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1 **Inhibition of fermentative hydrogen production by lignocellulose-derived**
2 **compounds in mixed cultures**

3

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20 **Abstract**

21

22 Dark fermentation using mixed cultures is an attractive biological process for producing
23 hydrogen (H₂) from lignocellulosic biomass at a low cost. Physico-chemical pretreatment is
24 generally used to convert lignocellulosic materials into monosaccharides. However, the
25 processes involved also release degradation by-products which can, in turn, inhibit microbial
26 growth and metabolism and, hence, impact substrate conversion. In this study, the impact on
27 H₂ production of lignocellulose-derived compounds (*i.e.* furan derivatives, phenolic
28 compounds and lignins) was assessed along with their effect on bacterial communities and
29 metabolisms. Batch tests were carried out using xylose as model substrate (1.67
30 mol_{H₂}.mol_{xylose}⁻¹ in the control test). All the putative inhibitory compounds showed a
31 significant negative impact on H₂ production performance (ranging from 0.34 to 1.39
32 mol_{H₂}.mol_{xylose}⁻¹). The H₂ yields were impacted more strongly by furan derivatives 0.40 - 0.51
33 mol_{H₂}.mol_{xylose}⁻¹) than by phenolic compounds (1.28 - 1.39 mol_{H₂}.mol_{xylose}⁻¹). Except for the
34 batch tests supplemented with lignins, the lag phase was shorter for inhibitors having the
35 highest molecular weight (8 days versus 22 days for the lowest MW). Variability of the lag
36 phase was clearly related to a shift in bacterial community structure, as shown by multivariate
37 ordination statistics. The decrease in H₂ yield was associated with a decrease in the relative
38 abundance of several H₂-producing clostridial species. Interestingly, *Clostridium beijerinckii*
39 was found to be more resistant to the inhibitors, making this bacterium an ideal candidate for
40 H₂ production from hydrolysates of lignocellulosic biomass.

41

42

43 **Keywords:** hydrogen, CE-SSCP, *hydA*, lignocellulose, anaerobic digestion, inhibitors

44 **1. Introduction**

45

46 Hydrogen (H₂) is one of the most promising energy carriers because it is energetically dense,
47 clean-burning and is sustainable since it can be produced from renewable sources. Among the
48 various kinds of H₂-producing biotechnology, dark fermentation using anaerobic microbial
49 communities is particularly attractive for its ability to produce H₂ at high rates and at a low
50 cost from complex and unsterilized substrates such as solid waste-derived carbon sources [1-
51 3]. Despite significant advances in research on dark fermentation, its commercial
52 development has been substantially limited by certain major challenges. These include the use
53 of renewable sources for H₂ production i.e. from pretreated biomass, and the improvement of
54 H₂ production yields through the development of optimal control of mixed-culture
55 fermentation [4-6].

56 Lignocellulosic biomass is an abundant renewable resource worldwide representing an
57 attractive, low-cost feedstock for H₂ production. Lignocellulosic biomass contains variable
58 amounts of cellulose (a glucose polymer), hemicellulose (a hexose/pentose heteropolymer
59 with xylose as the major constituent) and lignin (an aromatic organic polymer intertwined
60 amongst cellulosic and hemicellulosic compounds). Recently, H₂ production by dark
61 fermentation from lignocellulosic residues have been shown in mesophilic and thermophilic
62 mixed cultures [6-11]. Due to the complex structure of lignocellulosic materials, pretreatment
63 processes are nevertheless required prior to dark fermentation. Such processes make the main
64 constituents, cellulose and hemicellulose, more accessible to the enzymes that convert
65 carbohydrate polymers into monosaccharides such as glucose or xylose [8, 12, 13]. The
66 pretreatment methods used to improve dark fermentation of lignocellulosic biomass are
67 classified into four groups: physical (e.g. mechanical disruption), chemical (e.g. alkali, dilute

68 acid), thermal (e.g. steam explosion) and biological (e.g. lignin degradation by white rot
69 fungi) [8, 9, 13, 14].

70 The existing pretreatment processes for lignocellulosic biomass have a major drawback: the
71 lead to the formation of undesirable by-products, other than monosaccharides, which reduce
72 the fermentability of the hydrolyzates obtained. Indeed, a broad range of compounds
73 generated during hydrolysis have been previously identified as inhibitors of microbial growth
74 as well as their metabolism, i.e. ethanol fermentation [15-18]. Three groups of inhibitors have
75 been distinguished: organic acids such as acetate generated from hemicellulose degradation;
76 furan derivatives from sugar degradation such as furfural or 5-hydroxymethylfurfural (HMF);
77 and phenolic monomers such as vanillin or syringaldehyde derived from lignin microbial
78 alteration [6, 15, 19]. These inhibitors can affect microbial growth with three distinct modes
79 of action: undissociated weak organic acids penetrate microbial cells and decrease the
80 intracellular pH, furfural derivatives interfere with glycolytic and/or fermentative enzymes,
81 while macromolecules and phenolic compounds are damaging the microbial cellular
82 membranes [15, 19].

83 Clostridial species are generally found as the most abundant bacterial genus in H₂-producing
84 mixed cultures [5, 20-22]. In fact, the common use of heat-shock pretreatment of the
85 inoculum leads not only to the removal of H₂-consumers, i.e. methanogens, but also to the
86 selection of sporulating anaerobic H₂-producing bacteria, particularly *Clostridium* spp.
87 Clostridial species are metabolically versatile and degrade a wide range of organic materials
88 including carbohydrates, organic acids, alcohols and aromatic compounds [23]. In contrast to
89 ethanologenic microorganisms (e.g. *Saccharomyces* spp.), carboxylic acids such as acetate
90 have not been reported to significantly inhibit the growth of clostridial species and/or H₂
91 production [24, 25]. To date, the impact of furan derivatives and phenolic compounds on
92 mixed-culture fermentative H₂ production has not been investigated.

93 Inhibitory concentrations are quite specific to each microorganism, but, generally, 1 g.L⁻¹ can
94 be considered as the concentration at which a significant impact can be observed on the
95 growth of different types of microorganisms (i.e. yeast or bacteria) and on their H₂ or ethanol
96 production [16, 17, 25]. Moreover, a concentration of 1 g.L⁻¹ corresponds to an intermediate
97 concentration found in hydrolysates generated from lignocellulosic biomass after standard
98 pretreatment using dilute sulfuric acid and at high temperature. Furfural and HMF, for
99 instance, have been retrieved at concentrations ranging from 0 g.L⁻¹ for wheat straw [26],
100 about 1 g.L⁻¹ for corn stalks or cobs [26] to greater than 2 g.L⁻¹ for softwood [27, 28].
101 The aim of this study was to evaluate the impact on the performance of fermentative H₂
102 production processes of seven different molecules: furan derivatives (i.e. furfural and HMF),
103 phenolic compounds (i.e. phenol, syringaldehyde and vanillin) and lignins (i.e. kraft and
104 organosolv lignins), presenting different molecular weights at a common concentration of 1
105 g.L⁻¹. The metabolic routes and the bacterial communities involved were also assessed.

106 **2. Materials and methods**

107

108 **2.1. Hydrogen production in batch tests**

109

110 The H₂ production experiments were carried out in 500 mL glass bottles under batch
111 conditions. An anaerobically-digested sludge pretreated by heat shock (90°C, 10 min) was
112 used as inoculum. Two milliliters of this inoculum (final concentration of 250 mg-COD.L⁻¹)
113 were inoculated into the culture media (final working volume of 200 mL) containing 40 mM
114 of 2-(N-morpholino)ethanesulfonic acid (MES) buffer, 5 g.L⁻¹ of xylose and/or 1 g.L⁻¹ of the
115 following individual lignocellulose-derived compounds (Table 1): furfural, 5-
116 hydroxymethylfurfural (HMF), vanillin, syringaldehyde, phenol, kraft lignin, organosolv
117 lignin. The initial pH was adjusted to 5.5 using NaOH (1M). All batch tests were performed
118 in triplicate. After inoculation, each bottle was flushed with nitrogen for 5 minutes to create
119 anaerobic conditions. The bottles were then capped with a rubber stopper and incubated at
120 37°C for 50 days. Two milliliters of the mixed cultures were periodically collected and then
121 centrifuged (20,000 g, 10 min). The supernatants and the pellets were stored at -20°C, the
122 supernatants for further chemical analysis and the pellets for DNA extraction.

123

124 **2.2. Chemical data analysis (biogas, metabolic byproducts)**

125

126 Biogas volume was periodically measured using an acidified water displacement method.
127 Biogas composition (CH₄, CO₂, H₂ and N₂) was analyzed using a gas chromatograph (Clarus
128 280, Perkin Elmer) equipped with a HayeSep Q column and a molecular sieve. Operating
129 conditions were as follows: the carrier gas was argon at a pressure of 102kPa and a flow rate
130 of 4.5 mL.min⁻¹; temperature of the injector and the detector was fixed at 150 °C.

131 Volatile fatty acids (VFA) composition in the liquid phase, *i.e.* acetic (C2), propionic (C3),
132 butyric and iso-butyric (C4 and iC4), valeric and iso-valeric (C5 and iC5) and caproic (C6)
133 acids were determined using a gas chromatograph (GC-3900, Varian) equipped with a flame
134 ionization detector. The concentrations of xylose and non-VFA metabolic byproducts such as
135 organic acids (lactate), ethanol or acetone were measured by HPLC analysis and
136 refractometric detection (Waters R410). The components were separated in an Aminex HPX-
137 87H, 300 x 7.8 mm column (Biorad). The eluant corresponded to 0.005 M H₂SO₄. The
138 column temperature was maintained at 35°C and the flow rate at 0.4 mL.min⁻¹.

139 To determine H₂ production parameters after the addition of lignocellulose-derived
140 compounds (Table 1), the cumulative H₂ production (H) data for each inhibitor was fitted to a
141 modified Gompertz equation (Equation 1), as previously described by Quéméneur et al. [22].

$$142 \quad H(t) = P \cdot \exp \left\{ -\exp \left[\frac{R_m \cdot e}{P} (\lambda - t) + 1 \right] \right\} \quad (1)$$

143 where P is the maximum cumulative H₂ production (mL_{H₂}.L⁻¹), R_m is the maximum H₂
144 production rate (mL_{H₂}.L⁻¹.day⁻¹), λ is the lag-phase time (day), t is the incubation time (day)
145 and e is exp(1). The cumulative H₂ production was expressed in mL per L of culture taking
146 into account the variations in volume due to gas and liquid sampling. The values of P , R_m and
147 λ were estimated using a non-linear regression algorithm developed with Matlab software
148 (version 6.5, MathWorks®). The H₂ production yield was calculated by dividing the
149 maximum H₂ production (P) by the molar amount of xylose consumed.

150

151 **2.3. DNA extraction and PCR amplification**

152

153 For each experiment, molecular analyses of the bacterial communities were carried out at the
154 maximum H₂ production time (P).

155 Genomic DNA was extracted and purified from cell pellets (1 ml of culture) using the Qiagen
156 DNA stool mini kit. The amount and purity of DNA in the extracts were measured by
157 spectrophotometry (Infinite NanoQuant M200, Tecan).

158 The 16S rRNA genes were amplified using the universal primers W49 (5'-
159 ACGGTCCAGACTCCTACGGG-3', *Escherichia coli* position F331) [29] and 5'-fluorescein
160 phosphoramidite-labeled W104 (5'-TTACCGCGGCTGCTGGCAC-3', *E. coli* position R533)
161 [29]. Each PCR mixture (50 μ L) contained 1X *Pfu* Turbo DNA polymerase buffer, 200 μ M of
162 each dNTP, 130 ng of each primer, 0.5 U of *Pfu* Turbo DNA polymerase (Stratagene) and 5
163 ng of genomic DNA. Reactions were done in a Mastercycler thermal cycler (Eppendorf). The
164 16S rRNA genes were amplified as follows: 94 °C for 2 min, followed by 25 cycles
165 performed at 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C
166 for 10 min. The *Clostridium* genus was specifically analyzed by nested PCR using a bacterial-
167 domain forward primer W18 (5'- GAGTTTGATCMTGGCTCAG -3', *E. coli* position F9)
168 [30] and a *Clostridium*-specific reverse primer W109 (5'- CCCTTTACACCCAGTAA -3', *E.*
169 *coli* position R561) [31]. For cloning and sequencing 16S rRNA genes from the control
170 culture, fragments of about 500 bp were amplified using forward primer W18 and reverse
171 primer W31 (5'- TTACCGCGGCTGCTGGCAC-3', *E. coli* position R500), as described
172 above, except that primer hybridization at 50°C and an extension time of 1 min were applied.
173 The size of PCR products was determined by 2% gel electrophoresis stained with ethidium
174 bromide.

175

176 **2.4. CE-SSCP electrophoresis and statistical analysis**

177

178 One microliter of the appropriate dilution of PCR products was mixed with 18.8 μ L of
179 formamide and 0.2 μ L of internal standard GeneScan ROX (Applied Biosystems). Samples

180 were heat-denatured at 95°C for 5 min and immediately re-cooled in ice. CE-SSCP
181 electrophoresis was performed in an ABI Prism 3130 genetic analyser (Applied Biosystems)
182 with 50 cm-long capillary tubes filled with a non-denaturing 5.6% conformation analysis
183 polymer (Applied Biosystems). Samples were eluted at 12 kV and 32°C for either 30 min (for
184 16S rRNA gene) or 45 min (for *hydA* gene).

185 The CE-SSCP profiles were aligned with the internal standard to take into account inter-
186 sample electrophoretic variability. The CE-SSCP profiles were normalized using the
187 *StatFingerprints* library [32] from R version 2.9.2 [33] in accordance with standard procedure
188 [34]. The genetic distances between bacterial communities were assessed using Euclidean
189 distances. The effect of the inhibitors on the structure of bacterial communities was
190 investigated with a multivariate analysis of similarity (ANOSIM) [35] and genetic distances
191 between samples were displayed using principal component analysis (PCA). ANOSIM gave a
192 statistic R, ranging from 0 (dissimilar groups) to 1 (similar groups). The linear correlation
193 between the PCA ordination of the bacterial communities and key environmental parameters
194 was investigated using the *envfit* and *ordisurf* functions of the *vegan* library [36]. The
195 statistical significances were tested by Monte Carlo randomization.

196

197 **2.5. Assignment of the partial 16S rRNA gene sequences in mixed cultures**

198

199 The PCR products obtained from the control mixed cultures were purified using a PCR
200 purification kit (Qiagen). The 16S rRNA clone libraries were constructed using a TA cloning
201 kit (Invitrogen). The PCR-CE-SSCP profile of the PCR product of each clone, after
202 amplification with plasmid-targeted primers T7 and P13, was compared with the mixed-
203 culture profiles for peak assignment. The cloned inserts were then sent for sequencing
204 (MilleGen Company, Toulouse, France). To determine the identity percentage, the 16S rRNA

205 sequences of the clones were aligned with reference sequences retrieved from the Genbank
206 database using the CLUSTALW program [37] and further refined manually using the BioEdit
207 program [38]. The 16S rRNA gene sequences were deposited in the Genbank database under
208 the accession numbers JN116427 to JN116431.
209

210 **3. Results and discussion**

211

212 **3.1. Effect of inhibitor type on overall hydrogen production performance**

213

214 Throughout the batch experiments, only H₂ and CO₂ were observed as gaseous products. No
215 CH₄ was detected in the gas phase, indicating that the methanogenic activity was efficiently
216 suppressed after heat-shock treatment of the inoculum. No gas was produced from the
217 inhibitor compounds when added as sole carbon source (without xylose) at a concentration of
218 1 g.L⁻¹.

219 In the control cultures, H₂ production started after 3.2 (± 1.3) days and went on increasing for
220 50 days, attaining an H₂ yield of 1.67 (± 0.10) mol_{H₂}.mol_{xylose}⁻¹ (Table 2). This H₂ yield was
221 consistent with previously reported yields obtained from xylose with mesophilic or
222 thermophilic fermentative mixed cultures [39-41].

223 Whatever the lignocellulose-derived compounds added, a significant inhibition of H₂
224 production performances was observed in terms of lag phase, H₂ yield and maximum H₂
225 production. These results suggest that different inhibitory effects occurred according to the
226 nature of the compounds: furan derivatives, phenolic compounds or lignins (Table 2).

227 The addition of furan derivatives (furfural and HMF) led to the highest inhibition of H₂
228 production, with a dramatic drop in H₂ yields which decreased to 0.45 (± 0.10)
229 mol_{H₂}.mol_{xylose}⁻¹ (Figure 1). Furan derivatives have been previously reported to disturb the
230 membrane integrity of diverse microorganisms [19]. Nevertheless, both furfural and HMF (at
231 1 g.L⁻¹) have been reported to stimulate the growth of *Clostridium beijerinckii* BA101 as well
232 as the production of acetone-butanol-ethanol through non-H₂-producing pathways [24].
233 Despite having different characteristics (e.g. molecular mass), no significant difference was
234 found between the H₂ yield and maximum production for the cultures with furan derivatives

235 added. Nonetheless, the lag phase in furfural-added cultures was twice as long as in HMF-
236 added cultures: respectively 18.5 and 9.9 days. This result can be explained by the lower
237 molecular weight of furfural compared to HMF (Table 1). Indeed, the diffusion rate into the
238 cell should be higher for furfural than HMF which may explain the longer time needed for
239 microbial acclimation to furfural. In previous inhibition studies, furfural has been identified as
240 being a more efficient inhibitor than HMF of the growth of ethanol-producing yeasts [17]. In
241 contrast, Cao et al. [25] reported a higher decrease in H₂ yield of *Thermoanaerobacterium*
242 *thermosaccharolyticum* W16 with HMF (50.2%) than with furfural (17.9 %) at an initial
243 concentration of 1 g.L⁻¹.

244 The addition of phenolic compounds (phenol, syringaldehyde and vanillin) clearly had less
245 impact on the H₂ yields than the furan derivatives (1.33 vs. 0.45 mol_{H₂}.mol_{xylose}⁻¹,
246 respectively) (Figure 1). The highest H₂ production (1045 ± 26.6 mL.L⁻¹) and the shortest lag-
247 phase time (8.1 ± 2.6 days) were observed with syringaldehyde (Table 2). In contrast, the
248 longest lag-phase time (22.2 ± 1.2 days) was observed when phenol was added. As found in
249 the cultures with furan derivatives added, these differences in lag phase could be explained by
250 the intrinsic ability of these compounds to penetrate cell membranes: the higher the molecular
251 mass, the slower was the introduction into a cell and the shorter the lag phase. After this
252 acclimation period, which varied depending upon the type of phenolic compound (phenol,
253 syringaldehyde and vanillin), no significant difference in H₂ yield was observed between the
254 assays (Figure 1). Phenolic compounds are widely known to be toxic to microbial cells
255 because they increase membrane fluidity, which affects membrane permeability, and,
256 consequently, induce a longer acclimation of mixed cultures [15, 19]. Our results were in
257 agreement with the findings of Tai et al. [42] who observed significant inhibition of cell
258 metabolism of *Clostridium butyricum* at phenol concentrations higher than 1 g.L⁻¹. In
259 addition, no co-degradation phenomenon was detected with *C. butyricum* in pure cultures at

260 phenol concentrations higher than 0.6 g.L⁻¹ [42], as also shown in our study. Ezeji et al. [24]
261 reported a higher inhibition of *C. beijerinckii* cell growth by phenol than by syringaldehyde
262 (at a concentration of 1 g.L⁻¹). Contrary to our findings, Cao et al. [25] showed that vanillin at
263 a concentration ranging between 0 and 2 g.L⁻¹ caused no severe inhibition of *T.*
264 *thermosaccharolyticum* W16 for H₂ production. In the case of an increase of syringaldehyde
265 at 1 g.L⁻¹, the same authors indicated a reduction of H₂ production of 54.3% compared to 23.1
266 % for our mixed cultures. This result indicated that our mixed-culture inoculum had a higher
267 tolerance to syringaldehyde.

268 Lignins largely contributed to the decrease in H₂ production and yield, which can be
269 explained by their physicochemical properties, *e.g.* hydrophobicity, ramification,
270 methoxylation. Indeed, lignins can form lignin-carbohydrate complexes with xylose and, thus,
271 contribute to decreasing substrate availability in the medium. The differences in H₂ yield
272 between the lignins tested could be explained by different sorption capacity or different
273 release of phenolic compounds due to partial lignin microbial alteration. Contrary to furan
274 derivatives and phenol compounds, lignins only slightly affected the lag phase (Table 2).
275 Lignin is a complex chemical compound with a high molecular weight. Consequently, this
276 result is consistent with those obtained with furan derivatives and phenol compounds, *i.e.* the
277 lag phase decreased with increasing molecular weight. All these results together establish a
278 linear negative correlation between the lag phase and the monomer molecular mass of each
279 inhibitor (Pearson's product-moment correlation $r = -0.852$, $p < 0.001$) (Figure 2). For lignin-
280 added cultures, the molecular weight considered was the weight of the monomer, suggesting
281 that the inhibitory impact was due to monomers that can be released and not to polymers.

282

283

284 **3.2. Effect of the type of inhibitor on the fermentative routes**

285

286 Figure 3 shows the distributions of soluble metabolites concomitant to H₂ production in
287 mixed cultures. In the control, the major VFAs were acetate (33.3 %), butyrate (49.7 %),
288 caproate (6.0 %), propionate (10.5 %) and valerate (0.5 %), with butyrate as the main
289 component. Similar distribution of metabolic products was detected in anaerobic mixed
290 cultures, dominated by clostridial species, with xylose at 20 g.L⁻¹ as sole carbon source [43].

291 In the cultures supplemented with furan derivatives, metabolite distribution was similar to that
292 of the control cultures. Indeed, a similar butyrate/acetate ratio was found compared to the
293 control cultures (on average 1.35 vs 1.49, respectively) (Table 2). However, the addition of
294 furan derivatives led to the highest valerate levels (Figure 3). Valerate might be formed
295 through metabolic pathways requiring H₂ as electron donor and with the consumption of
296 propionate and CO₂, in accordance with the lower H₂ yields observed [44].

297 In the cultures supplemented with phenolic compounds, significant differences in metabolic
298 patterns were observed with all three compounds: phenol, vanillin and syringaldehyde (Figure
299 3). Among them, the least efficient phenol culture was characterized by the highest
300 butyrate/acetate ratio (Table 2), which was related to the lowest acetate levels. This result
301 suggests that phenolic compounds interfered differently with the bacterial community
302 structure (Figure 4) and/or with the activities of key enzymes involved in acetate and butyrate
303 formation.

304 In the cultures supplemented with lignin, the decrease in butyrate content was associated with
305 the lowest butyrate/acetate ratio and was concomitant with the formation of caproate.
306 Consequently, among the various possible metabolic pathways, caproate was most likely
307 produced directly from butyrate, H₂ and CO₂ [45]. An increase in caproate content with
308 substantial H₂ consumption was also reported in a previous study performed with
309 disaccharides in batch cultures [22]. Interestingly, the low H₂ production occurring in these

310 cultures was concomitant with the highest propionate levels (Figure 3). Propionate production
311 is known to have a negative effect on the efficiency of H₂ production. Cheng et al. [46]
312 reported a negative correlation between propionate and H₂ yield in starch-fed mixed cultures.
313 Zhang et al. [47] observed that an increase of the H₂ yield was concomitant with a decrease in
314 propionate production.

315 Whatever the mixed culture, the detection of high levels of acetate and butyrate suggested that
316 H₂ was produced during acetate-butyrate fermentation. The butyrate/acetate ratio can be used
317 as an indicator for monitoring H₂ production in dark fermentation process [46, 48]. However,
318 during our experiments, no correlation was observed between H₂ production yield and the
319 molar acetate/butyrate ratios (Pearson's $r = 0.467$, $p > 0.05$) (Table 2). Nevertheless, a positive
320 correlation was observed between the H₂ and butyrate production (Pearson's $r = 0.838$,
321 $p < 0.001$), indicating that the butyrate content was directly linked to H₂ production, whereas
322 acetate content resulted not only from the acetate pathway but also from the
323 homoacetogenesis (recombination of H₂ and CO₂) occurring in mixed cultures [49].

324

325 **3.3. Effect of the type of inhibitor on bacterial communities**

326

327 Figure 4 presents the averaged 16S rRNA gene-based CE-SSCP fingerprinting profiles of the
328 mixed cultures according to the presence of different inhibitors and obtained at the maximum
329 H₂ production time. The addition of an inhibitor had a significant effect on the bacterial
330 community structures (ANOSIM $R = 0.51$, $p < 0.005$). The differences in CE-SSCP profiles
331 were mostly related to the variation in the abundance ratio between clostridial and non-
332 clostridial species (represented in the left-hand and right-hand part of the CE-SSCP,
333 respectively). Nevertheless, clostridial species always remained the dominant bacteria in all of
334 the samples analyzed. On average, the clostridial species represented 70.7% of the bacterial

335 community compared to only 29.2% for non-clostridial species (Figure 4). Cultures with
336 phenol added, which presented the longest lag phase, were characterized by the highest
337 proportion of clostridial species (85.8%), mainly represented by *Clostridium beijerinckii*. In
338 contrast, cultures with lignin added showed the lowest proportion of clostridial species
339 (58.1%) associated with the shortest lag phase. A principal component analysis (PCA) was
340 used to compare the genetic distance of the species found in the mixed cultures at the time of
341 maximum H₂ production according to the experimental conditions [20-22] (Figure 5A). The
342 PCA showed no clustering of samples according to the type of inhibitor (while remaining
343 significant, $p < 0.001$). Interestingly, changes in the lag-phase ($p < 0.001$) and in the maximal H₂
344 production ($p < 0.025$) were significantly correlated with the genetic distance between samples
345 (Figure 5A). This result indicates that the genetic differences between the bacterial
346 populations were linked to the metabolic activity. Figure 5B highlighted the covariation of the
347 lag phase (larger rings corresponded to samples with larger values) and the genetic distance
348 (according to the physical distance between samples on the biplot). Interestingly, such linear
349 covariation is consistent with the linear negative correlation observed between the lag phase
350 and the monomer molecular mass of inhibitors (Figure 2). This result suggests that the initial
351 impact of inhibitors on bacterial cells is predominant in the selection of microorganisms.
352 Whatever the inhibitor tested, the CE-SSCP profiles were dominated by *Clostridium*
353 *beijerinckii* (peak 1), known to be an efficient H₂ producer [50, 51]. This species was less
354 sensitive to inhibitors than other clostridial and non-clostridial bacteria in our study. This
355 result is consistent with that from Ezeji et al. [24] who reported high resistance of *C.*
356 *beijerinckii* to furan derivatives and phenolic compounds in pure cultures. Here, similar results
357 were obtained in mixed culture with other species in competition, suggesting *C. beijerinckii* as
358 an ideal candidate for H₂ production from hydrolysates of lignocellulosic biomass.

359 The bacterial community of the control cultures was dominated by three clostridial species:
360 *Clostridium acetobutylicum*, *Clostridium beijerinckii* and *Clostridium cellulosi* (Figure 4).
361 Changes in the bacterial community due to the addition of an inhibitor was associated with
362 the decrease in the relative abundance of the clostridial species represented by peaks 2, 3 and
363 4 (Figure 4). Such species were likely to have been the support of H₂ production from xylose.
364 Indeed, *C. acetobutylicum* (peak 2) and *C. pasteurianum* (peak 4) are known as highly
365 efficient H₂ producers [52-53]. Consequently, the decrease in their relative abundance in
366 mixed cultures would directly affect H₂ production. Recently, *C. cellulosi* (peak 3) has been
367 reported as potentially favoring H₂ production by breaking down complex organic compounds
368 [54]. But *C. cellulosi* is a non-H₂-producer and should therefore be considered as a substrate
369 competitor. In lignin-added cultures, *C. cellulosi* could also be potentially involved in the
370 partial breaking down of lignin which might give rise to phenolic compounds (e.g.
371 syringaldehyde, vanillin) and thus decreased the H₂ yield, as previously suggested.
372 Interestingly, the same dominant H₂-producing clostridial species were found in both the
373 control cultures and those supplemented with organosolv lignin, suggesting that non-
374 clostridial species (shown in the right-hand part of the CE-SSCP profiles) negatively affected
375 the H₂ production by direct and/or indirect interaction: substrate competition, inhibitor
376 excretion and/or H₂ consumption. Lactic acid bacteria (LAB), such as *Sporolactobacillus* spp.
377 (peak 5), have been reported to decrease H₂ content in mixed cultures due to their inhibitory
378 effect caused by excreted bacteriocins which have an adverse effect on H₂ producing bacteria
379 [54, 55]. Lactate formation is also known to be unfavorable to H₂ production [3]. However,
380 no lactate was detected in our cultures. Since propionate accumulated, other bacterial species
381 such as *Clostridium* spp. (e.g. *Clostridium novyi*, *Clostridium propionicum*) or
382 *Propionibacterium* spp., which could have coexisted with LAB in our mixed cultures, might
383 have converted lactate to propionate [56] and thus contributed to reducing the H₂ yield.

384 Finally, the composition of the bacterial communities examined in this study was similar to
385 that reported in previous studies carried out in dark fermentation systems [20-22, 40, 46, 53,
386 57]. For example, Fang et al. [57] showed that 69.1% of the clones were affiliated to four
387 clostridial species and 13.5% were affiliated to *Sporolactobacillus racemicus* in the granular
388 sludge of a H₂ producing bioreactor using sucrose as substrate. In addition, clostridial species
389 have been reported to be responsible for H₂ production via the acetate/butyrate-type
390 fermentation [20-22]. Other fermentative pathways (e.g. propionate, lactate or caproate
391 fermentations) which reduce the H₂ yield can be carried out either by clostridia themselves or
392 by other non-clostridial species competing in mixed cultures. Nevertheless, further research is
393 still required to determine the exact role of non-dominant and non-clostridial species in the
394 improvement or the alteration of H₂ production performances in such fermentative mixed
395 cultures [53, 54].

396

397 **4. Conclusions**

398

399 The results of this study reveal that lignocellulose-derived compounds, i.e. furan derivatives,
400 phenol compounds or lignins, inhibit the H₂ production performance of mixed cultures. All
401 the compounds tested were unable to support fermentative H₂ production by mixed cultures in
402 the absence of an additional carbon source. Among the inhibitors tested, phenol compounds
403 were found to have less impact on H₂ production from xylose. Strong inhibition of H₂
404 production was observed in the presence of furan derivatives and lignins. A negative
405 correlation was observed between the lag phase and the molecular weight of the inhibitors.
406 Although the major VFAs in the soluble fraction were acetate and butyrate, significant
407 changes in metabolic profiles were observed, depending on the inhibitor. Based on 16S rDNA
408 analysis, clostridial species were found to be the dominant species in the mixed cultures
409 whatever the inhibitor supplemented. *Clostridium beijerinckii* was found to be more resistant
410 to inhibitors, making it the ideal candidate for H₂ production from hydrolysates from
411 lignocellulosic biomass. However, the relative abundance of some efficient H₂-producers such
412 as *C. acetobutylicum* and *C. pasteurianum* was dramatically affected by the addition of furan
413 derivatives and phenolic compounds. To conclude, our results indicate that fermentative H₂
414 production by mixed cultures is significantly inhibited by lignocellulose-derived compounds.
415 At all events, the efficiency of fermentative H₂-producing bioprocesses will also remain
416 dependant on the efficacy of delignification and depolymerisation processes in reducing the
417 levels of furan derivatives and phenolic compounds.

418

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420

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428

429 **References**

430

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- 592
593

594 **Captions to figures**

595

596 **Figure 1.** Effect of lignocellulose-derived compounds on dark fermentative H₂ production in
597 mixed cultures: (A) yields and (B) lag phase. Values correspond to means of three replicates
598 of independent values ± standard deviations (error bars). Control corresponds to mixed
599 cultures with xylose as sole carbon source.

600

601 **Figure 2.** Inverse negative correlation between the molecular weight of lignocellulose-derived
602 compounds and the lag phase.

603

604 **Figure 3.** Effect of lignocellulose-derived compounds on metabolite formation in
605 fermentative mixed cultures. Values correspond to means of three replicates of independent
606 values ± confidence intervals (error bars). Control corresponds to mixed cultures with xylose
607 as sole carbon source.

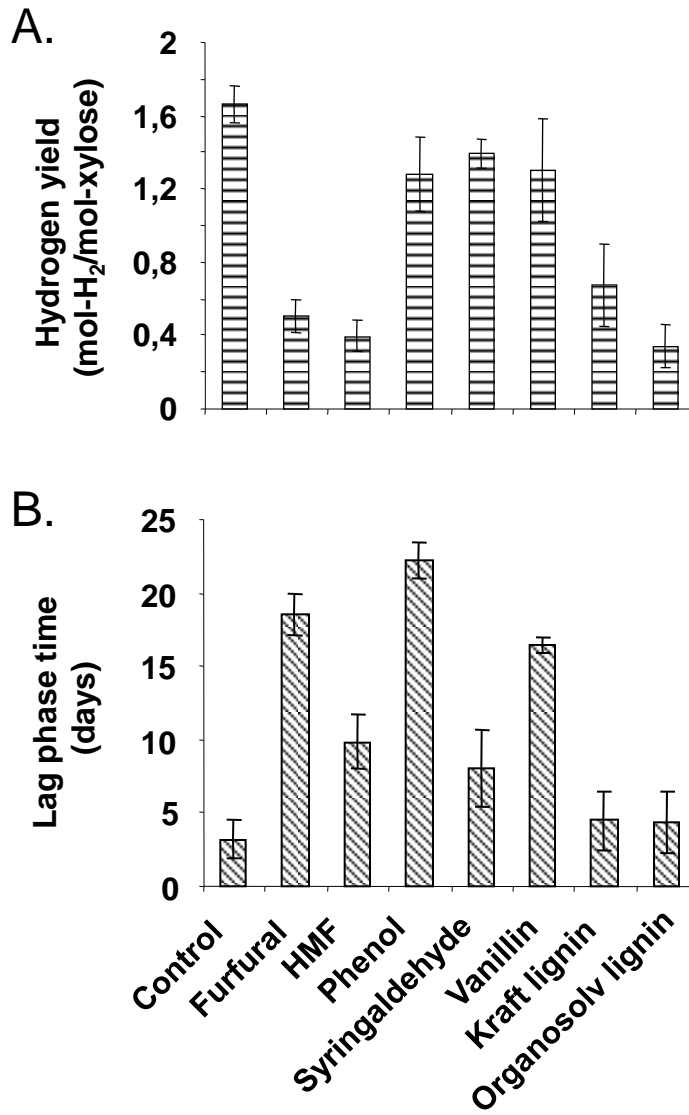
608

609 **Figure 4.** CE-SSCP profiles based on 16S rRNA gene fragments retrieved from H₂-producing
610 mixed cultures supplemented with individual lignocellulose-derived compounds, at the
611 maximum H₂ production time. Each CE-SSCP profile was first aligned using an internal
612 standard, and was then normalized. The X and Y axis of each CE-SSCP profile represent the
613 relative peak electrophoresis migration distance and the relative peak intensity, respectively.
614 A mean CE-SSCP profile of the three replicates is presented for each inhibitor supplement.
615 Control corresponds to mixed cultures from xylose as soel carbon source. The dominant peaks
616 of each CE-SSCP profile are marked with a number.

617

618 **Figure 5.** Principal Component Analysis (PCA) biplot of CE-SSCP profiles. (A) The first two
619 principal components axes explain 67.9% of the genetic variation. The different symbols
620 correspond to bacterial communities grown in the presence of different inhibitors (diamond
621 for positive control, filled triangle for furfural, open triangle for HMF, filled circle for phenol,
622 open circle for syringaldehyde, dashed circle for vanillin, filled square for Kraft lignin and
623 open square for organosolv lignin). Significant linear correlations between changes in genetic
624 profiles and changes in environmental parameters are indicated by arrows: lag phase
625 ($p < 0.001$) and H_2 production potential ($p < 0.025$). (B) Smooth surface response of the lag
626 phase values according to genetic distances between bacterial communities as shown by PCA
627 ordination. The size of the open circles (corresponding to bacterial community) is
628 proportional to the lag phase.

629



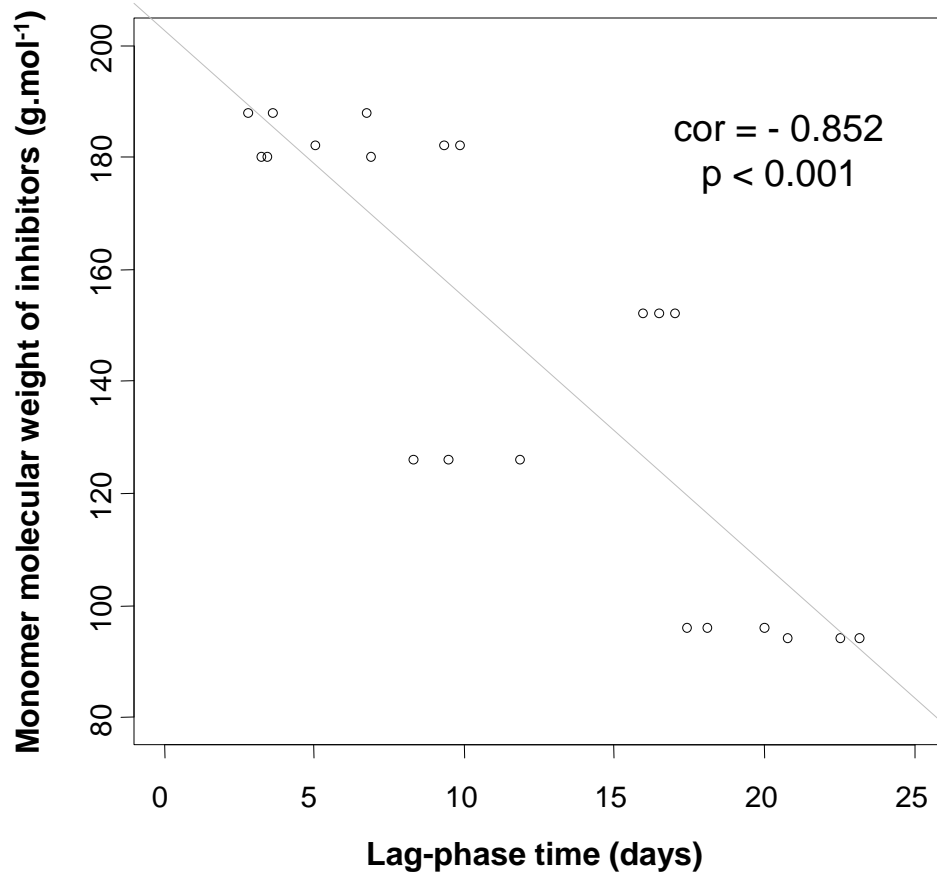
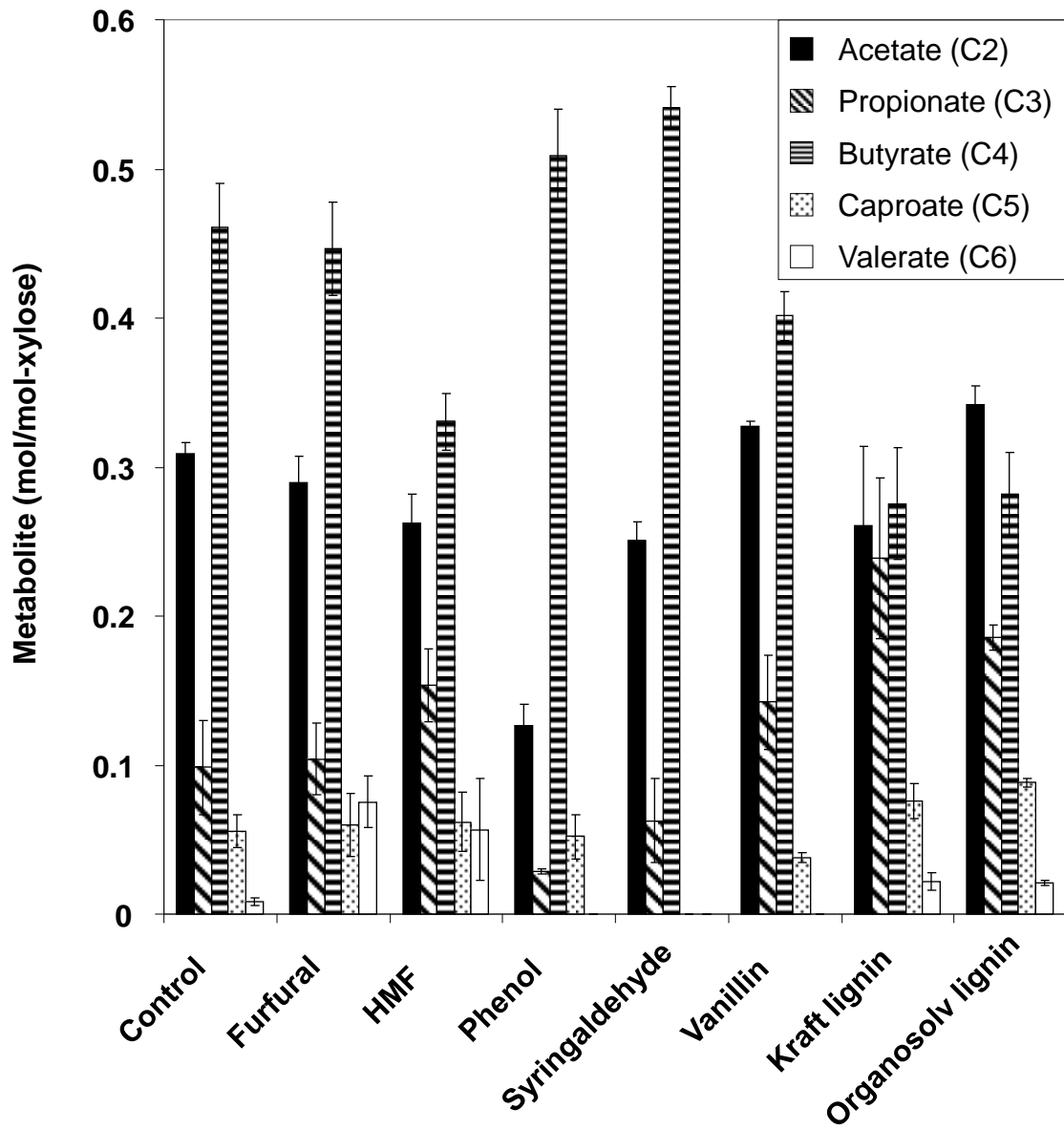
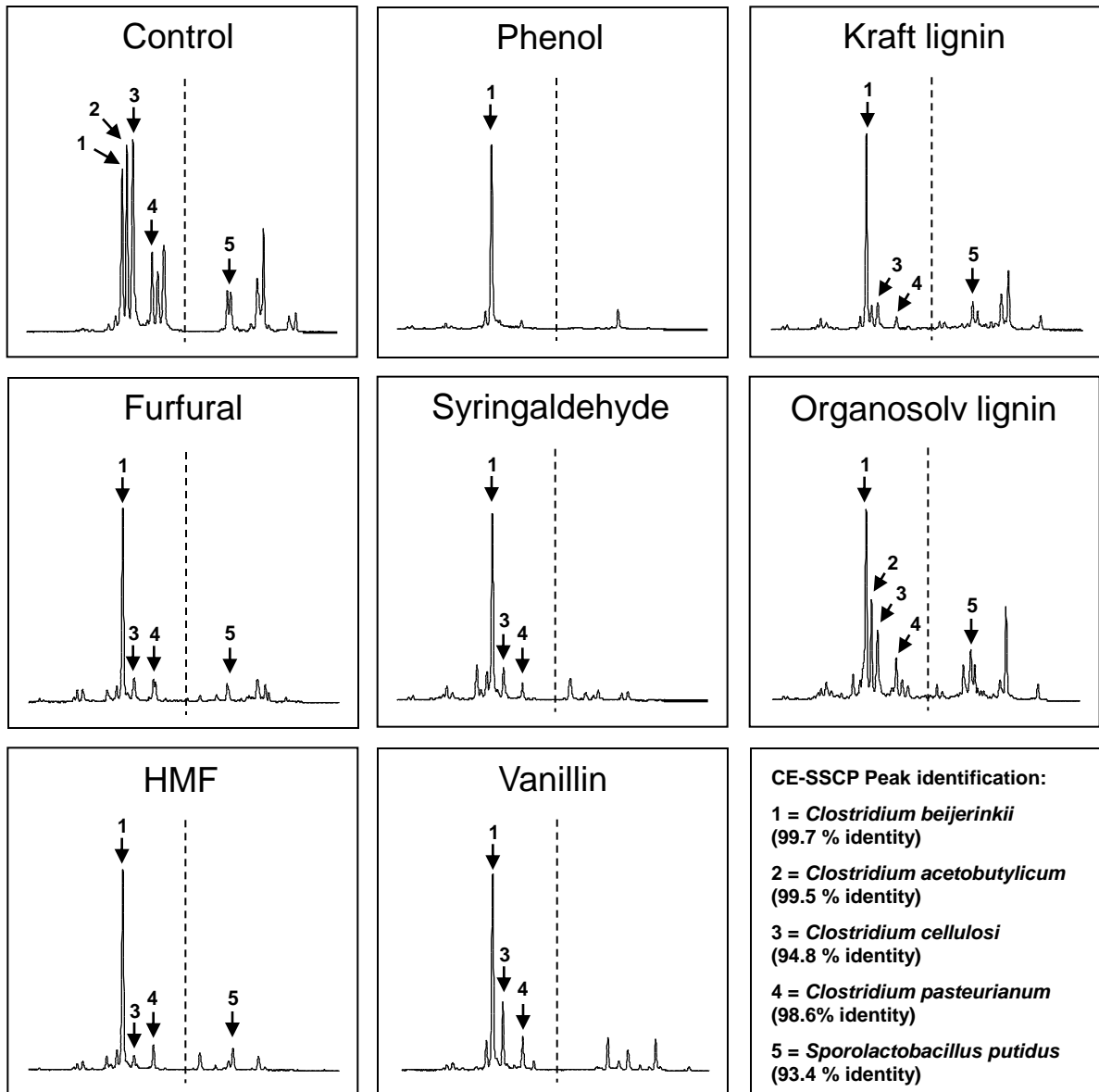


Figure3





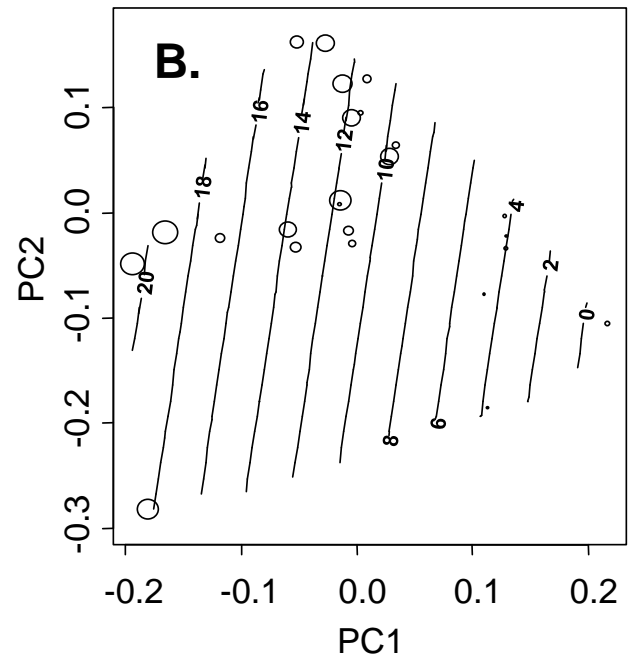
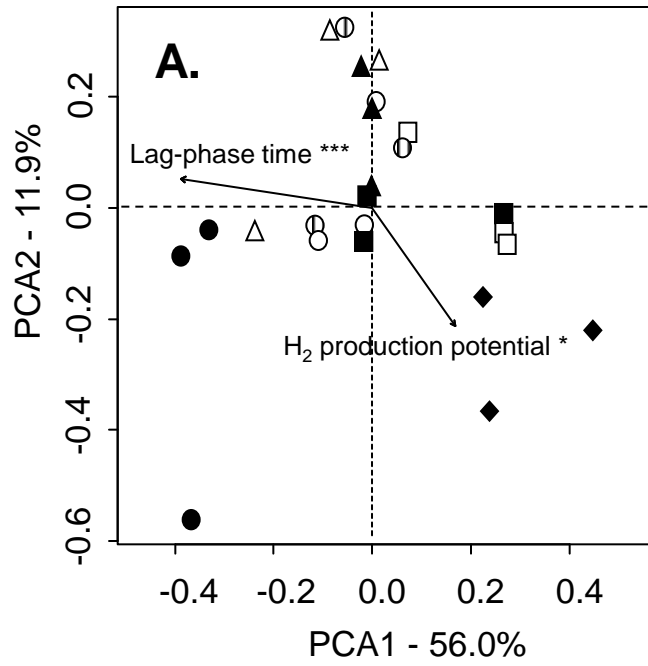


Table 1. Characteristics of the lignocellulose-derived inhibitors utilized in this study

Name	IUPAC name	Monomer molecular formula	Monomer molecular mass (g.mol ⁻¹)
Xylose	(2S,3R,4S,5R)-Oxane-2,3,4,5-Tetrol	C ₅ H ₁₀ O ₅	150
Furfural	Furan-2-carbaldehyde	C ₅ H ₄ O ₂	96.08
Hydroxymethylfurfural (HMF)	5-(hydroxymethyl)-2-furaldehyde	C ₆ H ₆ O ₃	126.11
Phenol	Phenol	C ₆ H ₆ O	94.11
Syringaldehyde	4-Hydroxy-3,5-dimethoxybenzaldehyde	C ₉ H ₁₀ O ₄	182.17
Vanillin	4-hydroxy-3-méthoxybenzaldéhyde	C ₈ H ₈ O ₃	152.14
Kraft lignin	n.a. ¹	C ₉ H _{8.5} O _{2.1} S _{0.1} (OCH ₃) _{0.8} (CO ₂ H) _{0.2}	180
Organosolv lignin	n.a. ¹	C ₉ H _{8.53} O _{2.45} (OCH ₃) _{1.04}	188

¹n.a.: not available

Table 2. Performances of mixed-culture fermentative H₂ production in batch tests after addition of an inhibitory compound

Added inhibitor	Modified Gompertz equation parameter values				Xylose consumed (mol)	Hydrogen yield (mol-H ₂ .mol-xylose ⁻¹)	Final pH	Molar butyrate/acetate ratio
	<i>P</i> (mL. L ⁻¹)	<i>R_m</i> (mL.L ⁻¹ .day ⁻¹)	<i>λ</i> (day)	<i>R</i> ²				
None (control – only xylose)	1367.7 (± 80.7)	42.8 (± 15.6)	3.2 (± 1.3)	0.99 (± 0.01)	7.3 (± 0.0)	1.67 (± 0.10)	4.2 (± 0.0)	1.49
Furfural	244.4 (± 45.9)	86.1 (± 98.0)	18.5 (± 1.4)	1.00 (± 0.00)	4.3 (± 0.0)	0.51 (± 0.09)	4.6 (± 0.1)	1.54
HMF	244.0 (± 34.3)	27.5 (± 12.7)	9.9 (± 1.8)	0.98 (± 0.00)	5.7 (± 0.0)	0.40 (± 0.08)	4.3 (± 0.1)	1.26
Phenol	480.5 (± 36.5)	49.6 (± 3.5)	22.2 (± 1.2)	1.00 (± 0.00)	3.4 (± 0.0)	1.28 (± 0.20)	4.8 (± 0.0)	1.23
Syringaldehyde	1045.0 (± 26.6)	43.0 (± 3.4)	8.1 (± 2.6)	0.99 (± 0.00)	6.7 (± 0.0)	1.39 (± 0.08)	4.2 (± 0.0)	2.16
Vanillin	449.1 (± 65.3)	37.8 (± 29.7)	16.5 (± 0.6)	1.00 (± 0.00)	3.1 (± 0.0)	1.30 (± 0.28)	4.7 (± 0.0)	4.04
Kraft lignin	305.1 (± 96.1)	37.6 (± 52.7)	4.5 (± 2.0)	0.93 (± 0.08)	4.1 (± 0.1)	0.67 (± 0.23)	4.8 (± 0.2)	1.06
Organosolv lignin	196.2 (± 57.8)	25.8 (± 32.0)	4.4 (± 2.1)	0.83 (± 0.13)	5.2 (± 0.1)	0.34 (± 0.11)	4.3 (± 0.0)	0.83