ,  $p < 0.05$ ). In addition, high initial relative  
302 divergences were observed for the *hydA* profiles, indicating a more sensitive response of the  
303 HPB population to the pH changes. Moreover, contrary to 16S rDNA structure, significant  
304 negative correlation was also observed between  $R_{\max}$  and the rate of *hydA* structure change.  
305 Consequently, our results indicated that the changes in the *hydA* community structure  
306 provided better information to monitor  $H_2$  production change than the total bacteria  
307 population structure.

308 Figure 3 shows, for each pH, the dynamics of the CE-SSCP fingerprints based on the *hydA*  
309 (Figure 3A) and 16S rRNA genes (Figure 3B) over the 24-day incubations. Both the *hydA* and  
310 16S rDNA CE-SSCP profiles showed pattern succession consisting of a simplification phase  
311 followed by stabilization and/or instability phase. This simplification was slower at high pH,  
312 and thus, at high  $R_{\max}$ . At the  $R_{\max}$  time, after 3 days (6 days in the case of pH 4), the gradual

313 disappearance of CE-SSCP peaks in relation to pH led to highest dissimilarities within the  
314 CE-SSCP profiles. At the  $H_{max}$  time (day 10 for pH 5.5-6 and day 24 for pH 4-5), the CE-  
315 SSCP fingerprints were both composed of only one major peak and several minor peaks.  
316 Interestingly, additional peaks appeared in CE-SSCP profiles at pH 5.5 and 6, at 24 days,  
317 when  $H_2$  consumption occurred. As previously observed [5, 6], the appearance and  
318 disappearance of CE-SSCP peaks, reflecting changes in the bacterial community composition,  
319 was correlated to  $H_2$  production performances. A simplification followed by a stabilization of  
320 the total bacterial communities have also been reported in similar batch tests of fermentative  
321  $H_2$  production [12] or photo- $H_2$  production [42]. These authors made the assumption that after  
322 a process of competition or cooperation, during the first days, the bacterial community  
323 structure tended to be stable for forming an optimal HPB population before  $H_{max}$  [10]. Our  
324 results were in agreement with the occurrence of such competition/cooperation processes. In  
325 addition, our findings were also consistent with previous studies showing that *hydA*  
326 population structure shifted over time [11, 43]. As *hydA* structure was correlated to  $H_2$  content  
327 shift, Tolvanen *et al.* [43] have reported that *hydA* qPCR melting curve analysis could be used  
328 as an indicator of  $H_2$  performance. Nevertheless, these authors have also indicated that  
329 fingerprinting techniques, such as DGGE or CE-SSCP, provide better resolution than qPCR  
330 melting curves for distinguishing different genes [43]. In this study, the *hydA* structure  
331 obtained by CE-SSCP analysis was thus a more reliable and functional tool for monitoring  
332 bioreactor performances. Moreover, this *hydA* functional fingerprint analysis was also more  
333 suitable than 16S rDNA fingerprint analysis for monitoring HPB populations in dark  
334 fermentation cultures.

335

### 336 **3.3. Bacterial population diversities**

337

338 Simpson's diversity indices (D) were calculated to assess the complexity of the CE-SSCP  
339 profiles and to compare the diversity of the H<sub>2</sub>-producing and total bacterial communities  
340 cultivated at different initial pH. Simpson's diversity index (D) corresponds to the probability  
341 that two individuals randomly selected from a community belong to the same species [29].  
342 Figure 4 presents the D values obtained from the *hydA* and 16S rDNA CE-SSCP profiles  
343 when the R<sub>max</sub> (Figure 4A) and the H<sub>max</sub> (Figure 4B) were reached. The D values obtained  
344 from the 16S rDNA CE-SSCP profiles were higher than those obtained from the *hydA* CE-  
345 SSCP profiles (Figures 4A and 4B). Whatever the gene tested the lowest D values were  
346 observed when the H<sub>max</sub> occurred (Figure 4B), while the highest values were obtained at the  
347 R<sub>max</sub> (Figure 4A). These results were in agreement with previous studies showing that the  
348 H<sub>max</sub> was associated with low bacterial diversity.

349 When H<sub>max</sub> was reached, no significant difference was observed between the D values  
350 obtained at different pH (Figures 4B). However, Fang *et al.* has reported that diversity  
351 obtained at the H<sub>max</sub> time increased with the rise of pH from 4.5 to 6.5 in an earlier study  
352 carried out using anaerobic digested sludge as the inoculum and rice slurry as the substrate  
353 (5.5 g-carbohydrate/L) [12]. This apparent difference is not contradictory with our results,  
354 because similar results could be obtained if these authors had used twice as much sugar as in  
355 our experience. Indeed, our results showed that the simplification of bacterial population was  
356 slower at higher pH (Figures 2 and 3).

357 When R<sub>max</sub> was reached, the *hydA* D values of the HPB communities ranged between 1.55  
358 and 2.67 and increased significantly with the increase in pH ( $p < 0.05$ ) (Figure 4A). The  
359 lowest D value was observed at pH 4, and was related to the lowest R<sub>max</sub>. In contrast, the  
360 highest D value observed at pH 6 was related to the highest R<sub>max</sub>. Correlation analysis  
361 highlighted strong positive correlation between the R<sub>max</sub> and the *hydA* D values. Thus, the  
362 present results indicated that greater *hydA* diversity (*i.e.* a slower simplification of the HPB



363 population) leads to higher  $R_{\max}$ , suggesting the establishment of positive bacterial  
364 interactions, such as co-metabolism or synergy. However, on a longer term, high *hydA*  
365 diversity, as observed at pH 5.5 and 6, affected the stability of the highest cumulated  $H_2$   
366 production value ( $H_{\max}$ ), through the increase in the  $H_2$  consumption activity. Similarly, the  
367 lowest 16S rDNA D value was found at the lowest pH while the highest D value was detected  
368 at the highest pH 6 (Figure 4A). Nevertheless, contrary to the *hydA* diversity, no significant  
369 correlation was found between 16S rDNA D values and the  $R_{\max}$ . Since the ecosystem  
370 functionality correlated with the *hydA* diversity, it can be concluded that the *hydA* functional  
371 marker was more informative than 16 rDNA phylogenetic marker and can be used as an  
372 indicator of the  $H_2$  production.

373

#### 374 **3.4. Affiliation of hydrogen producers**

375

376 Five dominant *hydA* CE-SSCP peaks were detected during the 24 days incubation (Figure 3).  
377 All peaks were assigned to *hydA* sequences affiliated with those of *Clostridium* cluster I  
378 species (Figure 5), supporting the predominant role of this cluster in HPB mixed cultures [5,  
379 16].

380 During the first days of the experiments and according to pH, from one to four different peaks  
381 were detected within the *hydA* CE-SSCP profiles. Peaks 1-3 were detected in both pH 5.5 and  
382 6 but were undetectable at lower pH. At pH 5.5 and 6, they were dominant after 3 days, when  
383  $R_{\max}$  was reached, but undetectable after 10 days, when  $H_{\max}$  occurred. The three *HydA*  
384 sequence of clone that matched with peaks 1-3 formed a group showing 86.2-87.5 % identity  
385 with that of *C. acetobutylicum* (Figure 5). Consequently, the increase of  $R_{\max}$  at highest pH  
386 (Table 1) may be associated with the growth of *C. acetobutylicum hydA* like gene-carrying  
387 strains known to be efficient  $H_2$  producers at pH higher than 5.5 in pure cultures [41, 44].

388 Peak 4 was observed whatever pH and time, showing a wider ecological niche compared to  
389 the other species of the seed inoculum. It dominated the *hydA* CE-SSCP at pH 4-5 and  
390 became the dominant H<sub>2</sub> producer after 10 days at pH 5.5 and 6. The HydA sequence of the  
391 clone matching with the peak 4 was affiliated with that of *Clostridium sporogenes* (70%  
392 identity) (Figure 5) whose optimum growth pH is around 6-7. HydA sequences affiliated to *C.*  
393 *sporogenes* were previously retrieved from mesophilic fermentative H<sub>2</sub>-producing batch  
394 cultures [6, 8]. In mesophilic continuous flow reactors using sucrose and heat-pretreated  
395 inocula, Duangmanee *et al.* [45] also found *C. sporogenes* among other strains as dominant  
396 species during H<sub>2</sub> production. This result confirms the key role of this species in H<sub>2</sub>-producing  
397 mixed cultures. Several studies with pure cultures of *C. sporogenes*, known as a non-toxicogenic  
398 variant of *C. botulinum*, have been previously conducted to inhibit growth of the toxicogenic  
399 species in food ecosystems [46]. However, to date no study has investigated biochemical  
400 kinetics of fermentative H<sub>2</sub> production by *C. sporogenes*, despite its repeated detection in H<sub>2</sub>-  
401 producing mixed cultures. Future studies should investigate this aspect in more detail.

402 Peak 5 was only detectable during the H<sub>2</sub> consumption phase at pH 5.5 and 6, suggesting that  
403 it could represent an H<sub>2</sub> consumer. Indeed, H<sub>2</sub> consumers, such as homoacetogens (*e.g.* *C.*  
404 *carboxidivorans*, *C. magnum*), can also contain *hydA* genes and their presence could explain  
405 the increase in acetate level at 24 days at pH 5.5 and 6. However, the HydA sequence of the  
406 clone matching with the peak 5 exhibited 88.2% identity with that of *C. acetobutylicum*  
407 (Figure 5), which has not yet been reported as  
408 homoacetogen. However, the clostridial *hydA* database needs to be improved for obtaining  
409 more accurate affiliation of clostridial H<sub>2</sub>-producers in dark fermentative bioreactors. It is  
410 noteworthy that the estimation of the clostridial strain function can be biased because  
411 clostridial fermenters and homoacetogens are phylogenetically closely related [47].  
412 Furthermore, some members of *Clostridium* genus, such as *C. acetobutylicum* or *C.*

413 *sporogenes*, detected during the H<sub>2</sub> consumption phase, are also known to harbor plasmid or  
414 chromosomal genes encoding [NiFe]-H<sub>2</sub>ases [48, 49]. Contrary to [FeFe]-H<sub>2</sub>ases, [NiFe]-  
415 H<sub>2</sub>ases from *Proteobacteria* are usually known to catalyze H<sub>2</sub> uptake in vivo, but [NiFe]-  
416 H<sub>2</sub>ases from *Clostridia* have not yet been studied and there is no information about their  
417 physiological function [48, 49]. Because this study focused on HPB, only clostridial genes  
418 encoding [FeFe]-H<sub>2</sub>ases have been targeted by specific functional primers [6]. Further  
419 functional studies on both [Fe-Fe]- and [NiFe]-H<sub>2</sub>ases have to be performed to investigate the  
420 potential metabolic flexibility of *Clostridia* and the H<sub>2</sub> recycling in dark fermentation  
421 processes.

422

423 **4. Conclusion**

424

425 In this study, the changes in both the taxonomic and functional bacterial community dynamics  
426 were compared during fermentative H<sub>2</sub> production. The main result of this work was that  
427 changes in *hydA* functional CE-SSCP fingerprints were better related to the H<sub>2</sub> production  
428 performances than 16S rDNA patterns. This work also demonstrated the usefulness of  
429 molecular descriptors, such as Simpson's diversity index and Euclidean distance, to visualize  
430 changes in bacterial community in relation to changes in environmental factors. Like the  
431 analysis of *hydA* gene expressions, the use of *hydA* fingerprinting CE-SSCP method coupled  
432 with the estimation of molecular descriptors offers valuable information for improving dark  
433 fermentation system monitoring and thus, provides a valuable tool for further studies on  
434 bacterial interactions in fermentative H<sub>2</sub>-producing bioreactors.

435

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437

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- 578

579 **Captions to figures**

580

581 **Figure 1.** Cumulative H<sub>2</sub> production of mixed cultures cultivated at different initial pHs.  
582 Values correspond to the average of triplicate analysis and error bars represent the standard  
583 deviations. The solid and dotted lines represent the H<sub>2</sub> production phase and the H<sub>2</sub>  
584 consumption phase, respectively.

585

586 **Figure 2.** Relative community divergence of the CE-SSCP profiles expressed by the relative  
587 Euclidean distance and based on the *hydA* genes (A) and the 16S rRNA genes (B) retrieved  
588 from the mixed cultures cultivated at various initial pHs. Points correspond to mean values (n  
589 = 3) and error bars to standard deviations.

590

591 **Figure 3.** Dynamics of the CE-SSCP profiles based on the *hydA* genes (A) and the 16S rRNA  
592 genes (B). The CE-SSCP profiles were aligned on the basis of the common ROX internal  
593 standard, and areas normalized. The x, y and z axis of each CE-SSCP profiles represent the  
594 relative peak migration distance, the relative peak intensity and the timeline, respectively. A  
595 representative CE-SSCP profile from the three replicates is presented for each pH.

596

597 **Figure 4.** Effect of the initial pH on the Simpson's diversity index obtained from the CE-  
598 SSCP profiles based on the *hydA* genes and the 16S rRNA genes at the maximum H<sub>2</sub>  
599 production rates (A) and at the maximum H<sub>2</sub> production potentials (B).

600

601 **Figure 5.** Neighbor-joining phylogenetic tree of the HydA sequences (134 amino acids). The  
602 Genbank accession numbers (in brackets) were obtained from the protein sequence databases.  
603 The sequences obtained in this study are in bold and highlighted. Circles at the branch nodes

604 represent bootstrap confidence level percentages obtained from 1000 replicates: black circles  
605 = 75-100%; white circles = 50-75%. The scale bar indicates 5% sequence divergence. The  
606 *Desulfovibrio vibrio* HydA sequences were used as outgroups.

Figure 1

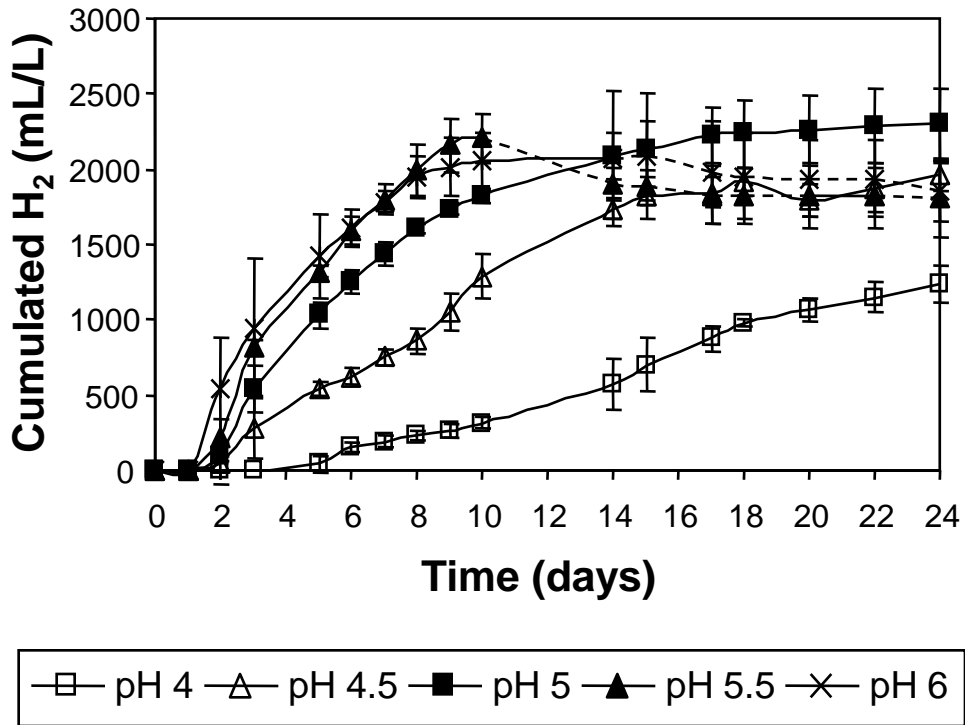
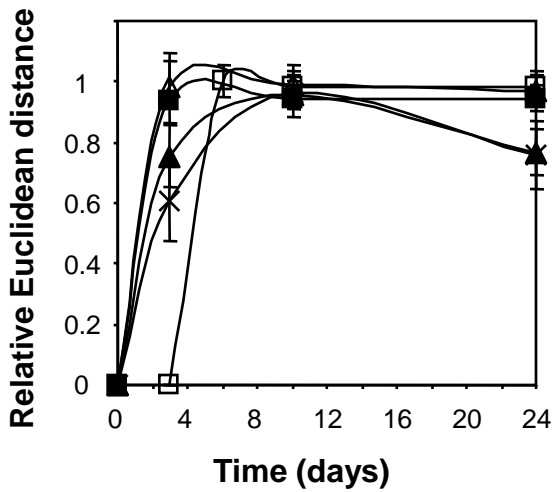
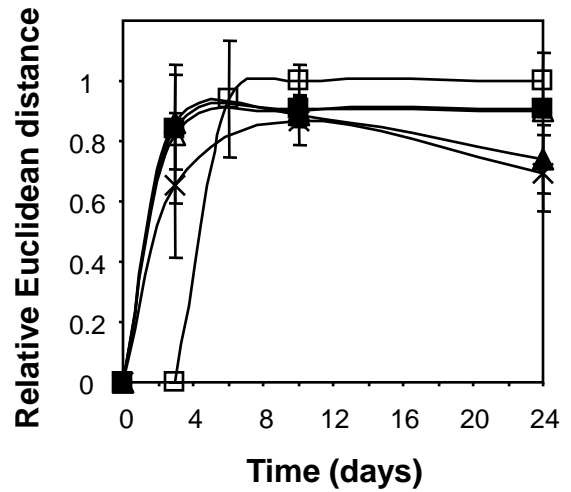


Figure 2

A. *hydA*

B. 16S rDNA



□ pH 4    △ pH 4.5    ■ pH 5    ▲ pH 5.5    × pH 6

Figure 3

A. *hydA*

B. 16S rDNA

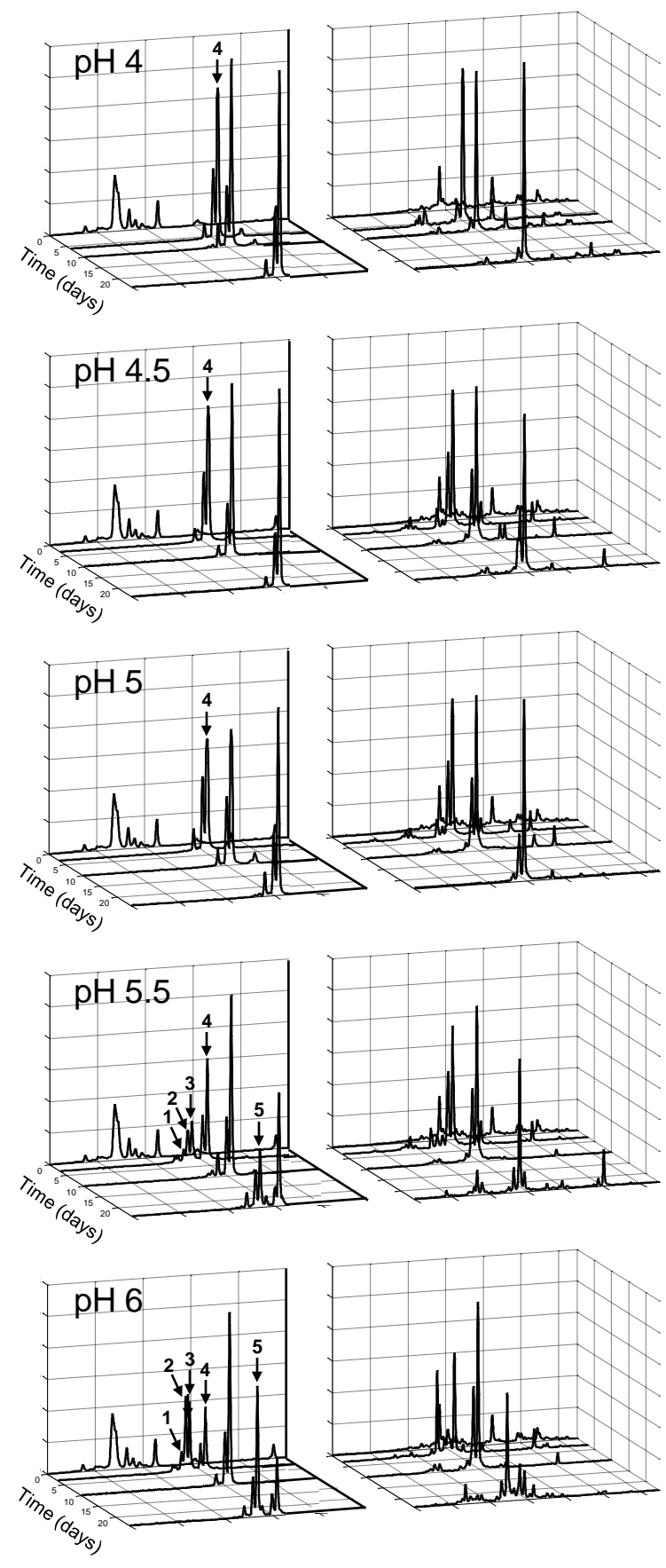




Figure 4

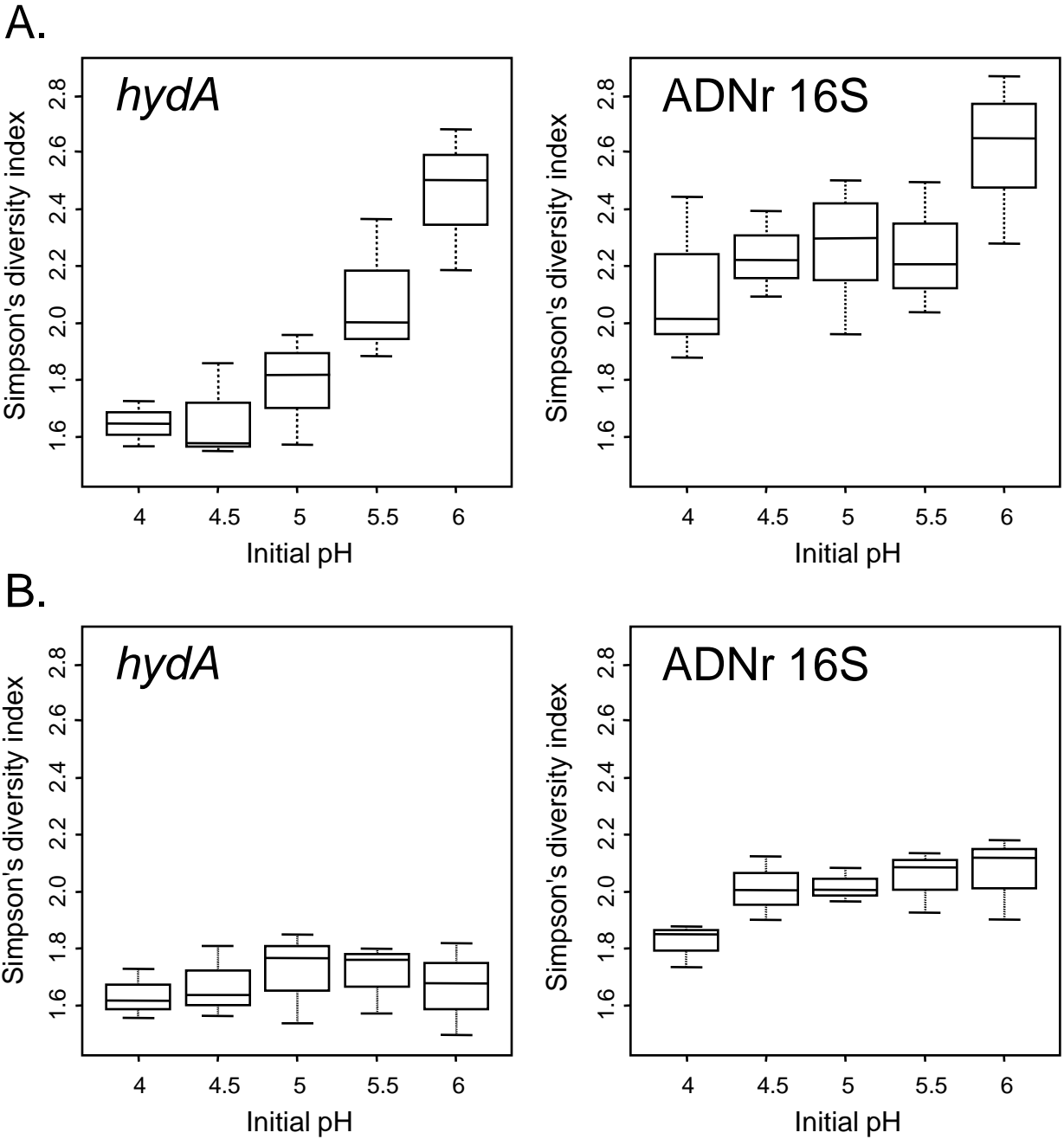
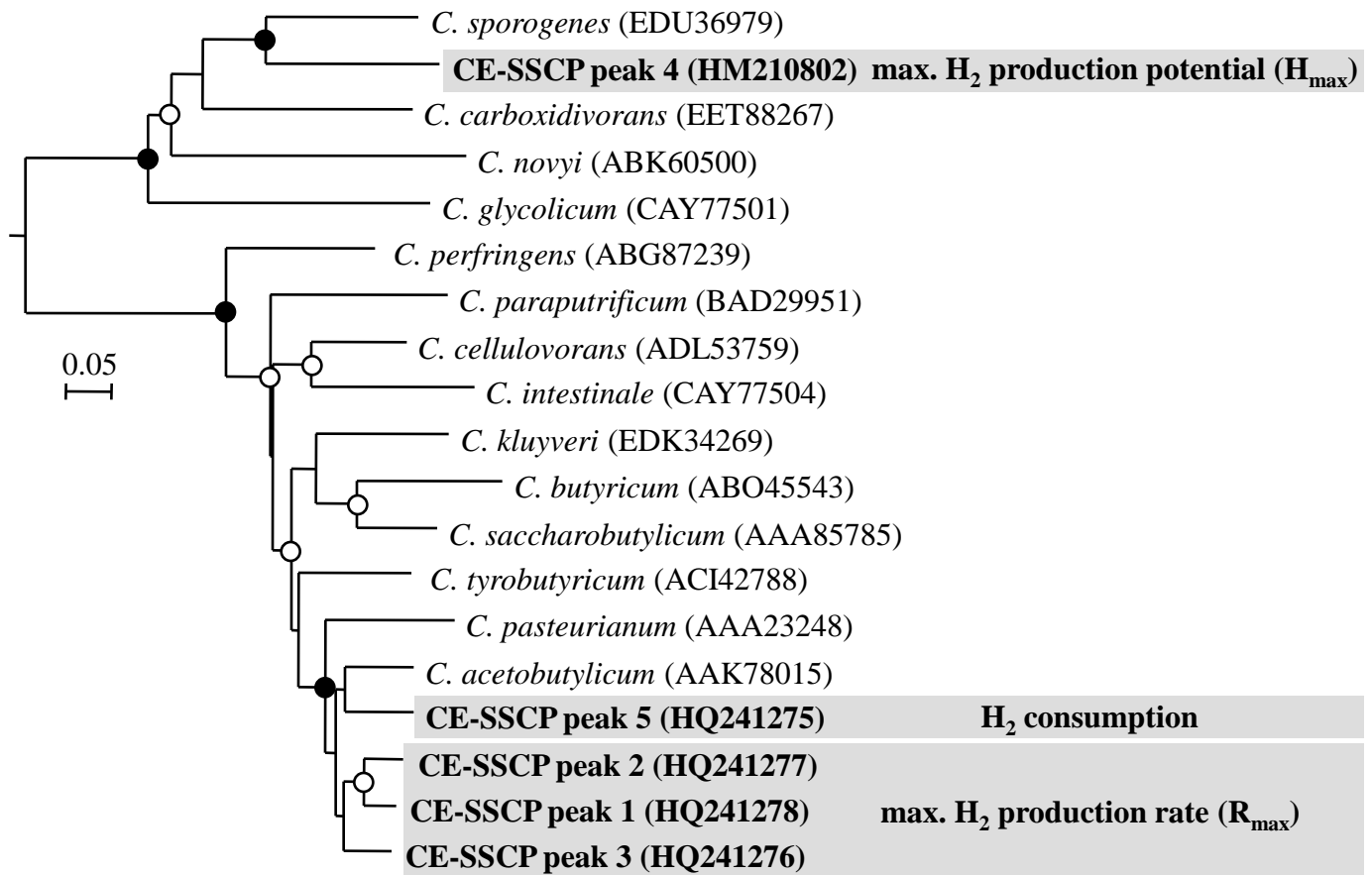


Figure 5



**Table 1**Table 1. Performance of fermentative H<sub>2</sub> production at different initial pH

Initial pH conditions	4	4.5	5	5.5	6	Statistical significance <sup>2</sup>
Final pH	3.3	3.4	3.6	3.9	4.3	***
Substrate utilization (%)	53.4	75.4	94.4	99.5	100.0	***
Max. H <sub>2</sub> content (%)	30.9	41.6	44.2	44.5	43.3	***
Final H <sub>2</sub> content (%)	30.9	41.6	43.5	28.5	27.4	***
Max. H <sub>2</sub> production rate = R <sub>max</sub> (mmol-H <sub>2</sub> /L/d)	16.4	20.6	24.4	28.7	38.3	***
Max. H <sub>2</sub> production potential = H <sub>max</sub> (mmol-H <sub>2</sub> /L)	55.2	87.7	102.7	98.6	99.9	***
Max. H <sub>2</sub> production potential = H <sub>max</sub> (mL/L)	1237	1965	2300	2209	2238	***
Soluble microbial products = SMP (g/L) <sup>1</sup>	2.1	3.3	4.6	4.5	4.6	**
Volatile fatty acids = VFA (g/L) <sup>1</sup>	1.9	3.3	4.2	4.1	4.4	**
H <sub>2</sub> yield (mol-H <sub>2</sub> /mol hexose)	1.8	2.0	1.9	1.7	1.7	n.s.
Acetate yield (mol-Ac/mol hexose)	0.28	0.35	0.33	0.32	0.35	*
Butyrate yield (mol-Bu/mol hexose)	0.48	0.62	0.61	0.58	0.59	**
Ethanol yield (mol-Eth /mol hexose)	0.17	0.00	0.18	0.14	0.10	**
Bu/Ac <sup>1</sup>	2.5	2.6	2.7	2.7	2.5	n.s.
Final Bu/Ac	2.5	2.6	2.7	1.8	2.1	**
Time when R <sub>max</sub> was reached (days)	6	3.7	3.7	3.7	3	n.s.
Time when H <sub>max</sub> was reached (days)	24	24	23.3	9.7	10.3	***

<sup>1</sup> Values obtained when maximal H<sub>2</sub> production was achieved

<sup>2</sup> Significance differences between pH conditions are indicated by asterisks: \*, p < 0.05; \*\*, p < 0.005; \*\*\*, p < 5 x 10<sup>-5</sup>; n.s., not significant (p > 0.05).