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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: H₂, 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^{1,2}. 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 activities³. To date, the main product considered in AD processes is biogas, consisting of a
37 mixture of methane and carbon dioxide. However, hydrogen gas (H₂) 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 future⁴. Several process control strategies and microbial selection procedures have been
41 explored over the past 15 years to promote H₂ production during the acidogenic phase of AD
42 in a process called dark fermentation (DF)⁵. During DF, H₂ 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 methane⁶. 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 H₂ 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 H₂). 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:

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

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:

$$104 \quad \text{COD}_{\text{substrate}} (\text{g}_{\text{COD}}/\text{g}_{\text{substrate}}) = 1.19x(\text{g}_{\text{carbohydrate}}/\text{g}_{\text{substrate}}) + 1.42x(\text{g}_{\text{protein}}/\text{g}_{\text{substrate}}) + 2.90x(\text{g}_{\text{lipid}}/\text{g}_{\text{substrate}}) \quad (2)$$

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 pvclust⁷, 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 mixOmics⁸.

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 H₂ yield higher than
133 0.01 g_{COD}·g_{COD}⁻¹ (based on total initial COD) were considered (400 articles). Patents
134 considered in this section were those explicitly claiming H₂ production (100 patents).

135 **3.1. The H₂ market**

136 Dihydrogen (H₂) 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 (~ 700·10⁹
139 Nm³) and is predicted to reach a near-exponential growth in the coming years. Indeed, the use
140 of H₂ as decarbonated energy carrier, both for energy storage or in the transportation sector,
141 could represent up to 30% of H₂ 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 CO₂ per kilogram of H₂ produced. Current H₂ 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 CO₂ 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 H₂ 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 H₂ production process
154 could be assessed between 1.5 and 3.5 €/kg_{H₂} 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^{9,10}, 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 bioH₂ production**

160 Three technologies have been developed for producing H₂ 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)⁵. 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 DF⁵. 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 hythane¹¹. 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 H₂ from organic substrates in presence of light¹². This additional energy input, which
179 can be artificial or natural (sun), makes thermodynamically favorable H₂-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 H₂ 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)¹³. External supply of electrical energy can here be used to make
191 thermodynamically favorable chemical reactions¹⁴. 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 CO₂. 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 H₂ production

206 One of the main benefits of using mixed-culture fermentation is its flexibility on
207 converting a wide range of substrates¹⁵. 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 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 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^{16,17}.

220 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 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^{18–21}. Municipal
244 waste may also contain recalcitrant biomass such as cardboard²² or bacterial cell walls in
245 sewage sludge²³. 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_{cornstarch}) was at
259 least doubled to ~ 180 €/t_{cornstarch} (equivalent to 330 €/t_{solubleCOD}) when the pretreatment cost
260 was taken into account²⁴. 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)²³.

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 H₂, 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^{21,25}. 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 microorganisms²¹, 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 activity²⁶. 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 time⁵. 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)²¹.

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 $22.2 \text{ g}_{\text{COD}}\cdot\text{L}^{-1}$ for 75% of the studies. Regarding hydrogen yields, values below
300 $0.16 \text{ g}_{\text{COD}_H_2}\cdot\text{g}_{\text{COD}}^{-1}$ 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 ($8.4 \text{ g}_{\text{COD}}\cdot\text{L}^{-1}$
302 ¹ on average) and mostly by photofermentation, microbial electrolysis technologies or by
303 coupling them with DF. When initial substrate concentrations were higher than $22.2 \text{ g}_{\text{COD}}\cdot\text{L}^{-1}$,

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 DF⁵. 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 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 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°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 maximum²⁷ 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 maximum²⁸ 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 maximum²⁹ 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. H₂ 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 H₂ and CO₂ 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 H₂ on the energy provided as light, does not exceed 10% under well-
363 controlled conditions¹². That makes this technology non-profitable if artificial light is used
364 and if H₂ 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 H_2 production was developed with a pure culture of *Rhodobacter capsulatus*³⁰, 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)³¹.

380 < Figure 10 >

381 In microbial electrolysis cells, electric energy is provided through an applied voltage
382 between two electrodes. When producing 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 processes¹⁴. The energy conversion efficiency of microbial electrolysis is defined as the ratio
385 of the energy recovered as 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 H_2 and
388 when acetate is used as substrate³². 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^{33,34}. In addition to the high conversion efficiencies, another
394 advantage of microbial electrolysis cells is the possibility to produce nearly pure 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_{COD}.L⁻¹ 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_{COD}.L⁻¹.d⁻¹) have been recently implemented and exhibit
404 very promising results³⁵⁻³⁸. 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)³⁹.

408 < Table 3 >

409 3.5. Downstream processes for H₂ production

410 Biohydrogen production during DF and photofermentation is always concomitant with
411 CO₂ production. If the biogas is not diluted, the proportions encountered range generally from
412 30 to 60% for H₂ and 40 to 70% for CO₂, with possible traces of CH₄ and /or H₂S. Mature
413 technologies for hydrogen separation that are currently used in petrochemical processes are
414 easily applicable to bioH₂ production processes^{40,41}. For instance, the Pressure Swing
415 Adsorption (PSA) process can produce H₂ at a purity of 99.999% with a H₂ recovery ranging
416 from 75 to 92% while a purity of 90-99% and a H₂ recovery of 85-95% can be achieved with
417 membrane permeation technologies⁴². Therefore, the biohydrogen separation step is not a
418 technological obstacle, but remains one of the most costly step of the overall process⁴³⁻⁴⁵.

419

420 4. Toward the waste-based biorefinery

421 Optimization of H₂ 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_{COD_H2}.g_{COD}⁻¹) 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 H₂ and CH₄ 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 H₂ and CH₄ (Section
432 3.2). This two-stage process presents several advantages¹¹:

- 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 H₂ in comparison with CH₄, *i.e.* heating value of 17.7 MJ/kg_{COD-H₂}
436 versus 12.5 MJ/kg_{COD-CH₄}, 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 H₂ and CH₄, at an average initial total COD concentration of
446 47.8 ± 38.2 g_{COD}.L⁻¹ (based on 34 articles). Average hydrogen and methane yields were 0.055
447 ± 0.032 and 0.670 ± 0.187 g_{COD}.g_{COD}⁻¹, 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 H₂ 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_{CODfed}) the economy of a one-stage
456 AD (14.5 - 36.5 € / t_{CODfed}) 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^{46,47}. For more detailed information about two-stage AD, readers may refer to Xia *et al.*
461 (2016)¹¹.

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 H₂ 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 ± 0.100
486 $\text{g}_{\text{COD}}\cdot\text{g}_{\text{COD}}^{-1}$) and H₂ ($0.117 \pm 0.062 \text{ g}_{\text{COD}}\cdot\text{g}_{\text{COD}}^{-1}$). 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 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 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).

516

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 2015⁴⁸ 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_{COD} (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)⁴⁹. 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 processes⁵⁰ or even $0.90 \text{ g}_{\text{COD}_{\text{acetate}}}\cdot\text{g}_{\text{COD}}^{-1}$ were
537 reached in microbial electrosynthesis processes⁵¹ (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)⁵².

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°C) carried out at low hydraulic retention time ($<3\text{j}$)⁵³⁻⁵⁵. Here, the acetate re-consumption
550 pathways were inhibited while all other compounds were converted into 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 acetate⁵⁶. 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 applicability⁵⁷. 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 esterified⁵⁸, converted
560 into lipids by yeasts⁵⁹, 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 nutrition⁶ 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 decrease⁶⁰.

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)⁶¹.
578 Butyrate production can also be promoted by chain elongation reactions⁶² 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)⁵².

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 extraction⁵⁶ 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^{58,63}.

589 < Table 6 >

590 4.3.3. Ethanol

591 Ethanol is a molecule with a large world market of 76,700 kt/year (2015)⁴⁸. 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%)⁶⁴, 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_{COD}, Table 4). It is noteworthy that ethanol market price rely heavily on support
599 policies, either through direct subsidies or fuel blend obligation⁶⁵.

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 $0.5 \text{ g}\cdot\text{L}^{-1}$ (Figure 13).
604 Nonetheless, several studies aimed at optimizing the ethanol/H₂ production from glycerol.
605 Using crude glycerol directly issued from the biodiesel industry, the highest concentration
606 achieved with mixed cultures reached $26.0 \text{ g}\cdot\text{L}^{-1}$, with a yield and productivity of 0.59
607 $\text{g}_{\text{COD}_{\text{ethanol}}}\cdot\text{g}_{\text{COD}}^{-1}$ and $1.6 \text{ g}_{\text{ethanol}}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$, respectively⁶⁶. This titer must be compared with
608 yeast-based fermentation of sugars which can typically attain titers as high as $150 \text{ g}\cdot\text{L}^{-1}$ ⁶⁷.
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 $4.8 \text{ g}_{\text{ethanol}}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$, respectively⁶⁸.
611 However, the productivity when using a complex substrate remained low with maximum
612 value around $9.5 \text{ g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ (Table 5)⁵².

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 MJ.kg^{-1}) for ethanol concentrations lower than $\sim 14 \text{ g.L}^{-1}$, thus making its
618 extraction clearly unsustainable in that case⁶⁹. 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 $> 40 \text{ g.L}^{-1}$ ⁶⁹. 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)⁶⁶ estimated that bioethanol
625 production costs using crude glycerol issued from a biodiesel production plant could be as
626 low as 0.27 €/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 impacts⁷⁰.

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 kt/yr and its selling price was estimated at 1.76 €/kg ,
638 equivalent to $1.05 \text{ €/kg}_{\text{COD}}$ ⁴⁸. This price could be raised to more than 3 €/kg , equivalent to
639 $1.79 \text{ €/kg}_{\text{COD}}$, in the coming years⁷¹.

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 ± 0.114
644 $\text{g}_{\text{COD_PDO}}.\text{g}_{\text{COD}}^{-1}$ (Cluster 5, Figure 12), a value close to the maximum theoretical yield (0.82
645 $\text{g}_{\text{COD_PDO}}.\text{g}_{\text{COD}}^{-1}$)⁷². Interestingly, the best production performances were achieved in a fed-
646 batch reactor fed with raw glycerol, with a final concentration of 82.7 g.L^{-1} , a yield of 0.75
647 $\text{g}_{\text{COD_PDO}}.\text{g}_{\text{COD}}^{-1}$ and a productivity of $73.7 \text{ g.L}^{-1}.\text{d}^{-1}$ (Table 5)⁷³. These results are comparable

648 with the best performances achieved so far during glycerol fermentation by pure culture of
649 unmodified strains^{74,75}.

650 Many methods have been developed for 1,3-propanediol extraction and purification
651 from fermentation media⁷⁴. 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%⁷⁶. The overall
655 extraction yield of the whole chain can be as high as 90%⁷⁶ but this method requires a large
656 amount of energy⁷⁴. 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 achieved⁷⁷. 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)⁷⁸. 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 precursor⁷⁹.
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 routes⁷⁹ and represents a market
671 of 400 kt/yr (2013)^{6,79}. Its market price is ranging between 1.25 and 1.38 €/kg, which is
672 equivalent to 0.83 - 0.91 €/kg_{COD} (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 H₂ production⁵. 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⁻¹ using food waste as
678 substrate⁸⁰. The best yields and productivities are 0.31 g_{COD_propionate}.g_{COD}⁻¹ and 22.0 g.L⁻¹.d⁻¹,
679 observed in two different studies (Table 5). Better yields up to 0.45 g_{COD_propionate}.g_{COD}⁻¹ were

680 attained when using defined fermentation media⁸¹, 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)⁷⁹.

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 ($> 2.9 \text{ g.L}^{-1}$)^{81,82}.
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_{COD} (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-sourced⁴⁸ as high isomeric purity lactic
696 acid can be produced by simple fermentation⁸³. This aspect is particularly important for PLA
697 production, which biodegradability depends on the L-isomer purity of the lactic acid⁸³. 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 H₂ production⁵. 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⁻¹, with a yield and productivity of 0.63 g_{COD_lactate}·g_{COD}⁻¹ and 12.8
705 g.L⁻¹·d⁻¹, respectively (Table 5)⁸⁴. Productivities as high as 40.0 g.L⁻¹·d⁻¹ were reached using
706 food waste as substrate⁸⁵.

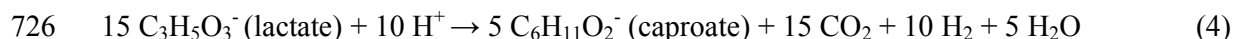
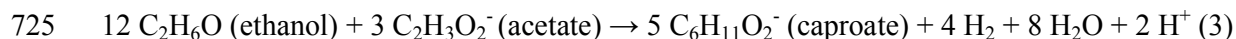
707 Similarly to carboxylic acids, lactate extraction is suitable when its selectivity is
708 sufficiently high. Some studies demonstrated the feasibility of lactate extraction after food
709 waste fermentation, using a process combining centrifugation, nanofiltration and
710 electro dialysis steps with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

711 *situ* from fermentation medium by adsorption on activated carbon that can be further desorbed
712 with acetone⁸⁶. 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)^{87,88}.
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_{COD} (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)⁸⁷:



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 minimized⁸⁹. Using complex substrates, the best
733 performances achieved so far are a maximum concentration of 11.9 g.L⁻¹, a yield of 0.81
734 g_{COD_caproate}.g_{COD}⁻¹ and a productivity of 26 g.L⁻¹.d⁻¹ (data issued from different studies, Table
735 5)^{90,91}. For more detailed information about caproate production, readers may refer to
736 Cavalcante *et al.* (2017)⁸⁷.

737 Caproate extraction is greatly facilitated by the low water solubility of its acid form (~
738 11 g.L⁻¹). 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 removed⁸⁸. Direct liquid-liquid extraction from the fermentation medium is also

741 possible⁹¹⁻⁹³. For instance, using trioctylphosphine oxide as solvent, caproate recovery yields
742 of 97.3% were achieved⁹³. Moreover, long-term caproate production (> 1 year) was
743 demonstrated⁹³ and at least one pilot-scale reactor was already implemented, incorporating an
744 extraction technology⁹⁴. 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 energy⁹⁵. 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.* surgery⁹⁵. 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-based⁴⁸. Its selling price is currently
757 varying between 2.20 and 5.00 €/kg, equivalent to 1.38 - 3.14 €/kg_{COD} (Table 4) but is not yet
758 economically competitive when compared to equivalent petro-based plastics (~1 €/kg)⁹⁶. As
759 one of the main contributor to the overall process operating costs is the carbon source⁹⁷, using
760 waste as substrate appears to be a promising way to reach economic viability⁹⁸. Moreover, it
761 would also improve environmental performances of PHA production, for instance when the
762 process is integrated in a wastewater treatment plant⁹⁹.

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_{PHA}.L⁻¹.d⁻¹ (Table 7)¹⁰⁰. The
770 best conversion efficiency was 0.41 g_{COD_PHA}.g_{COD}⁻¹ using paper industry effluents¹⁰¹. For
771 more detailed information about PHAs production from waste, readers may refer to Valentino
772 *et al.* (2017)⁹⁶.

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 ethanol⁹⁶. 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 particles⁹⁶. 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^{100,102}. 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 matter¹⁰³ 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, H₂ 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, H₂
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 H₂
803 production in AD plants, the territorial grid offered by the biogas plants would significantly

804 reduce the costs related to H₂ transportation. Research on biological H₂ 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 H₂ 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 bioH₂ production technologies conclude that DF is not economically competitive yet
817 with technologies such as biomass gasification or biogas reforming¹⁰. However, these
818 scenarios do not take into account the environmental benefits offered by DF when compared
819 to more traditional H₂-producing processes, as shown by recent LCA¹⁰, as well as products
820 other than H₂ 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 bioH₂ (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 bioH₂
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 H₂ production
845 (TRL3-4): because of their anti-correlation with H₂ 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 2030¹⁰³ 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 ₂
Biomolecules	List of 130 biomolecules according to Straathof (2014) ¹⁰⁴
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 _{COD} /g)
1,3-propanediol	C ₃ H ₈ O ₂	1.68
2,3-butanediol	C ₄ H ₁₀ O ₂	1.96
Acetate	C ₂ H ₄ O ₂	1.07
Butanol	C ₄ H ₁₀ O	2.59
Butyrate	C ₄ H ₈ O ₂	1.82
Caproate	C ₆ H ₁₂ O ₂	2.21
Cellulose	C ₆ H ₁₀ O ₅	1.19
Ethanol	C ₂ H ₆ O	2.09
Glucose/Fructose	C ₆ H ₁₂ O ₆	1.07
Glutamate	C ₅ H ₉ O ₄ N	0.98
Glycerol	C ₃ H ₈ O ₃	1.22
Hydrogen	H ₂	8.00
Lactate	C ₃ H ₆ O ₃	1.07
Methane	CH ₄	4.00
Methanol	CH ₄ O	1.50
PHA	(C ₄ H ₆ O ₂) _n	1.67
Propanol	C ₃ H ₈ O	2.40
Propionate	C ₃ H ₆ O ₂	1.51
Succinate	C ₄ H ₆ O ₄	0.95
Sucrose/Lactose	C ₁₂ H ₂₂ O ₁₁	1.12
Valerate	C ₅ H ₁₀ O ₂	2.04
Xylose/Arabinose	C ₅ H ₁₀ O ₅	1.07

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1167 **Table 3.** Conversion performances and features of the bioH₂ production technologies.

Technology	TRL	Substrate	Theoretical maximum yield (gCOD·gCOD ⁻¹)	Experimental average yield (gCOD·gCOD ⁻¹)	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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1169

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

Molecule	Market price (€/kg)*	Market price (€/kg _{COD})*	Global market volume (kt/yr)	References
PHA	2.20 – 5.00	1,38 – 3.14	17	⁹⁶ , this study
1,3-propanediol	1.76 – 3.00	1.05 – 1.79	128	^{48,71}
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	^{6,79} , this study
Lactic acid	0.84 – 1.51	0.79 – 1.41	472	⁴⁸ , this study
Acetic acid	0.33 – 0.67	0.31 – 0.63	13,570	⁴⁸ , this study
H ₂	1.50 – 5.00	0.19 – 0.63	60,000	This study
Ethanol	0.30 – 1.50	0.14 – 0.72	76,700	^{48,64} , this study
CH ₄	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 ⁻¹)	Yield* (g _{COB} ·g _{COB} ⁻¹)	Productivity* (g.L ⁻¹ ·d ⁻¹)	Process configuration	Process or start-up duration (d)**	Ref.
Highest concentrations							
Acetate	Sugar cane bagasse	35.3	0.24	1.1	Semi-continuous	90	⁴⁹
Butyrate	Food waste	21.4	0.26	2.7	Batch	8	⁸⁰
Caproate	Fermented municipal waste + ethanol	11.9	0.46	26.0	Continuous	140	⁹⁰
Ethanol	Raw glycerol	26.0	0.59	1.6	Fed-batch	15.8	⁶⁶
Lactate	Food waste	64.0	0.63	12.8	Batch	5	⁸⁴
Propionate	Food waste	15.8	0.16	2.0	Batch	8	⁸⁰
1,3-propanediol	Raw glycerol	82.7	0.75	73.4	Fed-batch	1.1	⁷³
Highest yields							
Acetate	Food waste	7.9	0.56	1.8	Batch	4.3	⁵⁰
Butyrate	Municipal waste	1.6	0.74	0.8	Batch	2.1	⁶¹
Caproate	Liquid phase of alcoholic fermentation	NA***	0.81	2.1	Continuous	350	⁹¹
Ethanol	Raw glycerol	8.0	0.91	4.8	Batch	1.7	⁶⁸
Lactate	Food waste	64.0	0.63	12.8	Batch	5	⁸⁴
Propionate	Sago starch wastewater	2.2	0.31	1.1	Semi-continuous	NA	¹⁰⁵
1,3-propanediol	Refined glycerol	82.7	0.75	73.4	Fed-batch	1.1	⁷³
Highest productivities							
Acetate	Beverage industry wastewater	3.6	0.18	57.0	Continuous	155	⁵²
Butyrate	Beverage industry wastewater	4.6	0.39	73.2	Continuous	155	⁵²
Caproate	Fermented municipal waste + ethanol	11.9	0.46	26.0	Continuous	140	⁹⁰
Ethanol	Beverage industry wastewater	0.6	0.06	9.5	Continuous	155	⁵²
Lactate	Food waste	40.0	0.41	40.0	Continuous	152	⁸⁵
Propionate	Cheese whey	6.9	0.26	27.6	Continuous	40	¹⁰⁶
1,3-propanediol	Raw glycerol	82.7	0.75	73.4	Fed-batch	1.1	⁷³

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

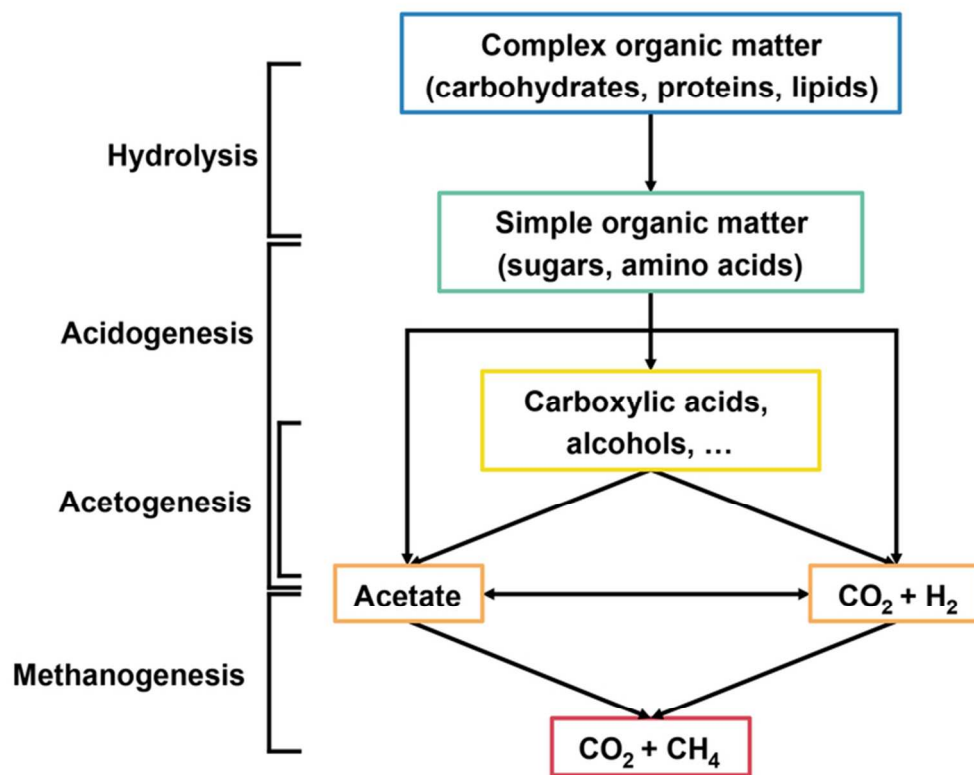
1192

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

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

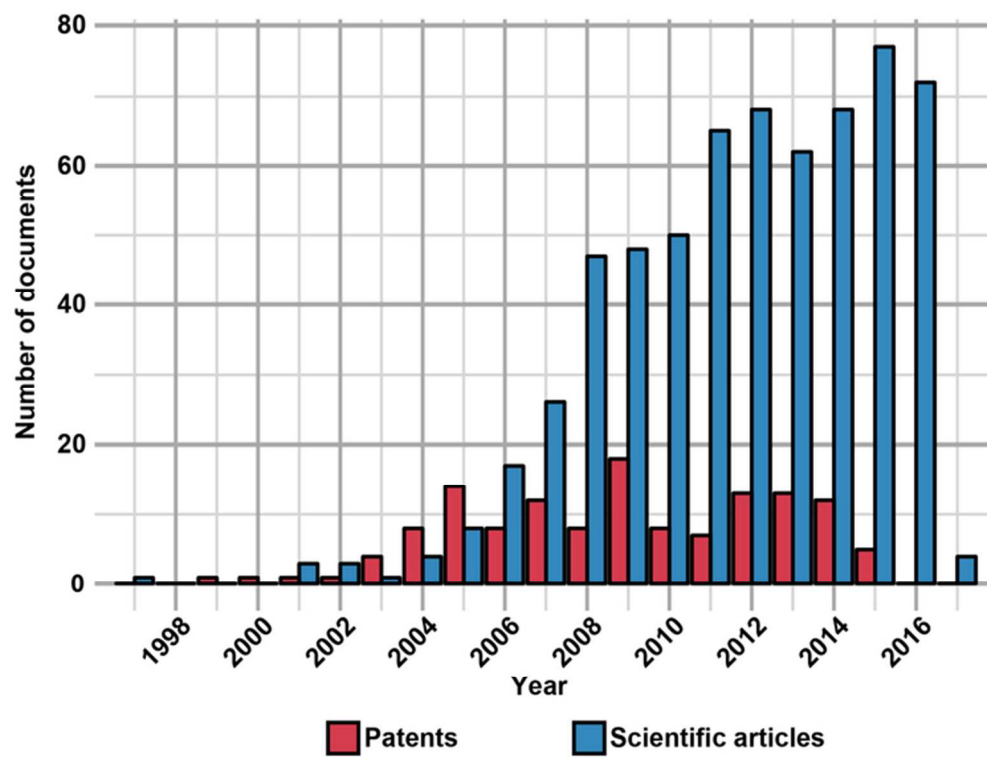
1194 * Yield calculated for the selection and accumulation steps

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



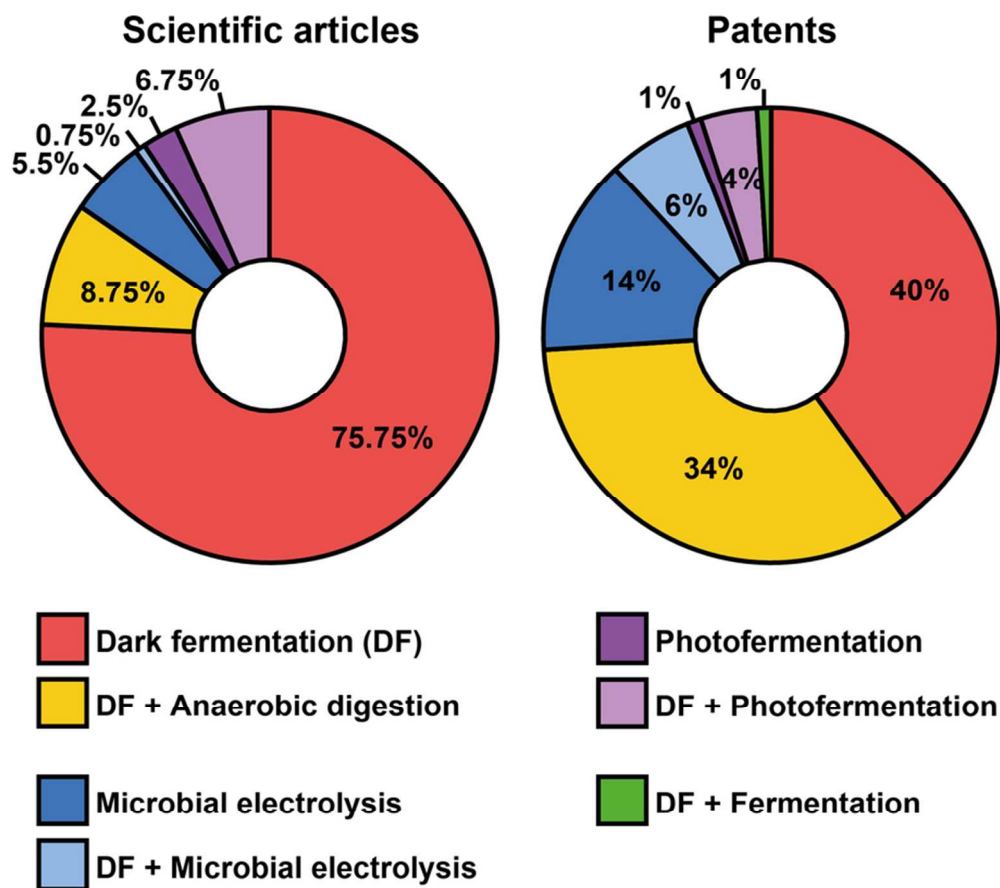
The different phases of anaerobic digestion.

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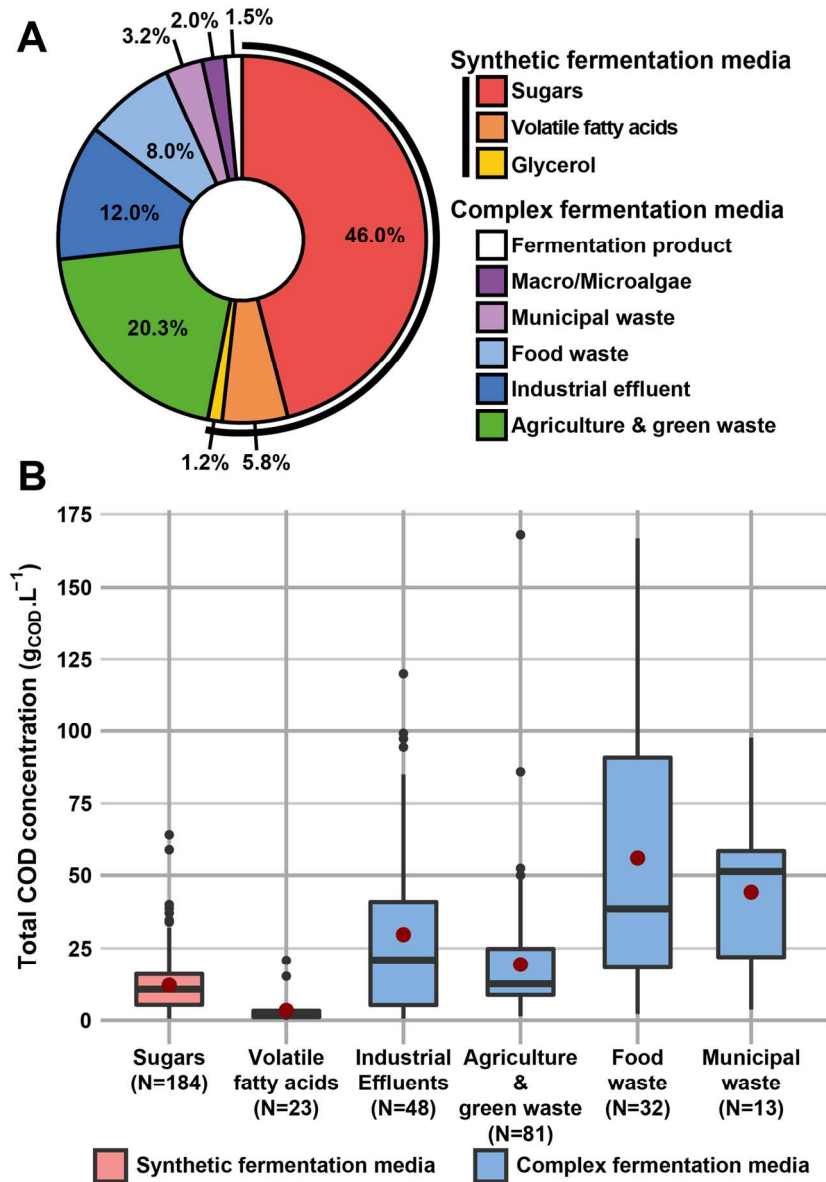
Publication of research articles and patents related to biomolecule production by mixed-culture fermentation between 1997 and January 2017. Information regarding patents after mid 2015 is incomplete due to the 18 month delay between patent filing and publication.

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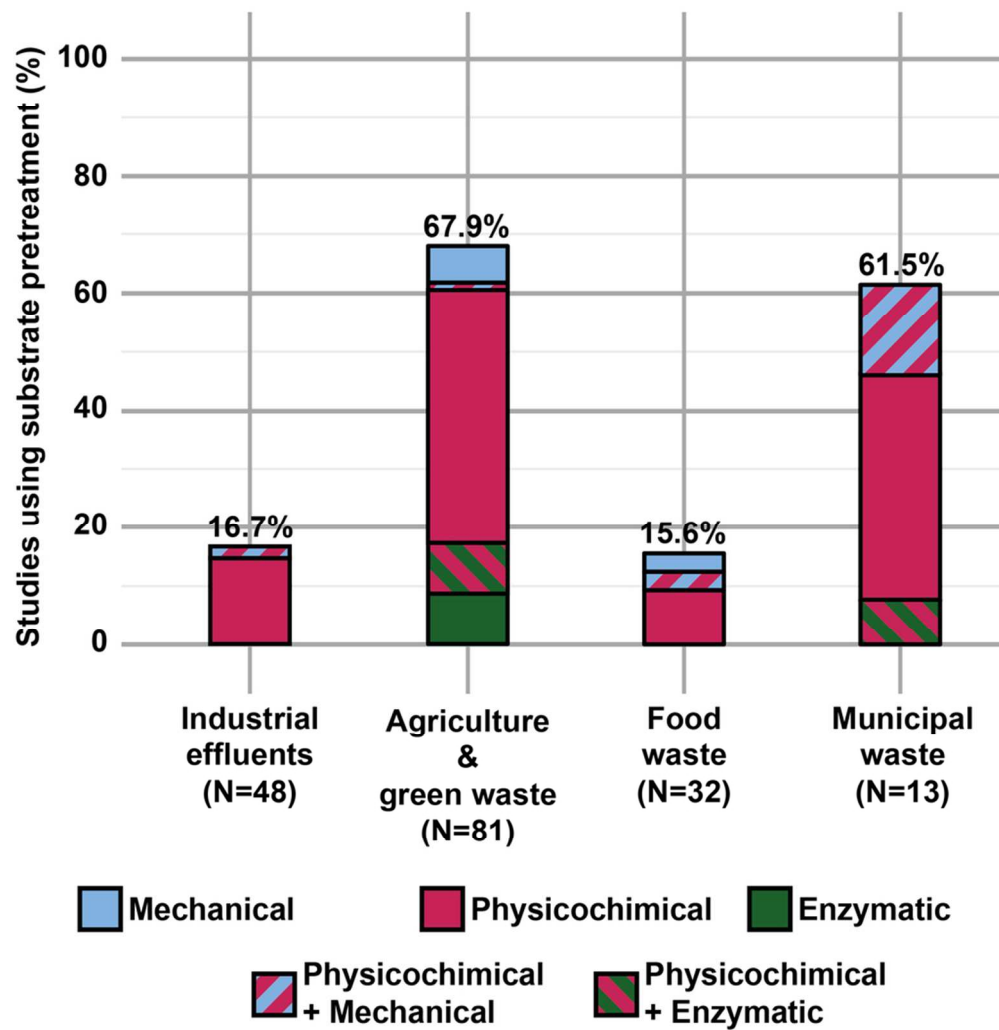
Technologies used for biological hydrogen production. The documents represented correspond to scientific articles displaying a hydrogen yield higher than $0.01 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$ (400 documents) and to the patents explicitly claiming hydrogen production (100 documents).

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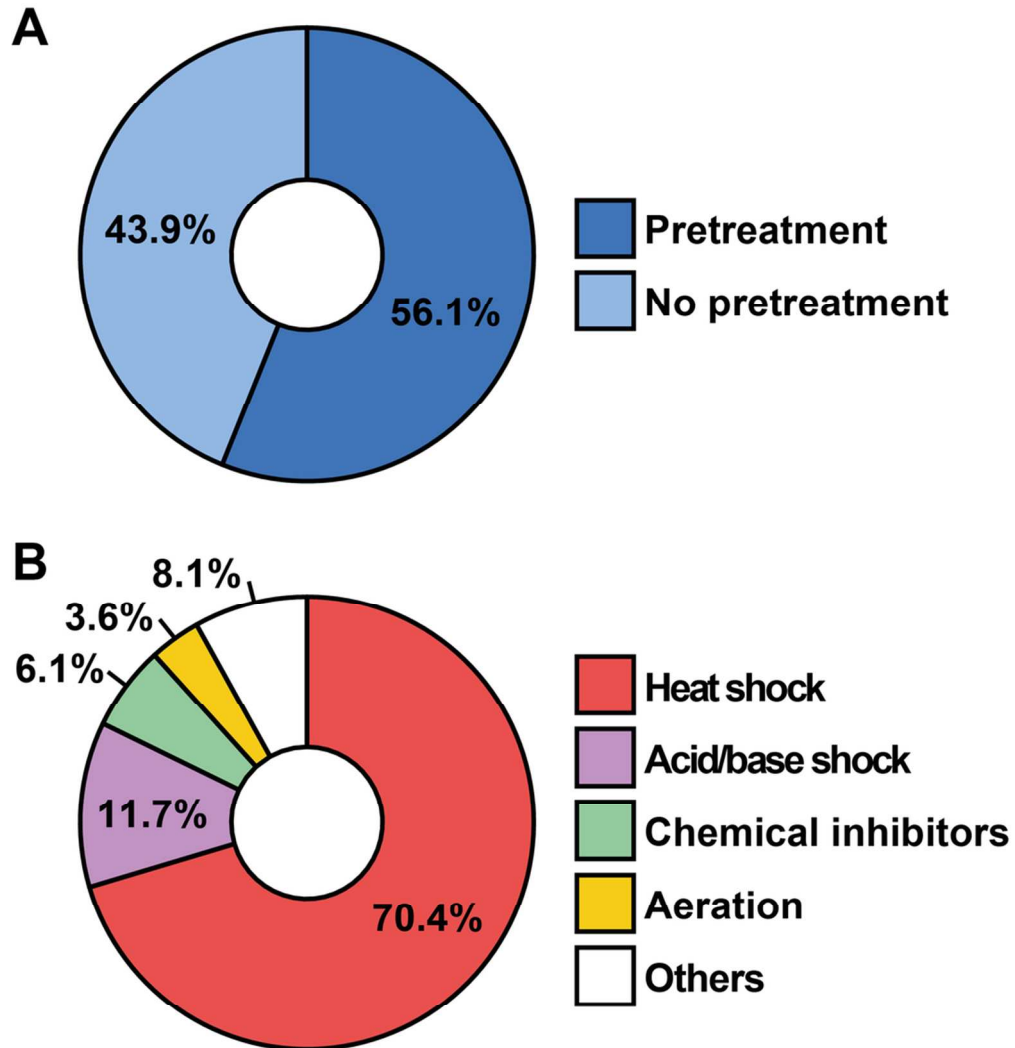
Substrates used for the biological production of hydrogen (A) and their total COD concentration (B). The total COD concentration corresponds to the initial concentration of the substrate for batch processes and to the concentration of the feed for continuous/semi-continuous processes. The documents represented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

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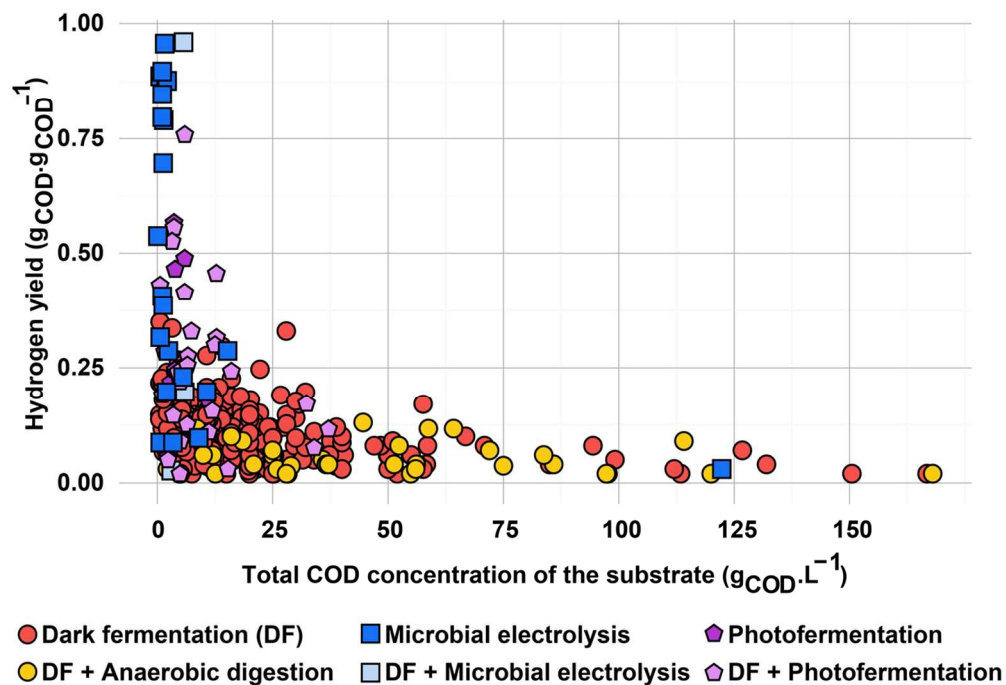
Substrate pretreatment carried out before biohydrogen production. N corresponds to the number of scientific articles taken into account for each category. Mechanical pretreatments: grinding, sonication; physicochemical pretreatments: acid/alkaline hydrolysis, heat treatment, steam explosion; enzymatic pretreatments: by microorganisms or enzyme cocktails.

86x90mm (300 x 300 DPI)



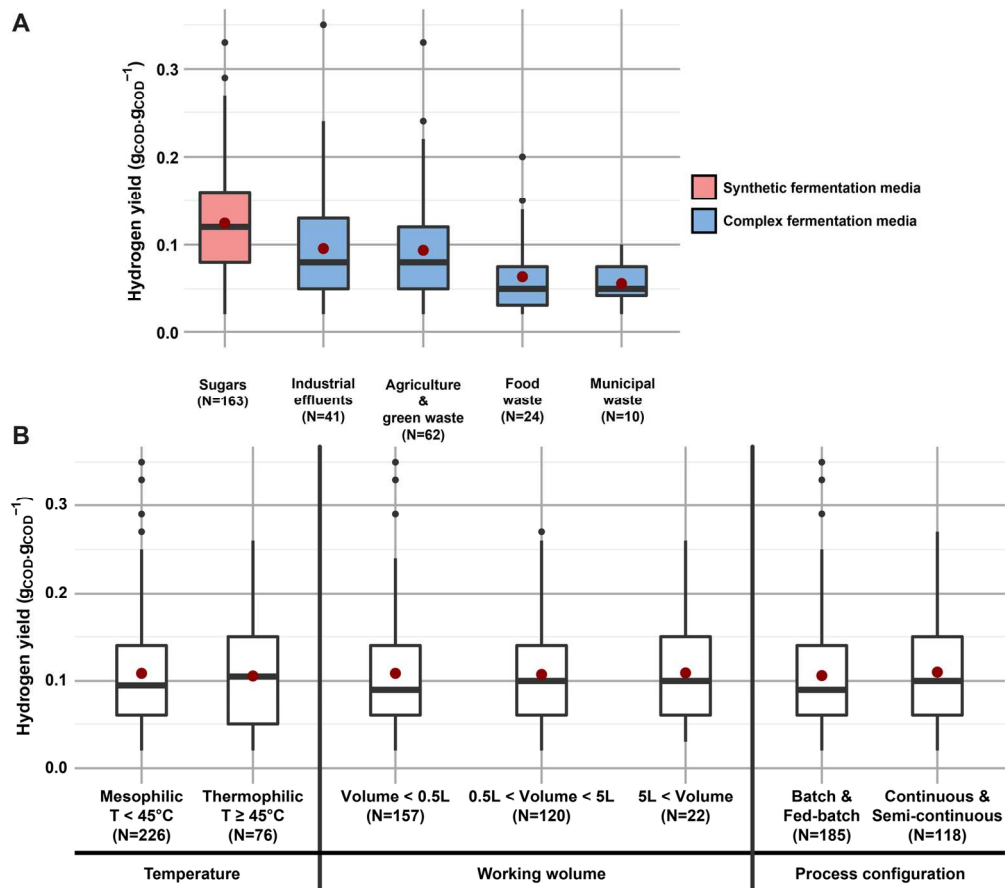
Part of the studies focused on dark fermentation using inoculum pretreatment (A) and the different pretreatment methods employed (B). The documents represented correspond to scientific articles for which the information concerning inoculum pretreatment is available, displaying a hydrogen yield higher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

91x100mm (300 x 300 DPI)



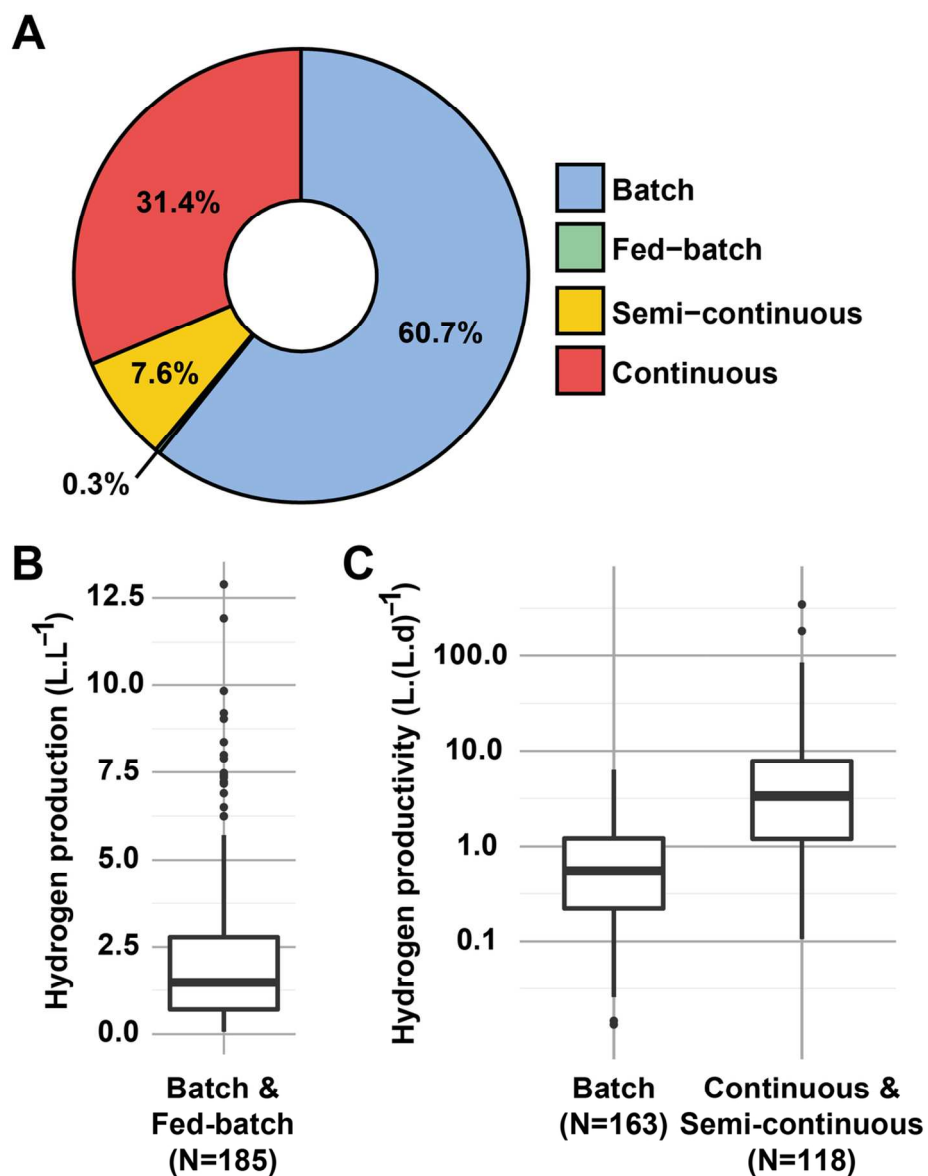
Hydrogen yield as a function of the total COD concentration of the substrates. The total COD concentration corresponds to the initial concentration of the substrate for batch processes and to the concentration of the feed for continuous/semi-continuous processes. The documents represented correspond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

119x83mm (300 x 300 DPI)



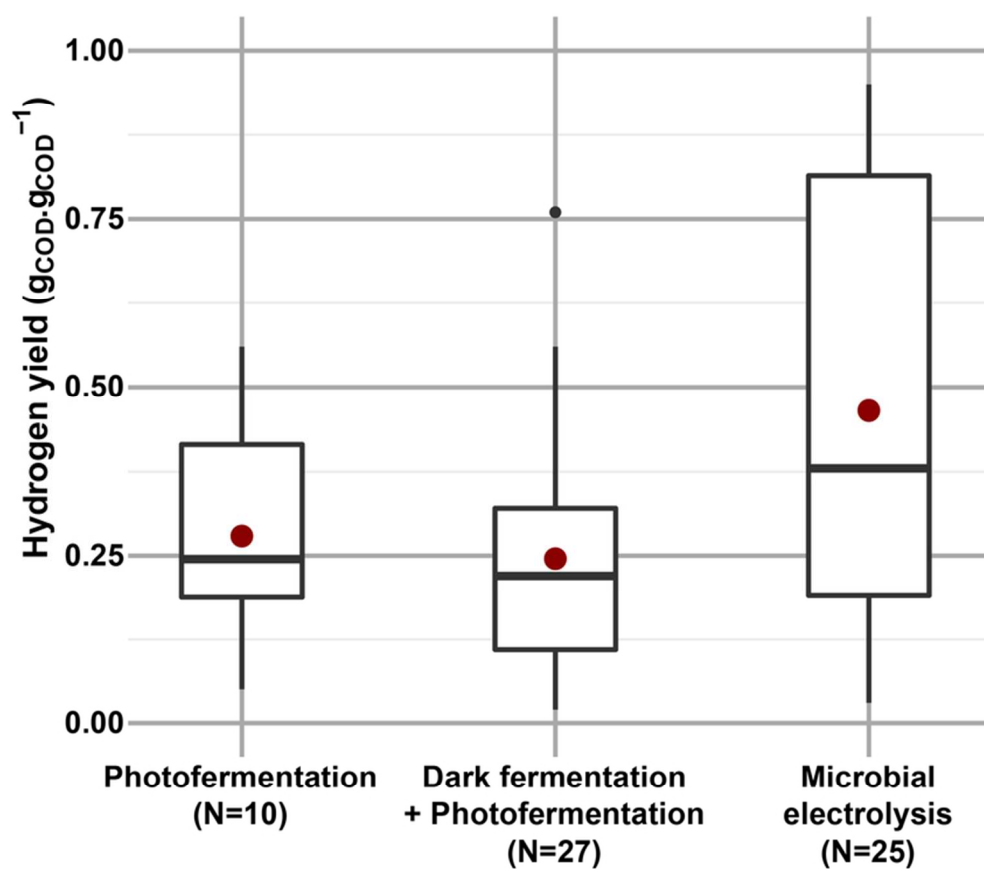
Hydrogen yield as a function of the substrates employed (A) and process parameters (B). The documents represented correspond to scientific articles displaying a hydrogen yield higher than $0.01 \text{ g}_{\text{COD}}\cdot\text{g}_{\text{COD}}^{-1}$ and focused on dark fermentation (303 documents). N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

154x138mm (300 x 300 DPI)



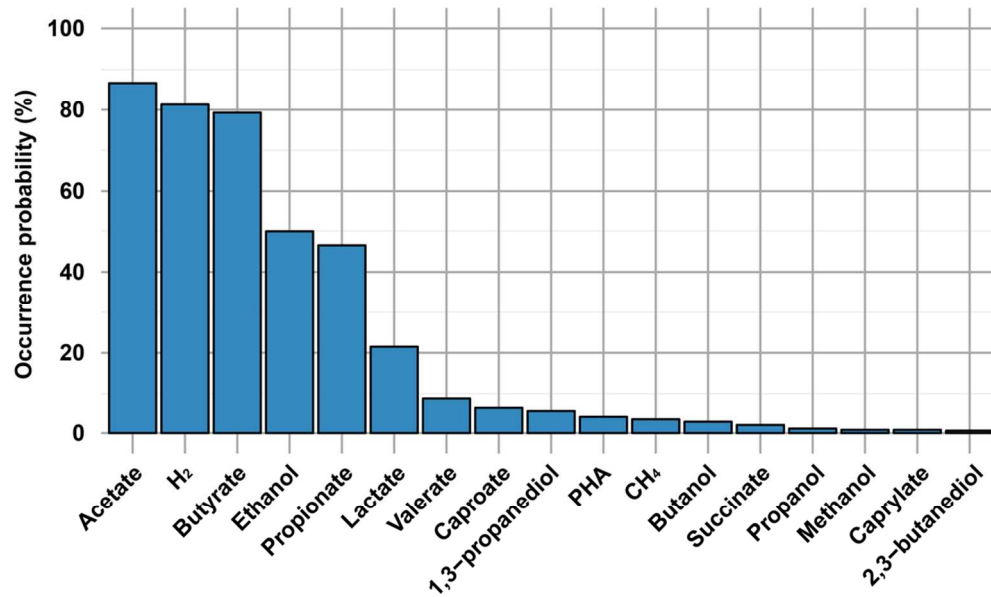
Frequency of use (A) and performance (B-C) of the different operation modes used for hydrogen production by dark fermentation. The documents represented correspond to scientific articles displaying a hydrogen yield higher than $0.01 \text{ g}_{\text{COD}} \cdot \text{g}_{\text{COD}}^{-1}$ and focused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

108x142mm (300 x 300 DPI)



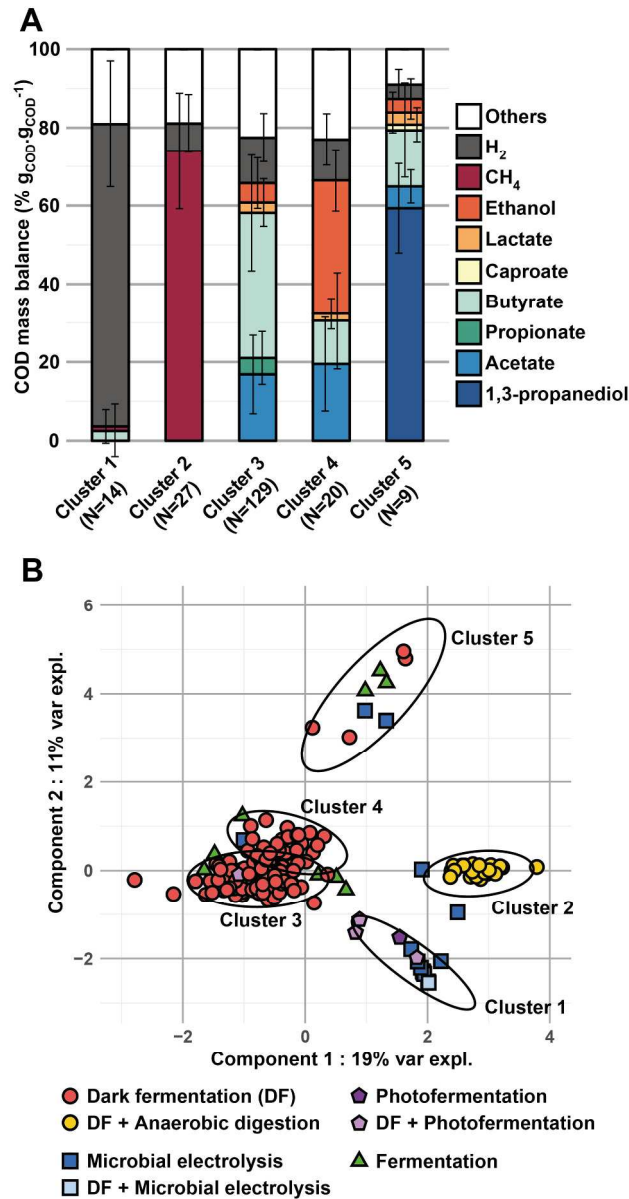
Hydrogen yields achieved by photofermentation and microbial electrolysis. The documents represented correspond to scientific articles displaying a hydrogen yield higher than $0.01 \text{ g}_{\text{COD}}.\text{g}_{\text{COD}}^{-1}$. N corresponds to the number of scientific articles taken into account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

72x63mm (300 x 300 DPI)



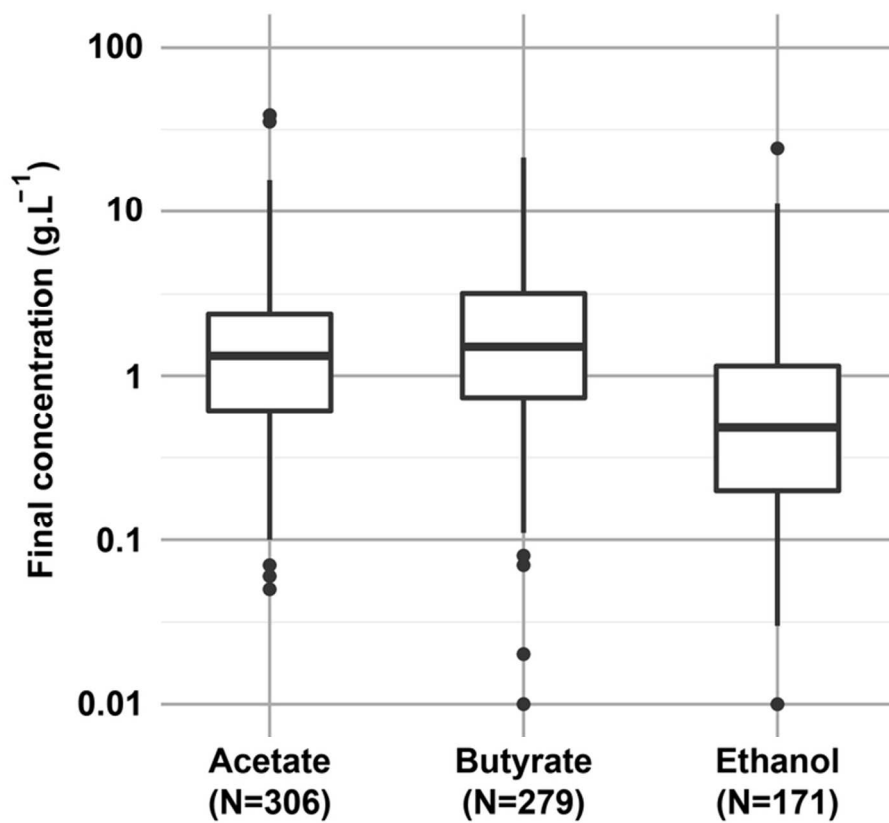
Occurrence probability of common metabolites within the database . Scientific articles in which only hydrogen was measured, as well as those dealing with the coupling between fermentation and anaerobic digestion were excluded. Metabolites were considered present when their respective yields were higher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

103x63mm (300 x 300 DPI)



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

158x303mm (300 x 300 DPI)

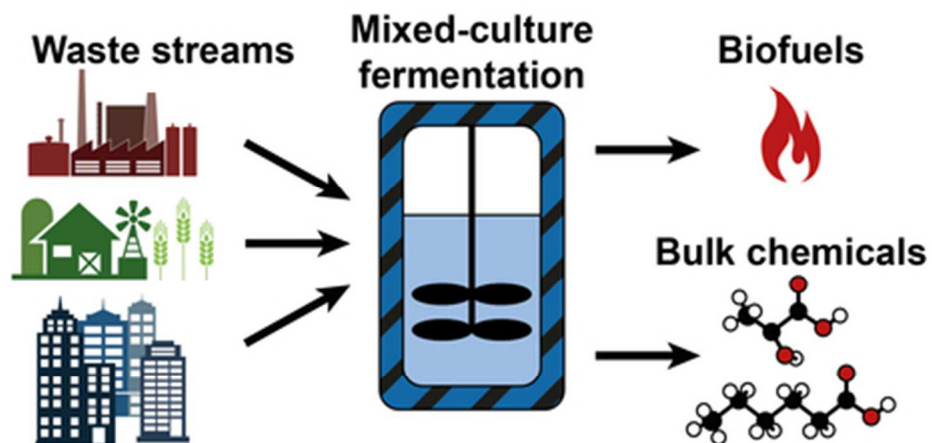


Final concentrations of acetate, butyrate and ethanol produced by mixed-culture fermentation processes. N corresponds to the number of scientific articles taken into account for each category.

71x61mm (300 x 300 DPI)

Table of contents entry

The production of energy carriers and bulk chemicals by mixed-culture fermentation is quantitatively analysed and discussed in a biorefinery context.



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39x19mm (300 x 300 DPI)