

# The environmental biorefinery: state-of-the-art on the production of hydrogen and value-added biomolecules in mixed-culture fermentation

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1 **The environmental biorefinery: state of the art on the production of hydrogen and**  
2 **value-added biomolecules in mixed-culture fermentation**

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10 **Abstract:**

11 The environmental biorefinery consists in recovering and adding value to waste,  
12 possibly through a multi-product approach. A first implementation of such concept is the  
13 production of methane and nutrient-rich digestate by anaerobic digestion in biogas plants.  
14 However, methane and digestate have only a low added-value and biogas plants still require  
15 feed-in tariff policies to be economically viable. The aim of this article is to provide a meta-  
16 analysis of current biomass recovery technologies compatible with environmental  
17 applications (*i.e.* non-sterile conditions and carried out by microbial mixed culture). A  
18 particular focus on those able to produce high value-added fermentation metabolites was  
19 made. To achieve this objective, both qualitative (*e.g.* substrates, pretreatments) and  
20 quantitative data (*e.g.* yields, productivities, process parameters) were retrieved from 624  
21 manually-checked research articles, excluding review papers, and 134 patents published after  
22 1997. In addition, a straightforward market study was carried out for nine promising  
23 biomolecules: H<sub>2</sub>, ethanol, acetate, propionate, lactate, 1,3-propanediol, butyrate, caproate and  
24 polyhydroxyalkanoates (PHAs). Finally, the feasibility of producing each biomolecules in the  
25 context of an environmental biorefinery is discussed in the light of the current process  
26 performances and their related bottlenecks.

27

28 **Keywords:** Biohydrogen; Bioeconomy; Biomolecules; Dark fermentation; Microbial  
29 electrosynthesis; Microbial consortia; Photofermentation; Pretreatment

## 30 1. Introduction

31 Anaerobic digestion (AD) is currently going through a strong industrial development in  
32 the renewable energy sector all around the world<sup>1,2</sup>. It is a mature biological process involving  
33 a complex association of microbial communities (*i.e.* mixed microbial cultures) able to  
34 convert into methane a variety of organic substrates including industrial and municipal  
35 wastewater, sewage sludge, municipal solid waste and residues and effluents from agricultural  
36 activities<sup>3</sup>. To date, the main product considered in AD processes is biogas, consisting of a  
37 mixture of methane and carbon dioxide. However, hydrogen gas (H<sub>2</sub>) which is an  
38 intermediate product of this process (Figure 1) has a higher added value than biogas and is  
39 predicted to become a wide energy carrier for transportation and energy storage in a near  
40 future<sup>4</sup>. Several process control strategies and microbial selection procedures have been  
41 explored over the past 15 years to promote H<sub>2</sub> production during the acidogenic phase of AD  
42 in a process called dark fermentation (DF)<sup>5</sup>. During DF, H<sub>2</sub> production is concomitant with  
43 the accumulation of soluble metabolites (carboxylic acids and alcohols, Figure 1), which have  
44 a higher added-value than biogas and could potentially be extracted prior to their conversion  
45 into methane<sup>6</sup>. Despite the numerous scientific studies published on mixed-culture DF and the  
46 few dozens on pilot-scale reactors, all showing great perspectives for this technology, there is  
47 still no industrial-scale implementation of such environmental biorefinery.

48 < Figure 1 >

49 The aim of this article is to provide a meta-analysis of the biomass recovery  
50 technologies operated under non-sterile conditions. Mixed-culture DF processes and, more  
51 broadly, mixed-culture bioprocesses used for the production of H<sub>2</sub> and value-added  
52 fermentation metabolites were considered. To achieve this objective, both qualitative (*e.g.*  
53 substrates, pretreatments) and quantitative data (*e.g.* yields, productivities, process  
54 parameters) were retrieved from 624 manually-checked research articles, except review  
55 articles, and 134 patents published after 1997 (Figure 2). In addition, a straightforward market  
56 study was carried out for nine promising biomolecules (including H<sub>2</sub>). Finally, the feasibility  
57 of producing each biomolecule in the context of environmental biorefinery is discussed in the  
58 light of the current process performances and their main bottlenecks.

59 < Figure 2 >

## 60 2. Methodology

### 61 2.1. Database building

62 The main database used in this study was established from the Scopus (title, abstract  
63 and keywords of research articles) and Orbit (full-text patents) databases and includes  
64 documents published from 1997 to January 2017. Documents were first selected by automatic  
65 queries based on a keyword list (Table 1) according to the following approach:

- 66 • Patents containing keywords from the “Hydrogen” AND “Process” lists
- 67 • Patents and research articles containing keywords from (“Hydrogen” OR  
68 “Biomolecules”) AND “Process” AND “Mixed culture” lists
- 69 • Patents and research articles containing keywords from “Biomolecules” AND  
70 “Combined processes”) AND “Mixed culture” lists

#### 71 < Table 1 >

72 This strategy led to the identification of 8853 research articles and 1654 patents (not  
73 considering non-extended Chinese patents). In a second step, the database was adjusted  
74 through the use of a keyword-based and automatic procedure and further manual evaluation to  
75 remove documents dealing with:

- 76 • methane production only
- 77 • ethanol production by yeasts
- 78 • microbial fuel cells
- 79 • pure cultures of either wild type or genetically modified organisms
- 80 • published results in non-peer-reviewed scientific journal
- 81 • state-of-the-art reviews or opinion articles

82

83 The final database includes 624 research articles and 134 patents (see Supplementary  
84 information). This database was then checked for the presence of a pre-established list of 25  
85 representative articles to ensure that no key publication was missing. The database was further  
86 manually enlarged with most recent articles (2017) to consider the most up-to-date  
87 observations about biomolecule production by mixed-culture fermentation.

88

## 89 2.2. Calculations

### 90 2.2.1. COD mass balance

91 In some research articles, the Chemical Oxygen Demand (COD) was experimentally  
92 measured and was directly retrieved. When this information was not available, the COD  
93 equivalents of the substrate (and the products) were assessed from the elemental composition  
94 of each compound. More precisely, for a compound  $C_wH_xO_yN_z^{n-}$ , the COD equivalent  
95 corresponds to:

$$96 \quad \text{COD}_{\text{molecule}} (\text{gCOD/gmolecule}) = 8 \cdot \frac{4 \cdot w + x - 2 \cdot y - 3z + n}{12 \cdot w + x + 16 \cdot y + 14 \cdot z} \quad (1)$$

97 The COD equivalents of the most often encountered molecules are provided in Table  
98 2. In addition, when the macromolecular characterization was provided (i.e. lipid, protein,  
99 carbohydrate and moisture content), the following molecular formulas were used:  $C_{57}H_{104}O_6$   
100 for lipids,  $C_5H_7O_2N$  for proteins and  $C_6H_{10}O_5$  for carbohydrates (corresponding to triolein,  
101 *Escherichia coli* and cellulose respectively). Additives used in fermentation media such as  
102 yeast or beef extract were also considered as pure proteins. According to these hypotheses, the  
103 COD equivalent calculation when macromolecular characterization was provided is:

$$104 \quad \text{COD}_{\text{substrate}} (\text{gCOD/gsubstrate}) = 1.19x(\text{gcarbohydrate/gsubstrate}) + 1.42x(\text{gprotein/gsubstrate}) + 2.90x(\text{glipid/gsubstrate}) \quad (2)$$

105 < Table 2 >

### 106 2.2.2. Productivities

107 All productivities reported in this article correspond to average productivities.  
108 Depending on the process configuration (i.e. continuous or discontinuous), the productivities  
109 of the different biomolecules were calculated as follows:

- 110 • For batch and fed-batch processes: average productivities were calculated by dividing  
111 final concentrations (or total gas production) by the total duration of the fermentation.
- 112 • For continuous and semi-continuous processes: in the case average productivities in  
113 the stationary phase were not provided, they were assessed by dividing concentrations  
114 of the biomolecules during the stationary phase by the hydraulic retention time.

115

### 116 2.2.3. Hierarchical clustering and PLS-DA

117 To obtain clusters based on metabolic profiles, all studies were first thoroughly  
118 examined to keep the results where more than 60% of initial COD was recovered as products  
119 at the end of the process or in the stationary phase, in discontinuous and continuous processes,  
120 respectively. The metabolic profiles were then considered as “successful” fermentation where  
121 results can be rigorously compared. COD profiles were then analysed by hierarchical  
122 clustering using the “pvclust” function of the R package pvclust<sup>7</sup>, using the “average” method  
123 and the Euclidean distance. The clusters retained were those gathering more than five  
124 fermentation profiles and for which the existence was statistically significant (p-values <  
125 0.05). Significance was assessed by bootstrap procedures (10,000 bootstraps). The five  
126 significant clusters were then graphically represented using a Partial Least Square  
127 Discriminant Analysis (PLS-DA) based on COD profiles. The PLS-DA was carried out using  
128 the “plsda” function of the R package mixOmics<sup>8</sup>.

129

### 130 **3. Bioprocesses for hydrogen production by mixed microbial consortia**

131 To strengthen the results and conclusions of this section, only the research articles for  
132 which it was possible to calculate COD mass balances and showing an H<sub>2</sub> yield higher than  
133 0.01 g<sub>COD</sub>·g<sub>COD</sub><sup>-1</sup> (based on total initial COD) were considered (400 articles). Patents  
134 considered in this section were those explicitly claiming H<sub>2</sub> production (100 patents).

#### 135 **3.1. The H<sub>2</sub> market**

136 Dihydrogen (H<sub>2</sub>) is a molecule used in industry as chemical reagent, especially for  
137 hydrogenation reactions as widely used in petrochemistry or for ammonia and methanol  
138 production. Currently, the global hydrogen consumption is about 60,000 kt/yr (~ 700·10<sup>9</sup>  
139 Nm<sup>3</sup>) and is predicted to reach a near-exponential growth in the coming years. Indeed, the use  
140 of H<sub>2</sub> as decarbonated energy carrier, both for energy storage or in the transportation sector,  
141 could represent up to 30% of H<sub>2</sub> world's consumption in 2030 and even reach more than 60%  
142 of the world consumption in 2050 (estimated to be ~ 480,000 kt/yr, source: Mcphy-energy).

143 Currently, bio-based dihydrogen is not present on the market. About 96% of the  
144 hydrogen currently present on the market is derived from fossil fuels such as natural gas  
145 through steam reforming, an environmental-impacting process, emitting more than 10 kg of  
146 fossil CO<sub>2</sub> per kilogram of H<sub>2</sub> produced. Current H<sub>2</sub> production costs by natural gas reforming

147 is between 1.0 and 2.0 €/kg but strongly depends on the hydrocarbons market price, and could  
148 also be negatively impacted by potential future regulations on CO<sub>2</sub> emissions.

149 The remaining 4% of worldwide hydrogen is produced by water electrolysis, an  
150 electrochemical process in which electric current is used to split water into dioxygen and  
151 dihydrogen. The cost of electrochemically-produced H<sub>2</sub> is estimated between 3.5 and 5.0  
152 €/kg. Environmental impacts are directly related to the source of the electricity used. Thus, a  
153 reasonable target for the overall production costs of a future biobased H<sub>2</sub> production process  
154 could be assessed between 1.5 and 3.5 €/kg<sub>H<sub>2</sub></sub> to be economically competitive with the  
155 existing market. It is likely that the environmental impact of biohydrogen production would  
156 be favourable when compared to the existing processes<sup>9,10</sup>, but it should be determined on a  
157 case-by-case basis through Life Cycle Assessments (LCAs).

158

### 159 **3.2. Current biological technologies for bioH<sub>2</sub> production**

160 Three technologies have been developed for producing H<sub>2</sub> with microbial consortia:  
161 DF, photofermentation and microbial electrolysis (Figure 3). DF corresponds to the anaerobic  
162 conversion of organic substrates through fermentation in the absence of light. DF leads to the  
163 production of hydrogen and soluble molecules such as short-chain carboxylic acids (acetic,  
164 propionic, butyric acids, Figure 1)<sup>5</sup>. DF is by far the most studied technology (75.75% of the  
165 scientific articles) but also the most patented (40% of the patents). The main advantage of this  
166 technology relies on the possibility to use complex low-cost substrates such as industrial or  
167 agricultural effluents or residues (Section 3.3). However, only a maximum of 33% of the  
168 substrate COD content can be converted into biohydrogen by DF<sup>5</sup>. A way to recover the  
169 energy remaining in the liquid phase is to inject the DF effluents into a digester to produce  
170 methane. Following such coupling, almost all of the COD of the feedstock can theoretically  
171 be converted into biohydrogen and methane, either separately or as a mixture that can be sold  
172 as hythane<sup>11</sup>. The studies reporting a coupling between DF and AD represent 8.75% of the  
173 scientific publications of the field, and 34% of the patents (Figure 3). This high patent  
174 proportion probably reflects a relative easiness of implementing DF upstream from a pre-  
175 existing AD plant, also known as two-step AD.

176 **< Figure 3 >**

177 Photofermentation is a technology involving photosynthetic organisms that can  
178 produce H<sub>2</sub> from organic substrates in presence of light<sup>12</sup>. This additional energy input, which  
179 can be artificial or natural (sun), makes thermodynamically favorable H<sub>2</sub>-producing reactions  
180 that are not possible in DF, thus allowing a more complete conversion of organic substrates  
181 into biohydrogen. However, this technology is less flexible than DF regarding the types of  
182 substrate. It is usually necessary to convert complex substrates into a mixture of carboxylic  
183 acids and alcohols prior to H<sub>2</sub> production by photofermentation. This feature makes possible  
184 the coupling between DF and photofermentation, in which DF effluents, rich in carboxylic  
185 acids, can be converted into biohydrogen. Overall, research on photofermentation and its  
186 coupling with DF accounts for 9.25% of scientific articles but only 5% of patents (Figure 3).

187 Finally, microbial electrolysis is the latest technology that has been investigated for  
188 the production of biohydrogen by mixed cultures. This technology requires specifically  
189 designed bioreactors compatible with the presence of electrodes (see Krieg et al. (2018) for  
190 more details)<sup>13</sup>. External supply of electrical energy can here be used to make  
191 thermodynamically favorable chemical reactions<sup>14</sup>. When used for hydrogen production,  
192 microbial electrolysis cells use the presence of electro-active microorganisms on the anodic  
193 surface to convert organic substrates into electric current, protons and CO<sub>2</sub>. This electric  
194 current can then be used on the cathode surface to abiotically convert protons into  
195 biohydrogen. When the anodic and cathodic compartments are separated by a membrane, the  
196 hydrogen produced at the cathode is nearly pure. Similarly to photofermentation, microbial  
197 electrolysis allows a more complete conversion of organic substrates into biohydrogen when  
198 compared to DF. Here, mixtures of carboxylic acids, as found in DF effluents, can be used as  
199 substrate at the anode. While microbial electrolysis accounts for only 6.25% of the published  
200 articles in the field, 20% of the patents are dealing with this technology, suggesting a high  
201 industrial interest.

202

### 203 **3.3. Substrates and pretreatments**

204

#### 205 3.3.1. Diversity of the substrates used for H<sub>2</sub> production

206 One of the main benefits of using mixed-culture fermentation is its flexibility on  
207 converting a wide range of substrates<sup>15</sup>. Within the scientific articles, synthetic fermentation



208 media are employed in 53% of the studies with the use of simple substrates such as glucose,  
209 sucrose, cellulose, mixtures of volatile fatty acids (*e.g.* acetate, propionate, butyrate) or  
210 purified glycerol (Figure 4A). The COD concentration of these simple substrates is usually  
211 low, with an average value of 12.1 and 3.4  $\text{g}_{\text{COD}}\cdot\text{L}^{-1}$  when sugars and volatile fatty acids are  
212 provided as substrates, respectively (Figure 4B). Usually, studies using synthetic fermentation  
213 media aim to elucidate the fundamentals of  $\text{bioH}_2$  production (*e.g.* effect of pH, effect of  
214 microbial population selection procedures) rather than demonstrating an actual feasibility of,  
215 for instance, a sugar-based  $\text{bioH}_2$  production process. Indeed, using such purified substrates  
216 represents a significant cost in biohydrogen production processes, particularly in comparison  
217 with food waste or co-products from the industrial sector. In addition, the use of readily edible  
218 sugars (mainly beet, sugar cane and maize crops) for commodity chemicals production  
219 competes with food production and raises societal debates<sup>16,17</sup>.

220  $\text{BioH}_2$  production from organic residues or agricultural/industrial process co-products  
221 concerns 47% of the scientific articles in the field (Figure 4A). Most of these substrates are  
222 issued from agriculture and green waste (20.3% of the scientific studies, 43.2% of the  
223 complex substrates). They include rice and wheat straws, corn stalks, sugar molasses or fruit  
224 production residues. Energy crops (*e.g.* sorghum, sugarcane, cassava) are only employed in  
225 22.9% of the studies using biomass from agriculture and green waste (*i.e.* 10.8% of the  
226 studies using complex substrates). On average, biomass from agriculture and green waste are  
227 used in  $\text{H}_2$  production process with a COD concentration of 19.4  $\text{g}_{\text{COD}}\cdot\text{L}^{-1}$  (Figure 4B). The  
228 second most important category of complex substrates gathers industrial effluents which are  
229 used in 12% of the studies reporting biohydrogen production (25.5% with complex  
230 substrates). These substrates are of various kinds and include, among others, residues from the  
231 paper, dairy and oilseed industries, as well as crude glycerol generated from the biodiesel  
232 industry. An average COD concentration of 29.7  $\text{g}_{\text{COD}}\cdot\text{L}^{-1}$  is reported in all the identified  
233 studies. For the rest of the complex substrates, four categories are distinguished: food waste  
234 (8% of the studies, average organic matter concentration of 55.9  $\text{g}_{\text{COD}}\cdot\text{L}^{-1}$ ), municipal waste  
235 such as sewage sludge or the organic fraction of municipal solid waste –OFMSW (3.2 % of  
236 the studies, average organic matter concentration of 44.2  $\text{g}_{\text{COD}}\cdot\text{L}^{-1}$ ), macro/ microalgae (2% of  
237 the studies) and dark or ethanol fermentation effluents (1.5% of the studies).

238 < **Figure 4** >

239 3.3.2. Substrate pretreatments

240 Due to their complex structure, some organic compounds cannot be directly and easily  
241 converted in biological processes. For instance, lignocellulosic materials, agriculture residues  
242 or urban green waste are composed of cellulose and hemicelluloses, but also of lignin, a  
243 polymer giving stiffness to plants and protecting them from microbial attack<sup>18–21</sup>. Municipal  
244 waste may also contain recalcitrant biomass such as cardboard<sup>22</sup> or bacterial cell walls in  
245 sewage sludge<sup>23</sup>. To exploit these biomasses in fermentation processes, pretreatments are  
246 applied to make their sugars more soluble and biologically more accessible. These  
247 pretreatments are mainly classified into three categories: mechanical (grinding, sonication),  
248 physico-chemical (acid/alkaline hydrolysis, heat treatment, steam explosion) or enzymatic (by  
249 microorganisms or enzyme cocktails) methods.

250 About 45% of the studies employing complex substrates reported the use of one or more  
251 biomass pretreatment methods. The most common methods are thermal pretreatments (19%),  
252 acid or alkaline hydrolysis (18%) and enzymatic pretreatments (8%), whether applied alone or  
253 in combination with other pretreatments. Biomasses from agriculture & green waste and  
254 municipal waste are the most preferred substrates to be pretreated, representing 67.9% and  
255 61.5% of the studies employing at least one pretreatment method, respectively (Figure 5). It is  
256 important to note that these pretreatments may represent significant costs prior to  
257 fermentation. As an illustration, the authors of a techno-economical study using corn stalks as  
258 substrate recently estimated that the price of raw substrate treatment (90 €/t<sub>cornstarch</sub>) was at  
259 least doubled to ~ 180 €/t<sub>cornstarch</sub> (equivalent to 330 €/t<sub>solubleCOD</sub>) when the pretreatment cost  
260 was taken into account<sup>24</sup>. Most industrial effluents and food waste do not require any  
261 pretreatment and are directly used as fermentation substrate (Figure 5). For more detailed  
262 information about substrate pretreatments, readers may refer to Carrere *et al.* (2016)<sup>23</sup>.

263 < **Figure 5** >

### 264 3.3.3. Inoculum pretreatments

265 In biological ecosystems, hydrogen is an energy vector favouring electron transfer  
266 between microorganisms. To optimize its production, it is not only recommended to favour  
267 microbial species that release their excess of electrons as H<sub>2</sub>, but also to prevent the growth of  
268 hydrogen-consuming microorganisms such as methanogenic archaea or acetogenic bacteria.  
269 The choice and adaptation of an inoculum is one of the most crucial elements when designing  
270 a biohydrogen production process. To obtain a suitable inoculum, the most common method  
271 (56.1% of the studies, Figure 6A) is to pretreat inocula originating from the environment or

272 from parent reactors to remove undesirable microorganisms<sup>21,25</sup>. The most widely used  
273 technique is thermal pretreatment (70.4% of the pretreatments, Figure 6B) which consists in  
274 applying a thermal shock to the inoculum. As a result, the microorganisms capable of  
275 surviving by forming spores are specifically selected, such as *Bacillus* and *Clostridium*  
276 species. These genera contain many efficient hydrogen-producing microorganisms<sup>21</sup>, as well  
277 as acetogenic species. However, thermal pretreatment is efficient to prevent methanogenic  
278 archaea growth and is usually sufficient for the start-up of hydrogen-producing processes.  
279 Following the same principle, other pretreatment methods aims to the elimination or  
280 inhibition of hydrogen-consuming bacteria and the indirect selection of hydrogen-producing  
281 bacteria, including acid or alkali treatments (11.7% of the pretreatments), addition of  
282 methanogenesis inhibitors such as chloroform and 2-bromoethanesulphonate (6.1%), or  
283 aeration (3.6 %) methods.

284 Studies that do not use pretreatment techniques (43.9% of the studies) employ other  
285 population selection pressure through process operating parameters. For example, it is  
286 possible to maintain acidic conditions in the fermentation medium to inhibit the methanogenic  
287 archaea activity<sup>26</sup>. In continuous processes, it is also possible to wash-out archaea, which have  
288 a lower growth rate than hydrogen-producing bacteria, by applying a short hydraulic retention  
289 time<sup>5</sup>. Operating parameters have also been widely optimized throughout the studies using  
290 pretreatments to prevent the re-emergence of hydrogen-consuming microorganisms during the  
291 process. For more detailed information about inoculum pretreatments, readers may refer to  
292 Rafieenia *et al.* (2017)<sup>21</sup>.

293 < **Figure 6** >

### 294 **3.4. Production performances**

295 As indicated in Sections 3.2 and 3.3, various technologies and a broad range of  
296 substrates can be used for biohydrogen production, thus leading to highly variable  
297 performances. Hydrogen yields as a function of the technologies and the initial COD are  
298 shown in Figure 7. Considering all the technologies, the total substrate COD concentration  
299 was lower than 22.2 g<sub>COD</sub>.L<sup>-1</sup> for 75% of the studies. Regarding hydrogen yields, values below  
300 0.16 g<sub>COD\_H2</sub>.g<sub>COD</sub><sup>-1</sup> were observed in more than 75% of the studies. Yields higher than this  
301 value were only reached when substrate with lower COD content were employed (8.4 g<sub>COD</sub>.L<sup>-1</sup>  
302 <sup>1</sup> on average) and mostly by photofermentation, microbial electrolysis technologies or by  
303 coupling them with DF. When initial substrate concentrations were higher than 22.2 g<sub>COD</sub>.L<sup>-1</sup>,

304 the maximum average hydrogen yield was only  $0.07 \text{ g}_{\text{COD}_H_2} \cdot \text{g}_{\text{COD}}^{-1}$ . These observations  
305 emphasize that, under the current state of the art, there is a compromise to be found between  
306 reaching high hydrogen yields and valorizing substrates at high COD content. In the  
307 following sections, the biohydrogen production performances obtained for each technology  
308 will be detailed and put into perspective with regard to this specific issue.

309 < **Figure 7** >

#### 310 3.4.1. Dark fermentation performances

311 One of the main advantages of DF processes is its flexibility regarding a wide range of  
312 substrates. However, hydrogen production performances can greatly vary depending on the  
313 nature and complexity of the substrates (Figure 8A). The average yield observed in DF was  
314 about  $0.108 \text{ g}_{\text{COD}_H_2} \cdot \text{g}_{\text{COD}}^{-1}$ . This value represents  $\sim 33\%$  of the maximum theoretical yield,  
315 *i.e.*  $0.33 \text{ g}_{\text{COD}_H_2} \cdot \text{g}_{\text{COD}}^{-1}$ , in DF<sup>5</sup>. Best performances were observed when synthetic  
316 fermentation media were employed rather than complex substrates ( $p$ -value  $< 0.0001$ ). The  
317 average yield reached then  $0.124$  and  $0.089 \text{ g}_{\text{COD}_H_2} \cdot \text{g}_{\text{COD}}^{-1}$  with purified sugars and complex  
318 substrates, respectively. Regarding complex substrates, the highest average yields were  
319 achieved with industrial effluents and biomass from agriculture and green waste (mostly after  
320 pretreatment) with average yields of  $0.096$  and  $0.094 \text{ g}_{\text{COD}_H_2} \cdot \text{g}_{\text{COD}}^{-1}$ , respectively.  
321 Interestingly, food and municipal waste are the substrates for which the lowest hydrogen  
322 yields were obtained, with average yields of only  $0.064$  and  $0.056 \text{ g}_{\text{COD}_H_2} \cdot \text{g}_{\text{COD}}^{-1}$ ,  
323 respectively. Surprisingly, when considering all the data retrieved from the studies dealing  
324 with DF for  $\text{H}_2$  production from organic biomass, only the intrinsic composition and the  
325 structural features of the organic substrates seem to have an influence on the  $\text{H}_2$  yields.  
326 Although each microbial community had its own optimal parameters, the observed hydrogen  
327 yields were not statistically different in all studies, whatever the process parameters (Figure  
328 8B) such as working volume (ranging from  $0.01$  to  $3,300 \text{ L}$ ) and temperature ( $15$  to  $80^\circ\text{C}$ ), or  
329 the mode of operation of the bioreactor (batch, semi-continuous or continuous).

330 < **Figure 8** >

331 Beyond the hydrogen yield, the choice of process parameters can strongly influence  
332 the composition of the microbial community (Section 3.3) and thus the hydrogen production  
333 kinetics or the stability of the process (Figure 9). In particular, the choice of operation mode  
334 (batch VS continuous) plays an important role, especially regarding the easiness of process

335 implementation and the related performances. The batch reactor is the simplest configuration.  
336 In this mode, all the substrate is added at start of reactor operation and no withdrawal of the  
337 medium is carried out before the end of fermentation. Because of its simplicity, most of the  
338 reactors have been carried out in batch mode (60.7% of the studies, Figure 9A) with hydrogen  
339 productions generally ranging from 0.70 to 2.76  $L_{H_2} \cdot L_{medium}^{-1}$  (1st and 3rd quartiles, Figure  
340 9B), with a maximum<sup>27</sup> of 12.88  $L_{H_2} \cdot L_{medium}^{-1}$ . In general, batch processes are not the most  
341 efficient from a kinetic point of view, because (i) a lag phase is often observed due to  
342 microbial inoculum storage and the time to adapt to the fermentation medium; (ii) batch tests  
343 are ended after a time chosen by the operator which is not necessarily optimal; (iii) most  
344 studies using batch reactors do not focus on microbial kinetics. Thus, relatively low hydrogen  
345 productivities were achieved in batch mode, with values generally ranging between 0.22 and  
346 1.22  $L_{H_2} \cdot L_{medium}^{-1} \cdot d^{-1}$  (1st and 3rd quartiles, Figure 9C), the median and maximum<sup>28</sup> values  
347 being 0.55 and 6.28  $L_{H_2} \cdot L_{medium}^{-1} \cdot d^{-1}$ , respectively. To attain higher productivities, solutions  
348 were to operate bioreactors in continuous (31.4% of the studies) or semi-continuous (7.6%)  
349 mode. In these cases, bioreactors have both inlet and outlet flows for feeding the substrate and  
350 withdrawing the products continuously or sequentially, respectively. Hydrogen production  
351 kinetics and productivities were optimized with these modes of operation and usually ranged  
352 between 1.20 and 7.80  $L_{H_2} \cdot L_{medium}^{-1} \cdot d^{-1}$  (1st and 3rd quartiles, Figure 9C), the median and  
353 maximum<sup>29</sup> values being 3.34 and 346.8  $L_{H_2} \cdot L_{medium}^{-1} \cdot d^{-1}$ , respectively.

354 < Figure 9 >

355 3.4.2. H<sub>2</sub> production performances of photo-fermentation and microbial electrolysis

356 To improve hydrogen yields, DF can be coupled with photofermentation or microbial  
357 electrolysis (Section 3.2). These two processes are based on a common principle: the  
358 conversion of volatile fatty acids and alcohols into H<sub>2</sub> and CO<sub>2</sub> is made thermodynamically  
359 favourable by providing an additional source of energy.

360 In the case of photofermentation, this external energy is provided by either artificial or  
361 natural light. However, the energy conversion efficiency of photofermentation, *i.e.* the ratio of  
362 the energy recovered as H<sub>2</sub> on the energy provided as light, does not exceed 10% under well-  
363 controlled conditions<sup>12</sup>. That makes this technology non-profitable if artificial light is used  
364 and if H<sub>2</sub> is the only product recovered. About 27% of the articles focusing on  
365 photofermentation concern the study of photofermentation alone while 73% of the articles  
366 deal with its coupling with DF. In both cases, the hydrogen yields were not significantly

367 different, with average values of 0.279 and 0.246  $\text{g}_{\text{COD}_{\text{H}_2}} \cdot \text{g}_{\text{COD}}^{-1}$  respectively (Figure 10).  
368 Overall, the average hydrogen yields of 0.255  $\text{g}_{\text{COD}_{\text{H}_2}} \cdot \text{g}_{\text{COD}}^{-1}$  obtained by both  
369 photofermentation or its coupling with DF were significantly higher than those obtained in  
370 DF alone, *i.e.* 0.108  $\text{g}_{\text{COD}_{\text{H}_2}} \cdot \text{g}_{\text{COD}}^{-1}$  (p-value < 0.0001). Nevertheless, these results do not  
371 necessarily reflect realistic working conditions, particularly regarding the use of small  
372 working volumes (< 0.25 L in 75% of the studies) and substrates with low COD concentration  
373 (< 11.2  $\text{g}_{\text{COD}} \cdot \text{L}^{-1}$  in 75% of the studies). A pilot-scale photofermentation process dedicated to  
374  $\text{H}_2$  production was developed with a pure culture of *Rhodobacter capsulatus*<sup>30</sup>, but no large  
375 scale mixed-culture process have been carried out so far. Adaptation to higher concentrations  
376 of organic matter or higher loading rates as well as the improvement of energy conversion  
377 efficiencies remain the main challenges of photofermentation prior to scaling up at larger  
378 scale. For more detailed information about photofermentation, readers may refer to  
379 Hallenbeck and Liu (2016)<sup>31</sup>.

380 < **Figure 10** >

381 In microbial electrolysis cells, electric energy is provided through an applied voltage  
382 between two electrodes. When producing  $\text{H}_2$ , these cells require voltage between 0.2 and 0.8  
383 V, which is much lower than the values of 1.8 to 3.5 V typically applied in water electrolysis  
384 processes<sup>14</sup>. The energy conversion efficiency of microbial electrolysis is defined as the ratio  
385 of the energy recovered as  $\text{H}_2$  over the electric energy provided to the system. As most of the  
386 energy is provided by the oxidation of organic matter at the cathode, efficiency calculated in  
387 this way can theoretically be as high as 1094% if based on the higher heating value of  $\text{H}_2$  and  
388 when acetate is used as substrate<sup>32</sup>. Experimentally, the average energy conversion efficiency  
389 is  $199 \pm 22\%$  in the scientific studies identified in the present article. Regarding the COD  
390 conversion efficiencies, an average hydrogen yield of 0.479  $\text{g}_{\text{COD}_{\text{H}_2}} \cdot \text{g}_{\text{COD}}^{-1}$  was reported in  
391 microbial electrolysis or by coupling it with DF. However, performances are extremely  
392 variable (Figure 10). Interestingly, yields higher than 0.950  $\text{g}_{\text{COD}_{\text{H}_2}} \cdot \text{g}_{\text{COD}}^{-1}$  were obtained in  
393 microbial electrolysis process<sup>33,34</sup>. In addition to the high conversion efficiencies, another  
394 advantage of microbial electrolysis cells is the possibility to produce nearly pure  $\text{H}_2$  when  
395 anodic and cathodic compartments are separated by a membrane.

396 The high performances of microbial electrolysis cells regarding hydrogen yields and  
397 energy efficiencies, as well as the high purity of the biohydrogen recovered, makes this  
398 technology particularly attractive as a complement to DF. However, this technology is still



399 mostly studied at a small scale (working volume < 0.50 L in 75% of the studies) and with  
400 substrates at low COD concentration (< 3.2 g<sub>COD</sub>.L<sup>-1</sup> in 75% of the studies) that mostly  
401 correspond to synthetic mixtures of volatile fatty acids (73% of the studies). Nevertheless,  
402 few pilot-scale reactors (volumes from 100 to 1000 L) operated with wastewaters at low  
403 organic loading rates (0.5 to 2.0 g<sub>COD</sub>.L<sup>-1</sup>.d<sup>-1</sup>) have been recently implemented and exhibit  
404 very promising results<sup>35-38</sup>. Similarly to photofermentation, research efforts are required to  
405 develop efficient microbial electrolysis cells at higher organic loading rates and treating real  
406 DF effluents, prior to its implementation at industrial scale. For more detailed information  
407 about microbial electrolysis, readers may refer to Zhen *et al.* (2017)<sup>39</sup>.

408 < Table 3 >

### 409 3.5. Downstream processes for H<sub>2</sub> production

410 Biohydrogen production during DF and photofermentation is always concomitant with  
411 CO<sub>2</sub> production. If the biogas is not diluted, the proportions encountered range generally from  
412 30 to 60% for H<sub>2</sub> and 40 to 70% for CO<sub>2</sub>, with possible traces of CH<sub>4</sub> and /or H<sub>2</sub>S. Mature  
413 technologies for hydrogen separation that are currently used in petrochemical processes are  
414 easily applicable to bioH<sub>2</sub> production processes<sup>40,41</sup>. For instance, the Pressure Swing  
415 Adsorption (PSA) process can produce H<sub>2</sub> at a purity of 99.999% with a H<sub>2</sub> recovery ranging  
416 from 75 to 92% while a purity of 90-99% and a H<sub>2</sub> recovery of 85-95% can be achieved with  
417 membrane permeation technologies<sup>42</sup>. Therefore, the biohydrogen separation step is not a  
418 technological obstacle, but remains one of the most costly step of the overall process<sup>43-45</sup>.

419

## 420 4. Toward the waste-based biorefinery

421 Optimization of H<sub>2</sub> production has been the main objective of the last 15 years of  
422 research concerning DF. However, the hydrogen yields achieved so far (average of 0.108  
423 g<sub>COD\_H2</sub>.g<sub>COD</sub><sup>-1</sup>) and the intrinsic metabolic limitations during DF severely limit the  
424 implementation of this process alone in a context of biomass recovery. A first solution, as  
425 presented in Section 3.4, is to couple DF with another hydrogen-producing process such as  
426 photofermentation or microbial electrolysis. An alternative is to implement a biorefinery  
427 approach in which several product streams are considered.

428

#### 429 **4.1. Two-stage anaerobic digestion for H<sub>2</sub> and CH<sub>4</sub> production**

430 The environmental biorefinery approach has been first and logically considered  
431 through the coupling between DF and AD leading to the production of H<sub>2</sub> and CH<sub>4</sub> (Section  
432 3.2). This two-stage process presents several advantages<sup>11</sup>:

- 433 • At equal COD conversion rates, an energy yield up to 10% higher than the  
434 one-stage AD can theoretically be achieved, because of the higher energy  
435 content of H<sub>2</sub> in comparison with CH<sub>4</sub>, *i.e.* heating value of 17.7 MJ/kg<sub>COD-H<sub>2</sub></sub>  
436 versus 12.5 MJ/kg<sub>COD-CH<sub>4</sub></sub>, respectively.
- 437 • The two-stage process is more stable than the one-stage AD and operating  
438 parameters can be more easily optimized as hydrolysis/acidogenesis and  
439 acetogenesis/methanogenesis steps are separated (Figure 1).
- 440 • Methane yields can be increased in the two-stage process due to better biomass  
441 hydrolysis in the first DF step
- 442 • The two-stage process can be successfully carried out at high organic loading  
443 rates, increasing subsequently the methane productivity.

444 In the studies focusing on two-stage AD, 72.5 ± 19.1% of the total COD content of  
445 substrates were recovered as H<sub>2</sub> and CH<sub>4</sub>, at an average initial total COD concentration of  
446 47.8 ± 38.2 g<sub>COD</sub>.L<sup>-1</sup> (based on 34 articles). Average hydrogen and methane yields were 0.055  
447 ± 0.032 and 0.670 ± 0.187 g<sub>COD</sub>.g<sub>COD</sub><sup>-1</sup>, respectively. Most studies used complex substrates  
448 (88%) that are representative of the categories presented in Figure 4, thus demonstrating the  
449 applicability of such coupling.

450 In addition, the two-stage AD could also increase, theoretically, the added value of the  
451 process. Indeed, the hydrogen market price is ranging between 1.5 and 5.0 €/kg (Section 3.1)  
452 while the feed-in tariff of methane is comprised within a range of 0.09 to 0.20 €/kg (through  
453 injection into the natural gas network in France). Even considering a low case scenario with  
454 an H<sub>2</sub> price of 1.5 €/kg and an identical COD recovery (*i.e.* 72.5%), the added-value of a two-  
455 stage process would increase by 23 to 65% (23.9 - 45.0 €/t<sub>CODfed</sub>) the economy of a one-stage  
456 AD (14.5 - 36.5 € / t<sub>CODfed</sub>) process. Such increase is mitigated by the costs related to  
457 hydrogen purification and DF reactor operation that should be evaluated on a case by case  
458 basis. Consistently, the first technical-economic studies showed that the two-stage process can  
459 be financially advantageous compared to single-stage AD for substrates such as food



460 waste<sup>46,47</sup>. For more detailed information about two-stage AD, readers may refer to Xia *et al.*  
461 (2016)<sup>11</sup>.

462

#### 463 4.2. Production of fermentation by-products

464

465 A wide range of molecules accumulate during AD (Figure 1) and mixed-culture  
466 fermentation processes. These compounds represent new opportunities for recycling waste  
467 into added-value molecules, such as short chain carboxylic acids and alcohols. Among the  
468 scientific articles identified in this study, the most present metabolites at the end of  
469 fermentation in batch and fed-batch reactors, or at the steady state in continuous and semi-  
470 continuous reactors were acetate (86.7% of the studies), butyrate (79.3%), ethanol (49.9%)  
471 and propionate (46.5%) (Figure 11). This result emphasizes that soluble metabolites are  
472 mostly produced as a mixture in mixed-culture fermentation. That represents a major  
473 challenge regarding the following separation/purification steps. To provide a better overview  
474 of the most commonly observed metabolites, a hierarchical clustering was performed based  
475 on the fermentation profiles reported in the literature (Figure 12). This clustering took into  
476 account the studies with more than 60% of the COD recovered as by-products (57% of the  
477 studies) and revealed five "standard" fermentation profiles (Figure 12A).

478 < **Figure 11** >

- 479 (1) The first cluster corresponds to the production of H<sub>2</sub> by photofermentation,  
480 microbial electrolysis and their coupling, as described in section 3.4.
- 481 (2) The second cluster brings together studies focused on two-stage AD as described in  
482 the previous section.
- 483 (3) Cluster 3 is predominantly composed of studies focusing on DF and includes 129  
484 scientific articles (Figure 12). This cluster is characterized by a fermentation profile  
485 dominated by butyrate ( $0.371 \pm 0.148 \text{ g}_{\text{COD}}\cdot\text{g}_{\text{COD}}^{-1}$ ), acetate ( $0.167 \pm 0.100$   
486  $\text{g}_{\text{COD}}\cdot\text{g}_{\text{COD}}^{-1}$ ) and H<sub>2</sub> ( $0.117 \pm 0.062 \text{ g}_{\text{COD}}\cdot\text{g}_{\text{COD}}^{-1}$ ). It is mainly observed during the  
487 fermentation of sugars (68% of the articles) or of sugar-rich complex substrates such  
488 as food waste, dairy or sugar industry wastewaters or hydrolysed lignocellulosic  
489 biomasses. The cluster 3 profile, characterized by a predominant production of  
490 butyrate and acetate, can be considered as a "typical" profile of DF.

- 491 (4) Similarly, cluster 4 essentially contains studies focusing on DF and includes 20  
492 scientific articles. In these studies, the dominant metabolites were ethanol ( $0.341 \pm$   
493  $0.079 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$ ), followed by acetate ( $0.195 \pm 0.120 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$ ), butyrate ( $0.111$   
494  $\pm 0.123 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$ ) and  $\text{H}_2$  ( $0.105 \pm 0.066 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$ ). The substrates mainly  
495 corresponded to sugars (55% of the articles) and effluents from the sugar and  
496 oleaginous (crude glycerol) industries or hydrolysed lignocellulosic biomasses. The  
497 main difference with cluster 3 lies in the mode of inoculum selection: in cluster 4,  
498 only 25% of the inocula were pretreated whereas 55.1% of the studies involved at  
499 least one inoculum pretreatment in cluster 3. Rather than selecting *Clostridiaceae*  
500 species using heat shocks, studies in cluster 4 mainly used aerotolerant inocula (*e.g.*  
501 activated sludge) and/or maintained an acidic pH ( $< 5$ ) during fermentation. These  
502 conditions allowed the enrichment of ethanol-producing bacteria such as species  
503 from the *Enterobacteriaceae* family.
- 504 (5) Finally, cluster 5 corresponds uniquely to studies that used glycerol (pure or crude)  
505 as substrate and gathers 9 studies. In this particular case, the main product is 1,3-  
506 propanediol ( $0.593 \pm 0.114 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$ ) which is accompanied by a variable  
507 mixture of carboxylic acids, ethanol and  $\text{H}_2$ .

### 508 < Figure 12 >

509 The results of this hierarchical clustering highlight that fermentation profiles are  
510 relatively stable and repeatable despite the great diversity of substrates and fermentation  
511 process conditions. In the following sections, production performance and contextual market  
512 elements are discussed for each of the main DF metabolites (acetate, butyrate, ethanol) as well  
513 as other molecules with high potential (1,3-propanediol, propionate, caproate, lactate, PHA).  
514 Other high-valued metabolites such as butanol and succinate will not be discussed as they are  
515 not commonly observed in mixed-culture fermentation (see Figure 11).

## 517 4.3. Applications and economy of dark fermentation co-products

### 518 4.3.1. Acetic acid

519 Acetic acid is a commodity chemical which has a very wide range of applications,  
520 including plastics manufacturing, its use as food additive or solvent. The total world market  
521 volume was 13,570 kt/yr in 2015<sup>48</sup> with more than 50% of the market located in Asia (mainly

522 China and India). The current market is relatively stable in Europe but growing in Asian  
523 countries and its annual growth rate has been estimated at 5% for the period from 2014 to  
524 2020. Its production is mostly oil-based while bio-based acetic acid represented only 10% of  
525 the global market in 2015. The market price of acetic acid is comprised between 0.33 and  
526 0.67 €/kg, equivalent to 0.31 to 0.63 €/kg<sub>COD</sub> (Table 4).

527 < **Table 4** >

528 A stable production of acetate can be achieved during DF at an average yield of  $0.167 \pm$   
529  $0.100 \text{ g}_{\text{COD}_{\text{acetate}}}\cdot\text{g}_{\text{COD}}^{-1}$  when a butyrate-dominated fermentation profile is observed (Cluster  
530 3, Figure 12). However, because substrates with low initial COD content are often used in  
531 DF, acetate concentrations in the fermentation medium are often low. A final acetate  
532 concentration higher than  $2.4 \text{ g}\cdot\text{L}^{-1}$  was reached in only 25% of the studies in which acetate  
533 production was observed (Figure 13). Nonetheless, acetate can be produced at higher  
534 concentrations and the best performance was reported with sugarcane bagasse, reaching a  
535 final concentration of  $35.3 \text{ g}\cdot\text{L}^{-1}$  (Table 5)<sup>49</sup>. By maximizing the acetate yields, values as high  
536 as  $0.56 \text{ g}_{\text{COD}_{\text{acetate}}}\cdot\text{g}_{\text{COD}}^{-1}$  in fermentation processes<sup>50</sup> or even  $0.90 \text{ g}_{\text{COD}_{\text{acetate}}}\cdot\text{g}_{\text{COD}}^{-1}$  were  
537 reached in microbial electrosynthesis processes<sup>51</sup> (Table 6). Finally, high acetate productivity  
538 values of  $57.0 \text{ g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$  were achieved even when employing complex substrates (Table 5)<sup>52</sup>.

539 The main limitation of acetate recovery when it is produced by mixed microbial  
540 cultures is the extraction/purification step of the molecule. Indeed, acetate is mostly produced  
541 together with other molecules having similar chemical characteristics (short chain carboxylic  
542 acids). That makes inefficient the extraction techniques traditionally used in fermentation  
543 processes. No scientific publication concerning the specific extraction of acetate from a  
544 mixture of carboxylic acids in fermentation processes was identified in the considered  
545 database. Moreover, no techno-economic study focused on acetate production by mixed-  
546 culture fermentation was carried out yet, making difficult to assess precisely the limits and the  
547 optimal operating range of potential recovery processes. Nonetheless, few strategies of *in-situ*  
548 acetate purification were proposed with, for instance, the case of hyper-thermophilic AD  
549 ( $70^\circ\text{C}$ ) carried out at low hydraulic retention time ( $<3\text{j}$ )<sup>53-55</sup>. Here, the acetate re-consumption  
550 pathways were inhibited while all other compounds were converted into  $\text{CH}_4$ . As only acetate  
551 remained in the liquid phase, traditional techniques such as liquid-liquid extraction processes  
552 were used to efficiently recover acetate<sup>56</sup>. However, the proof of concept of such process  
553 remains to be carried out on real effluents. Another example is the case of microbial

554 electrosynthesis, in which pure acetate can be produced in the cathodic compartment and then  
555 extracted by migration through membranes (electrodialysis). Although the principle is  
556 interesting, microbial electrolysis processes still suffer from low productivities that limit their  
557 applicability<sup>57</sup>. Instead of extracting acetate, it is also possible to upgrade an acetate-rich  
558 fermentation broth into a more easily-extractable compound using a secondary biological or  
559 chemical process. For instance, a mixture of carboxylic acids can be esterified<sup>58</sup>, converted  
560 into lipids by yeasts<sup>59</sup>, or used to produce caproic acid or polyhydroxyalkanoates (see Section  
561 4.4).

562 < **Figure 13** >

563 < **Table 5** >

#### 564 4.3.2. Butyric acid

565 Butyric acid is a precursor of esters used as food additives or in perfume formulation.  
566 It can also be directly used as antibacterial agent in the field of animal nutrition<sup>6</sup> and for  
567 bioplastic applications, although the former application would require approval of regulatory  
568 authorities such as the FDA in USA or EFSA in EU. The butyrate market is currently quite  
569 limited, with a current production capacity of about 30 kt/yr. This low capacity can be partly  
570 explained by selling prices that are still too high for the commodity market (from 1.67 to 2.09  
571 €/kg, Table 4). Nonetheless the butyrate market could reach an annual growth rate as high as  
572 12% between 2016 and 2020, depending on its availability (production volume) and the  
573 subsequent price decrease<sup>60</sup>.

574 Significant production of butyrate was observed in DF at an average yield of  $0.371 \pm$   
575  $0.148 \text{ g}_{\text{COD}_{\text{butyrate}}}\cdot\text{g}_{\text{COD}}^{-1}$  (Cluster 3, Figure 12). Although butyrate is generally produced at  
576 low concentration (Figure 13), final concentrations can reach up to  $21.4 \text{ g}\cdot\text{L}^{-1}$  (Table 5). The  
577 conversion yield in DF can be optimized to achieve  $0.74 \text{ g}_{\text{COD}_{\text{butyrate}}}\cdot\text{g}_{\text{COD}}^{-1}$  (Table 5)<sup>61</sup>.  
578 Butyrate production can also be promoted by chain elongation reactions<sup>62</sup> in which a mixture  
579 of acetate and ethanol is converted into butyrate (Table 6). Finally, butyrate productivities are  
580 high with  $73.2 \text{ g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$  as best value achieved with complex substrates (Table 5)<sup>52</sup>.

581 Butyrate is mostly produced together with other carboxylic acids in mixed-culture  
582 fermentation which represents a severe limitation to its specific extraction. However, the  
583 possible conversion of a mixture of lactate, acetate and ethanol by chain elongation (possibly  
584 requiring the addition of ethanol) makes theoretically possible the production of butyrate as

585 sole soluble carboxylic acid. Butyrate could then be extracted by liquid-liquid extraction<sup>56</sup> or  
586 electro dialysis. However such proof of concept remains to be demonstrated. Chemical routes  
587 such as esterification are also possible to facilitate extraction steps while upgrading butyrate  
588 into valued chemical such as butanol or butyl-butyrates<sup>58,63</sup>.

589 < Table 6 >

590 4.3.3. Ethanol

591 Ethanol is a molecule with a large world market of 76,700 kt/year (2015)<sup>48</sup>. Ethanol is  
592 predominantly a bio-based product (93%) and is mainly used as biofuel (80 to 85%), or in the  
593 food industry and as solvent. A strong market growth has occurred over the past ten years due  
594 to energy transition policies, particularly in Europe, Brazil and United States. The largest  
595 producers are the United States (59% of bioethanol) and Brazil (27%)<sup>64</sup>, which mainly use  
596 maize and sugar cane as raw materials, respectively. The ethanol market price is between 0.30  
597 and 1.50 €/kg depending on the product purity and the raw material (corresponding to 0.14 -  
598 0.72 €/kg<sub>COD</sub>, Table 4). It is noteworthy that ethanol market price rely heavily on support  
599 policies, either through direct subsidies or fuel blend obligation<sup>65</sup>.

600 Under certain conditions, ethanol can be produced by mixed cultures of fermentative  
601 bacteria at an average yield of  $0.341 \pm 0.079 \text{ g}_{\text{COD}_{\text{ethanol}}}\cdot\text{g}_{\text{COD}}^{-1}$  (Cluster 4, Figure 12).  
602 Similarly to acetate and butyrate, ethanol is generally not the targeted product and therefore  
603 accumulation is limited to low concentrations around a median of 0.5 g.L<sup>-1</sup> (Figure 13).  
604 Nonetheless, several studies aimed at optimizing the ethanol/H<sub>2</sub> production from glycerol.  
605 Using crude glycerol directly issued from the biodiesel industry, the highest concentration  
606 achieved with mixed cultures reached 26.0 g.L<sup>-1</sup>, with a yield and productivity of 0.59  
607  $\text{g}_{\text{COD}_{\text{ethanol}}}\cdot\text{g}_{\text{COD}}^{-1}$  and 1.6  $\text{g}_{\text{ethanol}}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ , respectively<sup>66</sup>. This titer must be compared with  
608 yeast-based fermentation of sugars which can typically attain titers as high as 150 g.L<sup>-1</sup><sup>67</sup>.  
609 The highest yield was observed from crude glycerol with a value of and concentration and  
610 productivity of 0.91  $\text{g}_{\text{COD}_{\text{ethanol}}}\cdot\text{g}_{\text{COD}}^{-1}$ , 8.0  $\text{g}_{\text{ethanol}}\cdot\text{L}^{-1}$  and 4.8  $\text{g}_{\text{ethanol}}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ , respectively<sup>68</sup>.  
611 However, the productivity when using a complex substrate remained low with maximum  
612 value around 9.5 g.L<sup>-1</sup>.d<sup>-1</sup> (Table 5)<sup>52</sup>.

613 The extraction and purification of ethanol from fermentation media is traditionally  
614 carried out by distillation followed by dehydration. However, a large amount of energy is  
615 required for these two purification steps, depending strongly on the ethanol concentration. In

616 particular, the energy required for the distillation step is higher than the heating value of  
617 ethanol ( $28.9 \text{ MJ.kg}^{-1}$ ) for ethanol concentrations lower than  $\sim 14 \text{ g.L}^{-1}$ , thus making its  
618 extraction clearly unsustainable in that case<sup>69</sup>. However, within an environmental biorefinery,  
619 a part of the energy required for distillation could be provided by recycling heat from the  
620 cogeneration biogas plant. Ethanol concentration remains the main parameter to be optimized  
621 prior to extraction, with an objective of, typically  $> 40 \text{ g.L}^{-1}$ <sup>69</sup>. Thus, considering the current  
622 state of the art, ethanol production by mixed-culture fermentation is far from being  
623 economically or even energetically competitive in most cases when compared with corn-  
624 based or sugar cane-based ethanol. However, Varrone et al. (2013)<sup>66</sup> estimated that bioethanol  
625 production costs using crude glycerol issued from a biodiesel production plant could be as  
626 low as  $0.27 \text{ €/kg}$  considering a mixed-culture fermentation reaching only  $26 \text{ g}_{\text{ethanol}}.\text{L}^{-1}$ ,  
627 making the process economically competitive. Thus, exploring mixed-culture ethanol  
628 production processes base on non-edible substrates that cannot be fermented by yeasts could  
629 reveal interesting niche with the potential of outcompeting current bioethanol production  
630 plants, especially regarding environmental and societal impacts<sup>70</sup>.

#### 631 4.4. Other value-added metabolites produced in mixed-culture fermentation

##### 632 4.4.1. 1,3-propanediol

633 1,3-propanediol (PDO) is a bio-based molecule entirely produced by biotechnological  
634 processes (currently by genetically modified organism cultures) from glucose or glycerol.  
635 PDO is mainly used as precursor of polytrimethylene terephthalate (PTT), a polymer used in  
636 the textile industry, or also directly used in the food, cosmetic and pharmaceutical sectors. In  
637 2015, its market volume was  $128 \text{ kt/yr}$  and its selling price was estimated at  $1.76 \text{ €/kg}$ ,  
638 equivalent to  $1.05 \text{ €/kg}_{\text{COD}}$ <sup>48</sup>. This price could be raised to more than  $3 \text{ €/kg}$ , equivalent to  
639  $1.79 \text{ €/kg}_{\text{COD}}$ , in the coming years<sup>71</sup>.

640 Only few studies are available on PDO production by mixed-culture fermentation but  
641 this is a growing research area, representing more than 50% of the articles published after  
642 2015. Among these studies, the substrate used for producing PDO is categorized as pure or  
643 crude glycerol. The yields are generally high, with an average of  $0.593 \pm 0.114$   
644  $\text{g}_{\text{COD}_{\text{PDO}}}\cdot\text{g}_{\text{COD}}^{-1}$  (Cluster 5, Figure 12), a value close to the maximum theoretical yield ( $0.82$   
645  $\text{g}_{\text{COD}_{\text{PDO}}}\cdot\text{g}_{\text{COD}}^{-1}$ )<sup>72</sup>. Interestingly, the best production performances were achieved in a fed-  
646 batch reactor fed with raw glycerol, with a final concentration of  $82.7 \text{ g.L}^{-1}$ , a yield of  $0.75$   
647  $\text{g}_{\text{COD}_{\text{PDO}}}\cdot\text{g}_{\text{COD}}^{-1}$  and a productivity of  $73.7 \text{ g.L}^{-1}\cdot\text{d}^{-1}$  (Table 5)<sup>73</sup>. These results are comparable



648 with the best performances achieved so far during glycerol fermentation by pure culture of  
649 unmodified strains<sup>74,75</sup>.

650 Many methods have been developed for 1,3-propanediol extraction and purification  
651 from fermentation media<sup>74</sup>. First, a three-step process is based on the high boiling point of  
652 1,3-propanediol (214 °C) and is composed of (1) a filtration step for biomass separation, (2)  
653 an evaporation step to remove compounds more volatile than PDO such as water and organic  
654 acids and, (3) a rectification step to produce PDO with a purity higher than 99%<sup>76</sup>. The overall  
655 extraction yield of the whole chain can be as high as 90%<sup>76</sup> but this method requires a large  
656 amount of energy<sup>74</sup>. An effective alternative is based on a succession of three successive  
657 steps, *i.e.* (1) biomass separation by microfiltration and activated carbon; (2) concentration by  
658 vacuum distillation and (3) final separation by silica gel chromatography. Following this  
659 procedure, a purity of 98% and an extraction yield of 75% were achieved<sup>77</sup>. An interesting  
660 improvement of this second downstream pipeline could be the implementation of a simulated  
661 moving bed as alternative chromatographic step, as described in a patent by Archer Daniels  
662 Midland Co (2001)<sup>78</sup>. Research on low-cost PDO extraction and purification is still active, but  
663 the mature existing technologies make possible the scaling up of a mixed-culture process for  
664 1,3-propanediol production.

#### 665 4.4.2. Propionic acid

666 Propionic acid can serve as food preservative in the fields of human and animal  
667 nutrition and is also a platform molecule that is used for example as flavour precursor<sup>79</sup>.  
668 However, similarly to butyrate, the use of waste-based propionic acid for food or feed  
669 applications would require approval of regulatory authorities such as FDA or EFSA in USA  
670 and UE, respectively. It is mainly produced by petrochemical routes<sup>79</sup> and represents a market  
671 of 400 kt/yr (2013)<sup>6,79</sup>. Its market price is ranging between 1.25 and 1.38 €/kg, which is  
672 equivalent to 0.83 - 0.91 €/kg<sub>COD</sub> (Table 4). However, a lower price would be necessary to  
673 meet the demand of the feed industry and expand the propionate market.

674 In DF processes, propionate production is generally avoided as this pathway is anti-  
675 correlated with H<sub>2</sub> production<sup>5</sup>. As a result, only very few studies have so far focused on the  
676 optimization of propionate production by microbial mixed culture and from complex  
677 substrates. The highest propionate final concentration found is 15.8 g.L<sup>-1</sup> using food waste as  
678 substrate<sup>80</sup>. The best yields and productivities are 0.31 g<sub>COD\_propionate</sub>.g<sub>COD</sub><sup>-1</sup> and 22.0 g.L<sup>-1</sup>.d<sup>-1</sup>,  
679 observed in two different studies (Table 5). Better yields up to 0.45 g<sub>COD\_propionate</sub>.g<sub>COD</sub><sup>-1</sup> were

680 attained when using defined fermentation media<sup>81</sup>, in particular when refined glycerol is used  
681 as substrate (Table 6). For more detailed information about propionate biological production,  
682 readers may refer to Es *et al.* (2017)<sup>79</sup>.

683 Similarly to acetate and butyrate, no low-cost process that could specifically extract  
684 propionate from mixtures of short-chain carboxylic acids has been developed. Nonetheless,  
685 AD can be used to convert into methane all soluble end-products generated by fermentation  
686 except propionate, and more particularly under high ammonium concentration ( $> 2.9 \text{ g.L}^{-1}$ )<sup>81,82</sup>.  
687 In that case, traditional carboxylic acid extraction techniques could be used to produce  
688 pure propionic acid, but the proof of concept remains to be demonstrated.

#### 689 4.4.3. Lactic acid

690 Lactic acid is an alpha-hydroxy acid widely used in the food industry (bacteriostatic,  
691 preservative, flavour enhancer), but also in the pharmaceutical sector and more recently in the  
692 polymer industry for polylactic acid manufacturing (PLA, bioplastic). The market value is  
693 high, ranging between 0.84 and 1.51 €/kg, equivalent to 0.79 – 1.41 €/kg<sub>COD</sub> (Table 4). The  
694 market volume is 472 kt/yr and is expected to grow in the coming years due to the increasing  
695 demand in PLA. The lactic acid is currently 100% bio-sourced<sup>48</sup> as high isomeric purity lactic  
696 acid can be produced by simple fermentation<sup>83</sup>. This aspect is particularly important for PLA  
697 production, which biodegradability depends on the L-isomer purity of the lactic acid<sup>83</sup>. First  
698 life cycle assessments have shown that

699 Similarly to propionate, lactic acid production is not desired in DF as it does not  
700 promote the H<sub>2</sub> production<sup>5</sup>. Nevertheless, very good performances have been achieved  
701 regarding lactate production by mixed-culture fermentation from both glucose and food waste  
702 (Table 5 and Table 6). In particular, lactate was produced with high selectivity even from  
703 complex substrates. Using food waste as the substrate, the highest lactate concentration  
704 achieved so far is 64.0 g.L<sup>-1</sup>, with a yield and productivity of 0.63 g<sub>COD\_lactate</sub>.g<sub>COD</sub><sup>-1</sup> and 12.8  
705 g.L<sup>-1</sup>.d<sup>-1</sup>, respectively (Table 5)<sup>84</sup>. Productivities as high as 40.0 g.L<sup>-1</sup>.d<sup>-1</sup> were reached using  
706 food waste as substrate<sup>85</sup>.

707 Similarly to carboxylic acids, lactate extraction is suitable when its selectivity is  
708 sufficiently high. Some studies demonstrated the feasibility of lactate extraction after food  
709 waste fermentation, using a process combining centrifugation, nanofiltration and  
710 electro dialysis steps with an overall recovery rate of 73%<sup>85</sup>. Lactate can also be extracted *in-*

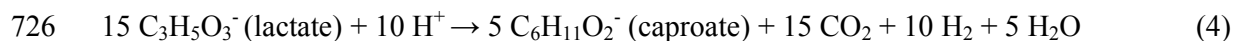
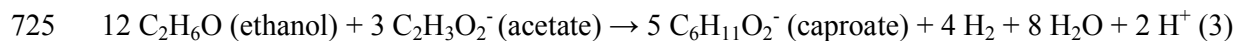


711 *situ* from fermentation medium by adsorption on activated carbon that can be further desorbed  
712 with acetone<sup>86</sup>. However, research on the improvement of such extraction processes, as well  
713 as on the techno-economic aspects of lactate production are still necessary before considering  
714 a scale-up of the process.

#### 715 4.4.4. Caproic acid

716 Caproic acid is a medium chain fatty acid (6 carbon atoms) used as antimicrobial  
717 agent, animal feed additive, food flavouring and potential biofuel precursor (e.g. decane)<sup>87,88</sup>.  
718 The industrial production of this molecule remains low, with a production capacity of only 25  
719 kt/yr. Its current selling price ranges between 1.88 and 2.09 €/kg, equivalent to 0.85-0.95  
720 €/kg<sub>COD</sub> (Table 4) that is far too high for a commodity product. This price would likely  
721 decrease if the caproic acid production is intensified.

722 Caproate can be produced from a mixture of acetate, butyrate, ethanol and lactate by  
723 chain elongation according to the following global reactions (butyrate being a reaction  
724 intermediate)<sup>87</sup>:



727 Caproate production can therefore be a way to recover value from a mixture of these  
728 metabolites. Caproate production by mixed cultures is a recent topic, with more than 90% of  
729 the identified articles having been published after 2013. In most studies, chain elongation  
730 reactions are favoured by ethanol addition (80% of the studies dealing with caproate).  
731 However, such external ethanol addition has a high environmental impact, and increase the  
732 costs of the process, and therefore should be minimized<sup>89</sup>. Using complex substrates, the best  
733 performances achieved so far are a maximum concentration of 11.9 g.L<sup>-1</sup>, a yield of 0.81  
734 g<sub>COD\_caproate</sub>.g<sub>COD</sub><sup>-1</sup> and a productivity of 26 g.L<sup>-1</sup>.d<sup>-1</sup> (data issued from different studies, Table  
735 5)<sup>90,91</sup>. For more detailed information about caproate production, readers may refer to  
736 Cavalcante *et al.* (2017)<sup>87</sup>.

737 Caproate extraction is greatly facilitated by the low water solubility of its acid form (~  
738 11 g.L<sup>-1</sup>). When caproate is concentrated in an acidic compartment by electrodialysis, caproic  
739 acid accumulation forms an organic phase on the top of the aqueous phase that can be  
740 physically removed<sup>88</sup>. Direct liquid-liquid extraction from the fermentation medium is also

741 possible<sup>91–93</sup>. For instance, using trioctylphosphine oxide as solvent, caproate recovery yields  
742 of 97.3% were achieved<sup>93</sup>. Moreover, long-term caproate production (> 1 year) was  
743 demonstrated<sup>93</sup> and at least one pilot-scale reactor was already implemented, incorporating an  
744 extraction technology<sup>94</sup>. In summary, caproate production by microbial mixed culture could  
745 reach a pre-industrial stage in the coming years, the main constraint being the reduction of the  
746 external supply of ethanol.

#### 747 4.4.5. Polyhydroxyalkanoates (PHAs)

748 Polyhydroxyalkanoates (PHAs) are a family of biodegradable polyesters that can be  
749 produced by fermentation. Indeed, in presence of an excess of carbon source content, some  
750 microorganisms are able to accumulate PHAs within their cell as a way to store carbon and  
751 energy<sup>95</sup>. Depending on the substrate available for the PHA-accumulating microorganisms,  
752 polymers have different physicochemical characteristics that can be exploited through  
753 numerous applications in the fields of packaging and health, *e.g.* surgery<sup>95</sup>. The most common  
754 PHAs are poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate)  
755 (PHBV) although a wide variety of PHAs can be produced. In 2015, the market volume of  
756 PHAs was only 17 kt/yr and its production was 100% bio-based<sup>48</sup>. Its selling price is currently  
757 varying between 2.20 and 5.00 €/kg, equivalent to 1.38 - 3.14 €/kg<sub>COD</sub> (Table 4) but is not yet  
758 economically competitive when compared to equivalent petro-based plastics (~1 €/kg)<sup>96</sup>. As  
759 one of the main contributor to the overall process operating costs is the carbon source<sup>97</sup>, using  
760 waste as substrate appears to be a promising way to reach economic viability<sup>98</sup>. Moreover, it  
761 would also improve environmental performances of PHA production, for instance when the  
762 process is integrated in a wastewater treatment plant<sup>99</sup>.

763 PHAs can be produced from a mixture of carboxylic acids (*i.e.* acetate, propionate,  
764 butyrate, valerate, caproate). Similarly to caproate, PHAs production can be used as a way to  
765 valorise DF effluents. When microbial mixed cultures are used, PHA production is generally  
766 carried out in three steps: (i) an acidogenesis step (*e.g.* DF) to produce carboxylic acids; (ii) a  
767 PHA-producing bacteria selection phase using feast/famine cycles and (iii) a PHA  
768 accumulation phase. The selection phase (ii) is generally kinetically limiting the whole  
769 process and explains the low productivities observed, typically 1 g<sub>PHA</sub>.L<sup>-1</sup>.d<sup>-1</sup> (Table 7)<sup>100</sup>. The  
770 best conversion efficiency was 0.41 g<sub>COD\_PHA</sub>.g<sub>COD</sub><sup>-1</sup> using paper industry effluents<sup>101</sup>. For  
771 more detailed information about PHAs production from waste, readers may refer to Valentino  
772 *et al.* (2017)<sup>96</sup>.

773 To date, several PHA extraction methods have been developed. After a biomass  
774 recovery phase (*e.g.* centrifugation), two strategies are generally employed: the most  
775 commonly used method consists in solubilizing PHAs in a solvent (*e.g.* chlorinated solvent)  
776 followed by a precipitation step, for example using ethanol<sup>96</sup>. High purities are achieved with  
777 this method but require high operating costs related to solvent recycling. The other method  
778 aims to disrupt or digest the cellular biomass using chemical or enzymatic treatment to release  
779 the PHAs as particles<sup>96</sup>. This second technique is still under development to obtain better  
780 extraction yields and a better stability of the PHA for thermoplastic applications. Pilot-scale  
781 reactors for PHAs production using mixed cultures and incorporating extraction/purification  
782 processes have recently been implemented<sup>100,102</sup>. The current technologies are however  
783 limited by their high production costs as well as their low productivities that does not yet fit  
784 with the demand of bioplastic purchasers.

785 < Table 7 >

786

## 787 5. Conclusions

788 Residual materials represent a significant source of organic matter<sup>103</sup> that can directly  
789 contribute to a circular and environmentally friendly economy. The development of AD is a  
790 first step towards residual materials conversion and recycling, particularly in a context of  
791 energy transition. However, methane has a limited added-value and requires feed-in tariffs to  
792 ensure the economic viability of the biogas plants. Recovery of other biomolecules upstream  
793 biogas plants could therefore be a way to improve the economic competitiveness of this sector  
794 while maintaining environmental and societal services, *i.e.* waste treatment and recycling.  
795 Thus, biogas plants have the potential to become environmental biorefinery in which biogas  
796 production would only be a final stage dedicated to the most recalcitrant organic fractions.

797 Biohydrogen is by far the most studied molecule that can be easily implemented in  
798 “second generation” biogas plants when considering DF processes. Moreover, H<sub>2</sub> should have  
799 an important place in the future economic landscape as chemical reagent and energy carrier,  
800 particularly as substitute for petro-based fuels in the transportation sector. Thus, H<sub>2</sub>  
801 production could be a first way to improve the economic viability of the biogas sector as there  
802 is both an emerging market demand and a high price for this molecule. By implementing H<sub>2</sub>  
803 production in AD plants, the territorial grid offered by the biogas plants would significantly

804 reduce the costs related to H<sub>2</sub> transportation. Research on biological H<sub>2</sub> production under non-  
805 sterile conditions has been active for more than 15 years, and mainly focused on the study of  
806 DF by mixed microbial communities. This process has been widely tested at laboratory scale  
807 for a wide range of operating conditions, such as temperature (from 15 to 80 °C), working  
808 volume (from 0.01 to 3300 L), operation modes (discontinuous, semi-continuous and  
809 continuous) and for a variety of complex substrates that are representative of most of the  
810 resources available for AD (Figure 4). However, an average of only 8.9% of the organic load  
811 is converted into H<sub>2</sub> when complex substrates are used in DF (9.6% for industrial effluents,  
812 9.4% for green and agricultural waste, 6.4 % for food waste and 5.6% for municipal waste).  
813 Therefore, there is still room for improvement to reach the theoretical maximum yield of  
814 33%, especially regarding easily biodegradable sugar rich substrates such as food and  
815 municipal waste. Because of these low yields, most economic scenarios comparing the  
816 different bioH<sub>2</sub> production technologies conclude that DF is not economically competitive yet  
817 with technologies such as biomass gasification or biogas reforming<sup>10</sup>. However, these  
818 scenarios do not take into account the environmental benefits offered by DF when compared  
819 to more traditional H<sub>2</sub>-producing processes, as shown by recent LCA<sup>10</sup>, as well as products  
820 other than H<sub>2</sub> that can be coproduced and the possible couplings within a biorefinery that  
821 could improve economic performances of DF.

822 Nonetheless, DF acts also as pretreatment of the complex organic matter and the  
823 effluents are more easily degradable than the initial materials. Thus, DF effluents can be  
824 directly injected into biogas plants to recover soluble COD content as methane. Such  
825 coupling, also known as two-stage AD, is a mature technology supported by numerous studies  
826 and patents that could be readily implemented in a short term (TRL7, see supplementary  
827 information for more information about TRL scale). In addition to this coupling, new  
828 complementary routes with better added-value could be implemented in a near future:

- 829 • Producing more bioH<sub>2</sub> (TRL5-6): it is possible to inject DF effluents in a  
830 microbial electrolysis cell or in a photofermentation reactor. However, the  
831 development of both processes is currently limited by high capital expenditures  
832 and the low organic load rates they can withstand.
- 833 • Extracting biomolecules from DF effluents or bioelectrochemical processes  
834 (TRL2-3): acetate, butyrate and ethanol can be stably co-produced with bioH<sub>2</sub>  
835 during mixed-culture DF or produced purely by bioelectrochemical processes.  
836 Specific and low-cost extraction methods still need to be developed for DF

837 effluents whereas bioelectrochemical systems are still limited by low  
838 productivities.

- 839 • Producing biomolecules from DF effluents (TRL6): additional fermentation  
840 processes can be carried out to produce more easily extractable molecules such  
841 as caproate or PHAs from DF effluents. Caproate production is undergoing a  
842 scale-up phase while PHA production processes still suffers from low  
843 productivities.
- 844 • Redirecting DF toward the production of metabolites without H<sub>2</sub> production  
845 (TRL3-4): because of their anti-correlation with H<sub>2</sub> production, propionate and  
846 lactate production by mixed cultures has received little attention. Nevertheless,  
847 these metabolites have a high market value and early studies show that they  
848 could be produced with good performance upstream of biogas plants. It is also  
849 possible to produce 1,3-propanediol in the case where glycerol is used as  
850 substrate.

851 All these complementary or alternative processes are at unequal levels of  
852 technological maturity ranging from laboratory-scale pre-studies (lactate, propionate and 1,3-  
853 propanediol production by mixed cultures and specific extraction of DF metabolites) to pilot-  
854 scale processes (microbial electrolysis, photofermentation, PHAs and caproate production by  
855 mixed cultures). Modelling approaches such as life cycle assessment and techno-economic  
856 studies would be helpful to further scaling up steps by providing boundaries for economic and  
857 environmental viability. In all cases, the higher added-value of fermentative products when  
858 compared to methane leaves room for the potential addition of fermenters and extraction  
859 systems to biogas plants when these technologies are more mature. Whatever the recovery  
860 scenarios considered, AD will act as a final way to recover the last part of non-valorised  
861 organic matter.

862 To conclude, biogas plants transformation into environmental biorefineries responds  
863 not only to a concern for better economic viability, but could also represent an interesting  
864 source of bio-based platform molecules for the future bioeconomy. By keeping easily  
865 fermentable substrates for biomolecules production (*e.g.* agro-food industries effluents, food  
866 waste) and recalcitrant waste for AD (*e.g.* manure, sludge), significant volumes of  
867 biomolecules could be produced. For instance, if 1% of the French methane production  
868 expected in 2030<sup>103</sup> is diverted to produce lactate, a total production of 130 kt/yr could be  
869 achieved, which represents nearly a quarter of current world consumption. Thus, the

870 environmental biorefinery has the potential to become a major supplier of biobased molecules  
871 with the lowest environmental impact and contribute as a sustainable way to the future  
872 bioeconomy.

873

874 **Abbreviations**

875 **AD:** Anaerobic digestion

876 **DF:** Dark fermentation

877 **COD:** Chemical oxygen demand

878 **LCA:** Life cycle assessment

879 **PDO:** 1,3-propanediol

880 **PHA:** Polyhydroxyalkanoate

881 **PLA:** Polylactic acid

882 **PLS-DA:** Partial least square discriminant analysis

883 **TRL:** Technology readiness level

884

885 **Competing interests**

886 The authors declare that they have no competing interests.

887

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893



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1099

1100 **Legends**

1101 **Figure 1.** The different phases of anaerobic digestion.

1102 **Figure 2.** Publication of research articles and patents related to biomolecule production by  
1103 mixed-culture fermentation between 1997 and January 2017. Information regarding patents  
1104 after mid 2015 is incomplete due to the 18 month delay between patent filing and publication.

1105 **Figure 3.** Technologies used for biological hydrogen production. The documents represented  
1106 correspond to scientific articles displaying a hydrogen yield higher than  $0.01 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$  (400  
1107 documents) and to the patents explicitly claiming hydrogen production (100 documents).

1108 **Figure 4.** Substrates used for the biological production of hydrogen (A) and their total COD  
1109 concentration (B). The total COD concentration corresponds to the initial concentration of the  
1110 substrate for batch processes and to the concentration of the feed for continuous/semi-  
1111 continuous processes. The documents represented correspond to scientific articles displaying  
1112 a hydrogen yield higher than  $0.01 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$  (400 documents). N corresponds to the number  
1113 of scientific articles taken into account for each category. Only the categories with  $N \geq 10$  are  
1114 represented. Red dots represent the average of the distributions.

1115 **Figure 5.** Substrate pretreatment carried out before biohydrogen production. N corresponds to  
1116 the number of scientific articles taken into account for each category. Mechanical  
1117 pretreatments: grinding, sonication; physico-chemical pretreatments: acid/alkaline hydrolysis,  
1118 heat treatment, steam explosion; enzymatic pretreatments: by microorganisms or enzyme  
1119 cocktails.

1120 **Figure 6.** Part of the studies focused on dark fermentation using inoculum pretreatment (A)  
1121 and the different pretreatment methods employed (B). The documents represented correspond  
1122 to scientific articles for which the information concerning inoculum pretreatment is available,  
1123 displaying a hydrogen yield higher than  $0.01 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$  and focused on dark fermentation,  
1124 or on a coupling between dark fermentation and another technology (346 documents).

1125 **Figure 7.** Hydrogen yield as a function of the total COD concentration of the substrates. The  
1126 total COD concentration corresponds to the initial concentration of the substrate for batch  
1127 processes and to the concentration of the feed for continuous/semi-continuous processes. The  
1128 documents represented correspond to scientific articles displaying a hydrogen yield higher  
1129 than  $0.01 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$  (400 documents).

1130 **Figure 8.** Hydrogen yield as a function of the substrates employed (A) and process  
1131 parameters (B). The documents represented correspond to scientific articles displaying a  
1132 hydrogen yield higher than  $0.01 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$  and focused on dark fermentation (303  
1133 documents). N corresponds to the number of scientific articles taken into account for each  
1134 category. Only the categories with  $N \geq 10$  are represented. Red dots represent the average of  
1135 the distributions.

1136 **Figure 9.** Frequency of use (A) and performance (B-C) of the different operation modes used  
1137 for hydrogen production by dark fermentation. The documents represented correspond to  
1138 scientific articles displaying a hydrogen yield higher than  $0.01 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$  and focused on  
1139 dark fermentation (303 documents). N corresponds to the number of scientific articles taken  
1140 into account for each category. Hydrogen production and productivities are normalized by the  
1141 working volumes.

1142 **Figure 10.** Hydrogen yields achieved by photofermentation and microbial electrolysis. The  
1143 documents represented correspond to scientific articles displaying a hydrogen yield higher  
1144 than  $0.01 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$ . N corresponds to the number of scientific articles taken into account  
1145 for each category.

1146 **Figure 11.** Occurrence probability of common metabolites within the database. Scientific  
1147 articles in which only hydrogen was measured, as well as those dealing with the coupling  
1148 between fermentation and anaerobic digestion were excluded. Metabolites were considered  
1149 present when their respective yields were higher than  $0.01 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$ . The result is  
1150 standardized on 353 documents.

1151 **Figure 12.** Standard fermentation profile identification by hierarchical clustering. Average  
1152 metabolic profiles of the clusters (A) and their representation by discriminant analysis (PLS-  
1153 DA)(B). Only scientific articles reporting more than 60% of the substrate total COD  
1154 recovered as products are taken into account (222 documents). N corresponds to the number  
1155 of scientific articles of each cluster identified by hierarchical clustering. Only the clusters with  
1156  $N \geq 5$  are represented. Error bars corresponds to standard deviations. Ellipses represent 95%  
1157 confidence intervals.

1158 **Figure 13.** Final concentrations of acetate, butyrate and ethanol produced by mixed-culture  
1159 fermentation processes. N corresponds to the number of scientific articles taken into account  
1160 for each category.



1161 **Table 1.** Keywords used for the database building.

Category	Keywords
Process	Fermentative process, Photofermentation, Photoautotrophy, Phototrophy, Fermentation / Light, Photosynthesis, MEC, Microbial electro*, Bioelectrochemistry, Bioelectrolysis, Electrofermentation, Electromicrob*, Dark Fermentation, Obscure fermentation, Anaerobic digestion, Anaerobic condition, Anaerobic process, Acidogenesis, Acetogenesis, Methanogenesis, Solventogenesis, Chain elongation
Combined processes	Association of two processes (see previous line)
Hydrogen	Hydrogen, Dihydrogen, H <sub>2</sub>
Biomolecules	List of 130 biomolecules according to Straathof (2014) <sup>104</sup>
Mixed culture	Consortium, Consortia, Co-culture, Microbiome, Microbiota, Microflora, open-culture, Symbiosis, Mixed culture, Community, Population, Dominant

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1164 **Table 2.** Molecular formulas and COD equivalents of commonly encountered biomolecules.

Biomolecule	Molecular formula	COD equivalent (g <sub>COD</sub> /g)
1,3-propanediol	C <sub>3</sub> H <sub>8</sub> O <sub>2</sub>	1.68
2,3-butanediol	C <sub>4</sub> H <sub>10</sub> O <sub>2</sub>	1.96
Acetate	C <sub>2</sub> H <sub>4</sub> O <sub>2</sub>	1.07
Butanol	C <sub>4</sub> H <sub>10</sub> O	2.59
Butyrate	C <sub>4</sub> H <sub>8</sub> O <sub>2</sub>	1.82
Caproate	C <sub>6</sub> H <sub>12</sub> O <sub>2</sub>	2.21
Cellulose	C <sub>6</sub> H <sub>10</sub> O <sub>5</sub>	1.19
Ethanol	C <sub>2</sub> H <sub>6</sub> O	2.09
Glucose/Fructose	C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	1.07
Glutamate	C <sub>5</sub> H <sub>9</sub> O <sub>4</sub> N	0.98
Glycerol	C <sub>3</sub> H <sub>8</sub> O <sub>3</sub>	1.22
Hydrogen	H <sub>2</sub>	8.00
Lactate	C <sub>3</sub> H <sub>6</sub> O <sub>3</sub>	1.07
Methane	CH <sub>4</sub>	4.00
Methanol	CH <sub>4</sub> O	1.50
PHA	(C <sub>4</sub> H <sub>6</sub> O <sub>2</sub> ) <sub>n</sub>	1.67
Propanol	C <sub>3</sub> H <sub>8</sub> O	2.40
Propionate	C <sub>3</sub> H <sub>6</sub> O <sub>2</sub>	1.51
Succinate	C <sub>4</sub> H <sub>6</sub> O <sub>4</sub>	0.95
Sucrose/Lactose	C <sub>12</sub> H <sub>22</sub> O <sub>11</sub>	1.12
Valerate	C <sub>5</sub> H <sub>10</sub> O <sub>2</sub>	2.04
Xylose/Arabinose	C <sub>5</sub> H <sub>10</sub> O <sub>5</sub>	1.07

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1167 **Table 3.** Conversion performances and features of the bioH<sub>2</sub> production technologies.

Technology	TRL	Substrate	Theoretical maximum yield (gCOD·gCOD <sup>-1</sup> )	Experimental average yield (gCOD·gCOD <sup>-1</sup> )	Energy conversion efficiency (%)
Dark fermentation	7	Sugars Waste/wastewater	0.333	0.124 (sugars) 0.089 (waste/wastewater)	-
Photofermentation	6	Volatile fatty acids Fermentation effluent	1.000	0.255	<10
Microbial electrolysis	6	Volatile fatty acids Fermentation effluent	1.000	0.479	199

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1170 **Table 4.** Market price and volume of biomolecules of interest.

Molecule	Market price (€/kg)*	Market price (€/kg <sub>COD</sub> )*	Global market volume (kt/yr)	References
PHA	2.20 – 5.00	1,38 – 3.14	17	<sup>96</sup> , this study
1,3-propanediol	1.76 – 3.00	1.05 – 1.79	128	<sup>48,71</sup>
Butyric acid	1.67 – 2.09	0.92 – 1.15	30**	This study
Caproic acid	1.88 – 2.09	0.85 – 0.95	25**	This study
Propionic acid	1.25 – 1.38	0.83 – 0.91	400	<sup>6,79</sup> , this study
Lactic acid	0.84 – 1.51	0.79 – 1.41	472	<sup>48</sup> , this study
Acetic acid	0.33 – 0.67	0.31 – 0.63	13,570	<sup>48</sup> , this study
H <sub>2</sub>	1.50 – 5.00	0.19 – 0.63	60,000	This study
Ethanol	0.30 – 1.50	0.14 – 0.72	76,700	<sup>48,64</sup> , this study
CH <sub>4</sub>	0.09 – 0.20***	0.02 – 0.05***	-	This study

1171 \* Excluding transport costs

1172 \*\* Production capacity

1173 \*\*\* Feed-in tariff for methane injection into the natural gas network in France

1174

1175 **Table 5.** Best performance of soluble metabolite production by mixed cultures from complex  
1176 substrates.

Metabolite	Substrate	Concentration (g.L <sup>-1</sup> )	Yield* (g <sub>cod</sub> ·g <sub>cod</sub> <sup>-1</sup> )	Productivity* (g.L <sup>-1</sup> ·d <sup>-1</sup> )	Process configuration	Process or start-up duration (d)**	Ref.
<b>Highest concentrations</b>							
Acetate	Sugar cane bagasse	<b>35.3</b>	0.24	1.1	Semi-continuous	90	<sup>49</sup>
Butyrate	Food waste	<b>21.4</b>	0.26	2.7	Batch	8	<sup>80</sup>
Caproate	Fermented municipal waste + ethanol	<b>11.9</b>	0.46	26.0	Continuous	140	<sup>90</sup>
Ethanol	Raw glycerol	<b>26.0</b>	0.59	1.6	Fed-batch	15.8	<sup>66</sup>
Lactate	Food waste	<b>64.0</b>	0.63	12.8	Batch	5	<sup>84</sup>
Propionate	Food waste	<b>15.8</b>	0.16	2.0	Batch	8	<sup>80</sup>
1,3-propanediol	Raw glycerol	<b>82.7</b>	0.75	73.4	Fed-batch	1.1	<sup>73</sup>
<b>Highest yields</b>							
Acetate	Food waste	7.9	<b>0.56</b>	1.8	Batch	4.3	<sup>50</sup>
Butyrate	Municipal waste	1.6	<b>0.74</b>	0.8	Batch	2.1	<sup>61</sup>
Caproate	Liquid phase of alcoholic fermentation	NA***	<b>0.81</b>	2.1	Continuous	350	<sup>91</sup>
Ethanol	Raw glycerol	8.0	<b>0.91</b>	4.8	Batch	1.7	<sup>68</sup>
Lactate	Food waste	64.0	<b>0.63</b>	12.8	Batch	5	<sup>84</sup>
Propionate	Sago starch wastewater	2.2	<b>0.31</b>	1.1	Semi-continuous	NA	<sup>105</sup>
1,3-propanediol	Refined glycerol	82.7	<b>0.75</b>	73.4	Fed-batch	1.1	<sup>73</sup>
<b>Highest productivities</b>							
Acetate	Beverage industry wastewater	3.6	0.18	<b>57.0</b>	Continuous	155	<sup>52</sup>
Butyrate	Beverage industry wastewater	4.6	0.39	<b>73.2</b>	Continuous	155	<sup>52</sup>
Caproate	Fermented municipal waste + ethanol	11.9	0.46	<b>26.0</b>	Continuous	140	<sup>90</sup>
Ethanol	Beverage industry wastewater	0.6	0.06	<b>9.5</b>	Continuous	155	<sup>52</sup>
Lactate	Food waste	40.0	0.41	<b>40.0</b>	Continuous	152	<sup>85</sup>
Propionate	Cheese whey	6.9	0.26	<b>27.6</b>	Continuous	40	<sup>106</sup>
1,3-propanediol	Raw glycerol	82.7	0.75	<b>73.4</b>	Fed-batch	1.1	<sup>73</sup>

1177 \* Yields are normalized on the total COD content of the substrate. Productivities correspond  
1178 to average productivities (batch, fed-batch and semi-continuous reactors) or productivities  
1179 during steady states (continuous reactors).

1180 \*\*Process duration stands for total batch/fed-batch duration if applicable. Start-up duration  
1181 corresponds to the time required to reach the best performing steady state in continuous/semi-  
1182 continuous processes. Both durations are not necessarily optimal due to experimental design.

1183 \*\*\* Continuous extraction

1184 **Table 6.** Best performance of soluble metabolite production by mixed cultures from synthetic  
1185 fermentation media.

Metabolite	Substrate	Concentration (g.L <sup>-1</sup> )	Yield* (g <sub>COB</sub> .g <sub>COB</sub> <sup>-1</sup> )	Productivity* (g.L <sup>-1</sup> .d <sup>-1</sup> )	Process configuration	Process or start-up duration (d)**	Ref.
<b>Highest concentrations</b>							
Acetate	Glucose	<b>34.4</b>	0.31	3.1	Fed-batch	11	53
Butyrate	Refined glycerol	<b>13.5</b>	0.19	0.5	Fed-batch	27	107
Caproate	Lactate	<b>23.4</b>	0.72	1.5	Fed-batch	16	108
Ethanol	Refined glycerol	<b>11.1</b>	0.42	3.7	Batch	3	109
Lactate	Glucose	<b>21.5</b>	0.92	43.0	Continuous	38	110
Propionate	Refined glycerol	<b>22.6</b>	0.45	0.8	Fed-batch	28	81
1,3-propanediol	Refined glycerol	<b>81.4</b>	0.56	23.8	Fed-batch	3.4	111
<b>Highest yields</b>							
Acetate	CO <sub>2</sub> + electricity	4.7	<b>0.90</b>	0.9	Fed-batch	5	51
Butyrate	Acetate + ethanol	11.5	<b>0.80</b>	20.0	Continuous	NA	62
Caproate	Lactate	23.4	<b>0.72</b>	1.5	Fed-batch	16	108
Ethanol	Xylose	3.1	<b>0.61</b>	1.0	Batch	3	112
Lactate	Glucose	21.5	<b>0.92</b>	43.0	Continuous	38	110
Propionate	Refined glycerol	22.6	<b>0.45</b>	0.8	Fed-batch	28	81
1,3-propanediol	Refined glycerol	1.7	<b>0.77</b>	2.0	Batch	0.8	113
<b>Highest productivities</b>							
Acetate	Sucrose	2.7	0.14	<b>127.9</b>	Continuous	27	114
Butyrate	Sucrose	4.9	0.30	<b>237.1</b>	Continuous	NA	29
Caproate	Acetate + ethanol	8.7	0.51	<b>52.2</b>	Continuous	75	115
Ethanol	Sucrose	0.5	0.05	<b>22.5</b>	Continuous	27	114
Lactate	Glucose	19.2	0.90	<b>115.0</b>	Continuous	35	110
Propionate	Starch	1.0	0.07	<b>46.1</b>	Continuous	NA	116
1,3-propanediol	Refined glycerol	81.4	0.56	<b>23.8</b>	Fed-batch	3.4	111

1186 \* Yields are normalized on the total COD content of the substrate. Productivities correspond  
1187 to average productivities (batch, fed-batch and semi-continuous reactors) or productivities  
1188 during steady states (continuous reactors).

1189 \*\*Process duration stands for total batch/fed-batch duration if applicable. Start-up duration  
1190 corresponds to the time required to reach the best performing steady state in continuous/semi-  
1191 continuous processes. Both durations are not necessarily optimal due to experimental design.

1192

1193 **Table 7.** Best performance of PHA production by mixed cultures.

Maximized variable	Substrate	Accumulation step		Global process		Ref.
		Yield (g <sub>COD</sub> ·g <sub>COD</sub> <sup>-1</sup> )	Productivity (g·L <sup>-1</sup> ·d <sup>-1</sup> )	Yield (g <sub>COD</sub> ·g <sub>COD</sub> <sup>-1</sup> )	Productivity (g·L <sup>-1</sup> ·d <sup>-1</sup> )	
<b>Synthetic fermentation media</b>						
Yields & productivities	Acetate + Propionate + Butyrate	<b>0.84</b>	<b>29.3</b>	<b>0.49*</b>	<b>1.2</b>	117
<b>Complex substrates</b>						
Yields	Paper mill wastewater	<b>0.75</b>	NA	<b>0.41**</b>	NA	101
Productivity (accumulation)	Sugar-cane molasses	0.63	<b>259.2</b>	NA	NA	118
Productivity (global)	Snack industry wastewater	NA	12.0	0.30**	<b>1.0</b>	100

1194 \* Yield calculated for the selection and accumulation steps

1195 \*\* Yields calculated for the fermentation, selection and accumulation steps