



**HAL**  
open science

## Dark fermentation and microalgae cultivation coupled systems: Outlook and challenges

Julien Lacroux, Mercedes Llamas, Kevin Dauplain, Romina Avila,  
Jean-Philippe Steyer, Robert van Lis, Eric Trably

### ► To cite this version:

Julien Lacroux, Mercedes Llamas, Kevin Dauplain, Romina Avila, Jean-Philippe Steyer, et al.. Dark fermentation and microalgae cultivation coupled systems: Outlook and challenges. *Science of the Total Environment*, 2023, 865, pp.161136. 10.1016/j.scitotenv.2022.161136 . hal-04007434

**HAL Id: hal-04007434**

**<https://hal.inrae.fr/hal-04007434>**

Submitted on 28 Jul 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# DARK FERMENTATION AND MICROALGAE CULTIVATION COUPLED SYSTEMS: OUTLOOK AND CHALLENGES

Julien Lacroux<sup>1</sup>, Mercedes Llamas<sup>1,2</sup>, Kevin Dauptain<sup>1</sup>, Romina Avila<sup>3</sup>, Jean-Philippe Steyer<sup>1</sup>, Robert van Lis<sup>1</sup>, Eric Trably<sup>1\*</sup>.

<sup>1</sup> LBE, Univ Montpellier, INRAE, 102 avenue des Etangs, F-11100 Narbonne, France.

<sup>2</sup> Instituto de la Grasa (C.S.I.C.), Campus Universidad Pablo de Olavide, Edificio 46., Ctra. de Utrera km. 1, 41013 Sevilla, Spain

<sup>3</sup> Chemical, Biological and Environmental Engineering Department, Escola d'Enginyeria, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, E-08193, Spain.

**Corresponding author: [eric.trably@inrae.fr](mailto:eric.trably@inrae.fr)**

11		
12	1. Introduction.....	2
13	2. The basis of Dark Fermentation process.....	6
14	3. Cultivation of microalgae on Dark Fermentation Effluents.....	9
15	3.1. Heterotrophic growth on Dark Fermentation Effluent compounds .....	10
16	3.1.1. Acetate: the favoured substrate .....	11
17	3.1.2. Other substrates.....	12
18	3.1.3. Behaviour in mixtures .....	13
19	3.1.4. Mechanisms of substrate inhibition.....	14
20	3.2. Tuning the mixotrophic process .....	16
21	4. Approaches to enhance microalgae cultivation on Dark Fermentation Effluents .....	19
22	4.1. Dark Fermentation strategies.....	19
23	4.1.1. Substrate and nutrients.....	19
24	4.1.2. Anaerobic inoculum .....	21
25	4.1.3. Operational parameters.....	21
26	4.2. Microalgae cultivation strategies .....	23
27	4.2.1. pH control.....	24
28	4.2.2. Initial microalgae inoculum .....	25
29	4.2.3. Light intensity .....	25
30	4.2.4. Isolation and screening of new microalgae.....	27
31	4.2.5. Genetic Modifications/ adaptive evolution.....	28
32	4.2.6. Microalgae co-cultivation.....	29
33	5. Microalgal biorefinery .....	30
34	5.1. Microalgae applications .....	30

35	5.2. Microalgae as a substrate for Dark Fermentation .....	32
36	6. Conclusions.....	34
37	7. Acknowledgements .....	35
38	REFERENCES .....	36

39

## 40 **KEYWORDS**

41 Dark fermentation; Hydrogen; Microalgal growth; Volatile fatty acids; Biorefinery; Waste  
42 valorization.

43

## 44 **ABSTRACT**

45 The implementation of a sustainable bio-based economy is considered a top priority today.

46 There is no doubt about the necessity to produce renewable bioenergy and bio-sourced  
47 chemicals to replace fossil-derived compounds. Under this scenario, strong efforts have been  
48 devoted to efficiently use organic waste as feedstock for biohydrogen production via dark  
49 fermentation. However, the technoeconomic viability of this process needs to be enhanced by  
50 the valorization of the residual streams generated. The use of dark fermentation effluents as  
51 low-cost carbon source for microalgae cultivation arises as an innovative approach for  
52 bioproducts generation (*e.g.*, biodiesel, bioactive compounds, pigments) that maximizes the  
53 carbon recovery. In a biorefinery context, after value-added product extraction, the spent  
54 microalgae biomass can be further valorised as feedstock for biohydrogen production. This  
55 integrated process would play a key role in the transition toward a circular economy.

56 This review covers recent advances in microalgal cultivation on dark fermentation effluents  
57 (DFE). BioH<sub>2</sub> via dark fermentation processes and the involved metabolic pathways are detailed  
58 with a special focus on the main aspects affecting the effluent composition. Interesting traits of  
59 microalgae and current approaches to solve the challenges associated to the integration of dark  
60 fermentation and microalgae cultivation are also discussed.

## 61 **1. Introduction**

62 Environmental damage and the finite petroleum supplies are two main global concerns of the  
63 21<sup>st</sup> century. To face those challenges, there is no doubt about the necessity to implement  
64 sustainable process to produce energy and products from non-fossil sources.

65 Hydrogen gas (H<sub>2</sub>) is considered as the most promising green fuel due to its high energy content  
66 (122 MJ/kg) and the lack of carbon dioxide (CO<sub>2</sub>) released during its combustion, which makes  
67 this technology a key-player to reach a carbon neutral economy (Balachandar et al., 2020). That  
68 is why a growing interest in H<sub>2</sub> production and storage has recently emerged globally. Analysts  
69 estimate that green H<sub>2</sub> could meet 24 % of energy world demand by 2050, with annual sales in  
70 the range of 630 billion € (BNEF, 2020). For its part, the European Commission has recently  
71 launched a Hydrogen Strategy targeting the promotion of H<sub>2</sub> technologies in order to address  
72 the Green Deal and Europe's clean energy transition. This strategy includes massive  
73 investments in the H<sub>2</sub> sector accounting for 180-470 cumulative billion € in the European Union  
74 by 2050 (European Commission, 2020).

75 So far, most of the H<sub>2</sub> is produced from traditional fossil sources. However, strong efforts are  
76 being made to develop cleaner H<sub>2</sub> productions such as water electrolysis from renewable  
77 electricity sources and thermo-chemical or biological processes (El-Emam and Özcan, 2019).  
78 Among the green H<sub>2</sub>-producing technologies, dark fermentation (DF) has emerged as one of  
79 the most sustainable alternatives. Dincer and Acar (2014) assessed 19 technologies for H<sub>2</sub>  
80 production, including environmental, technical, financial, and social impacts. They concluded  
81 that DF is the most economical method while exhibiting the lowest global warming potential  
82 (< 1 kgCO<sub>2</sub>/kgH<sub>2</sub>).

83 DF is a well-known technology where biodegradation of organic matter takes place leading to  
84 the production of gases (H<sub>2</sub> and CO<sub>2</sub>) and other soluble metabolites. One of the main advantages  
85 of this technology is the wide variety of feedstocks which can be employed such as activated

86 sludge, lignocellulosic biomass, food waste or microalgae. This process offers the dual  
87 advantage of generating bioproducts while valorising wastes that otherwise should be treated,  
88 clearly contributing to a circular economy. However, some concerns associated to BioH<sub>2</sub>  
89 production via DF should be tackled. H<sub>2</sub> is necessarily coproduced with CO<sub>2</sub> during DF, which  
90 results in a net loss of carbon in the gaseous fraction and therefore, one of the major challenges  
91 associated to the biological production of H<sub>2</sub> is the need of purification. This CO<sub>2</sub> contained in  
92 the gaseous fraction can finally be valorised as a synthon to produce chemicals such as  
93 polycarbonates, carbamates or polyurethanes either through chemical or biological routes, or  
94 directly used a substrate for autotrophic micro-organisms (Heffernan et al., 2023; Romans-  
95 Casas et al., 2021).

96 Additionally, the organic matter conversion into H<sub>2</sub> during this bioprocess is uncomplete  
97 leading to limited H<sub>2</sub> yields and organic matter-rich effluents. DF must therefore be associated  
98 with other processes to reduce the effluent organic matter content before disposal. On one hand,  
99 the solid fraction of the dark fermentation effluents contains the more recalcitrant organic  
100 matter that is not degraded by the microbial. This solid fraction can be valorized via  
101 conventional anaerobic digestion (AD) for biogas production (Llamas et al., 2021). On the other  
102 hand, the liquid supernatant is rich on metabolites with commercial value such as VFAs, lactate  
103 or ethanol (Dahiya et al., 2015). Those molecules represent an opportunity for the bioeconomy,  
104 as they could be a product by themselves or can be used as precursors in other processes  
105 (Bundhoo, 2017).

106 Despite the wide range of applications of those compounds, the major challenge associated with  
107 their use is the need of extraction or even purification which entail high costs and technological  
108 limitations. Therefore, the search of alternative applications with a direct use of those  
109 metabolites avoiding extraction/purification could present a major advantage for the  
110 development of feasible processes that serve the circular economy. Some of the processes that

111 use these organic acids-rich effluents directly include AD, photofermentation and  
112 bioelectrochemical systems for energy production (**Figure 1**).

113 Recently, the novel idea of coupling DF with microalgae culture has been suggested as an  
114 effective way to treat DFE and provide cheap substrates for heterotrophic or mixotrophic micro-  
115 algae production while maximizing the carbon recovery. Under this innovative biorefinery  
116 concept, microalgae could be employed as cell factories for the production of not only third-  
117 generation biofuels (biogas, bioethanol, biodiesel, bioH<sub>2</sub>) but also high value-added chemicals  
118 (i.e. cosmetics, pharmaceuticals, nutraceuticals, biofertilizers, pigments) (Dourou et al., 2021;  
119 Siddiki et al., 2022). Although microalgae exhibit lower cell densities and longer cultivation  
120 period than other microorganisms such as bacteria or yeast, the use of microalgae technologies  
121 presents crucial environmental advantages: CO<sub>2</sub> capture during their photosynthetic activity and  
122 shorter production periods than plants. Besides these interesting traits, microalgae present the  
123 ability to grow in a residual environment. Considering that the cost of the substrate is claimed  
124 to be a key issue for attaining economically competitive bioprocesses, the utilization of  
125 renewable waste streams as low-cost substrates for bioproducts generation arises as an attractive  
126 option. Therefore, the coupling of DF and microalgae cultivation supports not only DFE  
127 treatment but also carbon recovery maximization through an efficient multi-product generation  
128 (H<sub>2</sub> and high-value products) (Chong et al., 2022; Scarponi et al., 2021). Many studies have  
129 focused on DF coupled to other processes (*i.e.* AD, electrofermentation, photofermentation),  
130 but the integration with microalgae cultivation has seldom been reported. To cover this gap of  
131 knowledge, this review aims at evaluating the coupling of DF and microalgae cultivation in a  
132 biorefinery context, describing recent approaches and associated challenges that need to be  
133 faced to reach a viable industrial application.

134

## 135 2. The basis of Dark Fermentation process

136 DF is a promising technology designated to obtain bioenergy from organic substrates in the  
137 form of bioH<sub>2</sub>. This bioprocess corresponds to the intermediate fermentative steps of the AD  
138 process which ultimately leads to the production of methane (CH<sub>4</sub>). During DF, simple  
139 monomers (carbohydrates, proteins and lipids) are generated from the hydrolysis of complex  
140 organic matter. Subsequently, those monomers are converted into H<sub>2</sub> and CO<sub>2</sub> due to the activity  
141 of an anaerobic bacterial consortium.

142 One of the main drawbacks of H<sub>2</sub> production via DF is the economic viability of the process.  
143 The fermentative H<sub>2</sub> production costs (2.5 €/kg H<sub>2</sub>) need to be reduced to be competitive with  
144 fossil fuel technologies (<1€/kg H<sub>2</sub>) (Bundhoo, 2017). In addition, H<sub>2</sub> production by  
145 fermentative bacteria is limited by their metabolic constraints: the degradation of organic matter  
146 into H<sub>2</sub> is incomplete with a theoretical maximum yield of 33% of the initial organic matter.  
147 The rest is retrieved in the form of soluble metabolites in the DFE (Sharma et al., 2020). Those  
148 organic matter-rich DFE can serve as feedstock to other processes, allowing the valorization of  
149 wastes that otherwise should be treated before disposal.

150 The physico-chemical properties of the DFE may affect the downstream bioprocess that is  
151 chosen to be integrated in the biorefinery scheme as highlighted in **Figure 1**. For instance, when  
152 DF is coupled to AD, the effluent composition is not crucial, as long as the total acids  
153 concentration remain below the methanogenic bacterial inhibition thresholds. However, for  
154 other potential coupled processes such as photofermentation, bioelectrosystems or microbial  
155 cultivation, acetate enriched DFE should always be favoured since this molecule is the easiest  
156 assimilable metabolites among other ones that are produced.

157 The composition of the DFE mainly depends on the substrate and inoculum employed as well  
158 as the operating parameters applied. These factors strongly affect the bacterial communities

159 involved and the related fermentative metabolic pathways, which ultimately determine the fate  
160 of the organic matter, and therefore the H<sub>2</sub> yields and metabolite profiles (Greses et al., 2020).

161 BioH<sub>2</sub> generation takes place through sequential biochemical reactions as depicted in **Figure 2**.  
162 The DF bioprocess is carried out by anaerobic hydrogen producing bacteria (so- called HPB),  
163 that are usually members of the genus *Clostridium*. Other well described HPB genera include  
164 members of *Enterobacter* sp., *Escherichia-Shigella*, *Bacillus* sp., *Ethanoligenens* sp.,  
165 *Megasphaera* sp. or *Prevotella* sp. The relative abundance of those species in the bioprocess  
166 depends on the organic substrate employed and on the initial inoculum (Cabrol et al., 2017;  
167 Etchebehere et al., 2016b).

168 Metabolically, H<sub>2</sub> production pathways start with the conversion of glucose to pyruvate, which  
169 is further oxidized into acetyl-CoA via two different routes: the pyruvate ferredoxin oxydo-  
170 reductase (PFOR) and pyruvate formate lyase (PFL) pathways, depending on the bacterial  
171 species involved (**Figure 2**). In both cases, excess electrons resulting from these oxidation steps  
172 are used to produce H<sub>2</sub> through hydrogenases. The remaining acetyl-CoA can be converted into  
173 acetate for ATP generation.

174 The PFOR pathway is mainly followed by sporulating strict anaerobes related to *Clostridium*  
175 sp. (Hallenbeck and Benemann, 2002). Through this pathway, a maximum theoretical yield of  
176 4 mol H<sub>2</sub>/mol glucose can be obtained (**Equation 1, Table 1**). Together with H<sub>2</sub> and energy  
177 production, the purpose of the fermentation process is to regenerate oxidative power (NAD<sup>+</sup>).  
178 However, the NAD<sup>+</sup> generation in the PFOR pathway is not energetically favoured and thus,  
179 this should be alternative regenerated via fermentative metabolites production such as butyrate.  
180 Butyrate production pathway is the most thermodynamically favoured pathway for both energy  
181 and oxidative power production, generating 2 mol H<sub>2</sub>/mol glucose (**Equation 2, Table 1**).



182 In contrast, facultative anaerobic microorganisms, such as *Enterobacter sp.*, *E. coli* or  
183 *Ethanoligenens sp.*, preferentially follow the PFL pathway (Hallenbeck and Benemann, 2002).  
184 During PFL pathway the ATP production route must be derived to regenerate NAD<sup>+</sup> mainly by  
185 converting the acetyl-CoA into ethanol. In this case, 2 mol H<sub>2</sub>/mol glucose can also be obtained  
186 alongside with an equimolar mixture of acetate and ethanol (**Equation 3, Table 1**). In mixed  
187 cultures, substrate competition between HPB and other non-H<sub>2</sub> producer microorganisms can  
188 occur. Those bacteria are mainly propionate producers such as *Clostridium propionicum* and  
189 lactic acid bacteria such as *Lactobacillus sp.* or *Lactococcus sp.* The first ones can convert  
190 glucose into propionate according to **Equation 5** while the latter ones convert glucose into  
191 lactate according to **Equation 6 or 7**. Lactic acid bacteria may however impact DF processes  
192 positively since they can further convert lactate and acetate into butyrate and H<sub>2</sub> (**Equation 8**).  
193 Other metabolites such as succinate, caproate or valerate can sometimes also be found in DFE.  
194 However, those acids are produced from auxiliary metabolic pathways resulting generally in  
195 minor concentrations.

196 In practice, the H<sub>2</sub> yields obtained using mixed cultures range between 1 and 2.5 mol H<sub>2</sub>/mol  
197 glucose. Apart from H<sub>2</sub>, this process generates effluents rich in compounds including acetate,  
198 butyrate and ethanol. The profiles and concentration of those metabolites in DFE are very  
199 variable according to the fermentation pattern followed by the bacteria and organic substrate  
200 nature and concentration. A compilation of H<sub>2</sub> yields and metabolites profile generated during  
201 DF of different substrates are summarized in **Table 2**.

202 Moscoviz et al. (2018) distinguished two main fermentation clusters corresponding broadly to  
203 the aforementioned metabolic equations. The first group, corresponding to an average mixed  
204 culture typically found in DF, led to a balance between acetate and butyrate pathways  
205 (**Equation 4, Table 1**). Following this stoichiometry, a theoretical yield of 2.5 mol H<sub>2</sub> /mol  
206 glucose can be obtained, associated to a molar ratio butyrate/acetate of 0.66. This profile is the

207 most commonly found in literature, probably because fermentation conditions are mainly  
208 favouring it. The second group corresponds to fermentations following the PFL pathway,  
209 leading to concomitant acetate and ethanol formation. This kind of profile is usually observed  
210 when DF conditions favours the emergence of *Enterobacteriales* (Palomo-Briones et al., 2017).  
211 Interestingly, ethanol production can also be observed with *Clostridiales* when grown under  
212 stressful conditions, shifting from H<sub>2</sub> production to solventogenesis (Dauplain et al.,  
213 2021). Finally, a third group of fermentation patterns can be described that are characterized by  
214 low H<sub>2</sub> production with various amounts of lactate, formate or propionate.

215 All this considered, it seems apparent that fine-tuning of process parameters can be used to  
216 promote the activity of the microorganisms involved in a specific metabolic pathway.  
217 Therefore, the knowledge about substrate-microbiome-products relationship can be employed  
218 as a tool to optimize the generation of a targeted product.

219

### 220 **3. Cultivation of microalgae on Dark Fermentation Effluents**

221 Among all the potential microorganisms that can be employed in bioprocesses, microalgae are  
222 considered as one of the most promising for bioproducts generation. Indeed, they can be used  
223 as cell factories to produce not only third-generation biofuels (bioethanol, biodiesel, bioH<sub>2</sub>) but  
224 also higher value-added bioproducts (*i.e.*, cosmetics, pharmaceuticals, nutraceuticals,  
225 biofertilizers, pigments) (Dourou et al., 2021; Patel et al., 2017). The microalgae group gathers  
226 unicellular or multicellular organisms usually able to perform photosynthesis, converting CO<sub>2</sub>  
227 into organic matter using light energy. They can be broadly classified into prokaryotic  
228 (cyanobacteria) and eukaryotic microalgae such as green algae (chlorophyta), red algae  
229 (rhodophyta) and diatoms (Brennan and Owende, 2010). Some microalgae species can use

230 multiple metabolic pathways for growth, depending on the environmental conditions, substrate,  
231 and light availability. This metabolic flexibility enables different cultivation methods, *i.e.*,  
232 phototrophic, heterotrophic and mixotrophic culture.

233 Under phototrophic conditions, microalgae absorb light energy while fixing inorganic carbon  
234 for biosynthesis. Photoautotrophic microalgae cultivation is however usually more expensive  
235 than plant crops because the growth of microalgae requires appropriate light, mixing, pH and  
236 CO<sub>2</sub> and inorganic salt concentration (Yew et al., 2019). Furthermore, light availability and  
237 seasonality, self-shading effects and more generally photosynthetic constraints limit final  
238 biomass concentration and thus their productivity and commercial potential (Gouveia et al.,  
239 2016; Kenny and Flynn, 2017). For this reason, the use of organic carbon sources (under  
240 heterotrophic or mixotrophic cultivation modes) has been suggested to circumvent all those  
241 drawbacks (Hu et al., 2018). As a result, light is less essential while high cell concentrations  
242 and high volumetric productions can be achieved.

243 With all this in mind, VFAs-rich DFE arise as a potential carbon source for microalgae  
244 cultivation in heterotrophy or mixotrophy. Aside from VFAs, DFE also contain mineral  
245 nutrients such as ammonium and orthophosphate which can sustain microalgae growth (Turon  
246 et al., 2016). Therefore, coupling DF to microalgae cultivation would allow the conversion of  
247 mixed VFA from the effluents into valuable biomass, effectively maximizing the carbon  
248 recovery from wastes (**Figure 3**).

249

### 250 **3.1. Heterotrophic growth on Dark Fermentation Effluent compounds**

251 When microalgae are cultivated under heterotrophic conditions, organic compounds provide  
252 both carbon and energy sources to support the microbial growth. However, consumption rates

253 and biomass yields achieved heavily depend on the substrates employed as well as the  
254 considered species (**Table 3**).

255

### 256 ***3.1.1. Acetate: the favoured substrate***

257 Acetate has been consistently reported as the most easily assimilable substrate among the VFAs  
258 found in DFE, given its simple structure and low electron content (Fei et al., 2015; Turon et al.,  
259 2015a; Venkata Mohan and Prathima Devi, 2012). Acetate metabolisation requires thus only a  
260 few enzymatic steps which have been extensively described in detail by several authors  
261 (Johnson and Alric, 2013; Perez-Garcia and Escalante, 2011). However, its uptake and transport  
262 into the cell is not entirely clear. Acids in solution exists under two forms: the undissociated  
263 acid (RH) and the ionic acid form ( $R^-$ ). The undissociated acetic acid form (AcH) is liposoluble  
264 and can thus diffuse passively into the cells, without any active transport requirement. The  
265 anionic acetic acid form ( $Ac^-$ ), however, is thought to be actively imported via a  
266 monocarboxylate/proton (MCT) transporter as described in other eukaryotes (Perez-Garcia and  
267 Escalante, 2011), but no clear evidence can be found in literature. After transport into the cell,  
268 acetate is converted to acetyl-CoA, a central metabolite which can serve as precursor for major  
269 metabolic pathways such as the glyoxylate cycle and the Krebs cycle, which produce  
270 metabolites for further synthesis of amino acids, fatty acids and sugars. Heterotrophic acetate  
271 consumption has been widely evidenced for several microalgae strains including  
272 *Chlamydomonas reinhardtii* (Moon et al., 2013), *Chlorella vulgaris* (Shen et al., 2016), *C.*  
273 *sorokiniana* (Abiusi et al., 2020), *Scenedesmus sp.* (Ren et al., 2018), *Cryptocodinium cohnii*  
274 (Chalima et al., 2019), *Auxenochlorella protothecoïdes* (Fei et al., 2015) or *Euglena gracilis*  
275 (Nakazawa, 2017) In fact, growth rates and biomass yields obtained when using acetate as  
276 carbon source are generally higher than in pure autotrophy (Perez-Garcia and Bashan, 2015).

277

### 278 **3.1.2. Other substrates**

279 Generally, other substrates will not be as easily consumed as acetate, mainly due to the need of  
280 additional enzymatic steps before complete assimilation. Given that most of the research carried  
281 out on carbon metabolism in microalgae focused on acetate, limited information is available  
282 about mechanisms of other carbon compounds transportation and metabolism.

283 Only few studies investigated the effect of butyrate on microalgae growth in well-controlled  
284 conditions, *i.e.*, single strain, axenic conditions and butyrate as single substrate. In human  
285 colonocytes, butyrate is transported via a MCT (Cuff et al., 2005). Butyrate is then imported in  
286 the mitochondrion where it is oxidized into acetyl-CoA (Donohoe et al., 2011). In microalgae,  
287 butyrate metabolism is probably related to butyrate oxidation in the glyoxysomes into acetyl-  
288 CoA via a  $\beta$ -oxidation pathway (Baroukh et al., 2017). This pathway has been mostly  
289 deciphered in the heterotrophic alga *Polytomella* sp. (Lacroux et al., 2022) but remain to be  
290 confirmed in phototrophic algae. Heterotrophic butyrate consumption has been evidenced for  
291 few strains such as *C. sorokiniana* (Patel et al., 2021; Turon et al., 2015a), *Auxenochlorella*  
292 *protothecoïdes* (Patel et al., 2021) and the heterotrophic dinoflagellate *C. cohnii* (Chalima et  
293 al., 2019). Biomass productivity on butyrate is nevertheless about 5- to 10-fold lower than the  
294 one obtained on acetate as carbon source, within a range of 0.1 – 0.29 g/Ld (Lacroux et al.,  
295 2020; Turon et al., 2015a). The only known exception is the heterotrophic alga *Polytomella* sp.,  
296 which exhibits highly efficient butyrate assimilation (Lacroux et al., 2022). Biomass yields (in  
297 grams of biomass per gram of substrate) are, nevertheless, higher on butyrate than acetate since  
298 butyrate contains twice more carbon than acetate.

299 Similarly, little information on lactate metabolism in microalgae is available. Lactate is likely  
300 first converted into pyruvate via the enzyme lactate dehydrogenase (LDH), as found in *C.*  
301 *pyrenoidosa* (*C. sorokiniana*) and *C. reinhardtii* (Gruber et al., 1974; Husic and Tolbert, 1985).  
302 This enzyme can either produce (or oxidize) D-lactate from (or into) pyruvate and it is thought

303 to participate in NADH recycling notably in anaerobic growth (Burgess et al., 2016). However,  
304 several authors evidenced that external lactate is not consumed by *Chlorella* species either in  
305 heterotrophy (Turon et al., 2015a) or mixotrophy (Liu et al., 2013). By contrast, heterotrophic  
306 lactate consumption was reported by some other species such as *Euglena gracilis* (Fujita et al.,  
307 2008), and *Scenedesmus abundans* (Lin et al., 2020).

308 Finally, ethanol consumption also seems to be highly strain-specific even among the same  
309 genus. The ethanol molecule can diffuse passively through the cell membrane and is then  
310 oxidized in acetyl-CoA by an alcohol/aldehyde dehydrogenase (ADH) enzyme (Atteia et al.,  
311 2003). However, *C. reinhardtii* can neither consume ethanol nor butanol even in presence of  
312 the required enzymes (Catalanotti et al., 2013; Jiang et al., 2017). Ren et al. (2018b) cultivated  
313 an isolated *Scenedesmus* sp. in heterotrophic conditions on an effluent containing several  
314 substrates (acetate, butyrate, propionate, valerate, ethanol) resulting in the exhaustion of all  
315 substrates but not ethanol, even at the lowest concentration of 0.15 g/L. By opposite, other  
316 species such as *Polytomella* sp. has been shown to grow on ethanol as concentrated as 40 mM  
317 (Atteia et al., 2000).

### 318 **3.1.3. Behaviour in mixtures**

319 The profile and concentration of all these compounds may differ among DFE. In the presence  
320 of multiple carbon sources, microorganisms tend to use the simplest one first and then the more  
321 complex ones. The phenomenon is known as carbon catabolite repression and has been  
322 extensively discussed for bacteria (Görke and Stülke, 2008). In the case of microalgae, a diauxic  
323 growth behaviour, where acetate is always consumed before butyrate, was evidenced for *C.*  
324 *sorokiniana* and *Auxenochlorella protothecoïdes* growing in heterotrophy (Turon et al., 2015a)  
325 or mixotrophy (Lacroux et al., 2021a). Considering the easy acetate assimilation, the presence  
326 of this acid has always been reported to improve biomass productivities (Fei et al., 2015; Turon  
327 et al., 2015a, 2015c; Venkata Mohan and Prathima Devi, 2012). Diauxic behaviour was

328 evidenced by Fei et al. (2015) as well, who investigated the effect of VFA ratio on the growth  
329 of *A. protothecoïdes* in synthetic mixtures. An acetic:propionic:butyric acid mixture at an ratio  
330 8:1:1 was the most beneficial for growth, nearly doubling final biomass as compared to a 4:3:3  
331 ratio. Similarly, Kim et al. (2019) reported the maximal growth of *C. vulgaris* on a medium  
332 containing a 6:1:3 mixture ratio, concluding that the presence of acetate as major component  
333 also boosted butyrate consumption two-fold. Patel et al. (2022) recently cultivated *A.*  
334 *protothecoïdes* and *C. sorokiniana* on VFAs from acidogenic fermentation of waste  
335 lignocellulosic biomass from brewers' spent grain, initially containing high amounts of acetate,  
336 propionate and butyrate (10.07, 0.81 and 1.24 g/L respectively). Interestingly, they obtained  
337 high biomass and lipid productivities in comparison with other studies using similar mixtures  
338 (**Table 3**), probably owing to the high acetate concentration in the medium. A sequential VFAs  
339 assimilation was also observed by the authors, acetate being consumed first followed by  
340 butyrate and propionate.

#### 341 **3.1.4. Mechanisms of substrate inhibition**

342 The major DF compounds (*e.g.*, VFAs, ethanol) can exert an inhibitory effect on microalgae at  
343 high concentrations. The ethanol toxicity on microalgae highly depends on concentration and  
344 the species studied. Few strains such as *Dunaliella tertiolecta*, *Isochrysis galbana*, *Monodus*  
345 *subterraneus* or *Spirulina platensis* show a relatively high tolerance to ethanol concentrations  
346 above 15 g/L (Miazek et al., 2017). *C. reinhardtii* was not able to grow on 23.6 g/L ethanol and  
347 4 g/L butanol and exhibited a 50% growth reduction when cultivated in presence of 14.2 g/L  
348 ethanol or 2.4 g/L butanol. It should be mentioning that these ethanol concentrations are not  
349 commonly attained during DF. According to Moscoviz et al., unless operating under specific  
350 conditions, an average ethanol concentration of 0.5 g/L has been commonly found in DFE  
351 (Moscoviz et al., 2018).

352 Regarding acetate, Chen and Johns, (1994) reported for the first time a reduction of *C.*  
353 *reinhardtii* maximal heterotrophic growth rate on acetate at concentrations above 0.5 g/L. They  
354 concluded that acetate was an inhibitory substrate. Similarly, VFA mixtures over 8 g/L were  
355 found to be inhibitory for *A. protothecoïdes* and an increase in lag phase duration was observed  
356 when increasing VFAs concentration from 2 g/L to 4 g/L (Fei et al., 2015). Turon et al. (2015a)  
357 stated that *C. sorokiniana* and *A. prothotecoïdes* heterotrophic growth was negatively affected  
358 when using VFAs mixtures over 2.5 g/L acetate or 0.4 g/L butyrate. The sensitivity to VFAs is  
359 besides highly strain dependant. For example, Cheng et al. (2021) found that growth rate of  
360 *Scenedesmus obliquus* was increasingly lowered with increasing concentration of acetate as low  
361 as 50 mg/L. As a result, microalgae cultivation on these substrates has been generally performed  
362 at low total VFA concentrations (<4 g/L).

363 The acid inhibitory effect is caused by the undissociated form of the acid (RH) at high  
364 concentrations. Indeed, this undissociated form is liposoluble and can cross the cell membrane  
365 into the cell, where it will dissociate causing cytosolic acidification. The export of this internal  
366 dissociated form out of the cell is energy consuming, resulting in ATP depletion when RH  
367 concentration is in excess. As a result, a lack of energy available for division causes the cells to  
368 stop growing (Russell, 1992). RH concentration in solution directly depends on the acid  
369 concentration and the pH of the medium and it can be calculated by the Henderson-Hasselbalch  
370 equation (Eq.1) where  $C_t$  refers to the total acid concentration (g/L).

371 
$$[RH] = \frac{C_t}{1+10^{pH-pKa}} \quad (\text{Eq.1})$$

372 The detrimental effects of RH on bacterial or yeast cells have been well documented  
373 (Giannattasio et al., 2013). In contrast, the literature on microalgae is scarce, especially for  
374 molecules other than acetate. Lacroux et al. (2020) determined the acetate and butyrate  
375 inhibition threshold of four chlorophyte strains (*i.e.*, *Acutodesmus obliquus*, *Auxenochlorella*



376 *protothecoïdes*, *Chlamydomonas reinhardtii* and *Chlorella sorokiniana*). The growth of those  
377 strains was found to be inhibited at concentration ranges of 47 to 207 mg/L acetic acid and 12.5  
378 to 50 mg/L butyric acid depending on the studied strain.

379 Therefore, knowing the pH of the fermentation effluent and the metabolite concentration, RH  
380 concentration in DFE can be calculated. According to the determined concentration and the  
381 strain tolerance to RH, an accurate pH control or effluent dilution needs to be employed to avoid  
382 the inhibitory effect exerted by acids. For instance, the concentration of acetic acid and butyric  
383 acid in the experiments conducted by Fei et al. (2015) were of 147 mg/L and 73 mg/L  
384 respectively (pH of 6.3; total concentration of acetate 4.8 g/L and butyrate 2.4 g/L). These  
385 concentrations were in the range of the inhibitory thresholds given above, which could explain  
386 while inhibition occurred. Besides, the fact that these substrates were mixed could have further  
387 aggravated their toxic effects.

388

### 389 **3.2. Tuning the mixotrophic process**

390 Besides heterotrophy, microalgae can also be cultivated on organic substrate under mixotrophic  
391 conditions. Mixotrophic growth combines phototrophic and heterotrophic modes and therefore  
392 a simultaneous consumption of organic and inorganic carbon sources occurs in presence of light  
393 to obtain both carbon and energy (Perez-Garcia and Bashan, 2015). Mixotrophy usually results  
394 in enhanced growth rates and biomass yields when compared to purely autotrophic or  
395 heterotrophic conditions, as these two latter processes may occur non-competitively and  
396 simultaneously (Pang et al., 2019). For instance, when growing *C. sorokiniana* on glucose under  
397 mixotrophy, 4.57 g/L biomass was obtained while 1.7 g/L and 2.78 g/L biomass were reached  
398 under autotrophic and heterotrophic conditions, respectively (Li et al., 2016). However, care

399 should be taken when interpreting and extrapolating mixotrophic results, mainly due to the  
400 complex interactions occurring between the heterotrophic and autotrophic metabolisms.

401 In some instances, mixotrophic growth rates can indeed be higher to the sum of autotrophic and  
402 heterotrophic growth rates since synergic effects occur between the two metabolisms, allowing  
403 a more efficient use of carbon and energy (Zhang et al., 2017). Regarding CO<sub>2</sub> fixation, internal  
404 recirculation of the CO<sub>2</sub> coming from cellular respiration can occur in mixotrophic cultivation.  
405 This allows to reach biomass yields up to 1 gC<sub>X</sub>/gC<sub>S</sub> (Abiusi et al., 2020) while reducing net  
406 CO<sub>2</sub> emissions compared to heterotrophic cultures (Smith et al., 2015). This increased internal  
407 CO<sub>2</sub> concentration was evidenced on the gene expression level by Cecchin et al. (2018) who  
408 found that acetate presence caused upregulation of the phosphoenolpyruvate carboxylase  
409 enzyme. Aside from maximizing the carbon recovery, environmental assessments advocate  
410 mixotrophic cultivations where CO<sub>2</sub> is fixed contributing in the reduction of CO<sub>2</sub> emissions,  
411 instead of heterotrophy (Hu et al., 2018). Regarding light, microalgae cultivated in mixotrophy  
412 can withstand higher light intensities compared to autotrophy. Indeed, the presence of acetate  
413 reduces the photo-inhibition by interacting with the photosystem PSII, which reduces  
414 production of oxygen radicals (Roach et al., 2013). For instance, when *C. sorokiniana* was  
415 cultivated on acetate under mixotrophy, Xie et al. (2016) showed that high light intensity (up  
416 to 800 μE/m<sup>2</sup>/s) resulted in a positive effect on acetate assimilation and thus, in the microalgae  
417 growth rate. In contrast, autotrophic growth rate decreased by 20% under high light intensities  
418 (800 μE/m<sup>2</sup>/s) compared to the low intensity tested (90 μE/m<sup>2</sup>/s). In a specific light condition,  
419 growth rates in mixotrophy can thus be higher than expected (Xie et al., 2016). Finally, organic  
420 carbon uptake by some strains such as *C. sorokiniana* or *A. protothecoïdes*, especially butyrate,  
421 has been demonstrated to be light-dependent (Turon et al., 2015c). Although the reasons are  
422 not entirely deciphered, it is probable that light provides the necessary energy to metabolize

423 this organic substrate. Consequently, a null growth in heterotrophy could still result in a higher-  
424 than-expected growth rate in mixotrophy due to specific interactions.

425 On the contrary, mixotrophic growth rates and yields can also be lower than the sum of the  
426 autotrophic and heterotrophic one. For example, high level of easily assimilable substrates such  
427 as acetate may reduce photosynthetic activity. Heifetz et al. (2000) showed that the presence of  
428 29.4 mM acetate reduced external CO<sub>2</sub> fixation to 66% compared to the autotrophic control  
429 while not affecting the growth rate, indicating that organic carbon assimilation was favoured  
430 over inorganic carbon capture. This is probably due to the binding of acetate to PSII, which  
431 reduced its activity (Roach et al., 2013). Similarly, the influence of inorganic carbon on the  
432 organic carbon uptake is not completely elucidated. Liu et al (2013) also showed that  
433 supplementing a medium containing 2.72 g/L bicarbonate with several concentrations of  
434 butyrate (from 0.5 to 1.8 g/L), resulted in a concomitant increase in *C. vulgaris* ESP-6 biomass  
435 yield. These studies suggest that organic carbon assimilation always occurs compared to  
436 autotrophic growth when possible, irrespective of CO<sub>2</sub> or bicarbonate concentration. However,  
437 contradictory results found by other authors indicate that inorganic carbon does influence the  
438 mixotrophic process. For example, Liu et al. (2013) showed a microalgae growth inhibition on  
439 butyrate (0.5 g/L) when bicarbonate concentration is above 2.72 g/L. Sforza et al. (2012)  
440 showed that when bubbling 5% CO<sub>2</sub>, glycerol consumption by *Chlorella protothecoïdes* was  
441 reduced by 3.8-fold compared to cells grown under atmospheric CO<sub>2</sub> concentration (0.04%  
442 CO<sub>2</sub>). Similarly, in the case of *Nannochloropsis salina*, switching from atmospheric CO<sub>2</sub>  
443 concentration to 5% CO<sub>2</sub> conditions, almost a complete inhibition of the glycerol consumption  
444 was reported. Thus, the influence of inorganic carbon in the mixotrophic process should be still  
445 elucidated. More specifically, the ratio of inorganic/organic carbon regarding light availability  
446 and organic substrate nature, should be investigated to unravel the interactions between  
447 heterotrophic and autotrophic metabolisms.

## 448 4. Approaches to enhance microalgae cultivation on Dark Fermentation

### 449 Effluents

450 Two major challenges should be faced in order to reach an optimal integration of DF and  
451 microalgae cultivation and attaining sustainable and economically competitive combination of  
452 these two bioprocesses. Firstly, microalgal biomass productivity is relatively low, and the VFA  
453 assimilation rate by microalgae is projected to be lower than the VFA production rate by DF.  
454 Indeed, between 6-15 days are usually needed for the algae to grow and completely remove  
455 VFAs (Lacroux et al., 2021a), while DF is usually carried out with HRT around 12 hours (Ren  
456 et al., 2018). Secondly, high concentrations of the major DF compounds (e.g., acetate, butyrate,  
457 ethanol, lactate) can inhibit proper microalgae growth. This limits microalgae cultivation to low  
458 VFA concentration, usually in the range of 1-2 g/L (Li et al., 2020). The following sections aim  
459 to describe the causes of these limitations and the proposed solutions to overcome these  
460 challenges.

### 461 4.1. Dark Fermentation strategies

462 DFE often present higher concentration of VFA with chains longer than acetate (Moscoviz et  
463 al., 2018). Therefore, to optimize the coupling between DF and microalgae cultivation, special  
464 attention must be paid to the factors affecting the microbial activity during DF (as previously  
465 explained in Section 2) which ultimately determines the fate of the organic matter and the acid  
466 concentration and profile. However, those factors (*i.e.* feedstock and inoculum employed or  
467 operational parameters applied) should be optimized to drive DF towards suitable metabolites  
468 without sacrificing H<sub>2</sub> yields.

#### 469 4.1.1. Substrate and nutrients

470 Widely available and low-cost organic waste are potential feedstock sources for DF, such as  
471 waste activated sludge, algal biomass, lignocellulosic-based biomass or food waste (Guo et al.,  
472 2010). The macromolecular composition of a given substrate affects bioconversion yields due  
473 to the different hydrolysis rates of carbohydrates, proteins and lipids (Angelidaki and Sanders,  
474 2004). Although the nature of the organic substrates is complex and their composition diverse,  
475 it is well known that H<sub>2</sub> yield is directly correlated to the soluble carbohydrates content  
476 (Jarunglumert et al., 2021) while proteins and lipids contributions are not significant. Besides,  
477 it has been demonstrated that the macromolecular substrate composition can significantly affect  
478 the metabolite profile obtained. Regueira et al. (2020a) stated that odd-chain acids (especially  
479 propionic acid) are mainly associated to protein-rich substrates such as microalgae biomass. By  
480 contrast, even-carbon number VFAs (acetic and butyric acid) have been reported to prevail  
481 during DF of carbohydrate-rich substrates. A compilation of H<sub>2</sub> yields and metabolites profile  
482 generated during DF of different substrates are summarized in **Table 2**.

483 The C:N ratio has been reported as key parameter in DF as well. Optimal C:N ratios ranging  
484 between 5 and 200 have been reported in the literature for DF using different configurations  
485 and operational parameters (Elbeshbishy et al., 2017). For instance, after testing several C:N  
486 ratios (40-130) using sucrose as substrate and *Clostridium pasteurianum* as inoculum. Lin and  
487 Lay (2004) reported an optimum H<sub>2</sub> production (4.8 mol H<sub>2</sub>/mol sucrose) applying C:N ratio  
488 of 47. Those authors concluded that higher C:N ratios (> 47) lead to a low H<sub>2</sub> production due  
489 to nitrogen-limited growth while lower ratios (C:N < 47) lead to potential free ammonia  
490 inhibition. Regarding the metabolic profiles, acetate and propionate fractions increased by 75%  
491 and 90% when C:N ratio increased from 47 to 130, respectively, suggesting that butyrate  
492 fermentation shifted towards acetate fermentation. Conversely, Anzola-Rojas and co-workers  
493 (2015) studied C:N ratios from 40 to 190, reporting an optimal C:N ratio of 137 for a maximum  
494 H<sub>2</sub> yield of 3.5 mol H<sub>2</sub>/mol sucrose. These authors did not find a clear influence of the C:N ratio

495 on the fermentation pathways, since similar metabolic profiles in terms of ethanol, acetic acid  
496 and butyric acid were produced.

#### 497 **4.1.2. Anaerobic inoculum**

498 To ensure H<sub>2</sub> production from organic matter, fermenters are often inoculated with HPB issued  
499 from various environments (anaerobic sludge, aerobic sludge, sediments) (Etchebehere et al.,  
500 2016b). These inocula also host various non-HPB such as lactic acid or propionic bacteria as  
501 well as H<sub>2</sub>-consuming organisms such as homoacetogenic bacteria and methanogenic archaea,  
502 that inevitably reduce the DF yield by either outcompeting for the substrate or consuming the  
503 desired product. Therefore, HPB inoculum enrichment methods have been investigated to  
504 improve H<sub>2</sub> yields. A way of selecting some HPB relies on their ability to form spores, such as  
505 *Clostridium sp.* Thus, inoculum are commonly pre-treated by applying a physical (thermal  
506 shock, micro-aeration, irradiation, sonication) or chemical stress (pH, 2-  
507 bromoethanesulphonate) (Rafieenia et al., 2018). Recently, Luo et al. (2022) compared different  
508 inoculum pretreatment methods aiming to maximize the bioconversion of food waste into H<sub>2</sub>.  
509 They concluded that the alkali-treated inoculum exhibited the highest H<sub>2</sub> yield (157 mL H<sub>2</sub>/VS)  
510 corresponding to a 70% improvement in comparison to control experiment.

511 At this point it is worth to highlight that different microbial populations lead to different  
512 distribution of soluble products (as described in Section 2). It has been reported that acetic-  
513 butyric acid rich effluents were obtained when using heat-shock and alkaline pre-treated  
514 inocula, while acetic and propionic acid were the main products when and acid pre-treated  
515 inoculum was employed, and ethanol is produced when aeration pre-treatment is applied (Ren  
516 et al., 2008)

#### 517 **4.1.3. Operational parameters**

518 H<sub>2</sub> yields and production rates can be enhanced by optimizing the operational and design  
519 parameters of DF bioreactors (*i.e.*, temperature, pH, hydraulic retention time (HRT), organic  
520 loading rate (OLR) and partial pressure of H<sub>2</sub>) which, in turn, can control the microbial's  
521 metabolism.

522 Several studies have correlated H<sub>2</sub> production performances with the microbial population  
523 density. Therefore, different cell retention strategies such as the use of granulated sludge and  
524 biofilm in those reactors have been employed (Etchebehere et al., 2016a). Besides the specific  
525 advantages of these different bioreactor configurations, HPB selection can also be ensured by  
526 taking advantage of the differences in growth of the various microorganisms. In continuous  
527 operation, differences in growth rates among the microorganisms enable selection of HPB by  
528 shortening the hydraulic retention time (HRT). Short HRT (6-12 h) are indeed more favourable  
529 for H<sub>2</sub> producers, while longer HRT (18-24 h) negatively affects the H<sub>2</sub> yields due to microbial  
530 community shifts (Palomo-Briones et al., 2017). In the same way, pH and temperature are  
531 crucial parameters for H<sub>2</sub> synthesis. Acidic pH values (around 5.5) inhibits the methanogenic  
532 archaea activity while allowing maximum H<sub>2</sub> production (Liu et al., 2008). However, pH lower  
533 than 4.5 tends to cause metabolic shifts in *Clostridium* sp. towards solventogenesis (*i.e.*,  
534 acetone-butanol-ethanol) due to the accumulation of undissociated VFAs (Van Ginkel and  
535 Logan, 2005). Operational pH affects the metabolic by-products as well. In most of the studies,  
536 neutral pH favours the acetate pathways, while acidic pH conditions favour the butyrate  
537 production pathways (Ghimire et al., 2015). Temperature affects not only the microbial activity  
538 shifting the DF products, but also the physical state of the organic matter. Several authors  
539 reported higher H<sub>2</sub> yields at thermophilic than mesophilic temperatures when using organic  
540 wastes as substrate (Shin et al., 2004; Valdez-Vazquez et al., 2005). In fact, it is known that  
541 inhibition of the H<sub>2</sub>-consuming homoacetogenic activity can be achieved under thermophilic  
542 conditions (Luo et al., 2011). In terms of VFAs production, acetic acid was reported as a

543 dominant by-product in thermophilic digestion, whereas butyrate is mainly formed in  
544 mesophilic conditions (Liu et al., 2008).

545 Butyrate inhibition can be reduced by increasing the acetate content in the medium (Baroukh  
546 et al., 2017; Fei et al., 2015). In this way, microalgae can quickly build up biomass from acetate  
547 leading to a decrease in the butyrate to biomass ratio. Nevertheless, the potential inhibition by  
548 acetate should not be neglected when using this strategy. During the DF process, acetate can be  
549 also produced via homoacetogenesis (Pavlostathis and Giraldo-Gomez, 1991). Therefore,  
550 maximizing acetate concentration in DFE could be done by applying the proper favourable  
551 conditions to ensure homoacetogenic bacteria activity (i.e. increasing HRT) (Siriwongrungronson  
552 et al., 2007). However, this strategy likely decreases the H<sub>2</sub> yield obtained during the process  
553 (Saady, 2013).

554 Kim et al. (2019) investigated the growth of *C. vulgaris* on different effluents produced from  
555 the DF of algal biomass. By adjusting DF operational parameters (*e.g.* temperature, pH and  
556 HRT), they obtained different fermentation profiles, either propionate- (ratio 5:4:1) or acetate-  
557 rich (ratio 6:1:3). Maximum algal biomass was achieved for the latter ratio, mainly because the  
558 strain was unable to consume propionate.

559 Since changes in DF conditions can affect the H<sub>2</sub> productivities, major efforts must be made to  
560 optimize both DF and the microalgae cultivation bioprocess.

## 561 **4.2. Microalgae cultivation strategies**

562 Microalgae cultivation on DFE can be improved by optimizing the reactor design, operational  
563 parameters or product-recovery techniques. Additionally, the use of newly isolated or  
564 developed microalgae strains resistant to the metabolites usually found in DFE as well as co-



565 cultures with heterotrophic organisms, can also be promising approaches to circumvent  
566 challenges associated with DFEs.

#### 567 **4.2.1. pH control**

568 Although an effluent can be simply diluted to decrease the total acid concentration and avoid  
569 inhibition, the resulting lower final biomass concentration increases the biomass harvesting  
570 costs. Lowering RH to below toxicity levels can also be achieved by increasing the initial pH.  
571 For example, growth of *C. sorokiniana* was completely inhibited at pH 6.0 by 0.8 g/L butyrate,  
572 while increasing the pH to 7 allowed growth at the same total butyrate concentration (Lacroux  
573 et al., 2020). When further raising the pH to 8.0, Lacroux and co-workers (2021) could cultivate  
574 *C. sorokiniana* on 8 g/L butyrate without any inhibition. On the opposite side, when growth and  
575 organic acid consumption occur, pH of the medium will inevitably rise (Chalima et al., 2019;  
576 Lacroux et al., 2021b). From these studies, the upper pH value tolerated by the algae seems to  
577 be around 9, with pH values around 10 causing the complete inhibition of acetate or butyrate  
578 consumption. pH should thus be tightly controlled to remain around neutral values during  
579 growth. For example, acetate removal by *C. sorokiniana* was found to decrease by 70% when  
580 increasing the pH from 8 to 9 (Lacroux et al., 2020). Lacroux et al. (2021) almost doubled both  
581 biomass production and substrate consumption during batch cultivation of *C. sorokiniana* on  
582 2.5 g/L acetate using buffer mediated pH control (0 mM to 100 mM). For the cultivation of *C.*  
583 *cohnii*, a fed-batch pH-auxostat strategy was adopted since the pH was found to increase up to  
584 8.9 in spite the presence of buffer (Chalima et al., 2019). Since VFAs-rich effluent is acidic, pH  
585 could be lowered through VFA addition. Using a similar strategy, a final biomass of 22 g/L was  
586 obtained using acetate in fed-batch, compared to a maximum of around 6 g/L achieved in batch  
587 (Chalima et al., 2019). Cho et al. (2015a) found that the biomass production rate of *C. vulgaris*  
588 on a concentrated effluent (13.7 gCOD/L as VFA) improved from 296 to 433 mg/L/d when  
589 maintaining the pH between 7 and 8.5.

590

#### 591 **4.2.2. Initial microalgae inoculum**

592 The inhibitory effect of the acids can also be reduced by increasing initial microalgae density  
593 of the inoculum or by decreasing the substrate to biomass ratio S/X. As an illustration, *C.*  
594 *vulgaris* butyrate consumption and biomass production rate were increased by respectively 2.5-  
595 fold and 2-fold when decreasing S/X from 8.0 to 1.5 (Liu et al., 2013b). Similarly, the growth  
596 of *C. reinhardtii* could not be observed on DFE effluent containing more than 2 g/L total VFA  
597 when S/X was above 20. Increasing inoculum density 21 times enabled growth on 2.5 g/L total  
598 VFA (Radhakrishnan et al., 2021).

599

#### 600 **4.2.3. Light intensity**

601 Light can provide the necessary energy to deal with inhibitory effects: biomass can grow based  
602 on autotrophic metabolism and, once sufficient biomass concentration had been reached,  
603 butyrate consumption can start. In fact, light has been shown to alleviate heterotrophic growth  
604 inhibition of *C. sorokiniana* on butyrate (Turon et al., 2015c). Butyrate uptake by *C.*  
605 *sorokiniana* was not observed in darkness, while it was promoted under 100  $\mu\text{E}/\text{m}^2/\text{s}$  continuous  
606 illumination (Turon et al., 2015c). Similarly, butyrate consumption by *C. vulgaris* increased  
607 from 10% to almost 100% when switching from dark conditions to illumination with 150  
608  $\mu\text{E}/\text{m}^2/\text{s}$  (Liu et al., 2013b). It should be highlighted that raw fermentation effluents may present  
609 a dark colour as well as a high solid content. These specific characteristics may severely reduce  
610 light penetration in the reactor bulk and could thus prevent mixotrophic growth. DFE also likely  
611 contains dissolved inorganic carbon as a result of bacterial respiration (Liu et al., 2013a).  
612 Therefore, since light presence is essential for the consumption of organic substrates and  
613 microalgae growth, DFE pre-treatments or dilution may have to be considered.

614 Besides light intensity, light wavelength is another important factor to be considered during  
615 mixotrophic cultivation. Although the exact wavelength required for photosynthesis depends  
616 on the species and its internal pigment composition, the blue (420-470 nm) and red (660 nm)  
617 lights usually promotes best autotrophic microalgae growth (Schulze et al., 2014). However,  
618 very few studies investigated the influence of light colours on DFE substrates assimilation. In  
619 the case of acetate, *S. abundans* final biomass increased to 0.82 g/L using red light as compared  
620 to 0.52 g/L using white light (Gupta and Pawar, 2018). Similarly, optimum *Dunaliella salina*  
621 biomass productivity was obtained on 4 g/L using a combination of 65% blue and 35% green  
622 light (Bredda et al., 2020). As discussed in section 3.2, it remains unclear whether the boost in  
623 productivity is only due to the increase of autotrophic activity or to potential positive  
624 interactions.

625

#### 626 **4.2.4. Nutrients requirements (C:N:P balancing)**

627 Alongside carbon, microalgae need mineral nutrients such as nitrogen (N) and phosphorus (P)  
628 for the synthesis of various biomolecules such as nucleic acids and amino-acids. Optimum  
629 nutrient requirements of microalgae can be determined by estimating their elemental  
630 composition under non-limiting conditions in which their maximum growth rate is achieved. A  
631 common molar C:N:P given for microalgae is the Redfield ratio 106:16:1 (Redfield, 1958).  
632 This ratio is however an average and strong variations can be found among species and  
633 cultivation conditions.

634 Under high C:N:P conditions, growth rates tend to diminish to benefit carbon storage  
635 compounds such as lipids and carbohydrates. For example, *C. vulgaris* biomass productivity  
636 dropped from 137 to 70 mg/L/d when switched from N- and P- rich conditions to N- and P-  
637 limited conditions while a nearly 3-fold increase in lipid yield was attained (Shen et al., 2016).  
638 That strain could still assimilate up to 362.8 mg/L acetate even under complete N and P

639 depletion. When cultivating *A. prothotecoïdes* and *C. sorokiniana* on a mixture of VFAs in  
640 heterotrophy, Patel et al. (2021) reported an increase in lipid content from 10% to 30% for both  
641 strains when increasing the C:N ratio from 20 to 60. The C:N ratio increment did not affect the  
642 acetate, propionate and butyrate consumption rates which were exhausted at the end of the  
643 culture. However, longer-chain VFAs (valerate and caproate) could not be further consumed.

644 Adjusting C:N:P ratio in the medium to the biomass requirements could be done by mixing  
645 DFE with other N or P sources with a poor C content (*e.g.* AD effluent or another wastewater).  
646 For example, Chiranjeevi and Mohan (2017) produced lipids from microalgae using a dual  
647 growth phase strategy, using a nutrient-rich effluent for a growth step followed by a lipid  
648 accumulation step on a nutrient-poor effluent. They observed an increase in carbohydrate  
649 content during growth phase from 0.15 up to 0.4 g/g. As the stress phase was extended, the  
650 amount of carbohydrates later decreased down to 0.3 g/g while microalgae accumulated lipids  
651 up to 0.35 g/g.

652

#### 653 ***4.2.4. Isolation and screening of new microalgae***

654 Most of the studies published on the coupling of DF and microalgae cultivation processes  
655 focused on photosynthetic strains such as *Chlorella* or *Scenedesmus* species (**Table 3**). As  
656 detailed earlier, these species seem limited in their ability to grow on DFE. Expanding  
657 collection screening to other species and especially phyla other than chlorophytes appear  
658 necessary. Currently, selecting a microalgal strain for growth on DFE based on metabolic and  
659 biochemical traits is rather challenging due to the little data accumulated on the subject and the  
660 great diversity in algal phylogeny. However, some species may appear more suitable than  
661 others. For example, *Euglena gracilis* and related species could be promising candidates given  
662 their ability to consume organic acids and ethanol while presenting photosynthetic properties

663 and accumulating paramylon, a valuable polysaccharide which structure is similar to starch  
664 (Santek et al., 2012). Alternatively, purely heterotrophic species could be more adapted to grow  
665 on organic acids. For instance, (Chalima et al., 2019) cultivated the heterotrophic marine  
666 microalgae dinoflagellate *C. cohnii* on various single VFA (acetic, propionic and butyric acid)  
667 for the production of docosahexaenoic acid (DHA). The strain was able to grow and consume  
668 acetic, propionic and butyric acids at 30 g/L, 10 g/L and 15 g/L, respectively. The microalgae  
669 performance was further evaluated on a DFE permeate: the strain could remove all organic  
670 carbon in only 60 h, which is relatively fast considering the growth rates of green strains.  
671 Similarly, when screening for butyrate consuming strains, Lacroux et al, 2022, found that the  
672 heterotrophic strain *Polytomella* sp. grew at constant growth rates of 3.8 d<sup>-1</sup> up to 38 g/L  
673 acetate and 2.5 d<sup>-1</sup> up to 18 g/L butyrate. However, the main advantage of using microalgae  
674 *i.e.* CO<sub>2</sub> fixation is lost. Secondly, isolation of strains from the environment could be another  
675 step in improving the coupling (Lacroux et al., 2022). Ren et al. (2013) could for example  
676 isolate a new *Scenedesmus* strain by screening the lipid content of 88 isolates using a Nile red  
677 staining method. They could isolate a lipid accumulating strain able to consume most of the  
678 organic compounds except ethanol when cultivated on DFE (Ren et al., 2018). Isolation criteria  
679 should not only be based on the type of storage compound but also on the ability of the strain  
680 to consume the organic acids present in DFE.

681

#### 682 **4.2.5. Genetic Modifications/ adaptive evolution**

683 The model strains could be improved through adaptive laboratory evolution experiments  
684 (ALE). ALE is a powerful tool enabling the selection of microorganisms with higher fitness to  
685 a given environment. Besides, the phenotypic adaptation can be further linked to genotypic  
686 changes thanks to omics techniques, allowing to unravel the mechanisms leading to the desired  
687 traits (Dragosits and Mattanovich, 2013). This technique has for example been used to generate

688 various microalgae strains resistant to different environmental stresses (Zhang et al., 2021). In  
689 the case of DF coupling, the growth rate of *Auxenochlorella protothecoïdes* on butyrate  
690 improved by nearly 3-fold after three growth cycles on this substrate (Turon et al., 2015a).  
691 Although hardly qualifiable as evolution, these results show that adaptation to VFA is possible  
692 using the consumption of one of the organic acids as a selection factor.

693

#### 694 **4.2.6. Microalgae co-cultivation**

695 Finally, microalgae could be co-cultivated with heterotrophic organisms, more adapted to  
696 degrade complex organic compounds present in the DFE compared to microalga. The  
697 fermentative communities have for example been advantageously used to increase VFA  
698 removal rates while reducing the need of sterilization. As an example, Turon et al. (2015b)  
699 cultivated *C. sorokiniana* in heterotrophy on a VFA rich effluent (0.74 g/L acetate, 1.25 g/L  
700 butyrate) containing the fermentative bacteria. They showed that, in heterotrophy, the algae  
701 could outcompete bacteria for acetate due to drastic change from anaerobic to aerobic  
702 conditions. Once the aerobic community developed, butyrate was however only consumed by  
703 bacteria (Turon et al., 2015a).

704 Mixotrophic cultivation can further promote microalgae growth, by taking advantage of the  
705 synergetic interactions between phototrophic and heterotrophic species. Indeed, under light, the  
706 CO<sub>2</sub> produced by heterotrophic respiration can be further photosynthetically fixed by  
707 microalgae (Sial et al., 2021). For example, Qi et al., (2018), cultivated *C. sorokiniana* on a  
708 synthetic fermentation effluent containing ethanol (0.16 g/L), butanol (0.11 g/L), acetate (0.21  
709 g/L) and butyrate (0.93 g/L) with three different bacterial species (*Exiguobacterium*  
710 *aurantiacum*, *Stenotrophomonas acidaminiphila* and *Chryseobacterium scophthalmus*). The  
711 presence of bacteria always improved the final microalgal biomass concentration by around

712 40% compared to the control. This result was mainly explained by the increased total COD  
713 removal rate in presence of bacteria, which simultaneously increased the dissolved CO<sub>2</sub>  
714 concentration.

715 Algal mixotrophy can also be used to design an anaerobic-microaerobic consortium. Indeed, in  
716 mixed culture, bacteria and microalgae should compete not only for organic substrates but also  
717 for oxygen (Sforza et al., 2018). By controlling the amount of dissolved oxygen produced by  
718 microalgae and in absence of external oxygen, simultaneous cultivation of fermentative bacteria  
719 and microalgae is possible. This strategy was followed by Ren et al. (2015) who could  
720 simultaneously produce hydrogen and lipids from various starch-rich wastewaters using an  
721 anaerobic sludge - *Scenedesmus* consortium. The use of the symbiotic consortium always  
722 resulted in an improved COD and mineral nutrient removal efficiency by almost 4-fold  
723 compared to anaerobic sludge alone. As a result, residual VFA concentration was minimal and  
724 total energy conversion efficiency was almost doubled.

725

## 726 **5. Microalgal biorefinery**

### 727 **5.1. Microalgae applications**

728 Microalgae have been the focus of a large body of research due to their capacity to produce not  
729 only biofuels but also high value-added products (Siddiki et al., 2022). However, when  
730 microalgae are cultivated on residual effluents, this biomass cannot be used for human  
731 consumption but another type of lower cost product biorefinery can be envisaged.

732 Microalgae have been successfully cultivated on various DFE, mainly for biolipids production  
733 purposes (Sajjadi et al., 2018). The main strains used for such a coupling are *Auxenochlorella*

734 *protothecoïdes*, *Chlorella* sp. and *Scenedesmus* sp. either for their high lipid content, their  
735 ability to grow in hetero- or mixotrophy on VFA and their overall robustness.

736 Recent studies on the coupling of DF and microalgae cultivation were focused on the use of  
737 synthetic VFA as model substrates or the use of effluent obtained from glucose DF as model  
738 effluent. These studies demonstrated the economic potentialities of algae production from DFE  
739 (Fei et al., 2015). Since then, microalgae cultivation has been successfully carried out on real  
740 waste streams, either from food waste, starch-rich wastewater or lignocellulosic biomass. For  
741 example, Ren et al. (2018) compared the performances of the coupled system with three  
742 simulated wastewaters (protein, fat, or carbohydrates rich). Overall, starch-rich wastewater was  
743 the most appropriate for both H<sub>2</sub> production (134 mg/L substrate) and microalgae cultivation  
744 (100% VFA removal, 52.6 mg/L/d lipid productivity). The coupled system improved the global  
745 energy conversion efficiency by two-fold compared to DF alone. Similarly, Ren et al. (2019)  
746 obtained a 17% increase in energy conversion efficiency (in comparison to DF alone) when  
747 applying a two-step process for DF of agricultural waste and further microalgae cultivation on  
748 DFE rich in acetate and butyrate, obtaining a co-production of 811 mL H<sub>2</sub>/L and 58 mg/L/d of  
749 algal lipids. Mu et al. (2020) used duckweed biomass as a DF substrate to produce 170 mL H<sub>2</sub>/g  
750 substrate. *C. saccharophila* was subsequently cultivated on the acidogenic effluent, effectively  
751 removing 70% VFA and all residual nitrogen while producing up to 0.27 g/L lipids. In another  
752 study, high starch wastewater was co-digested with poultry manure to produce a maximum of  
753 5.03 mol H<sub>2</sub>/kg COD reduced. The liquid effluent was then used to cultivate *C. reinhardtii*,  
754 yielding a biomass concentration of up to 1.45 g/L associated with a lipid yield of 0.29 g/L  
755 (Radhakrishnan et al., 2021).

756 Besides biofuel application, microalgae biomass from VFA has been suggested as a source of  
757 protein (Patel et al., 2022) or docosahexaenoic acid (DHA) (Chalima et al., 2019). Even though  
758 these applications would have higher added value than biolipids, health and safety issues should



759 first be properly addressed if these products were to be used for human consumption (Vilas-  
760 Boas et al., 2021). In addition, carbohydrates obtained from mixotrophic microalgae cultivation  
761 can be subsequently employed as feedstock to synthesize more chemicals and energy via DF in  
762 a closed loop system as reported by Liu et al. (2013b).

763

## 764 **5.2. Microalgae as a substrate for Dark Fermentation**

765 The macromolecular composition of microalgae (carbohydrates up to 65 % DW and protein up  
766 to 70% DW depending on the species) along with the lack of lignin, make this biomass a  
767 suitable substrate for bioH<sub>2</sub> production (Tyagi, 2017).

768 In addition, microalgae biomass constitutes a versatile substrate since it can be used as raw  
769 biomass, lipid-extracted microalgae biomass as well as residual microalgae biomass after the  
770 production of value-added compounds (Nobre et al., 2013) (**Figure 4**). After lipid extraction,  
771 microalgae biomass generates between 60 - 70% of residue (Ghimire et al., 2017), becoming  
772 a rich-carbohydrate feedstock. The lipid extraction during that process contributes to the  
773 biodegradability of the cellular structure, and therefore facilitates employing this residue as a  
774 substrate enhance the accessibility of HPB to intracellular content (Nobre et al., 2013). For  
775 instance, *Nannochloropsis* sp. residual biomass after lipids and carotenoids extraction was  
776 employed for H<sub>2</sub> production obtaining a H<sub>2</sub> yield 26% higher than the one from raw microalgae  
777 biomass (Nobre et al., 2013). Likewise, a high fermentative H<sub>2</sub> production yield (192 mL H<sub>2</sub>/g  
778 VS) was reported for *Dunaliella* lipid-extracted biomass (Chen et al., 2020).

779 However, when using microalgae biomass as a potential substrate for DF, diverse limiting  
780 factors should be considered.

781 Firstly, microalgae have a complex layered cell wall structure consisting of an inner and an  
782 outer layer, typically formed by polymers such as cellulose, hemicellulose, pectin and starch  
783 (Hallenbeck and Benemann, 2002). This structure can hamper bacterial hydrolysis and affect  
784 the release of intracellular compounds. To tackle this challenge, different pretreatments have  
785 been intensively studied in terms of microalgae disruption and organic matter solubilization for  
786 microbial degradation (e.g. thermal, electromagnetic radiation, acid/alkali and enzymatic  
787 pretreatments). However, cell disintegration does not necessarily translate into H<sub>2</sub> production  
788 and other techniques are necessary for polysaccharide hydrolysis into simple monomers to be  
789 available for HPB. For example, the combination of ultrasonication (20 min, 200 W) with  
790 enzymatic hydrolysis ( $\alpha$ -amylase and glucoamylase) of cyanobacteria *Arthrospira platensis*  
791 increased by 47% the fermentative H<sub>2</sub> yield (82.4 mL H<sub>2</sub>/g DW) in comparison to  
792 ultrasonication as the sole pretreatment (55.9 mL H<sub>2</sub>/g DW) (Cheng et al., 2012).

793 H<sub>2</sub> production yield declines with carbohydrate chain length (Quéméneur et al., 2011). **Table 4**  
794 presents the carbohydrate profile that can be obtained from some microalgae species. Similar  
795 H<sub>2</sub> yields (1.84-2.2 mol H<sub>2</sub>/mol substrate) were reported for monosaccharides such as glucose,  
796 arabinose, xylose, and fructose (Masset et al., 2012; Quéméneur et al., 2011; Taguchi et al.,  
797 1994) while lower values (1.65-1.67 mol H<sub>2</sub>/mol hexose) were obtained for disaccharides such  
798 as maltose and sucrose (Quéméneur et al., 2011). When cellulose was used as a substrate, H<sub>2</sub>  
799 yield as low as 0.48 mol H<sub>2</sub>/mol hexose was reported (Zagrodnik and Seifert, 2020). In contrast,  
800 starch fermentation yielded 1.5 mol H<sub>2</sub>/mol glucose equivalent when using cultures of the  
801 hyperthermophilic bacterium *Thermotoga neapolitana* (Nguyen et al., 2010).

802 Secondly, the high protein content in microalgae biomass (**Table 4**) causes ammonium release  
803 during DF process. Excess of NH<sub>3</sub> can be inhibitory since this unionized form of nitrogen can  
804 easily penetrate the microbial cell wall, changing the intracellular pH, increasing the

805 maintenance energy and finally inhibiting specific enzymes involved in H<sub>2</sub> production  
806 inhibiting the HPB activity (Ramos-Suárez and Carreras, 2014). Besides, excess ammonium  
807 can lead to an unbalanced C:N ratio.

808 One alternative to obtain a suitable C:N ratio is to increase the microalgae carbohydrate content  
809 via optimization of the environmental conditions during microalgae cultivation (temperature,  
810 nutrients starvation, CO<sub>2</sub> concentration) (Brányiková et al., 2011; Izumo et al., 2007; Markou  
811 et al., 2012). However, playing on environmental conditions is not always feasible. In this sense,  
812 despite the low H<sub>2</sub> potential of proteins (N-rich) in comparison to carbohydrates (C-rich), the  
813 co-fermentation of microalgae with other substrates containing a different macromolecular  
814 composition can be a strategy that contributes to a balanced carbon to nitrogen (C:N) ratio is  
815 essential to optimize H<sub>2</sub> production in a fermentative process (Sun et al., 2018; Xia et al., 2016).

816 Lastly, sodium inhibition can occur when using marine strains. Despite sodium being an  
817 essential trace element for the synthesis and metabolism of anaerobic microorganisms, sodium  
818 excess (above 2 mg/L) increases osmotic pressure in the solution, leading to inactivation or  
819 death of bacteria (Lee et al., 2012). Some alternatives to address sodium inhibition include the  
820 use of salinity-tolerant inoculum (Riffat and Krongthamchat, 2007) or the acclimation of  
821 anaerobic microorganisms to gradually higher concentrations of sodium (Lefebvre et al., 2007).

822

## 823 **6. Conclusions**

824 The effluents derived from organic wastes produced during dark fermentation arise as a  
825 potential carbon source for microalgae cultivation that can boost the viability of bioproduct  
826 generation. The present review brings to the forefront the efficient multi-product generation  
827 (bioH<sub>2</sub>, biofuel and bioproducts) from a single waste by integrating different bioprocesses.

828 The increasing number of research studies in biorefinery approaches during the recent years is  
829 indicative of the significant progress and expectations to attain efficient bioproduct generation  
830 from low cost carbon sources in the near future. For that purpose, major efforts should be still  
831 made to optimize the bioprocesses of dark fermentation and microalgae cultivation and tackle  
832 the main challenges associated to their integration.

## 833 **7. Acknowledgements**

834 This study was funded by the National Research Institute of Agriculture, Alimentation and  
835 Environment (INRAE), and partly supported by the FermALip project, funded by the Carnot  
836 institute 3BCAR. JL received a PhD fellowship from European Union from the Occitanie  
837 region, France, with complementary funding from FEDER.

838

## 839 **CRedit authorship contribution statement**

840 **Julien Lacroux:** Conceptualization, Writing – original draft; Writing - review & editing.

841 **Mercedes Llamas:** Conceptualization, Writing – original draft, Writing - review & editing.

842 **Kevin Dauplain:** Writing – original draft. **Romina Avila:** Writing – original draft. **Jean-**

843 **Philippe Steyer:** Conceptualization, Funding acquisition, Supervision. **Robert van Lis:**

844 Conceptualization, Supervision, Funding acquisition. **Eric Trably:** Conceptualization, Funding

845 acquisition, Supervision.

## 846 **Declaration of competing interest**

847 The authors declare that they have no known competing financial interests or personal

848 relationships that could have appeared to influence the work reported in this review.

849

## 850 REFERENCES

- 851 Abiusi, F., Wijffels, R.H., Janssen, M., 2020. Doubling of microalgae productivity by oxygen balanced  
852 mixotrophy. *ACS Sustain. Chem. Eng.* 8, 6065–6074.
- 853 Angelidaki, I., Sanders, W., 2004. Assessment of the anaerobic biodegradability of macropollutants.  
854 *Re/Views Environ. Sci. Bio/Technology* 3, 117–129. <https://doi.org/10.1007/s11157-004-2502-3>
- 855 Anzola-Rojas, M. del P., Gonçalves da Fonseca, S., Canedo da Silva, C., Maia de Oliveira, V., Zaiat, M.,  
856 2015. The use of the carbon/nitrogen ratio and specific organic loading rate as tools for  
857 improving biohydrogen production in fixed-bed reactors. *Biotechnol. Reports* 5, 46–54.  
858 <https://doi.org/https://doi.org/10.1016/j.btre.2014.10.010>
- 859 Atteia, A., van Lis, R., Mendoza-Hernández, G., Henze, K., Martin, W., Riveros-Rosas, H., González-  
860 Halphen, D., 2003. Bifunctional aldehyde/alcohol dehydrogenase (ADHE) in chlorophyte algal  
861 mitochondria. *Plant Mol. Biol.* 53, 175–188.
- 862 Atteia, A., van Lis, R., Ramírez, J., González-Halphen, D., 2000. *Polytomella* spp. growth on ethananol:  
863 Extracellular pH affects the accumulation of mitochondrial cytochrome c550. *Eur. J. Biochem.*  
864 267, 2850–2858.
- 865 Balachandar, G., Varanasi, J.L., Singh, V., Singh, H., Das, D., 2020. Biological hydrogen production via  
866 dark fermentation: A holistic approach from lab-scale to pilot-scale. *Int. J. Hydrogen Energy* 45,  
867 5202–5215. <https://doi.org/10.1016/j.ijhydene.2019.09.006>
- 868 Baroukh, C., Turon, V., Bernard, O., 2017. Dynamic metabolic modeling of heterotrophic and  
869 mixotrophic microalgal growth on fermentative wastes. *PLoS Comput. Biol.* 13, e1005590.
- 870 BNEF, 2020. *Hydrogen Economy Outlook*. Bloom. *New Energy Financ.* 12.
- 871 Brányiková, I., Maršáľková, B., Doucha, J., Brányik, T., Bišová, K., Zachleder, V., Vítová, M., 2011.  
872 *Microalgae—novel highly efficient starch producers*. *Biotechnol. Bioeng.* 108, 766–776.
- 873 Bredda, E.H., Da Silva, A.F., Silva, M.B., Da Rós, P.C.M., 2020. Mixture design as a potential tool in  
874 modeling the effect of light wavelength on *Dunaliella salina* cultivation: an alternative solution  
875 to increase microalgae lipid productivity for biodiesel production. *Prep. Biochem. Biotechnol.*  
876 50, 379–389. <https://doi.org/10.1080/10826068.2019.1697936>
- 877 Brennan, L., Owende, P., 2010. Biofuels from microalgae—A review of technologies for production,  
878 processing, and extractions of biofuels and co-products. *Renew. Sustain. Energy Rev.* 14, 557–  
879 577. <https://doi.org/10.1016/j.rser.2009.10.009>
- 880 Bundhoo, Z.M.A., 2017. Coupling dark fermentation with biochemical or bioelectrochemical systems  
881 for enhanced bio-energy production: A review. *Int. J. Hydrogen Energy* 42, 26667–26686.  
882 <https://doi.org/10.1016/j.ijhydene.2017.09.050>
- 883 Burgess, S.J., Taha, H., Yeoman, J.A., Iamshanova, O., Chan, K.X., Boehm, M., Behrends, V., Bundy,  
884 J.G., Bialek, W., Murray, J.W., 2016. Identification of the elusive pyruvate reductase of  
885 *Chlamydomonas reinhardtii* chloroplasts. *Plant Cell Physiol.* 57, 82–94.
- 886 Cabrol, L., Marone, A., Tapia-Venegas, E., Steyer, J.P., Ruiz-Filippi, G., Trably, E., 2017. Microbial  
887 ecology of fermentative hydrogen producing bioprocesses: Useful insights for driving the  
888 ecosystem function. *FEMS Microbiol. Rev.* 41, 158–181.  
889 <https://doi.org/10.1093/femsre/fuw043>
- 890 Catalanotti, C., Yang, W., Posewitz, M.C., Grossman, A.R., 2013. Fermentation metabolism and its  
891 evolution in algae. *Front. Plant Sci.* 4, 150.

- 892 Cecchin, M., Benfatto, S., Griggio, F., Mori, A., Cazzaniga, S., Vitulo, N., Delledonne, M., Ballottari, M.,  
893 2018. Molecular basis of autotrophic vs mixotrophic growth in *Chlorella sorokiniana*. *Sci. Rep.* 8,  
894 1–13.
- 895 Chalima, A., Hatzidaki, A., Karnaouri, A., Topakas, E., 2019. Integration of a dark fermentation  
896 effluent in a microalgal-based biorefinery for the production of high-added value omega-3 fatty  
897 acids. *Appl. Energy* 241, 130–138. <https://doi.org/10.1016/J.APENERGY.2019.03.058>
- 898 Chen, F., Johns, M.R., 1994. Substrate inhibition of *Chlamydomonas reinhardtii* by acetate in  
899 heterotrophic culture. *Process Biochem.* 29, 245–252. [https://doi.org/10.1016/0032-](https://doi.org/10.1016/0032-9592(94)80064-2)  
900 9592(94)80064-2
- 901 Chen, S., Qu, D., Xiao, X., Miao, X., 2020. Biohydrogen production with lipid-extracted *Dunaliella*  
902 biomass and a new strain of hyper-thermophilic archaeon *Thermococcus eurythermalis* A501.  
903 *Int. J. Hydrogen Energy* 45, 12721–12730.
- 904 Cheng, J., Fan, W., Zheng, L., 2021. Development of a mixotrophic cultivation strategy for  
905 simultaneous improvement of biomass and photosynthetic efficiency in freshwater microalga  
906 *Scenedesmus obliquus* by adding appropriate concentration of sodium acetate. *Biochem. Eng. J.*  
907 176, 108177. <https://doi.org/10.1016/j.bej.2021.108177>
- 908 Cheng, J., Xia, A., Song, W., Su, H., Zhou, J., Cen, K., 2012. Comparison between heterofermentation  
909 and autofermentation in hydrogen production from *Arthrospira (Spirulina) platensis* wet  
910 biomass. *Int. J. Hydrogen Energy* 37, 6536–6544.
- 911 Chiranjeevi, P., Mohan, S.V., 2017. Diverse acidogenic effluents as feedstock for microalgae  
912 cultivation: dual phase metabolic transition on biomass growth and lipid synthesis. *Bioresour.*  
913 *Technol.* 242, 191–196.
- 914 Cho, H.U., Kim, Y.M., Choi, Y.N., Xu, X., Shin, D.Y., Park, J.M., 2015. Effects of pH control and  
915 concentration on microbial oil production from *Chlorella vulgaris* cultivated in the effluent of a  
916 low-cost organic waste fermentation system producing volatile fatty acids. *Bioresour. Technol.*  
917 184, 245–250. <https://doi.org/10.1016/j.biortech.2014.09.069>
- 918 Chong, C.C., Cheng, Y.W., Ishak, S., Lam, M.K., Lim, J.W., Tan, I.S., Show, P.L., Lee, K.T., 2022.  
919 Anaerobic digestate as a low-cost nutrient source for sustainable microalgae cultivation: A way  
920 forward through waste valorization approach. *Sci. Total Environ.* 803, 150070.  
921 <https://doi.org/10.1016/j.scitotenv.2021.150070>
- 922 Cuff, M., Dyer, J., Jones, M., Shirazi-Beechey, S., 2005. The human colonic monocarboxylate  
923 transporter Isoform 1: its potential importance to colonic tissue homeostasis. *Gastroenterology*  
924 128, 676–686.
- 925 Dahiya, S., Sarkar, O., Swamy, Y. V., Venkata Mohan, S., 2015. Acidogenic fermentation of food waste  
926 for volatile fatty acid production with co-generation of biohydrogen. *Bioresour. Technol.* 182,  
927 103–113. <https://doi.org/10.1016/j.biortech.2015.01.007>
- 928 Dauptain, K., Schneider, A., Noguer, M., Fontanille, P., Escudie, R., Carrere, H., Trably, E., 2021.  
929 Impact of microbial inoculum storage on dark fermentative H<sub>2</sub> production. *Bioresour. Technol.*  
930 319, 124234. <https://doi.org/10.1016/j.biortech.2020.124234>
- 931 Dincer, I., Acar, C., 2014. Review and evaluation of hydrogen production methods for better  
932 sustainability. *Int. J. Hydrogen Energy* 40, 11094–11111.  
933 <https://doi.org/10.1016/j.ijhydene.2014.12.035>
- 934 Donohoe, D.R., Garge, N., Zhang, X., Sun, W., O'Connell, T.M., Bungler, M.K., Bultman, S.J., 2011. The  
935 microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon.

- 936 Cell Metab. 13, 517–526.
- 937 Dourou, M., Dritsas, P., Baeshen, M.N., Elazzazy, A., Al-Farga, A., Aggelis, G., 2021. High-added value  
938 products from microalgae and prospects of aquaculture wastewaters as microalgae growth  
939 media. FEMS Microbiol. Lett. 367, 1–14. <https://doi.org/10.1093/FEMSLE/FNAA081>
- 940 Dragosits, M., Mattanovich, D., 2013. Adaptive laboratory evolution—principles and applications for  
941 biotechnology. Microb. Cell Fact. 12, 1–17.
- 942 El-Emam, R.S., Özcan, H., 2019. Comprehensive review on the techno-economics of sustainable large-  
943 scale clean hydrogen production. J. Clean. Prod. 220, 593–609.  
944 <https://doi.org/10.1016/j.jclepro.2019.01.309>
- 945 Elbeshbishy, E., Dhar, B.R., Nakhla, G., Lee, H.-S., 2017. A critical review on inhibition of dark  
946 biohydrogen fermentation. Renew. Sustain. Energy Rev. 79, 656–668.  
947 <https://doi.org/https://doi.org/10.1016/j.rser.2017.05.075>
- 948 Etchebehere, C., Castelló, E., Wenzel, J., del Pilar Anzola-Rojas, M., Borzacconi, L., Buitrón, G., Cabrol,  
949 L., Carminato, V.M., Carrillo-Reyes, J., Cisneros-Pérez, C., 2016a. Microbial communities from 20  
950 different hydrogen-producing reactors studied by 454 pyrosequencing. Appl. Microbiol.  
951 Biotechnol. 100, 3371–3384.
- 952 Etchebehere, C., Castelló, E., Wenzel, J., del Pilar Anzola-Rojas, M., Borzacconi, L., Buitrón, G., Cabrol,  
953 L., Carminato, V.M., Carrillo-Reyes, J., Cisneros-Pérez, C., Fuentes, L., Moreno-Andrade, I., Razo-  
954 Flores, E., Filippi, G.R., Tapia-Venegas, E., Toledo-Alarcón, J., Zaiat, M., 2016b. Microbial  
955 communities from 20 different hydrogen-producing reactors studied by 454 pyrosequencing.  
956 Appl. Microbiol. Biotechnol. 100, 3371–3384. <https://doi.org/10.1007/s00253-016-7325-y>
- 957 European Commission, 2020. The hydrogen strategy for a climate-neutral Europe. Dk 53, 1689–1699.
- 958 Fei, Q., Fu, R., Shang, L., Brigham, C.J., Chang, H.N., 2015. Lipid production by microalgae *Chlorella*  
959 *protothecoides* with volatile fatty acids (VFAs) as carbon sources in heterotrophic cultivation  
960 and its economic assessment. Bioprocess Biosyst. Eng. 38, 691–700.  
961 <https://doi.org/10.1007/s00449-014-1308-0>
- 962 Fujita, T., Aoyagi, H., Ogbonna, J.C., Tanaka, H., 2008. Effect of mixed organic substrate on  $\alpha$ -  
963 tocopherol production by *Euglena gracilis* in photoheterotrophic culture. Appl. Microbiol.  
964 Biotechnol. 79, 371–378.
- 965 Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N.L., Esposito, G., 2015. A review on  
966 dark fermentative biohydrogen production from organic biomass: Process parameters and use  
967 of by-products. Appl. Energy 144, 73–95. <https://doi.org/10.1016/j.apenergy.2015.01.045>
- 968 Ghimire, A., Kumar, G., Sivagurunathan, P., Shobana, S., Saratale, G.D., Kim, H.W., Luongo, V.,  
969 Esposito, G., Munoz, R., 2017. Bio-hythane production from microalgae biomass: key challenges  
970 and potential opportunities for algal bio-refineries. Bioresour. Technol. 241, 525–536.
- 971 Giannattasio, S., Guaragnella, N., Ždravlević, M., Marra, E., 2013. Molecular mechanisms of  
972 *Saccharomyces cerevisiae* stress adaptation and programmed cell death in response to acetic  
973 acid. Front. Microbiol. 4, 33.
- 974 Görke, B., Stülke, J., 2008. Carbon catabolite repression in bacteria: many ways to make the most out  
975 of nutrients. Nat. Rev. Microbiol. 6, 613–624.
- 976 Gouveia, L., Graça, S., Sousa, C., Ambrosano, L., Ribeiro, B., Botrel, E.P., Neto, P.C., Ferreira, A.F.,  
977 Silva, C.M., 2016. Microalgae biomass production using wastewater: Treatment and costs:  
978 Scale-up considerations. Algal Res. 16, 167–176. <https://doi.org/10.1016/J.ALGAL.2016.03.010>

- 979 Greses, S., Tomás-pejó, E., González-fernandez, C., 2020. Short-chain fatty acids and hydrogen  
980 production in one single anaerobic fermentation stage using carbohydrate-rich food waste. J.  
981 Clean. Prod. <https://doi.org/10.1016/j.jclepro.2020.124727>
- 982 Gruber, P.J., Frederick, S.E., Tolbert, N.E., 1974. Enzymes related to lactate metabolism in green algae  
983 and lower land plants. *Plant Physiol.* 53, 167–170.
- 984 Guo, X.M., Trably, E., Latrille, E., Carrère, H., Steyer, J.-P., 2010. Hydrogen production from  
985 agricultural waste by dark fermentation: A review. *Int. J. Hydrogen Energy* 35, 10660–10673.  
986 <https://doi.org/https://doi.org/10.1016/j.ijhydene.2010.03.008>
- 987 Gupta, S., Pawar, S.B., 2018. Mixotrophic cultivation of microalgae to enhance the quality of lipid for  
988 biodiesel application: effects of scale of cultivation and light spectrum on reduction of A-  
989 linolenic acid. *Bioprocess Biosyst. Eng.* 41, 531–542. [https://doi.org/10.1007/s00449-017-1888-](https://doi.org/10.1007/s00449-017-1888-6)  
990 6
- 991 Hallenbeck, P.C., Benemann, J.R., 2002. Biological hydrogen production; fundamentals and limiting  
992 processes. *Int. J. Hydrogen Energy* 27, 1185–1193.
- 993 Heffernan, J.K., Lai, C.Y., Gonzalez-Garcia, R.A., Keld Nielsen, L., Guo, J., Marcellin, E., 2023. Biogas  
994 upgrading using *Clostridium autoethanogenum* for value-added products. *Chem. Eng. J.* 452,  
995 138950. <https://doi.org/10.1016/j.cej.2022.138950>
- 996 Heifetz, P.B., Forster, B., Osmond, C.B., Giles, L.J., Boynton, J.E., 2000. Effects of acetate on  
997 facultative autotrophy in *Chlamydomonas reinhardtii* assessed by photosynthetic  
998 measurements and stable isotope analyses. *Plant Physiol.* 122, 1439–1446.
- 999 Hu, J., Nagarajan, D., Zhang, Q., Chang, J.S., Lee, D.J., 2018. Heterotrophic cultivation of microalgae  
1000 for pigment production: A review. *Biotechnol. Adv.* 36, 54–67.  
1001 <https://doi.org/10.1016/j.biotechadv.2017.09.009>
- 1002 Husic, D.W., Tolbert, N.E., 1985. Anaerobic formation of D-lactate and partial purification and  
1003 characterization of a pyruvate reductase from *Chlamydomonas reinhardtii*. *Plant Physiol.* 78,  
1004 277–284.
- 1005 Izumo, A., Fujiwara, S., Oyama, Y., Satoh, A., Fujita, N., Nakamura, Y., Tsuzuki, M., 2007.  
1006 Physicochemical properties of starch in *Chlorella* change depending on the CO<sub>2</sub> concentration  
1007 during growth: comparison of structure and properties of pyrenoid and stroma starch. *Plant Sci.*  
1008 172, 1138–1147.
- 1009 Jarunglumert, T., Bampenrat, A., Sukkathanyawat, H., Prommuak, C., 2021. Enhanced energy  
1010 recovery from food waste by co-production of bioethanol and biomethane process.  
1011 *Fermentation* 7, 1–12. <https://doi.org/10.3390/fermentation7040265>
- 1012 Jiang, Y., Xiao, P., Shao, Q., Qin, H., Hu, Z., Lei, A., Wang, J., 2017. Metabolic responses to ethanol and  
1013 butanol in *Chlamydomonas reinhardtii*. *Biotechnol. Biofuels* 10, 1–16.
- 1014 Johnson, X., Alric, J., 2013. Central carbon metabolism and electron transport in *Chlamydomonas*  
1015 *reinhardtii*: metabolic constraints for carbon partitioning between oil and starch. *Eukaryot. Cell*  
1016 12, 776–793.
- 1017 Kenny, P., Flynn, K.J., 2017. Physiology limits commercially viable photoautotrophic production of  
1018 microalgal biofuels. *J. Appl. Phycol.* 29, 2713–2727.
- 1019 Kim, D., Kim, S., Han, J.I., Yang, J.W., Chang, Y.K., Ryu, B.G., 2019. Carbon balance of major volatile  
1020 fatty acids (VFAs) in recycling algal residue via a VFA-platform for reproduction of algal biomass.  
1021 *J. Environ. Manage.* 237, 228–234. <https://doi.org/10.1016/j.jenvman.2019.02.040>



- 1022 Lacroux, J., Jouannais, P., Atteia, A., Bonnafous, A., Trably, E., Steyer, J.-P., van Lis, R., 2022.  
 1023 Microalgae screening for heterotrophic and mixotrophic growth on butyrate. *Algal Res.* 67,  
 1024 102843. <https://doi.org/10.1016/j.algal.2022.102843>
- 1025 Lacroux, J., Seira, J., Trably, E., Bernet, N., Steyer, J., van Lis, R., 2021a. Mixotrophic Growth of  
 1026 *Chlorella sorokiniana* on Acetate and Butyrate : Interplay Between Substrate , C : N Ratio and  
 1027 pH. *Front. Microbiol.* 12, 1–16. <https://doi.org/10.3389/fmicb.2021.703614>
- 1028 Lacroux, J., Seira, J., Trably, E., Bernet, N., Steyer, J.P., van Lis, R., 2021b. Mixotrophic Growth of  
 1029 *Chlorella sorokiniana* on Acetate and Butyrate: Interplay Between Substrate, C:N Ratio and pH.  
 1030 *Front. Microbiol.* 12, 1–16. <https://doi.org/10.3389/fmicb.2021.703614>
- 1031 Lacroux, J., Trably, E., Bernet, N., Steyer, J.P., van Lis, R., 2020. Mixotrophic growth of microalgae on  
 1032 volatile fatty acids is determined by their undissociated form. *Algal Res.* 47, 101870.  
 1033 <https://doi.org/10.1016/j.algal.2020.101870>
- 1034 Lefebvre, O., Quentin, S., Torrijos, M., Godon, J.-J., Delgenes, J.P., Moletta, R., 2007. Impact of  
 1035 increasing NaCl concentrations on the performance and community composition of two  
 1036 anaerobic reactors. *Appl. Microbiol. Biotechnol.* 75, 61–69.
- 1037 Li, T., Kirchhoff, H., Gargouri, M., Feng, J., Cousins, A.B., Pienkos, P.T., Gang, D.R., Chen, S., 2016.  
 1038 Assessment of photosynthesis regulation in mixotrophically cultured microalga *Chlorella*  
 1039 *sorokiniana*. *Algal Res.* 19, 30–38. <https://doi.org/10.1016/j.algal.2016.07.012>
- 1040 Li, T., Yang, F., Xu, J., Wu, H., Mo, J., Dai, L., Xiang, W., 2020. Evaluating differences in growth,  
 1041 photosynthetic efficiency, and transcriptome of *Asterarcys* sp. SCS-1881 under autotrophic,  
 1042 mixotrophic, and heterotrophic culturing conditions. *Algal Res.* 45, 101753.  
 1043 <https://doi.org/10.1016/j.algal.2019.101753>
- 1044 Lin, C.Y., Lay, C.H., 2004. Carbon/nitrogen-ratio effect on fermentative hydrogen production by  
 1045 mixed microflora. *Int. J. Hydrogen Energy* 29, 41–45.  
 1046 [https://doi.org/https://doi.org/10.1016/S0360-3199\(03\)00083-1](https://doi.org/https://doi.org/10.1016/S0360-3199(03)00083-1)
- 1047 Lin, Y.-S., Yuwono, W., Wang, H.-Y., 2020. Lipid Induction in *Scenedesmus abundans* GH-D11 by  
 1048 Reusing the Volatile Fatty Acids in the Effluent of Dark Anaerobic Fermentation of Biohydrogen.  
 1049 *Appl. Biochem. Biotechnol.* 191, 258–272.
- 1050 Liu, C.-H., Chang, C.-Y., Liao, Q., Zhu, X., Chang, J.-S., 2013. Photoheterotrophic growth of *Chlorella*  
 1051 *vulgaris* ESP6 on organic acids from dark hydrogen fermentation effluents. *Bioresour. Technol.*  
 1052 145, 331–336. <https://doi.org/https://doi.org/10.1016/j.biortech.2012.12.111>
- 1053 Liu, C.H., Chang, C.Y., Liao, Q., Zhu, X., Chang, J.S., 2013a. Photoheterotrophic growth of *Chlorella*  
 1054 *vulgaris* ESP6 on organic acids from dark hydrogen fermentation effluents. *Bioresour. Technol.*  
 1055 145, 331–336. <https://doi.org/10.1016/J.BIORTECH.2012.12.111>
- 1056 Liu, C.H., Chang, C.Y., Liao, Q., Zhu, X., Liao, C.F., Chang, J.S., 2013b. Biohydrogen production by a  
 1057 novel integration of dark fermentation and mixotrophic microalgae cultivation. *Int. J. Hydrogen*  
 1058 *Energy* 38, 15807–15814. <https://doi.org/10.1016/J.IJHYDENE.2013.05.104>
- 1059 Liu, D., Zeng, R.J., Angelidaki, I., 2008. Effects of pH and hydraulic retention time on hydrogen  
 1060 production versus methanogenesis during anaerobic fermentation of organic household solid  
 1061 waste under extreme-thermophilic temperature (70° C). *Biotechnol. Bioeng.* 100, 1108–1114.
- 1062 Liu, D.W., Zeng, R.J., Angelidaki, I., 2008. Enrichment and adaptation of extreme-thermophilic (70 C)  
 1063 hydrogen producing bacteria to organic household solid waste by repeated batch cultivation.  
 1064 *Int. J. Hydrogen Energy* 33, 6492–6497.

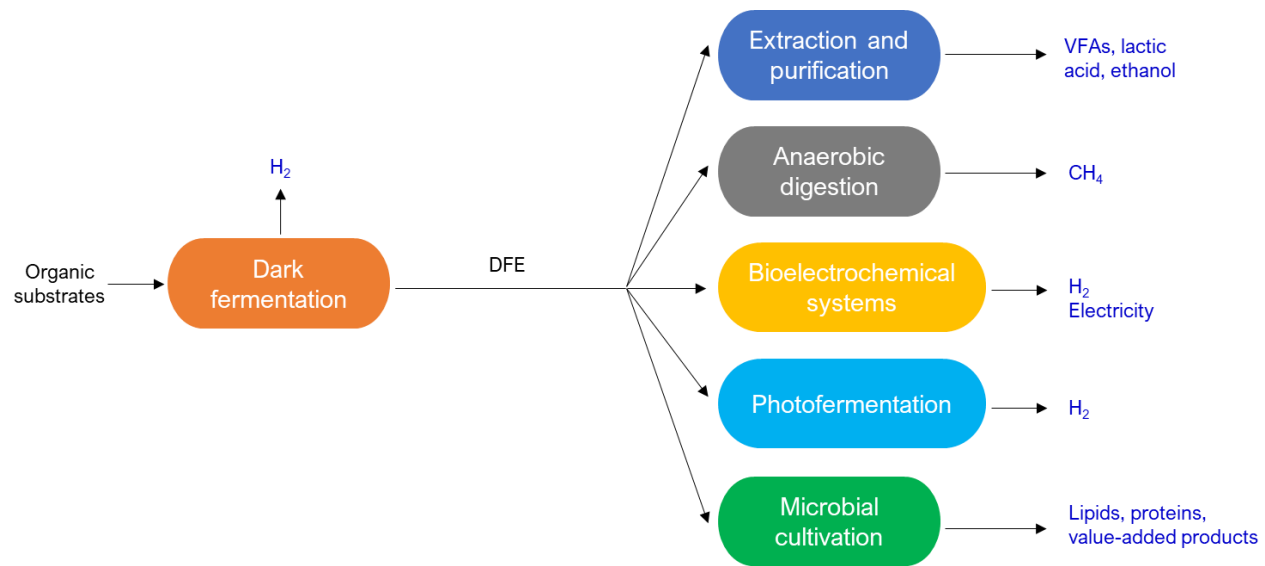
- 1065 Llamas, M., Greses, S., Tomás-Pejó, E., González-Fernández, C., 2021. Tuning microbial community in  
 1066 non-conventional two-stage anaerobic bioprocess for microalgae biomass valorization into  
 1067 targeted bioproducts. *Bioresour. Technol.* 337. <https://doi.org/10.1016/j.biortech.2021.125387>
- 1068 Luo, G., Karakashev, D., Xie, L., Zhou, Q., Angelidaki, I., 2011. Long-term effect of inoculum  
 1069 pretreatment on fermentative hydrogen production by repeated batch cultivations:  
 1070 Homoacetogenesis and methanogenesis as competitors to hydrogen production. *Biotechnol.*  
 1071 *Bioeng.* 108, 1816–1827.
- 1072 Luo, L., Sriram, S., Johnravindar, D., Louis Philippe Martin, T., Wong, J.W.C., Pradhan, N., 2022. Effect  
 1073 of inoculum pretreatment on the microbial and metabolic dynamics of food waste dark  
 1074 fermentation. *Bioresour. Technol.* 358, 127404.  
 1075 <https://doi.org/https://doi.org/10.1016/j.biortech.2022.127404>
- 1076 Markou, G., Angelidaki, I., Georgakakis, D., 2012. Microalgal carbohydrates: an overview of the  
 1077 factors influencing carbohydrates production, and of main bioconversion technologies for  
 1078 production of biofuels. *Appl. Microbiol. Biotechnol.* 96, 631–645.
- 1079 Masset, J., Calusinska, M., Hamilton, C., Hilgsmann, S., Joris, B., Wilmotte, A., Thonart, P., 2012.  
 1080 Fermentative hydrogen production from glucose and starch using pure strains and artificial co-  
 1081 cultures of *Clostridium* spp. *Biotechnol. Biofuels* 5, 1–15.
- 1082 Miazek, K., Kratky, L., Sulc, R., Jirout, T., Aguedo, M., Richel, A., Goffin, D., 2017. Effect of organic  
 1083 solvents on microalgae growth, metabolism and industrial bioproduct extraction: a review. *Int.*  
 1084 *J. Mol. Sci.* 18, 1429.
- 1085 Moon, M., Kim, C.W., Park, W.K., Yoo, G., Choi, Y.E., Yang, J.W., 2013. Mixotrophic growth with  
 1086 acetate or volatile fatty acids maximizes growth and lipid production in *Chlamydomonas*  
 1087 *reinhardtii*. *Algal Res.* 2, 352–357. <https://doi.org/10.1016/J.ALGAL.2013.09.003>
- 1088 Moscoviz, R., Trably, E., Bernet, N., Carrère, H., 2018. The environmental biorefinery: State-of-the-art  
 1089 on the production of hydrogen and value-added biomolecules in mixed-culture fermentation.  
 1090 *Green Chem.* 20, 3159–3179. <https://doi.org/10.1039/c8gc00572a>
- 1091 Mu, D., Liu, H., Lin, W., Shukla, P., Luo, J., 2020. Simultaneous biohydrogen production from dark  
 1092 fermentation of duckweed and waste utilization for microalgal lipid production. *Bioresour.*  
 1093 *Technol.* 302, 122879. <https://doi.org/10.1016/J.BIORTECH.2020.122879>
- 1094 Nakazawa, M., 2017. C2 metabolism in *Euglena*. *Euglena Biochem. Cell Mol. Biol.* 39–45.
- 1095 Nguyen, T.-A.D., Kim, K.-R., Nguyen, M.-T., Kim, M.S., Kim, D., Sim, S.J., 2010. Enhancement of  
 1096 fermentative hydrogen production from green algal biomass of *Thermotoga neapolitana* by  
 1097 various pretreatment methods. *Int. J. Hydrogen Energy* 35, 13035–13040.
- 1098 Nobre, B.P., Villalobos, F., Barragan, B.E., Oliveira, A.C., Batista, A.P., Marques, P., Mendes, R.L.,  
 1099 Sovová, H., Palavra, A.F., Gouveia, L., 2013. A biorefinery from *Nannochloropsis* sp. microalga-  
 1100 extraction of oils and pigments. Production of biohydrogen from the leftover biomass.  
 1101 *Bioresour. Technol.* 135, 128–136.
- 1102 Palomo-Briones, R., Razo-Flores, E., Bernet, N., Trably, E., 2017. Dark-fermentative biohydrogen  
 1103 pathways and microbial networks in continuous stirred tank reactors: Novel insights on their  
 1104 control. *Appl. Energy* 198, 77–87. <https://doi.org/10.1016/j.apenergy.2017.04.051>
- 1105 Pang, N., Gu, X., Chen, S., Kirchoff, H., Lei, H., Roje, S., 2019. Exploiting mixotrophy for improving  
 1106 productivities of biomass and co-products of microalgae. *Renew. Sustain. Energy Rev.* 112, 450–  
 1107 460. <https://doi.org/10.1016/j.rser.2019.06.001>

- 1108 Patel, A., Gami, B., Patel, P., Patel, B., 2017. Microalgae: Antiquity to era of integrated technology.  
1109 *Renew. Sustain. Energy Rev.* 71, 535–547.
- 1110 Patel, A., Krikigianni, E., Rova, U., Christakopoulos, P., Matsakas, L., 2022. Bioprocessing of volatile  
1111 fatty acids by oleaginous freshwater microalgae and their potential for biofuel and protein  
1112 production. *Chem. Eng. J.* 438, 135529. <https://doi.org/10.1016/J.CEJ.2022.135529>
- 1113 Patel, A., Mahboubi, A., Horváth, I.S., Taherzadeh, M.J., Rova, U., Christakopoulos, P., Matsakas, L.,  
1114 2021. Volatile fatty acids (VFAs) generated by anaerobic digestion serve as feedstock for  
1115 freshwater and marine oleaginous microorganisms to produce biodiesel and added-value  
1116 compounds. *Front. Microbiol.* 72.
- 1117 Pavlostathis, S.G., Giraldo-Gomez, E., 1991. Kinetics of anaerobic treatment: A critical review. *Crit.*  
1118 *Rev. Environ. Control.* <https://doi.org/10.1080/10643389109388424>
- 1119 Perez-Garcia, O., Bashan, Y., 2015. Microalgal heterotrophic and mixotrophic culturing for bio-  
1120 refining: from metabolic routes to techno-economics. *Algal biorefineries* 61–131.
- 1121 Perez-Garcia, O., Escalante, F.M., 2011. "Heterotrophic Cultures of Microalgae". *Metab. Potential*  
1122 *Prod. Water Res* 45, 11–36.
- 1123 Qi, W., Mei, S., Yuan, Y., Li, X., Tang, T., Zhao, Q., Wu, M., Wei, W., Sun, Y., 2018. Enhancing  
1124 fermentation wastewater treatment by co-culture of microalgae with volatile fatty acid- and  
1125 alcohol-degrading bacteria. *Algal Res.* 31, 31–39. <https://doi.org/10.1016/J.ALGAL.2018.01.012>
- 1126 Quéméneur, M., Hamelin, J., Benomar, S., Guidici-Orticoni, M.-T., Latrille, E., Steyer, J.-P., Trably, E.,  
1127 2011. Changes in hydrogenase genetic diversity and proteomic patterns in mixed-culture dark  
1128 fermentation of mono-, di- and tri-saccharides. *Int. J. Hydrogen Energy* 36, 11654–11665.
- 1129 Radhakrishnan, R., Banerjee, Sanjukta, Banerjee, Srijoni, Singh, V., Das, D., 2021. Sustainable  
1130 approach for the treatment of poultry manure and starchy wastewater by integrating dark  
1131 fermentation and microalgal cultivation. *J. Mater. Cycles Waste Manag.* 23, 790–803.
- 1132 Rafieenia, R., Lavagnolo, M.C., Pivato, A., 2018. Pre-treatment technologies for dark fermentative  
1133 hydrogen production: Current advances and future directions. *Waste Manag.* 71, 734–748.  
1134 <https://doi.org/https://doi.org/10.1016/j.wasman.2017.05.024>
- 1135 Ramos-Suárez, J.L., Carreras, N., 2014. Use of microalgae residues for biogas production. *Chem. Eng.*  
1136 *J.* 242, 86–95. <https://doi.org/10.1016/j.cej.2013.12.053>
- 1137 Redfield, A.C., 1958. The biological control of chemical factors in the environment. *Am. Sci.* 46, 230A–  
1138 221.
- 1139 Regueira, A., Bevilacqua, R., Lema, J.M., Carballa, M., Mauricio-Iglesias, M., 2020. A metabolic model  
1140 for targeted volatile fatty acids production by cofermentation of carbohydrates and proteins.  
1141 *Bioresour. Technol.* 298, 122535. <https://doi.org/10.1016/j.biortech.2019.122535>
- 1142 Ren, H.-Y., Liu, B.-F., Kong, F., Zhao, L., Ma, J., Ren, N.-Q., 2018. Favorable energy conversion  
1143 efficiency of coupling dark fermentation and microalgae production from food wastes. *Energy*  
1144 *Convers. Manag.* 166, 156–162.  
1145 <https://doi.org/https://doi.org/10.1016/j.enconman.2018.04.032>
- 1146 Ren, H.-Y., Liu, B.-F., Kong, F., Zhao, L., Ren, N., 2015. Hydrogen and lipid production from starch  
1147 wastewater by co-culture of anaerobic sludge and oleaginous microalgae with simultaneous  
1148 COD, nitrogen and phosphorus removal. *Water Res.* 85, 404–412.
- 1149 Ren, H.Y., Liu, B.F., Kong, F., Zhao, L., Ma, J., Ren, N.Q., 2018. Favorable energy conversion efficiency  
1150 of coupling dark fermentation and microalgae production from food wastes. *Energy Convers.*

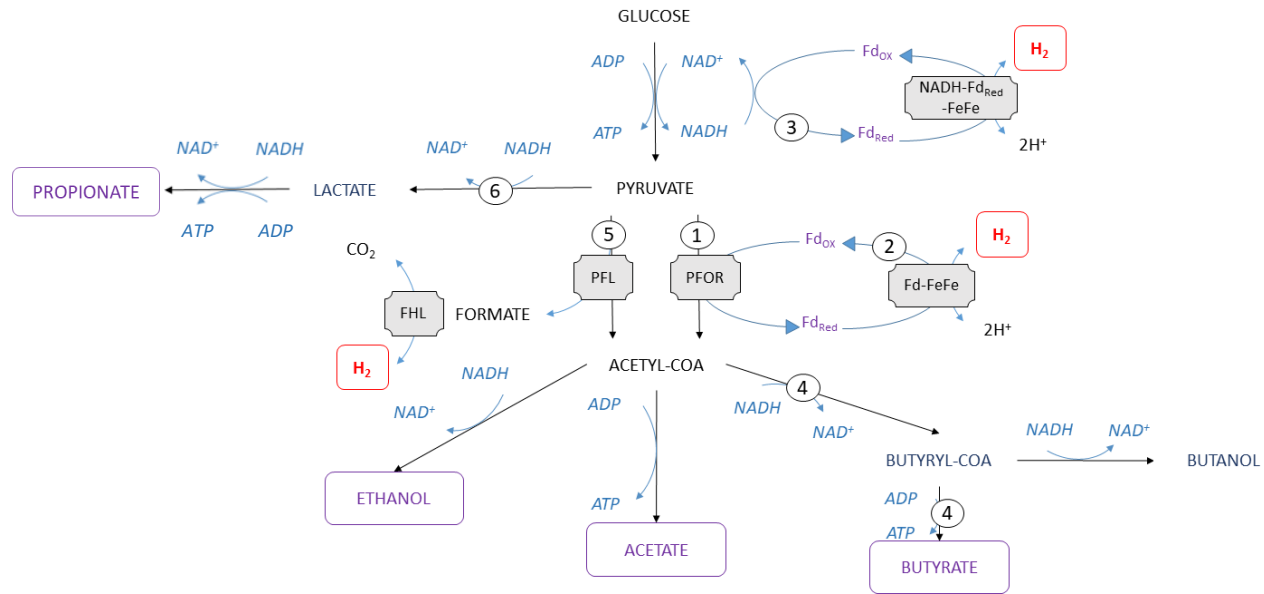
- 1151 Manag. 166, 156–162. <https://doi.org/10.1016/J.ENCONMAN.2018.04.032>
- 1152 Ren, H.Y., Liu, B.F., Ma, C., Zhao, L., Ren, N.Q., 2013. sp. strain R-16 isolated using Nile red staining:  
1153 effects of carbon and nitrogen sources and initial pH on the biomass and lipid production.  
1154 Biotechnol. Biofuels 6.
- 1155 Ren, N.-Q., Guo, W.-Q., Wang, X.-J., Xiang, W.-S., Liu, B.-F., Wang, X.-Z., Ding, J., Chen, Z.-B., 2008.  
1156 Effects of different pretreatment methods on fermentation types and dominant bacteria for  
1157 hydrogen production. Int. J. Hydrogen Energy 33, 4318–4324.  
1158 <https://doi.org/https://doi.org/10.1016/j.ijhydene.2008.06.003>
- 1159 Riffat, R., Krongthamchat, K., 2007. Anaerobic treatment of high-saline wastewater using halophilic  
1160 methanogens in laboratory-scale anaerobic filters. Water Environ. Res. 79, 191–198.
- 1161 Roach, T., Sedoud, A., Krieger-Liszka, A., 2013. Acetate in mixotrophic growth medium affects  
1162 photosystem II in *Chlamydomonas reinhardtii* and protects against photoinhibition. Biochim.  
1163 Biophys. Acta - Bioenerg. 1827, 1183–1190. <https://doi.org/10.1016/J.BBABIO.2013.06.004>
- 1164 Romans-Casas, M., Blasco-Gómez, R., Colprim, J., Balaguer, M.D., Puig, S., 2021. Bio-electro CO<sub>2</sub>  
1165 recycling platform based on two separated steps. J. Environ. Chem. Eng. 9.  
1166 <https://doi.org/10.1016/j.jece.2021.105909>
- 1167 Russell, J.B., 1992. Another explanation for the toxicity of fermentation acids at low pH: anion  
1168 accumulation versus uncoupling. J. Appl. Bacteriol. 73, 363–370.
- 1169 Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark  
1170 fermentation: unresolved challenge. Int. J. Hydrogen Energy 38, 13172–13191.
- 1171 Sajjadi, B., Chen, W.Y., Raman, A.A.A., Ibrahim, S., 2018. Microalgae lipid and biomass for biofuel  
1172 production: A comprehensive review on lipid enhancement strategies and their effects on fatty  
1173 acid composition. Renew. Sustain. Energy Rev. 97, 200–232.  
1174 <https://doi.org/10.1016/j.rser.2018.07.050>
- 1175 Santek, B., Friehs, K., Lotz, M., Flaschel, E., 2012. Production of paramylon, a ss-1, 3-glucan, by  
1176 heterotrophic growth of *Euglena gracilis* on potato liquor in fed-batch and repeated-batch  
1177 mode of cultivation. Eng. Life Sci. 12.
- 1178 Scarponi, P., Izzo, F.C., Bravi, M., Cavinato, C., 2021. *C. vulgaris* growth batch tests using winery waste  
1179 digestate as promising raw material for biodiesel and stearin production. Waste Manag. 136,  
1180 266–272. <https://doi.org/10.1016/j.wasman.2021.10.014>
- 1181 Schulze, P.S.C., Barreira, L.A., Pereira, H.G.C., Perales, J.A., Varela, J.C.S., 2014. Light emitting diodes  
1182 (LEDs) applied to microalgal production. Trends Biotechnol. 32, 422–430.
- 1183 Sforza, E., Cipriani, R., Morosinotto, T., Bertucco, A., Giacometti, G.M., 2012. Excess CO<sub>2</sub> supply  
1184 inhibits mixotrophic growth of *Chlorella protothecoides* and *Nannochloropsis salina*. Bioresour.  
1185 Technol. 104, 523–529. <https://doi.org/10.1016/j.biortech.2011.10.025>
- 1186 Sforza, E., Pastore, M., Spagni, A., Bertucco, A., 2018. Microalgae-bacteria gas exchange in  
1187 wastewater: how mixotrophy may reduce the oxygen supply for bacteria. Environ. Sci. Pollut.  
1188 Res. 25, 28004–28014.
- 1189 Sharma, S., Basu, S., Shetti, N.P., Aminabhavi, T.M., 2020. Waste-to-energy nexus for circular  
1190 economy and environmental protection: Recent trends in hydrogen energy. Sci. Total Environ.  
1191 713, 136633. <https://doi.org/10.1016/j.scitotenv.2020.136633>
- 1192 Shen, X.F., Liu, J.J., Chauhan, A.S., Hu, H., Ma, L.L., Lam, P.K.S., Zeng, R.J., 2016. Combining nitrogen  
1193 starvation with sufficient phosphorus supply for enhanced biodiesel productivity of *Chlorella*

- 1194 vulgaris fed on acetate. *Algal Res.* 17, 261–267. <https://doi.org/10.1016/J.ALGAL.2016.05.018>
- 1195 Shin, H.-S., Youn, J.-H., Kim, S.-H., 2004. Hydrogen production from food waste in anaerobic  
1196 mesophilic and thermophilic acidogenesis. *Int. J. Hydrogen Energy* 29, 1355–1363.
- 1197 Sial, A., Zhang, B., Zhang, A., Liu, K., Imtiaz, S.A., Yashir, N., 2021. Microalgal–bacterial synergistic  
1198 interactions and their potential influence in wastewater treatment: a review. *BioEnergy Res.* 14,  
1199 723–738.
- 1200 Sialve, B., Bernet, N., Bernard, O., 2009. Anaerobic digestion of microalgae as a necessary step to  
1201 make microalgal biodiesel sustainable. *Biotechnol. Adv.* 27, 409–416.  
1202 <https://doi.org/http://dx.doi.org/10.1016/j.biotechadv.2009.03.001>
- 1203 Siddiki, S.Y.A., Mofijur, M., Kumar, P.S., Ahmed, S.F., Inayat, A., Kusumo, F., Badruddin, I.A., Khan,  
1204 T.M.Y., Nghiem, L.D., Ong, H.C., Mahlia, T.M.I., 2022. Microalgae biomass as a sustainable  
1205 source for biofuel, biochemical and biobased value-added products: An integrated biorefinery  
1206 concept. *Fuel* 307, 121782. <https://doi.org/10.1016/j.fuel.2021.121782>
- 1207 Siriwongrungson, V., Zeng, R.J., Angelidaki, I., 2007. Homoacetogenesis as the alternative pathway  
1208 for H<sub>2</sub> sink during thermophilic anaerobic degradation of butyrate under suppressed  
1209 methanogenesis. *Water Res.* 41, 4204–4210. <https://doi.org/10.1016/j.watres.2007.05.037>
- 1210 Smith, R.T., Bangert, K., Wilkinson, S.J., Gilmour, D.J., 2015. Synergistic carbon metabolism in a fast  
1211 growing mixotrophic freshwater microalgal species *Micractinium inermum*. *Biomass and  
1212 Bioenergy* 82, 73–86. <https://doi.org/10.1016/J.BIOMBIOE.2015.04.023>
- 1213 Sun, C., Xia, A., Liao, Q., Fu, Q., Huang, Y., Zhu, X., Wei, P., Lin, R., Murphy, J.D., 2018. Improving  
1214 production of volatile fatty acids and hydrogen from microalgae and rice residue: effects of  
1215 physicochemical characteristics and mix ratios. *Appl. Energy* 230, 1082–1092.
- 1216 Taguchi, F., Mizukami, N., Hasegawa, K., Saito-Taki, T., 1994. Microbial conversion of arabinose and  
1217 xylose to hydrogen by a newly isolated *Clostridium* sp. No. 2. *Can. J. Microbiol.* 40, 228–233.
- 1218 Turon, V., Baroukh, C., Trably, E., Latrille, E., Fouilland, E., Steyer, J.P., 2015a. Use of fermentative  
1219 metabolites for heterotrophic microalgae growth: Yields and kinetics. *Bioresour. Technol.* 175,  
1220 342–349. <https://doi.org/10.1016/J.BIORTECH.2014.10.114>
- 1221 Turon, V., Trably, E., Fayet, A., Fouilland, E., Steyer, J.P., 2015b. Raw dark fermentation effluent to  
1222 support heterotrophic microalgae growth: Microalgae successfully outcompete bacteria for  
1223 acetate. *Algal Res.* 12, 119–125. <https://doi.org/10.1016/j.algal.2015.08.011>
- 1224 Turon, V., Trably, E., Fouilland, E., Steyer, J.P., 2016. Potentialities of dark fermentation effluents as  
1225 substrates for microalgae growth: A review. *Process Biochem.* 51, 1843–1854.  
1226 <https://doi.org/10.1016/j.procbio.2016.03.018>
- 1227 Turon, V., Trably, E., Fouilland, E., Steyer, J.P., 2015c. Growth of *Chlorella sorokiniana* on a mixture of  
1228 volatile fatty acids: The effects of light and temperature. *Bioresour. Technol.* 198, 852–860.  
1229 <https://doi.org/10.1016/J.BIORTECH.2015.10.001>
- 1230 Tyagi, A.C., 2017. Drainage and Environmental Sustainability. *Irrig. Drain.* 66, 451–452.
- 1231 Valdez-Vazquez, I., Ríos-Leal, E., Esparza-García, F., Cecchi, F., Poggi-Varaldo, H.M., 2005. Semi-  
1232 continuous solid substrate anaerobic reactors for H<sub>2</sub> production from organic waste: mesophilic  
1233 versus thermophilic regime. *Int. J. Hydrogen Energy* 30, 1383–1391.
- 1234 Van Ginkel, S., Logan, B.E., 2005. Inhibition of biohydrogen production by undissociated acetic and  
1235 butyric acids. *Environ. Sci. Technol.* 39, 9351–9356.

- 1236 Venkata Mohan, S., Prathima Devi, M., 2012. Fatty acid rich effluent from acidogenic biohydrogen  
1237 reactor as substrate for lipid accumulation in heterotrophic microalgae with simultaneous  
1238 treatment. *Bioresour. Technol.* 123, 627–635. <https://doi.org/10.1016/j.biortech.2012.07.004>
- 1239 Vilas-Boas, A.A., Pintado, M., Oliveira, A.L.S., 2021. Natural bioactive compounds from food waste:  
1240 Toxicity and safety concerns. *Foods* 10, 1564.
- 1241 Xia, A., Jacob, A., Tabassum, M.R., Herrmann, C., Murphy, J.D., 2016. Production of hydrogen,  
1242 ethanol and volatile fatty acids through co-fermentation of macro-and micro-algae. *Bioresour.*  
1243 *Technol.* 205, 118–125.
- 1244 Xie, X., Huang, A., Gu, W., Zang, Z., Pan, G., Gao, S., He, L., Zhang, B., Niu, J., Lin, A., 2016.  
1245 Photorespiration participates in the assimilation of acetate in *Chlorella sorokiniana* under high  
1246 light. *New Phytol.* 209, 987–998.
- 1247 Yew, G.Y., Lee, S.Y., Show, P.L., Tao, Y., Law, C.L., Nguyen, T.T.C., Chang, J.S., 2019. Recent advances  
1248 in algae biodiesel production: From upstream cultivation to downstream processing. *Bioresour.*  
1249 *Technol. Reports* 7, 100227. <https://doi.org/10.1016/J.BITEB.2019.100227>
- 1250 Zagrodnik, R., Seifert, K., 2020. Direct fermentative hydrogen production from cellulose and starch  
1251 with mesophilic bacterial consortia. *Polish J. Microbiol.* 69, 109–120.
- 1252 Zhang, B., Wu, J., Meng, F., 2021. Adaptive Laboratory Evolution of Microalgae: A Review of the  
1253 Regulation of Growth, Stress Resistance, Metabolic Processes, and Biodegradation of  
1254 Pollutants. *Front. Microbiol.* 2401.
- 1255 Zhang, Z., Sun, D., Wu, T., Li, Y., Lee, Y., Liu, J., Chen, F., 2017. The synergistic energy and carbon  
1256 metabolism under mixotrophic cultivation reveals the coordination between photosynthesis  
1257 and aerobic respiration in *Chlorella zofingiensis*. *Algal Res.* 25, 109–116.  
1258 <https://doi.org/https://doi.org/10.1016/j.algal.2017.05.007>
- 1259

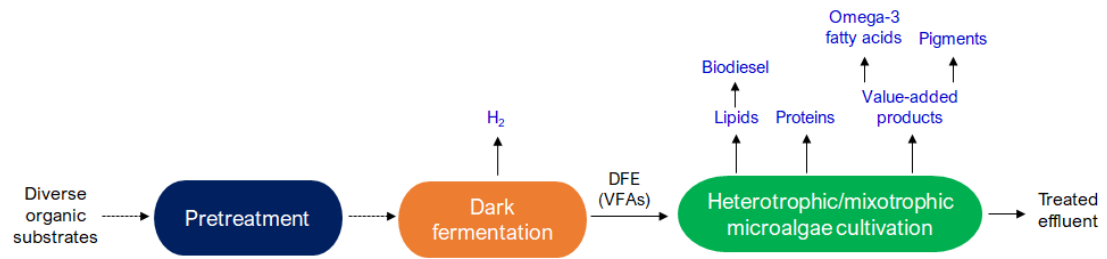


**Figure 1.** Dark fermentation effluents utilization in coupled technologies under a biorefinery concept.

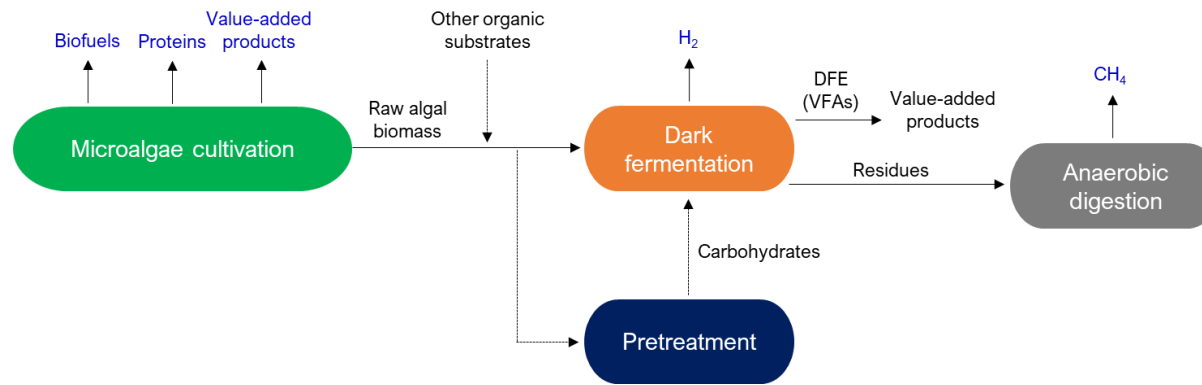


**Figure 2. Metabolic pathways in dark fermentation.** Pyruvate is the key intermediate of the metabolic pathways. Pyruvate can be converted in acetyl-CoA via the pyruvate ferredoxin oxidoreductase (PFOR) pathway (1), which leads to the production of reduced ferredoxin (Fd<sub>red</sub>). The oxidation of Fd<sub>red</sub> into oxidised ferredoxin (Fd<sub>ox</sub>) through Fe-Fe hydrogenases (Fd-FeFe) leads to the production of H<sub>2</sub> (2). Organisms following the PFOR pathway are able to regenerate NAD<sup>+</sup> via NADH by the NADH-ferredoxin oxidoreductase (NADH-Fd<sub>red</sub>-FeFe) with concomitant H<sub>2</sub> production (3). To regenerate NAD<sup>+</sup>, acetyl-CoA can also be converted to butyrate (4). Alternatively, pyruvate can be cleaved into formate and acetyl-CoA via the pyruvate formate lyase (PFL) pathway (5). The formate is subsequently converted into H<sub>2</sub> and CO<sub>2</sub> via the formate hydrogenase complex (FHL). Pyruvate can also be converted to lactate via homolactic fermentation (6).





**Figure 3. Coupling dark fermentation and microalgae cultivation.** Dark fermentation leads to the concomitant production of H<sub>2</sub> and VFAs while treating various organic wastes. These VFAs can serve as low-cost organic substrates for the cultivation of microalgae and production of valuable compounds



**Figure 4. Microalgae as DF substrate.**

**Table 1. Equations of the main metabolic pathways occurring during dark fermentation.** Equations 1 and 2 correspond to the theoretical pathways followed by HPB using the PFOR pathway while equation 3 correspond to the theoretical pathway followed by HPB using the PFL pathway. Equation 4 correspond to the mixed culture assumption equation. Equation 5 is a H<sub>2</sub> consuming pathway followed by propionic acid bacteria. Equation 6 and 7 are respectively the homo- and hetero-lactic fermentation pathways followed by LAB. In some cases, LAB can produce H<sub>2</sub> through equation 8. *Glu*: Glucose; *AcA*: Acetic acid; *ProA*: Propionic acid; *ButA*: Butyric acid; *Eth*: Ethanol; *LA*: Lactic acid

Equations	N°
$C_6H_{12}O_6 (Glu) + 2 H_2O \rightarrow 2CH_3COOH (AcA) + 2 CO_2 + 4 H_2$	1
$C_6H_{12}O_6 (Glu) \rightarrow CH_3CH_2CH_2COOH(ButA) + 2 CO_2 + 2 H_2$	2
$C_6H_{12}O_6 (Glu) + 2 H_2O \rightarrow CH_3COOH(AcA) + CH_3CH_2OH(Eth) + 2 CO_2 + 2 H_2$	3
$4 C_6H_{12}O_6 (Glu) + 2 H_2O \rightarrow 2 CH_3COOH(AcA) + 3 CH_3CH_2CH_2COOH(ButA) + 8 CO_2 + 10 H_2$	4
$C_6H_{12}O_6(Glu) + 2 H_2 \rightarrow 2 CH_3CH_2COOH(ProA) + 2 H_2O$	5
$C_6H_{12}O_6 (Glu) \rightarrow 2 CH_3CH(OH)COOH(LA)$	6
$C_6H_{12}O_6 (Glu) \rightarrow CH_3CH(OH)COOH (LA) + CH_3CH_2OH(Eth) + CO_2$	7
$4 CH_3CH(OH)COOH (LA) + 2 CH_3COOH (Ac) \rightarrow 3 CH_3CH_2CH_2COOH(ButA) + 4 CO_2 + 2 H_2$	8

**Table 2. Fermentation profiles of various substrates.** The table provides the H<sub>2</sub> yields (in mmol H<sub>2</sub>/molhexose or mmol H<sub>2</sub>/gCOD) of simple sugars or complex substrates as well as the main metabolites (g COD/L) obtained at the end of the fermentation.

Fermentation parameters			H <sub>2</sub> yields		Metabolites (g COD/L)					References
Substrate	Substrate concentration (g COD/L)	Inoculum and pretreatment	mmol H <sub>2</sub> /mmol hexose	mmol H <sub>2</sub> /g COD	Acetate	Propionate	Butyrate	Lactate	Ethanol	
Starch	20.0	AS (HT)	0.9	4.7	1.7	1.2	12.7	0.5	-	(Arooj et al., 2008)
Raw cassava starch	10.0	AS (U)	1.7	-	0.35	-	2.7	-	0.33	(Wang et al., 2017)
Lactose	12.3	AS (HT)	2.1	5.4	1.1	-	4.7	0.4	-	(Palomo-Briones et al., 2018)
Glucose	17.1	AS (HT)	2.6	15.7	4.4	0.15	5.5	-	0.13	(Hafez et al., 2010)
Glucose	5.5	WWTPS (U)	-	5.1	0.9	0.3	0.4	-	3.1	(Song et al., 2011)
Cellulose	2.1	AS (HT)	1.1	5.7	0.2	-	0.2	0.1	1.0	Santos-Lopes et al (2020)
Molasses	5.0	WWTPS (AE)	-	8.5	0.75	0.2	0.2	-	1.3	(Ren et al., 2018)
Molasses	8.0	WWTPS (AE)	-	10.7	0.6	0.2	0.9	-	3.8	(Wang et al., 2014)
Food waste	20.0	AS (U)	-	3.02	5.2	3.9	6.93	-	-	(Micolucci et al., 2020)
Food waste	76.4	AS (U)	-	4.1	6.1	1.94	28.3	-	22.8	(Greses et al., 2022)
Food waste	30.1	AS (HT)	-	0.02	0.2	0.6	0.9	4.9	47.4	(Santiago et al., 2019)
Food waste	12.0	AS (HT)	-	4.3	6.2	0.8	7.5	6.7	3.1	(Moreno-Andrade et al., 2015)

AS: Anaerobic sludge; WWTPS: Wastewater treatment plant sludge; DSS: Domestic sewage sediments; HT: Heat-chock pretreatment; U: untreated; AE: Aerobic pretreatment.

**Table 3. Growth characteristics of pure microalgae strains on single VFA (acetate or butyrate).**

Strain	Substrate	Substrate concentration (g/L)	Metabolism	Growth rate (d <sup>-1</sup> )	Biomass productivity (g/L/d)	Biomass yield (gX / gS)	Reference
<i>Acutodesmus obliquus</i>	Acetate	0.6	H	0.4	-	-	(Combres et al., 1994)
	Acetate	0.6	M	1.2	-	-	(Combres et al., 1994)
	Acetate	1.25	M	-	0.46	1.06	(Lacroux et al., 2020)
	Butyrate	0.9	M	-	0.11	3.72	(Lacroux et al., 2020)
<i>Auxenochlorella protothecoides</i>	Acetate	0.25 - 2.5	H	2.05	-	0.3	(Turon et al., 2015a)
	Acetate	20.5	M	-	0.54	0.08	(Heredia-Arroyo et al., 2011)
	Acetate	1.25	M	-	0.38	1.46	(Lacroux et al., 2020)
	Butyrate	0.18 - 0.45	H	0.22	-	0.53	(Turon et al., 2015a)
	Butyrate	0.9	M	-	0.1	1.07	(Lacroux et al., 2020)
<i>Cryptocodinium cohnii</i>	Acetate	3.5**	H	-	2.1	0.72	(Chalima et al., 2019)
	Butyrate	2.4**	H	-	2.8	0.29	(Chalima et al., 2019)
<i>Chlamydomonas reinhardtii</i>	Acetate	1.25	M	-	0.56	1.16	(Lacroux et al., 2020)
	Acetate	1	H	0.84	-	0.52	(Boyle and John, 2009)
	Butyrate	0.9	M	-	0.1	3.28	(Lacroux et al., 2020)
<i>Chlorella sorokiniana</i>	Acetate	0.25 - 2.5	H	2.23	-	0.336	(Turon et al., 2015a)
	Acetate	3	H	4.32	-	0.4	(Abiusi et al., 2020)
	Acetate	0.75	M	4.14	-	0.64	(Turon et al., 2015c)
	Acetate	2	M	-	0.789	0.75	(Wang et al., 2016)
	Butyrate	0.18	H	0.16	-	0.62	(Turon et al., 2015a)
	Butyrate	0.55	M	-	0.14	0.952	(Turon et al., 2015c)
	Butyrate	0.9	M	-	0.23	2.11	(Lacroux et al., 2020)
<i>Chlorella vulgaris</i>	Acetate	13.7	M	-	0.3	0.29	(Yeh et al., 2012)
	Acetate	1	M	-	0.4	0.8	(Liu et al., 2013)
	Butyrate	1	M	-	0.29	2.67	(Liu et al., 2013)

H: heterotrophic metabolism; M; mixotrophic metabolism; \*Substrate concentration in the fed-batch feed

**Table 4. Macromolecular composition and carbohydrate profile of some microalgae species.** Polysaccharides and monosaccharides content in algal biomass are presented for selected microalgae and cyanobacteria due to their diverse effect on H<sub>2</sub> production.

Microalgae/Cyanobacteria species	Proteins (%)	Lipids (%)	Carbohydrates (%)	Major carbohydrates	Other carbohydrates	Cultivation conditions	References
<i>Chlamydomonas reinhardtii</i>	9.2	-	59.7	43.6% starch 44.7% glucose	2.7% galactose 1.9% arabinose 1.4% mannose 0.9% rhamnose 0.4% fucose	Operation mode: fed-batch (1 M acetic acid). Cultivation time: 4 d.	(Choi et al., 2010)
<i>Scenedesmus obliquus</i>	-	19	51.8	78% glucose	22% xylose + galactose	Operation mode: batch, nitrogen starvation. Cultivation time: 3 d.	(Ho et al., 2012)
<i>Dunaliella tertiolecta</i>	20	15	12.2	85.3% glucose	5.5% rhamnose 4.5% mannose 2% ribose 1.1% galactose 1% xylose 0.65% arabinose	Operation mode: batch, growth media.	(Brown, 1991)
<i>Chlorella vulgaris</i>	51-58	14-22	12-17	42-50% rhamnose 22-30% galactose	5-17% xylose 2-10% mannose 4-9% arabinose 0-4% glucose	Not specified.	(Pieper et al., 2012; Wang and Yin, 2018)
<i>Isochrysis galbana</i>	29	23	12.9	76.5% glucose	19% galactose 5.7% arabinose 3.6% mannose 2.3% xylose 2% ribose	Operation mode: batch, growth media.	(Brown, 1991)
<i>Anabaena variabilis</i> (cyanobacteria)	-	-	46.2	27.6% reducing sugar	11.6% glycogen 2.5% starch 2.1% cellulose 1.2% hemicellulose	Operation mode: BG-11 medium without N source. Cultivation time: 24 d.	(Deb et al., 2019)
<i>Microcystis aeruginosa</i>	-	-	41.1	23.4% reducing sugar	9.7% glycogen 3.1% starch 2.6% cellulose 0.7% hemicellulose	Operation mode: BG-11 medium. Cultivation time: 24 d.	(Deb et al., 2019)
<i>Spirulina platensis</i> (cyanobacteria)	55	-	13.6	54.4% glucose 22.3% rhamnose	9.3% mannose 7% xylose 2.6% galactose	Not specified.	(Shekharam et al., 1987; Soto-Sierra et al., 2018)
<i>Nannochloropsis oculata</i>	35	18	7.8	68.2% glucose	4-8% of rhamnose, mannose, ribose, xylose, fucose, and galactose	Not specified.	(Brown, 1991)