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A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products

Anish Ghimire, Luigi Frunzo, Francesco Pirozzi, Eric Trably, Renaud Escudié, Piet N. L. Lens, Giovanni Esposito

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15 **Abstract**

16 Dark fermentation of organic biomass, i.e. agricultural residues, agro-industrial wastes and
17 organic municipal waste is a promising technology for producing renewable biohydrogen. In
18 spite of its future potential, this technology needs further research and development to improve
19 the biohydrogen yield by optimizing substrate utilization, microbial community enrichment and
20 bioreactor operational parameters such as pH, temperature and H₂ partial pressure. On the other
21 hand, the technical and economic viability of the processes need to be enhanced by the use of
22 valuable by-products from dark fermentation, which mostly includes volatile fatty acids. This
23 paper reviews a range of different organic biomasses and their biohydrogen potential from
24 laboratory to pilot-scale systems. A review of the advances in H₂ yield and production rates
25 through different seed inocula enrichment methods, bioreactor design modifications and
26 operational conditions optimization inside the dark fermentation bioreactor is presented. The
27 prospects of valorizing the co-produced volatile fatty acids in photofermentation and
28 bioelectrochemical systems for further H₂ production, methane generation and other useful
29 applications have been highlighted. A brief review on the simulation and modeling of the dark
30 fermentation processes and their energy balance has been provided. Future prospects of solid
31 state dark fermentation are discussed.

32

33 **Keywords:** Biofuels, biohydrogen, dark fermentation, H₂ yield, biomass valorization,
34 fermentation metabolites

35 **1. Introduction**

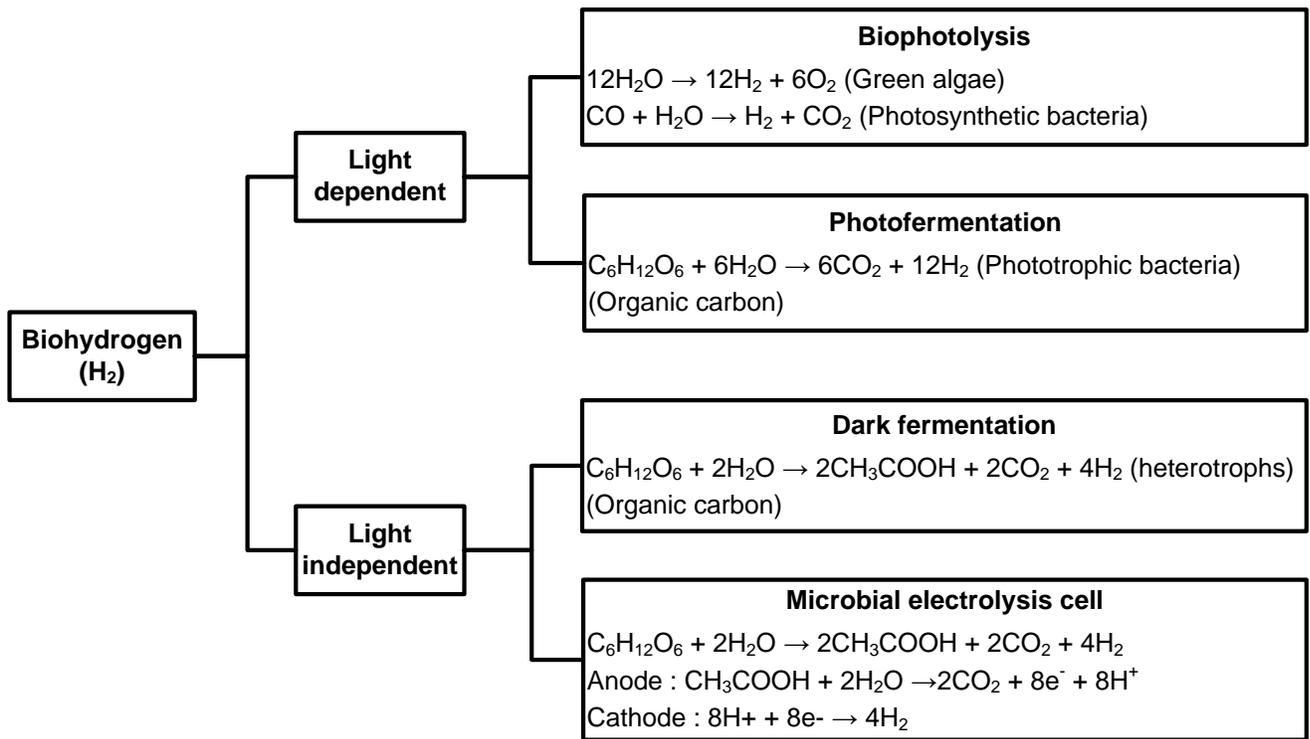
36 Environmental friendly energy carriers and sources are the most highlighted topic in the energy
37 and environmental sector. The current global energy demand is mostly dependent on reserves of
38 fossil fuels, which are depleting, and the world is facing severe pollution problems from the by-
39 products of fossil fuels uses [1]. The scientific community has widely accepted the fact that the
40 increasing CO₂ level due to the use of fossil resources is impacting the greenhouse gas effect and
41 global warming. Therefore, different ways to harness the energy from clean renewable sources
42 are being developed but the search for reliable energy sources is still on.

43 In the past years, the research and development interests have been directed towards renewable
44 energy technologies like the anaerobic digestion (AD) of organic biomass and waste. For
45 alternative energy carrier, hydrogen could be a fuel of the future because of its high energy
46 content, environmental friendliness of production, and also because it can give substantial social,
47 economic and environmental credentials [2]. Hydrogen is a carbon-free clean fuel, as the final
48 by-product of its combustion is only water [2]. Hydrogen can also be helpful in addressing global
49 warming and increasing pollution problems. Furthermore, it is preferred over methane owing to
50 its wider industrial applications, i.e. H₂ is used in the synthesis of ammonia and hydrogenation of
51 edible oil, petroleum, coal and shale oil [3]. Hydrogen can be directly used either in combustion
52 engines because of its highest energy per unit weight, i.e. 143 GJ per ton [2] among known
53 gaseous biofuels or to produce electricity via fuel cell technologies [4]. Thus, the creation of a
54 hydrogen economy which incorporates the production and use of hydrogen as an energy carrier
55 could in the future lead to sustainable energy systems [1,5].

56 The major challenge in the use of this promising energy carrier lies in its sustainable production
57 and storage. In commercial applications, hydrogen has been produced from natural gas (48%)
58 and oil (30%) by steam reforming processes, and also by other industrial methods such as coal
59 gasification (18%) and water electrolysis (4%) [6]. However, these processes are highly energy
60 intensive and use non-renewable sources of energy which makes them less attractive from an
61 environmental point of view. In order to produce a cleaner and more sustainable fuel, the
62 hydrogen should come from processes that could avoid or minimize CO₂ emissions.

63 Hydrogen can be produced from biological processes which are less energy intensive and more
64 environmental friendly in terms of global reduction of CO₂. These renewable biohydrogen

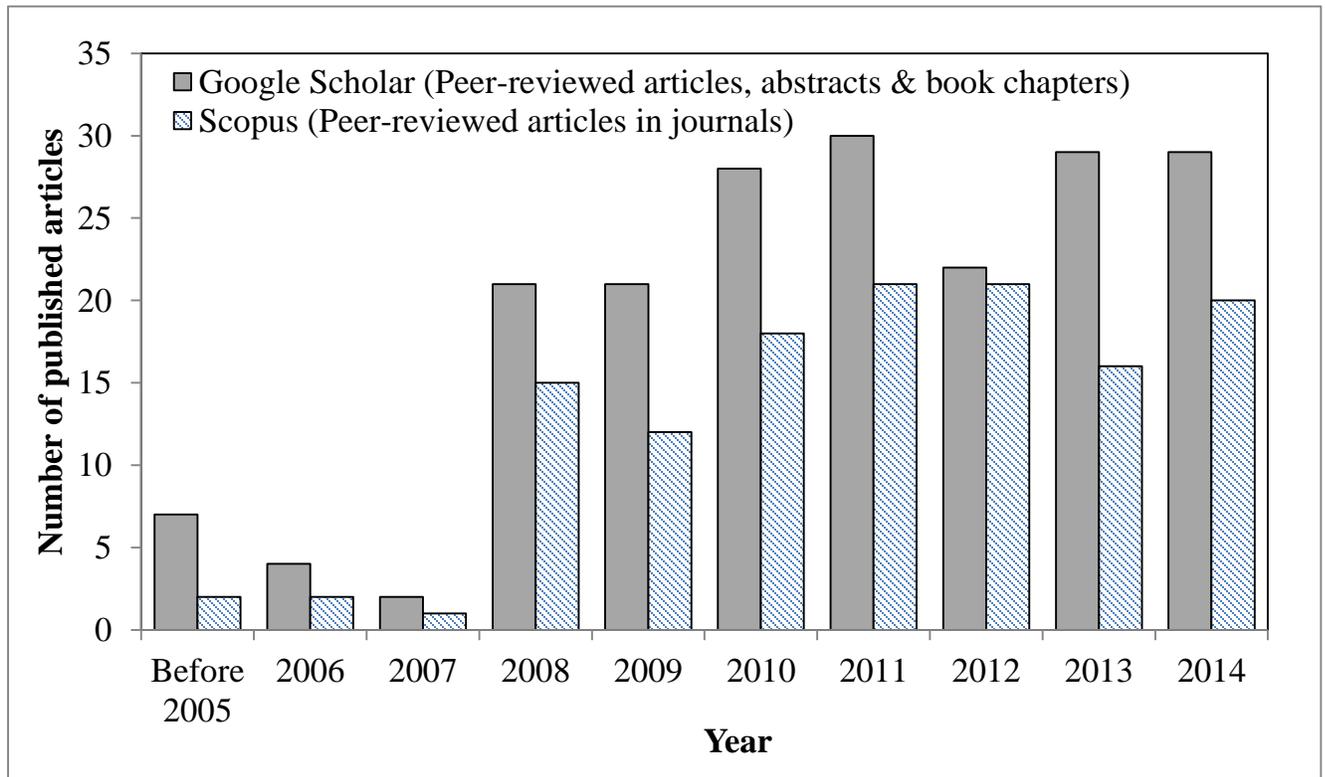
65 producing technologies have potential to become cost competitive as they can use low value
 66 waste biomass as feedstock [2], e.g. municipal, agricultural and industrial organic and
 67 wastewater. Biohydrogen can be produced by both autotrophic and heterotrophic
 68 microorganisms (Fig. 1) [7]. In autotrophic conversions (also known as direct or indirect
 69 biophotolysis), solar energy is directly converted to hydrogen via photosynthetic reactions
 70 mediated by photosynthetic microorganisms, i.e. microalgae, protists and photosynthetic
 71 bacteria. Under heterotrophic conditions, the organic substrates are transformed into simpler
 72 organic compounds with simultaneous production of molecular hydrogen [7,8]. There are two
 73 types of heterotrophic conversions, photo-fermentation carried out by photosynthetic bacteria
 74 and dark fermentation (DF) carried out by anaerobic bacteria that convert carbohydrates into
 75 biohydrogen.



76
 77 Fig. 1. Biological pathways to produce hydrogen

78
 79 DF is the most studied and promising technology for biohydrogen production owing to its higher
 80 production rates and treatment capacity for organic waste. Several substrates rich in
 81 carbohydrates are also usable, such as first generation fuel crops (e.g. sugar cane, wheat, corn,

82 and sugar beets) as well as second generation biomass like agricultural residues and industrial
83 waste and wastewater [7]. In recent years, there are still increasing research activities in this
84 domain, as shown by the increasing number of peer-reviewed articles with “dark fermentation”
85 in the title (Fig. 2).



86
87 Fig. 2. Number of peer reviewed publications on DF published in the last decade [9,10]

88
89 At present, DF process development at industrial scale is limited by its lower hydrogen yield
90 compared to its theoretical maximum yield of 4 moles of H₂ per moles of hexose, as well as the
91 estimated costs associated with the H₂ production. There are areas for improvement to achieve
92 higher H₂ yields and production rates by optimizing the design and operation of DF bioreactors
93 [11]. The H₂ production cost in scaled-up systems can be minimized by using low cost renewable
94 materials such as waste biomass as feedstock [12,13]. Inoculum enrichment methods [8,14–17]
95 can improve the H₂ yield, and pre-treatment of substrates can also enhance the biohydrogen
96 production by improving the biodegradability of substrates [18–20]. Recently, there has been
97 growing interest on coupled processes to obtain a higher H₂ yield by integrating DF with

98 processes like photofermentation (PF) [21,22] or bioelectrochemical systems [23–25]. Because
99 of the profitable production of biomethane, a coupled DF-methanogenic stage has also been a
100 popular choice which increases the sustainability of the coupled-process by improving the energy
101 recovery from DF residues [26–28].

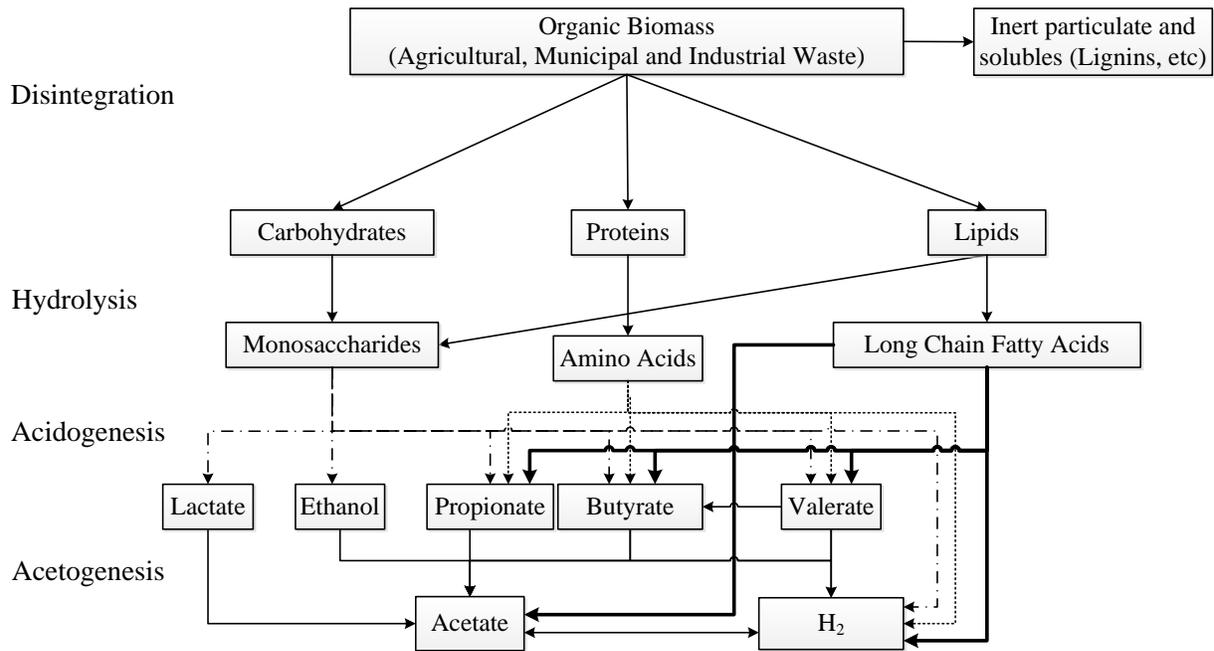
102 The aim of this paper is to provide an updated overview of advancements in biohydrogen
103 production via DF of organic biomass. Regardless of the increasing number of research articles
104 and reviews published, there is a need to provide an extended overview of dark fermentative
105 biohydrogen production with the utilization of by-products and the future challenges and
106 prospects for its up-scaled development. This review provides an insight on the factors that
107 influence the biochemical pathways in dark fermentative biohydrogen production to increase the
108 H₂ yield and post-utilization of DF residues to realize its future sustainability. To summarize, this
109 review provides an extended insight on a) possible feedstock or substrate sources and their
110 biohydrogen potential (BHP), b) factors that influence the fermentative H₂ yield: (i) inoculum
111 sources and enrichment methods, (ii) pre-treatment of substrates and (iii) bioreactor operation
112 and design (culture pH, temperature and OLR, HRT, H₂ partial pressure, nutrients and elements
113 addition), c) utilization of DF residues, d) pilot scale systems and e) challenges and future
114 prospects: (i) modeling and simulation of DF process, (ii) energy balance and conversion of
115 organic carbon, (iii) natural pH control and (iv) future prospects of solid state dark fermentation.

116 **2. Microbiology and biochemical pathways of DF**

117 In DF processes, carbohydrate-rich substrates are broken down anaerobically by hydrogen-
118 producing microorganisms, such as facultative anaerobes and obligate anaerobes. Molecular
119 hydrogen (H₂) is produced in the process of disposing the excess electrons through the activity of
120 the hydrogenase enzyme [8,29]. Under anaerobic environments, protons (H⁺) can act as electron
121 acceptors to neutralize the electrons generated by oxidation of organic substrates, consequently
122 producing H₂. In contrast with aerobic respiration, where oxygen is reduced and water is the final
123 product [7,30].

124 In the DF of glucose as the model substrate, H₂ -producing bacteria initially convert glucose to
125 pyruvate through glycolytic pathways producing adenosine triphosphate (ATP) from adenosine
126 diphosphate (ADP) and the reduced form of nicotinamide adenine dinucleotide (NADH) [8].

127 Pyruvate is further oxidized to acetyl coenzyme A (acetyl-CoA), carbon dioxide (CO₂) and H₂ by
 128 pyruvate ferredoxin oxidoreductase and hydrogenase. Depending on the type of microorganism
 129 and environmental conditions, pyruvate may also be converted to acetyl-CoA and formate which
 130 may be further converted into H₂ and CO₂. Also, acetyl-CoA might be converted to acetate,
 131 butyrate, and ethanol [8]. DF of complex carbohydrates by mixed anaerobic microbiota can
 132 result in a wide range of intermediates and by-products depending on the operational parameters,
 133 such as substrate type, substrate loading rate, pH, temperature and other operating and
 134 environmental conditions, as they also influence the microbial community structure in
 135 bioreactors. A representative biochemical pathway for DF of complex organic biomass is
 136 presented in Fig. 3.



137
 138 Fig. 3. Microbiological pathway of fermentative waste biomass degradation (Adapted and
 139 modified from [31])

140
 141 These biochemical pathways (Fig. 3) can be mediated by strict anaerobes (*Clostridia*,
 142 methylotrophs, rumen bacteria, methanogenic bacteria, archaea, etc.), facultative anaerobes
 143 (*Escherichia coli*, *Enterobacter*, *Citrobacter*), and even aerobes (*Alcaligenes*, *Bacillus*) [8].

144 Acetate and butyrate are the most common products of DF [32]. Common biochemical reactions
145 during DF undertaken by facultative anaerobes are the following:



147 (Acetic Acid)



149 (Butyric Acid)

150 When the metabolic pathway is such that it favors the production of acetic acid, the
151 stoichiometric yield of H₂ is 4 moles for each mole of glucose (i.e. 544 mL H₂/g hexose at 25 °C)
152 as seen in equation 1, whereas the yield of H₂ is 2 moles for a mole of glucose (i.e. 272 mL H₂/g
153 hexose at 25 °C) when the final product is butyric acid (equation 2). However, the actual
154 hydrogen yield is lower than the theoretical yield as part of the substrate is utilized for biomass
155 production and the degradation of the substrates might follow other biochemical pathways
156 without hydrogen production [33,34]. Under some conditions, the metabolic pathways lead to
157 ethanol and acetate production, lowering the stoichiometric hydrogen yield to 2 moles of H₂ for a
158 mole of glucose (i.e. 272 mL H₂/g hexose at 25 °C) as represented in equation 3 [8]:



160 A widely studied clostridia species, *Clostridium butyricum*, is responsible for the production of
161 butyric acid as a major product of fermentation together with acetate and hydrogen [32]. Another
162 fermentation pathway is the production of propionate by *Clostridium articum* which is a
163 hydrogen consuming pathway (equation 4). Similarly, metabolic pathways leading to only
164 ethanol and lactic acid production by *Clostridium barkeri* yield no hydrogen (equations 5 and 6)
165 [35]:



169 Hawkes [32] and Kim [36] proposed the molar ratio of butyric to acetic acid (B/A ratio) as a
170 quantitative indicator to biohydrogen yield associated with microbial metabolic pathways. Kim
171 et al. [36] found that B/A ratios were directly proportional to H₂ yields (mol H₂/mol hexose
172 consumed) during DF of sucrose in CSTR reactors operated at an organic loading rate (OLR) of

173 10.60 g Chemical Oxygen Demand (COD)/L, pH 5.5 and 12 h hydraulic retention time (HRT).
174 They also reported that a B/A ratio higher than 2.6 indicated an efficient H₂ production by
175 anaerobic microbiota. In DF with mixed cultures, a B/A ratio of 3:2 is generally observed,
176 resulting in a H₂ yield of 2.5 moles H₂ per mole of hexose fermented as given in equation 7 [32]:



178 In contrast, Guo et al. [37] showed in their study performed with lignocellulosic substrates that
179 this ratio might not give a good indication, particularly in batch tests where homoacetogenic
180 activity prevails. Therefore, higher acetate concentrations cannot always give an indication of a
181 higher H₂ yield. Some homoacetogens belonging to the genus *Clostridium* (e.g. *C. aceticum*) can
182 lower the H₂ yield by converting H₂ and CO₂ to acetate or can convert hexose directly to acetate
183 [32,36]. However, analysis of soluble metabolites can give an indication of the fermentation
184 pathways and thus the H₂ production performance.

185 Clostridia have been identified as the dominant hydrogen producing microorganisms in DF
186 operated with mesophilic mixed cultures at a pH of 5.5 [38]. Fang and Zhang [38] identified that
187 64.6% of all the microorganisms were affiliated with three *Clostridium* species (*Clostridiaceae*),
188 18.8% with *Enterobacteriaceae*, and 3.1% with *Streptococcus bovis* (*Streptococcaceae*) based
189 on the phylogenetic analysis of the rDNA sequences. Interestingly, Rafrafi et al. [39] reported
190 recently that sub-dominant species, in spite of their low abundance, can also have substantial
191 impact on the hydrogen production performance. The presence of some species like *E. coli* can
192 aid in increasing the H₂ yield by diverting the metabolic pathways to acetate and butyrate
193 hydrogen producing pathways (equation 7), while some communities such as *Bacillus spp.* and
194 *Lactobacillus spp.* can lower the H₂ yield by diverting the pathway to lactate accumulation
195 (equation 6).

196 Other results of the identification of the microbial diversity by community fingerprinting
197 techniques in the thermophilic DF of rice straw showed that hydrolytic and fermentative bacteria
198 such as *Clostridium pasteurianum*, *Clostridium stercorarium* and *Thermoanaerobacterium*
199 *saccharolyticum* dominated in the sludge of a repeated fed-batch reactor [40]. Shin et al. [41]
200 detected the hydrogen producing microorganisms *Thermoanaerobacterium*
201 *thermosaccharolyticum* and *Desulfotomaculum geothermicum* in a thermophilic acidogenic
202 culture, while *Thermotogales* strains and *Bacillus* species were detected in a mesophilic

203 acidogenic culture by Polymerase Chain Reaction (PCR)-Denaturing Gradient Gel
204 Electrophoresis (DDGE) analysis during DF of food waste. In another study, Quemeneur et al.
205 [42] investigated the potential of a molecular capillary electrophoresis-single strand
206 conformation polymorphism (CE-SSCP) fingerprinting method based on the *hydA* functional
207 genes to better describe the bacterial community dynamics in a mixed dark fermentative culture
208 at different pH conditions.

209 Some undesirable microorganisms which lower the total H₂ yield might be present in mixed
210 cultures of fermentative microorganisms, either by consuming the H₂ produced or by altering the
211 biochemical pathways of the H₂ synthesis [8]. The main H₂ consumers include methanogens,
212 homoacetogenic bacteria and sulfate reducing bacteria (SRB). The activity of these hydrogen
213 consumers can be controlled by inoculum pre-treatment methods and bioreactor operating
214 conditions [30,43]. The activity of methanogens and SRB can be significantly inhibited by
215 operating at a pH below 6 along with the control of the HRT and OLR. Therefore, hydrogen
216 production via a mixed dark fermentative culture is a complex system, influenced by a number of
217 parameters such as substrate types, substrate pretreatment, inoculum type, inoculum enrichment
218 method, bioreactor design and operation.

219 **3. Potential sources of organic biomass for fermentative biohydrogen production**

220 The substrate plays an important role in the H₂ yield, H₂ production rate and the overall economy
221 of the process. These are mainly dependent on the substrate's carbohydrate content,
222 biodegradation rate and bioavailability [12,13,16,43,44]. Carbohydrate rich substrates have been
223 extensively used in DF studies, in particular pure glucose, sucrose and starch mixtures [30]. But
224 renewable biohydrogen production requires the substrate or feedstock to come from renewable
225 resources [12,32]. Second generation biomass sources, such as waste biomass, are abundant and
226 thus can fulfill the promise for the supply of renewable substrates [13,14,43]. Besides
227 biohydrogen and volatile fatty acids as valuable by-products, DF also offers biological treatment
228 of the organic waste.

229 In more recent dark fermentative studies, complex substrates have been considered, such as the
230 organic fraction of municipal solid waste (OFMSW) [40,45–48], agricultural residues like
231 lignocellulosic biomasses (e.g. rice straw, wheat straw and corn stalks), agro-industrial wastes

232 like those from food processing industries (e.g. olive mill wastewater and cheese whey),
233 effluents from livestock farms and aquatic plants [13,14]. With the integration of DF within a
234 biorefinery concept, the waste generated from biofuel production such as crude glycerol [25,49],
235 de-oiled algal cake [50], cotton seed cake [51] can be utilized as a substrate, while dark
236 fermentative metabolites can be utilized in the production of micro-algal biomass [52–54] and
237 biodiesel [55], which in turn can be serve as feedstock for DF processes.

238 Based on their availability, novel low-cost substrate sources need to be explored and assessed for
239 their biohydrogen potential (BHP). Table 2 presents the biohydrogen production potential of
240 different organic biomasses by dark fermentative process. The fermentation pathways depend on
241 substrates and on microbial metabolism [8]. It has been well established that the type of substrate
242 influences biohydrogen yields [37,56]. Monlau [57] and Guo [37] in their studies reported that
243 the soluble and readily accessible sugars represent the main fraction of biomass that can be
244 converted into hydrogen. However, the biohydrogen production also depends on a number of
245 parameters such as inoculum type and enrichment methods, bioreactor design and operation
246 conditions. The latter are covered in the next sections.

247 **3.1 Agricultural residues**

248 Agricultural residues, which mainly include lignocellulosic wastes, are an economically viable
249 and renewable source of second generation carbon neutral biofuels [58]. These include plant
250 biomass waste, which generally contains cellulose, hemicellulose and lignin formed as products
251 of photosynthesis. Agricultural residues are produced when economically valuable products of
252 the crops are harvested and the residues such as straw, stover, peelings, cobs stalks, bagasse and
253 others are left over [58]. The 2010 global annual production of agricultural residues was around
254 5.1 billion dry tonnes [59]. The waste generated by the agricultural, forestry and aquaculture
255 sectors is increasing with the increasing population and thus the waste from this sector will be
256 increasing further in future. Guo et al. [43] have reported the potentials and challenges of
257 agricultural wastes as substrates for biohydrogen production. Examples of agricultural residues
258 as a potential feedstock sources for DF processes and recent advancements in their application
259 are discussed below.

260 **3.1.1 Lignocellulosic waste**

261 Rice straw is an example of a typical agricultural residue. It is the world's third largest
262 agricultural residue, after maize and wheat, with a reported global yearly production of
263 approximately 916 million tons in 2009 [60]. Thus, the use of this abundant biomass as a
264 feedstock in dark fermentative hydrogen production might hold future potential for feedstock
265 supply. Similarly, wheat straw, barley straw, corn stalk, corn cobs and others could be potential
266 DF feedstock. The cellulose and hemicellulose part of these wastes can be hydrolyzed into
267 carbohydrates which are further biologically converted to organic acids and biohydrogen in DF
268 processes (Table 1). The composition of typical crop residues in terms of cellulose,
269 hemicellulose and lignin content is presented in Table 1.

270 **Table 1**

271 Composition of typical agricultural waste (% of dry matter)

Component	Rice straw ^a (%)	Wheat straw ^b (%)	Barley straw ^b (%)	Corn stalk ^b (%)	Corn cob ^b (%)
Cellulose	38.6	35.4	37.2	36.7	35.3
Hemicellulose	19.7	24.6	24.4	26.2	37.1
Lignin	13.6	17.7 ^c	16.1 ^c	16.9 ^c	16.4 ^c
Ash	-	6.9	6.4	4.1	1.5

272 ^a Data obtained from [61]; ^b Data obtained from [62]; ^c Acid insoluble lignin

273 The main limitation in the utilization of these valuable resources lies in the complex structure of
274 lignocellulosic materials: a cross-linking between polysaccharides (cellulose and hemicellulose)
275 and lignin via ester and ether linkages, which decreases their biodegradability [58,63–65].
276 Therefore, prior to DF, these biomasses are often subjected to physical, chemical and biological
277 pre-treatment to increase their digestibility [58,63–69].

278 **3.1.2 Livestock waste (manure)**

279 Livestock wastes include solid animal manure waste, fodder waste (which generally contains a
280 lignocellulosic fraction) and wastewater which includes urine and feces. A large quantity of
281 livestock manure comes from cattle feedlots, poultry and swine buildings, identified as pollution

282 sources, which pose threats to the atmospheric and water environment [70]. The current practices
283 of management of livestock waste include its application in agricultural fields as well as
284 biological stabilization or treatment such as composting and AD. The first management practice
285 contributes in uncontrolled greenhouse gas emissions (mainly CH₄) from land applications.
286 Manure management practices can reduce direct and indirect greenhouse gas emissions by
287 generating energy in the form of biogas from the manure prior to its land application [70–72].
288 However, manure substrates need physical and chemical treatment to inhibit the methanogenic
289 activity that consumes H₂ [71,73]. Another, problem that might occur during the use of this
290 feedstock type is the inhibition of the biohydrogen production by ammonia as their high nitrogen
291 content might cause failure of the bioreactor: swine, poultry and dairy manure have a low C/N
292 ratio (C/N ratio of swine manure: 12.8) [74] and higher levels of ammoniacal nitrogen (cattle
293 slurry: 1040 -1925 mg/l and chicken manure 7000 - 12,800 mg/L) [75]. Salerno et al. [76]
294 reported that hydrogen production is possible at high concentrations up to 7.8 g N/L in
295 continuous flow systems if the microbial culture initially acclimated to a lower ammonia
296 concentration of 0.8 g N/L. total ammonia) However, the biohydrogen production decreases
297 when the total ammonia concentration would be increased to above 2 g N/L, which was in
298 agreement with the studies of Cavinato et al. [77]. Also, high sulfate concentrations in swine
299 manure can inhibit biohydrogen production due to the presence of hydrogen consuming sulfate
300 reducers [43].

301 Because of the high nitrogen content of animal manure, it can be used as a co-digestion substrate
302 for nitrogen supplementation of other agricultural residues to maintain a suitable carbon to
303 nitrogen ratio. Wu et al. [71] reported a H₂ yield of 1.18 and 1.63 mol H₂/mol glucose in a
304 fermentation of swine manure supplemented with glucose. Xing et al. [72] achieved an enhanced
305 H₂ yield of 31.5 ml/g Volatile Solids (VS) with acidification pretreated dairy manures while
306 treating 70 g/L of substrate at operating pH 5.0.

307 **3.2 Industrial waste**

308 Agro-industries waste such as palm oil mill wastewater [78–81] and olive mill wastewater
309 (OMWW) [82,83], tapioca industries and food industries such as brewery and dairy industries
310 [84–87] produce large quantities of carbohydrate rich non-toxic waste in the form of solid waste

311 and wastewater. It can be potential substrates for dark fermentative biohydrogen production. Ren
312 et al. [88] demonstrated that waste molasses are an excellent substrate in a pilot scale system
313 operated under mesophilic conditions (35 °C) where very good results were obtained in terms of
314 H₂ production rate (232 mL H₂/L/h) and yield (5.016 mol H₂/mol glucose equivalent). The
315 production of large quantities of this type of waste biomass supports its utilization in up-scaled
316 DF systems for continuous biohydrogen production. Similarly, cheese whey, a waste by-product
317 generated from cheese manufacturing industries and characterized by high organic loads,
318 comprising mainly carbohydrates (lactose), protein and lipids, is a very good potential substrate
319 for biohydrogen production [24,89,90].

320 **3.3 Organic fraction of municipal waste (OFMSW)**

321 OFMSW generally constitutes food waste which contains a high biodegradable carbohydrates
322 fraction with 85-95% volatile solids and 75-85% moisture content making it a good substrate for
323 DF [43]. Food waste present in municipal waste is mainly responsible for methane emissions and
324 leachate production from landfills [91]. AD has been proposed as the most suitable treatment
325 option for OFMSW or food waste with energy recovery and other environmental credentials
326 [92]. Thus, food waste has been used extensively in DF experiments [41,77,93–95]. Gioannis et
327 al. [17] have reviewed the studies of DF processes utilizing OFMW or food waste for dark
328 fermentative biohydrogen production.

329 Large quantities of waste biosolids or sludge are generated from municipal wastewater treatment
330 plants which generally contain carbohydrates or polysaccharides and proteins [96]. Several
331 researchers have used the available carbohydrates present in these biosolids in fermentative
332 hydrogen production [97,98]. However, the sludge needs pre-treatment, such as ultrasonication,
333 acidification, sterilization, freezing-thawing or alkaline pre-treatment, to facilitate the
334 fermentative process [96,98]. Besides, Kim et al. [97] demonstrated the usefulness of sewage
335 sludge as co-substrate in the DF of food waste.

336 **Table 2.**

337 Dark fermentative biohydrogen potential of different waste biomass under varying operating conditions

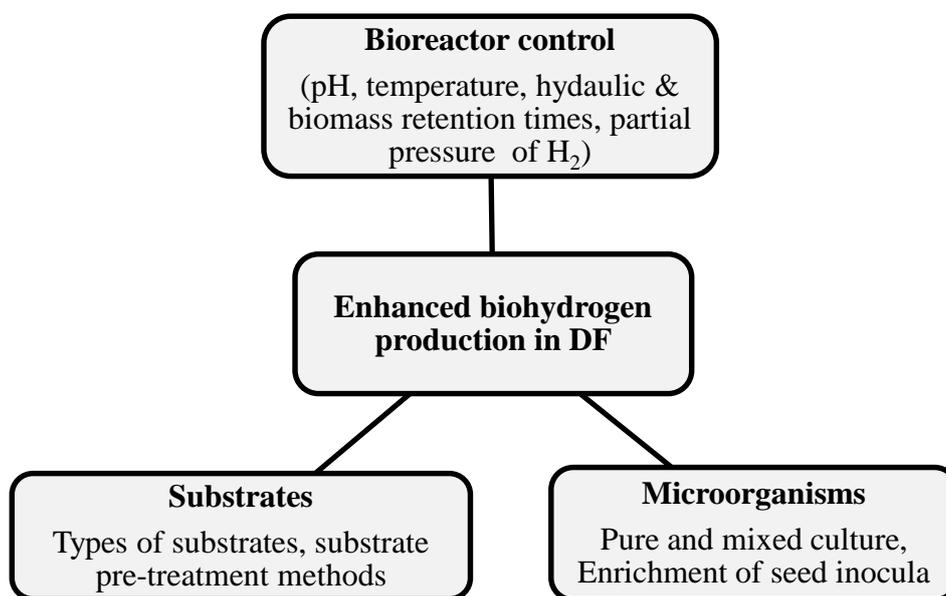
Substrate type	Microbial inoculum source	Reactor type	Temperature (°C)	pH	Maximum assessed H ₂ yield (mL H ₂ /g VS)	Maximum H ₂ production rate	H ₂ in biogas (%)	Reference
Food waste	Heat shock treated anaerobic sludge	Leaching Bed Reactor	37	-	310	91 L H ₂ /L/h	10 - 55	[99]
Food waste	Thermophilic acidogenic culture	Batch	55	4.5	46.3	3 mL H ₂ /g VS/h	23	[41]
Vegetable kitchen waste	Kitchen waste compost	Intermittent-CSTR	55	6.0	1.7 mmol H ₂ /g COD	1.0 L H ₂ /L/d	40	[100]
Food waste and sewage sludge	Anaerobic digester sludge	Batch	35	5.0–6.0	122.9 ^a	111:2 mL H ₂ /g VSS/h	-	[97]
OFMSW	Anaerobic digestate	Semi-continuous CSTR	55	6.4	360	-	58	[45]
OFMSW	Non-anaerobic inocula (soil, pig excreta)	Packed bed reactor	38	5.6	99	-	47	[101]
Wheat straw	Cow dung compost	Batch	36	6.5	68.1	-	52	[102]
Rice straw	Wastewater treatment plant sludge	Batch CSTR	55	6.5 (initial)	24.8 mL/g TS	-	-	[40]

Corn stalk wastes with acidification pre-treatment	Enriched cow dung composts	Batch CSTR	55	5.5	149.69	7.6 mL H ₂ /h	45–56	[46]
Rice slurry (5.5 g carbohydrate/L)	Anaerobic digester sludge	Batch	37	4.5 (initial)	346 ^b	2.1 L/g VS/d	45–56	[103]
Cheese whey	Adapted anaerobic sludge	Batch	55	-	111 ^c	3.46 mL H ₂ /L/h	-	[104]
Pig slurry	Mesophilic methanogenic sludge	CSTR	70	-	3.65	-	-	[105]
De-oiled algae cake	Anaerobic digester sludge	Batch	29 2	6 (initial)	66	0.08 ml/h	-	[50]

338 ^a mL H₂/g carbohydrate COD, ^b mL H₂/g carbohydrate, ^c mL H₂/g total sugar, ^d mL H₂/g algal biomass

339 **4. Factors affecting DF pathways and H₂ yield**

340 DF via mixed cultures is a complex system where environmental factors and bioreactor
341 operation conditions such as temperature, pH and H₂ partial pressure regulate metabolic
342 pathways of hydrogen producing microorganisms [8,30,43,106]. In addition, substrate types
343 and their pre-treatment methods, bioreactors configurations, inoculum sources and
344 enrichments also influence the biohydrogen production. Three categories of parameters that
345 influence the DF pathways, and thus the yield of biohydrogen, can be distinguished (Fig. 4).
346 These parameters are reviewed below and compared in relation to H₂ yield and production
347 rate.



348
349 Fig. 4. Strategies to enhance the biohydrogen yield in DF of organic biomass

350

351 **4.1 Inoculum and enrichment methods**

352 The hydrogen producing seed inoculum or culture is very important for the startup of the
353 hydrogen production process. Several studies using pure cultures have been done using a
354 range of substrates (Table 3). Various species of *Clostridia* and *Enterobacter* are widely used
355 in pure cultures (Table 3). Lee and Show [107] and Elsharnouby et al. [108] have reviewed
356 the studies of DF performed with pure cultures. Table 3 presents some of the dark
357 fermentative biohydrogen studies done with pure cultures.

358

359

360 **Table 3**
 361 Biohydrogen production studies using pure culture

Culture	Substrate	Culture type	Temp. (°C)	pH	Optimum H ₂ yield (mol H ₂ /mol glucose eqv.) ^a	Reference
<i>Enterobacter cloacae</i> IIT-BT08	Glucose	Batch	36	6	2.2	[109]
<i>Clostridium thermolacticum</i> DSM 2910	Lactose	Continuous	58	7	1.5	[110]
<i>Enterobacter cloacae</i> DM 11	Malt, yeast extract & glucose	Continuous	37	6	3.9	[111]
<i>Caldicellulosiruptor saccharolyticus</i> DSM 8903	Hydrolyzed potato steam peels	Batch	70	6.9	3.4	[112]
<i>Thermotoga neapolitana</i> DSM 4349	Hydrolyzed potato steam peels	Batch	80	6.9	3.3	[112]
<i>C. thermocellum</i> DSM 1237 and <i>C. thermopalmarium</i> DSM 5974	Cellulose	Batch	55	7	1.36	[113]
<i>Clostridium thermocellum</i> 7072	Corn stalk	Continuous (100 Liters)	55	7.2	0.45	[114]

362 ^a mol H₂/mol glucose equivalent was calculated based on the information provided from
 363 references at Standard Temperature and Pressure (STP) (0°C and Pressure 1 atm)

364

365 H₂ synthesizing bacteria exist commonly in environments such as soil, wastewater sludge and
 366 compost. All these materials can thus be used as an inoculum for fermentative H₂ production
 367 [8]. Indeed, cow dung, anaerobic sludge, municipal solid waste, soil and compost are some of
 368 the common sources of mixed cultures. A mixed culture of hydrogen producers is generally
 369 preferred over a pure culture due to its practicability for environmental engineering
 370 applications, economic benefits in operation (as it can economize asepsis costs), easiness in
 371 control based on differential kinetics of microbial subgroups and broader feedstock choice
 372 [8,30,45]. However, enrichment of mixed cultures becomes necessary to enhance the
 373 biohydrogen production on the one hand and inhibit hydrogen consumers such as

374 methanogens and homoacetogens on the other hand, as other trophic groups that readily
375 consume the produced hydrogen, such as *Clostridium* and *Enterobacter*, are often present in
376 these mixed inocula [30].

377 Pre-treatment of the inoculum to obtain an enrichment of hydrogen producers often relies on
378 the spore forming characteristics of H₂ producers such as *Clostridium*, which is ubiquitous in
379 anaerobic sludge and sediments [8,84,115,116]. These organisms have a better chance to
380 survive the harsh conditions during the pre-treatment of the inoculum than the non-spore
381 forming bacteria such as methanogens and can germinate under favorable conditions [8,15].
382 Table 4 summarizes the common pre-treatment measures adopted for enrichment of hydrogen
383 producers.

384 Heat treatment of mixed cultures for the enrichment of H₂ producers is a simple, inexpensive
385 and effective method [8,30]. However, the effect of heat treatment might be different
386 depending on the inoculum source such as activated sludge or anaerobic sludge [30]. Some
387 studies [117,118] reported a lower hydrogen yield by a heat shock (HS) treated seed
388 inoculum than obtained by other pre-treatment methods. This could be due to the inhibition
389 of other non-spore forming hydrogen producing bacteria which might destabilize the main
390 hydrogen production pathways. Similarly, acid or base treatment is based on the notion that
391 the activity of methanogens drops sharply at a pH below 6.3 or above 7.8 [8], while the
392 activity of *Clostridia sp.* and other hydrogen producers is not affected by an acidic pH (less
393 than pH 6).

394 Other pre-treatment methods such as chemical pretreatment and aeration are directed towards
395 the selective inhibition of methanogens present in anaerobic sludge, which are very sensitive
396 to changes in environmental conditions. Besides being strict anaerobes, methanogens are
397 sensitive to many chemicals [8]. Thus, oxygen can inhibit their activity during aeration
398 [116,117]. Wang and Wan [116] aerated the inoculum sludge with air for 24 hours to inhibit
399 the activity of methanogens. Likewise, chemical inhibitors like sodium 2-bromoethanesulfonic
400 acid (BESA), iodopropane, chloroform and acetylene are used to inhibit methanogens
401 [8,84,117–119]. Thus, selective inhibitors like chemical chloroform or BESA selectively
402 inhibit the activity of H₂ consumer methanogens. In methanogens, BESA functions by
403 inhibiting the activity of co-enzyme M reductase complex, which is a key co-enzyme of
404 methanogenesis [84,117].

405

406

407 **Table 4**408 Pre-treatment methods used to enriching hydrogen producing microorganisms in anaerobic
409 sludge

Treatment	Description	Inoculum source	Reference
Heat	100 °C for 15 min	Anaerobic digested sludge	[116]
Heat	80°C, 90°C and 100°C for 15-30 min	Anaerobic sludge	[119]
Heat	Heating in boiling water bath for 10-30 min	Anaerobic granular sludge	[74, 75]
Heat	105 °C for 4 hour	Anaerobic granular sludge	[120]
Heat	Incubation at 90 °C for 1 hour	Anaerobic granular sludge	[121]
Heat	100–105 °C in oven for 2 hour	Cow dung compost	[122]
Acid	pH to 2 for 24 h and increasing pH to 5.5 by adding a 2 N NaOH solution	Anaerobic digested sludge	[123] [79]
Acid	pH 3 with 2 N HCl for 24 hours	Anaerobic digested sludge	[124]
Acid	pH to 3 with 1 N HCl for 30 min	Anaerobic digested sludge	[117]
Acid	pH 3 with 0.1 N HCl solution for 24 hours and adjusting back to pH 7	Anaerobic granular sludge	[125]
Base	pH of the sludge to 3 with 1 mol/L of NaOH for 24 hours	Anaerobic digested sludge	[116]
Base	pH 8, 9 and 10 with 1 mol/L of NaOH for 3 hours	Anaerobic sludge	[119]
Base	pH 12 with 1 M NaOH for 24 hours and adjusting back to pH 7 using 1 M HCl	Anaerobic digested sludge	[118]

Load shock	Sludge (50 ml) spiked with 40 g of sucrose and acidification for 2 d	Anaerobic granular sludge	[121]
Load shock	Sludge (50 ml) spiked with 500 mL of sucrose (50 g/L) and acidification for 2 d	Anaerobic digested sludge	[118]
Chemical inhibition	10 mmol of BESA for 30 min and gravity separation for 2 h	Anaerobic digested sludge	[69, 70]
Chemical inhibition	0.2 g/l BESA for 24 h	Anaerobic granular sludge	[84]
Chemical inhibition	0.1% (v/v) chloroform for 24 h	Anaerobic digested sludge	[79]
Aeration	Aerate with air for 24 hours	Anaerobic sludge	[116]
Aeration	Flushing with air for 30 min	Anaerobic sludge	[117]
Microwave irradiation	Microwave radiation for 1.5 min	Cow dung compost	[126]

410

411

412 **Table 5**

413 Comparison of various inoculum pre-treatment methods for enriching hydrogen producing inocula

Inoculum source	Inoculum treatment methods	Substrate	Culture Temperature (°C)	Culture pH	Optimal pre-treatment method	Maximum H ₂ Yield (mol H ₂ /mol glucose eqv.) ^a	Maximum H ₂ Production Rate (mL H ₂ /L/h)	Reference
Anaerobic digested sludge	HS ^b , aeration, acid, base, BESA ^c and iodopropane	Sucrose	35	-	Base treatment	3.06	-	[117]
Anaerobic granular sludge	HS, acid and base	Glucose	35	-	Chloroform	1.55	-	[125]
Anaerobic sludge (UASB)	Acid, BESA, HS and their four combination	Dairy wastewater	29	-	BESA	0.0317 ^d	-	[84]
Anaerobic digested sludge	Acid, base, HS, aeration and chloroform	Glucose	36	7	Heat shock treatment	1.9	120.4 mL H ₂ /h	[116]
Anaerobic digested sludge	Acid, base, LS ^e , HS and BESA	Sucrose	60	5.5	Load shock treatment	1.96	11.2 mmol H ₂ /L/h	[118]
Suspended & granular anaerobic sludge mixture	HS, chloroform and combination of both	Ground wheat solution	37	7	Repeated heat shock treatment	25.7 ^f	-	[127]
Enriched inoculum (<i>Clostridium pasteurianum</i>)	Combination of acid with boiling and enzyme Viscozyme L ^g	Poplar leaves	35	7	2% Viscozyme L	44.92 mL H ₂ /g-dry poplar leaves	-	[128]
Anaerobic sludge	HS, acid and base	Glucose	35	6.2	Heat treatment at 80°C for 30 min	3.84	-	[119]

414 ^aCalculated based on the information provided from references at Standard Temperature (0⁰C and Pressure 1 atm), ^bHS = Heat shock, ^cBESA= 2-
415 bromoethanesulfonic acid, ^dmmol H₂/g COD, ^eLS : Load shock, ^fmL H₂/g cells/h, ^gViscozyme L is a mixture of arabanase, cellulase, b-glucanase,
416 hemicellulose and xylanase

417 **Table 6**

418 Evaluation of inoculum pre-treatment methods to enhance the DF capacity of the inoculum
 419 sludge

Pretreatment Method	Energy Requirement	Chemical Requirement	Economic cost	Scale-up application
Heat shock treatment	+++	+	+++	++
Acid treatment	+	+++	++	+++
Chemical Treatment	+	+++	+++	++
Aeration	+++	+	++	+++
Load shock treatment	++	++	+	+++

420 + Less intensive; ++ Moderately intensive; +++ Very Intensive

421 The effect of inoculum enrichment methods on H₂ production is different based on the source of
 422 inoculum (Table 5). However, in order to select an inoculum pre-treatment method for scaled-up
 423 systems, several parameters needs to be considered, such as operational costs, feasibility or
 424 complexity of the methods, time for the enrichment of the hydrogen producing seed, use of the
 425 DF residues in the post treatment processes. Table 6 gives a simple assessment of the commonly
 426 applied inoculum pre-treatment methods based on the authors' information from the literature.
 427 The selection of chemical treatment methods such as using BESA inhibits the methanogens if
 428 DF residues are to be used in AD. In addition, BESA is not environmental friendly and
 429 expensive to use in large industrial scale [8]. Likewise, heat shock treatment requires large
 430 energy inputs, which makes it less attractive for large scale applications. Acid and load shock
 431 pre-treatment can be applied at large scale to select the hydrogen producing inocula without net
 432 energy concerns.

433 In addition, there have been some dark fermentative studies done without the addition of seed
 434 inoculum, utilizing the microorganisms present in the waste itself [129,130]. The fermentative
 435 hydrogen production took longer than in the tests with inoculum supply. Nonetheless, inoculum
 436 pre-treatment is important in the batch tests or process start-up, selection of hydrogen producing
 437 microbial communities in the fermentative bioreactors are determined by the reactor operating
 438 conditions or environmental conditions [87,131–133].

439 **4.2 Design and operation of bioreactors**

440 The process design for dark fermentation depends mostly on substrates which limits the
 441 operational conditions of bioreactors such as culture temperature (mesophilic or thermophilic),
 442 reactor configurations (reactor types, wet, semi-dry or dry conditions, etc.) and feeding modes

443 (mono substrate or co-substrates) [134]. Weiland [135] reported the several types of bioreactors
444 used for the conversion of agricultural biomass to energy through upscale AD systems.
445 Although these bioreactors are designed for biomethanation by AD, these can be used for
446 biohydrogen production after modification of some operational parameters [43].

447

448 **4.2.1 Bioreactor configuration**

449 Different DF bioreactor configurations have been used in laboratory studies for a wide range of
450 substrates (see Tables 2, 7 and 8). Most of the dark fermentative hydrogen production studies
451 are carried out in a batch CSTR under wet conditions (<10 % total solids, TS). Besides CSTR,
452 many studies have been carried out in anaerobic fluidized bed reactor (AFBR), anaerobic
453 sequencing batch reactor (ASBR), fixed or packed bed reactors, UASB reactor, leaching bed
454 reactor, anaerobic baffled reactor, plug flow reactors, membrane bioreactor (MBR) and others
455 with an objective to enhance the biohydrogen yield and production rate. Recent research
456 [134,136–138] has been focusing on the future implications of high solids processes such as
457 semi-dry (10–20 % TS) and dry (>20 % TS) DF processes for biohydrogen production, as the
458 interests in the conversion of second generation lignocellulosic biomass (mostly agro-industrial
459 residues) is growing.

460 Studies have correlated the production of biohydrogen with microorganism density and different
461 cell retention strategies in bioreactors have been investigated [11,139,140]. The latter include
462 sludge granulation and biofilm systems to increase the microorganism content in the reactor.
463 The results of the studies showed that the volumetric hydrogen production rate of a bioreactor
464 depends on the ability to maintain a high microbial density. Gravala et al. [141] showed higher
465 hydrogen production rates in a UASB (which has a granular biomass retention) than a CSTR at
466 low retention times (19.05 and 8.42 mmol H₂/h/l, respectively at 2 h HRT), while the CSTR
467 reactor gave higher hydrogen yields at all HRTs tested.

468 Show et al. [142] compared the performance of a CSTR and an AFBR for biohydrogen
469 production using different biomass growth strategies with glucose as the substrate. The different
470 bioreactor configurations used in their research were: suspended sludge CSTR system, granular
471 sludge CSTR system, granular sludge AFBR system and biofilm AFBR system. The maximum
472 H₂ yield of their suspended sludge CSTR system, granule reactor and biofilm amounted to,
473 respectively, 1.92 mol H₂/mol glucose at a HRT of 6-12 hours, 1.83±0.09 mol H₂/mol glucose at
474 a HRT of 0.5 hours and 1.81 ± 0.08 mol H₂/mol glucose at a HRT of 0.5 hours.

475 Besides the specific advantages of these different bioreactor systems, the major drawbacks are
476 the washout of hydrogen-producing bacteria at short HRT in CSTR systems, low conversion
477 rates in granular reactor systems and rapid biofilm development leading to fragmentation and
478 separation from supporting media in biofilm systems [140]. In another study by Zhang et al.
479 [139], the biofilm based and granule-based reactors were compared for their biohydrogen
480 production potential. The results of the study concluded that the granule-based system was
481 advantageous as it could give better results in terms of biomass retention without subjecting to
482 wash-out of the biomass support carriers.

483 The incompatibility of the use in high organic loading rates and rapid growth of microorganisms
484 in biofilm systems makes them thus less attractive than granular systems. Show et al. [140]
485 recommended the column-shaped granular reactor for fermentative biohydrogen production
486 from wastewater though the system is not suitable for digestion of substrates with high solids
487 content or for a longer retention time in which anaerobic granules may disaggregate. High rate
488 bioreactors are necessary to convert complex organic biomass like OFMSW and agricultural
489 waste.

490

491 **4.2.2 Hydraulic retention time (HRT)**

492 The HRT can affect the hydrolysis of substrates and thus the production of intermediates and
493 products, thus affecting fermentative H₂ production. Besides, the hydrolysis HRT can also be
494 used as controlling parameter of the methanogenic activity. Some studies have demonstrated the
495 effect of the HRT on the biohydrogen production in DF process [143–146]. The different
496 growth rates of hydrogen producers and consumers make it possible to use the HRT as a
497 controlling parameter to inhibit the activity of H₂ consumers in the DF. It has been reported that
498 low HRTs favor hydrogen production as the methanogens are washed out, and hydrogen
499 production increases as the HRT decreases [143–145,147]. However, the optimum HRT for
500 biohydrogen production in DF depends on the type of substrates used as the hydrolysis rate
501 depends on the biodegradability of the substrates (Tables 2, 6 and 7).

502 However, the HRT alone is not sufficient to fully suppress the activity of methanogens [144].

503 Liu [144] investigated the effects of pH and HRT on hydrogen production using household solid
504 waste as a substrate in hyperthermophilic (70 °C) CSTR. The effect of the HRT (1, 2, 3, 4 and 6
505 days) at a constant pH of 7 and the effect of pH (5, 5.5, 6, 6.5 and 7) at constant HRT of 3 days
506 was investigated. The results of the experiments at different HRTs and constant pH 7 showed

507 unstable H₂ production with subsequent methanogenic activities at the end. However, a
508 combination of pH 5.5 and HRT of 3 days gave the optimum biohydrogen production condition.

509 **4.2.3 pH and temperature**

510 The operational pH and temperature are the most crucial parameters that determine the optimum
511 metabolic pathways of hydrogen synthesis as well as the inhibition of the hydrogen
512 consumption process which may occur simultaneously [35,148]. A acidic operational pH (below
513 6) mainly inhibits the methanogenic activity both under mesophilic and thermophilic conditions,
514 but the inhibition of hydrogen consuming homoacetogenic activity could only be achieved under
515 thermophilic conditions at the initial pH of 5.5 [124]. Thus, the control of the process pH and
516 temperature plays an important role in achieving high biohydrogen conversion rates by
517 minimizing the activity of hydrogen consumers.

518 The pH is one of the key parameters that can influence the metabolic pathway as it may directly
519 affect the hydrogenase activity, an iron containing enzyme which plays a major role in DF
520 [149]. A acidic pH affects the activity of the hydrogenase enzyme while it is one of the
521 important parameters for the inhibition of methanogenic activities in a mixed culture system
522 [8,35].

523 The optimum pH range for biohydrogen production varies from pH 4.5 [35] to 9 [150] in DF of
524 sucrose. Table 8 provides optimum operating pH ranges in different studies. The possible
525 explanations for these disagreements of optimum pH in various researches can be explained by
526 the differences in inoculum sources, inoculum enrichment methods and substrates types and
527 OLR used in the different studies [30].

528 The culture pH influences the metabolic by-products and biohydrogen yields. In most of the
529 studies, acetate and butyrate are the major end products of favorable hydrogen synthesis
530 (equations 1, 2 and 7). From the Table 8, it can be seen that the neutral operational pH favors
531 acetate pathways while acidic pH favors the butyrate pathways.. However, Khanal et al. [35]
532 concluded the independence of acetate and butyrate levels on different initial pH ranges studied
533 (4.5-7.5). Similarly, Luo et al. [151] reported butyrate as major acid in the DF of cassava stillage
534 in both BHP tests carried at the initial pH 5 and 7. Nevertheless, in another study by Luo et al.
535 [124], acetate was found as a major metabolic product when cultivation pH was 7 while butyrate
536 dominated at initial pH 5.5 in the BHP tests carried at mesophilic (37°C) conditions using acid
537 pre-treated inoculum. Luo et al. [124] also reported the inhibition of homoacetogenesis can be
538 achieved at pH 5.5 and thermophilic culture temperature. In a recent study of the DF of cheese

539 whey (from mozzarella production) at different pH ranges (5.5-7.7) and temperature 39 °C,
540 Gioannis [152] reported pH 6 as an optimal pH and acetate levels were higher in all the tests
541 except at pH 6.5 where butyrate and propionate levels were higher than acetate.

542 A lower pH (≤ 4.5) favors the solvent production [153]. In DF of glucose by *Clostridium*
543 *pasteurianum*, a pH below 5 favors the production of butanol and acetone [149]. Selection of the
544 operational pH also depends on the substrate type and OLR which determines the concentration
545 of VFA and thus the pH of the solution. The optimum temperature for DF processes varies with
546 the type of substrates and the operational pH (Table 8). The optimum pH for organic food waste
547 varies from 4.5 to 7, for lignocellulosic waste it varies from 6.5-7, whereas a neutral pH is
548 optimal for animal manure [43]. However, Tang et al. [154] reported the optimum pH of 5.5 at
549 45 °C for the DF of cattle wastewater. Thus, it becomes important to determine the optimum pH
550 conditions for the selected substrate type, loading rate and operational temperature.

551 A range of operational temperatures, i.e. mesophilic (35°C), thermophilic (55°C) and extreme
552 thermophilic (>65°C) has been studied to determine its effect on biohydrogen production
553 [41,45,155]. The studies have shown that the temperature can affect the metabolic pathway, thus
554 shifting the composition of the by-products of DF (Table 8). Valdez-Vazquez et al. [45] reported
555 higher H₂ yields in thermophilic fermentation than in the mesophilic temperature range. Also
556 acetic acid was a dominant by-product in thermophilic digestion, whereas butyrate was in
557 formed in a higher proportion in mesophilic digestion. Similarly, results of the extreme-
558 thermophilic (70°C) DF of household organic waste done by Liu et al. [156] also showed acetic
559 acid as the dominant by product in DF tests conducted at pH 7. In contrast, Shin et al. [41]
560 showed acetate as major at mesophilic culture while butyrate levels and hydrogen production
561 was higher at thermophilic culture, obtained in DF of food waste carried at pH 5.5. In other
562 study, Wang & Wan (2011) found the maximum substrate degradation efficiency, maximum H₂
563 yield and production rate at 37.8 °C in DF of glucose. These studies suggest temperature
564 influences biochemical pathways, although other factors such as culture pH, substrates types and
565 loading rates are equally important.

566 The H₂ yields depend on temperature as it affects the hydrolysis rate [41,45,143]. Biomass such
567 as agricultural residues require a high temperature to achieve a higher H₂ yield because a better
568 hydrolysis of lignocellulosic compounds is needed [43]. Kongjan & Angelidaki [155]
569 demonstrated biohydrogen production from extreme-thermophilic DF of wheat straw
570 hydrolysate. Similarly, thermophilic temperatures are favored in the DF of food waste [41]. In
571 contrast, easily biodegradable substrates prefer mesophilic temperatures for optimal H₂ yield.

572 The difference between the optimum operational temperatures is due to the difference in the
573 fraction of easily biodegradable compounds present in the feed substrate and the difference in
574 the inoculum used. Table 8 reports ranges of optimum temperatures which vary depending on
575 the type of substrate and inoculum used.

576 There are some techno-economic studies done, which compare the mesophilic and thermophilic
577 operation of DF processes. A thermophilic process seems to be more economical because of its
578 higher yield and lower requirement of feedstock in comparison to mesophilic DF processes
579 [158]. Foglia et al. [158] reported better economic performance for thermophilic DF in
580 comparison to mesophilic operation of the process converting sugars to produce hydrogen, CO₂
581 and organic acids followed by a photo-heterotrophic fermentation in two step process.

582

583 **4.2.4 H₂ partial pressure**

584 The partial pressure of hydrogen inside a biohydrogen reactor can influence the dark
585 fermentative biohydrogen production as a lower partial pressure in the head space of the reactors
586 facilitates the mass transfer of hydrogen from the liquid to gas phase [111,159]. During the
587 fermentation process, the hydrogenase is involved in reversibly oxidizing and reducing
588 ferredoxin. If the hydrogen concentration in the liquid phase increases, the oxidation of
589 ferredoxin becomes less favorable and the reduction of ferredoxin takes place [44], thus
590 reducing the H₂ production..

591 Lee et al. [160] studied the effect of the reduced partial pressure on the hydrogen production in a
592 CSTR reactor. Reduction in the partial pressure during the DF could lead to improvement in H₂
593 production. At a HRT of 6 h, they found an optimum hydrogen yield and hydrogen production
594 efficiency of 4.50 mol H₂/mol sucrose and 56.2% respectively. Similarly, the reduced pressure
595 of 380 mmHg gave a higher yield than the partial pressure of 760 mm Hg in another study done
596 by Mandal et al. [111].

597 In the AD process, the H₂ and CO₂ partial pressure is reduced by methanogens by their
598 conversion into CH₄. Jung et al. [161] reported strategies to remove dissolved H₂ from the
599 mixed liquor, including avoiding supersaturation by strong mixing, sparging with N₂ and CO₂
600 and application of a H₂-permeable membrane to withdraw dissolved H₂ from the mixed liquor.
601 Similarly, the partial pressure of H₂ could be reduced directly by decreasing the operating
602 pressure in the reactor using a vacuum pump [160]. Mandal et al. [111] reduced the partial
603 pressure of H₂ in a methanogenic reactor by adjusting the saline level of the gas collector using a

604 peristaltic pump. However, the use of vacuum pumps increases the cost of the process, while the
605 sparging with N₂ and CO₂ might render the recovery of H₂ difficult due to the dilution of the H₂
606 stream. An effective way to reduce the H₂ partial pressure would be to continuously collect the
607 produced gas phase from the reactor.

608

609 **Table 7**

610 Examples of innovative continuous DF bioreactors

Major substrate	Biomass retention system	Reactor type	Optimum HRT (hours)	Optimum Organic Loading Rate (OLR)	Optimum H ₂ production index	Reference
Glucose	Granule	CSTR	0.5	10 g glucose/L	H ₂ yield 1.81 mol H ₂ /mol glucose	[162]
Cheese whey	Granule	UASB reactor	6	20 g COD/L/d	H ₂ Production Rate 0.36-0.38 L H ₂ /L/d	[163]
Food waste	Biofilm	Batch pilot scale up-flow reactor (packed with coir pith)	0.50 m/day ^a	50 g COD/L	H ₂ Production Rate 9.67 LH ₂ /L/h	[164]
Food waste (pre-treated with alkali)	Suspended	ASBR (fill: 0.5 h; reaction: 8 h; settle: 3 h & discharge: 0.5 h)	36	30 g COD/L	H ₂ yield 0.69 mol H ₂ /mol hexose _{added}	[165]
Tequila vinasse	Suspended	ASBR (fill: 3 min; reaction: 5.33 h; settle: 30 min & discharge: 7 min) with 50% volumetric exchange rate	12	3 g COD/L	H ₂ Production Rate 50.5 mL H ₂ /L/h	[166]
Kitchen waste	Suspended	Inclined plug-flow reactor (inclined at 20°)	168 (7 days)	6.5 kgVS/m ³ /d	H ₂ yield 72 mL H ₂ /g VS	[167]
Municipal food waste & kitchen wastewater	Suspended	Anaerobic baffled reactor (ABR)	38.4 (1.6 days)	29 g COD _{total} /L/d	H ₂ Production Rate 6 L H ₂ /d	[48]
Glucose	Suspended	Anaerobic membrane bioreactor (MBR)	12 (SRT ^a)	5.8 g glucose/L	H ₂ Production Rate 640 mL H ₂ /h	[168]

611 ^aUp-flow velocity, ^bSRT= Solid Retention Time

612 **Table 8**

613 Effects of operational temperature and pH on fermentative hydrogen production

Substrate type	Microbial inoculum	Optimum pH	Optimum Temperature (°C)	Reactor type	HRT (days)	Maximum H ₂ Yield (mL H ₂ /g VS)	Major acid type produced	Reference
Food waste	Heat shock treated anaerobic sludge	6.3	35	Leaching Bed Reactor	25	310	Acetate	[99]
Cassava stillage	Heat treated UASB sludge	7	60	Batch CSTR	3.5	53.8	Butyrate	[151]
Cassava stillage	Heat treated UASB sludge	5	60	Batch CSTR	3.5	66.3	Butyrate	[151]
OFMSW	Untreated anaerobic digestate	5.5	37	Semi-continuous CSTR	21	165 ^a	Butyrate	[45]
OFMSW	Untreated anaerobic digestate	6.4	55	Semi-continuous CSTR	21	360 ^a	Acetate	[45]
Wheat Straw	Cow dung compost	7.0	36	Batch CSTR	6.25	68.1	Acetate and butyrate	[102]
Vegetable kitchen waste	Enriched from kitchen waste compost	7.0	55	Batch CSTR	7.0	0.57 ^b	Butyrate and lactate	[133]
Cattle wastewater	Mixed wastewater sludge, cow dung compost, chicken manure compost, river sludge	5.5	45	Batch CSTR	1.25	319 ^c	Butyrate	[154]
Rice straw	Heat treated wastewater sludge	6.5	55	Batch CSTR	6.5	24.8 ^d	Acetate, butyrate	[40]

614 ^a mL H₂/g VS_{removed}, ^b mmol H₂/g COD, ^c mL H₂/g COD_{consumed}, ^d mL/g TS

615 **4.3 Substrate pre-treatment for enhanced H₂ yield**

616 Fermentative biohydrogen production from lignocellulosic substrates is limited by biological
617 hydrolysis [19]. The complex organic substrates cited earlier, such as lignocellulosic biomasses,
618 demand physical, chemical, biological or a combination of these pre-treatments to enhance the
619 degradation process, system performance and biogas production [60,64,65,69]. These pre-
620 treatment methods reduce the crystallinity of the cellulose and increase the surface area of the
621 materials to improve the separation of the lignin and hemicellulose fractions [169]. There have
622 been some studies on the effect of the pre-treatment on fermentative biohydrogen production
623 [61,155,170,171]. These pre-treatment methods have in most cases a positive influence on the H₂
624 yield, as the biohydrogen production depends on the soluble fraction of sugars or carbohydrates.

625 Physical pre-treatment methods which generally include mechanical comminution (chopping,
626 grinding, milling), irradiation with gamma-rays, electro-beam or microwaves, hydrothermal
627 treatment, high pressure steaming or pyrolysis are effective in breaking the crystallinity,
628 increasing the accessible surface area and decreasing the degree of polymerization [69].

629 Chemical methods such as ozonolysis, acid or alkaline hydrolysis, solvent extraction, explosion
630 with steam ammonia fiber or CO₂ are effective in increasing the surface area, delignification and
631 also decreasing the crystallinity and rendering the partial or complete hydrolysis of
632 hemicelluloses. These physical and chemical treatment methods can be promising for industrial
633 applications as they are rapid. However, these methods demand energy and chemical inputs.

634 Moreover, lignocellulosic substrates can also be biologically treated with fungi and
635 actinomycetes which provide delignification and partial hydrolysis of cellulose, while some
636 enzymes (hemicellulase and cellulase) can aid in the hydrolysis and degradation of the
637 lignocellulosic materials [60].

638 The physical pre-treatment, especially the reduction of substrate particle size, has an effect on the
639 biogas yield and process kinetics [172–174]. Chen et al. [40] investigated the effects of the rice
640 straw particle size and concentration on cumulative dark fermentative biohydrogen production.
641 They used a meshed rice straw concentration of 30 g TS/L with five particle sizes (<0.297,
642 0.297-0.58, 0.58-1.19, 1.19-10 and >10 mm) as the substrate at an initial cultivation pH 6.5 and
643 temperature of 55 °C. The results of the study showed that rice straw of a particle size <0.297
644 mm gave the highest cumulative H₂ production (191 mL H₂/L) with a H₂ yield of 6.4 mL/g TS.

645 The substrate with a larger particle size had an extended lag phase and lower hydrogen
646 production. This can be explained by the fact that decreasing the particle size increases the
647 substrate availability for microbial hydrolysis and fermentation. Kongjan & Angelidaki [155]
648 pretreated wheat straw at 180 °C for 15 min to obtain a hydrolysate which mostly contained
649 hemicellulose leaving the cellulose and lignin in solid form. The hydrolysate was used as the
650 substrate for fermentative hydrogen production. Similarly, Zhang et al. [46] reported the use of
651 acid pretreated corn stalks for fermentative biohydrogen production. The biohydrogen yield from
652 acid pretreated corn stalks was higher than of that of untreated waste. However, mostly physical
653 pretreatment methods are applied in combination with chemical or biological pre-treatment
654 methods to obtain better and rapid hydrolysis of substrates (Table 9).

655 The effect of pre-treatment methods for different lignocellulosic substrates have a diverse effect
656 on the hydrolysis of soluble sugars and release of inhibitory products [175–178]. This needs to
657 be further investigated for the selection of suitable pre-treatment methods. These studies have
658 shown that the pre-treatment methods can enhance the system performance enhancing the biogas
659 production. However, the selection of a pre-treatment process should be based on effectiveness,
660 energy balance, economic feasibility and environmental sustainability [18]. In addition, some
661 studies have reported that during the pre-treatment of lignocellulosic biomass, various
662 undesirable compounds are released which exert inhibitory effects on microorganisms [63,175–
663 177]. The most commonly reported inhibiting substances which are released during the pre-
664 treatment processes are furfural, hydroxyl-furfural and phenolic substances. Quéméneur et al.
665 [63] and Monlau et al. [179] investigated the inhibition and control of these inhibitors on
666 biohydrogen production. Thus, the selection of pre-treatment methods for lignocellulosic
667 substrates should also consider these aspects.

668

669 **Table 9**

670 Examples of different pre-treatment methods applied to complex substrates used in DF

Substrate	H ₂ yield (mL H ₂ /g VS)	Pretreatment methods	Reactor mode	Temperature (°C)	Reference
Rice straw	24.8 ^a	Size reduction, <0.297mm	Bath	55	[40]
Wheat stalks	17.6	Size reduction, 1 mm	Batch	35	[180]
Wheat straw	212 ^b	Hydrothermal (180 °C for 15 min)	Continuous UASB reactor	70	[155]
Corn Stover	2.84 ^c	Steam explosion, 190.220 °C for 3-5 min	Batch	35	[181]
Corn Stover	3.0 ^c	Acidic steam explosion (1.2% H ₂ SO), 180 and 200 °C for 1–3 min	Batch	35	[181]
Beet-pulp	66.7 ±10.1 ^d	Alkaline at pH 12 using 2 M NaOH for 30 min. + Microwaves (170 °C for 30 min)	Batch	35±2	[182]
Bagasse	300	100 °C for 2 h +4% NaOH (w/v) + cellulase (20 FPU/g)	Batch	55	[170]
Grass	72.2 ^e	4% HCl (w/v), boiled 30 min.	Batch	35	[183]
Grass	39.5 ^e	4% NaOH (w/v), boiled 30 min.	Batch	35	[183]
Corn stalks	209.8	1.5% H ₂ SO ₄ , 121 °C for 60 min+ 9.4 IU/g of cellulase 52 °C at pH4.8 in 0.1 M sodium citrate buffer at 5% (w/v)	Batch	36±1	[184]
Corn cobs	107.9	100 °C, 30 min and 1% HCl (w/w)	Batch	36	[171]

671 ^amL/g TS, ^bmL H₂/g sugars, ^cmol H₂/mol glucose, ^dmL H₂/g COD, ^emL H₂/g dry grass

672 **4.3.1 Addition of nutrients and trace elements**

673 Microorganisms in fermentation processes require nutrients for bacterial activity and growth.
674 Thus, nutrients such as nitrogen, phosphate, metal ions and other micronutrients are needed in
675 fermentation processes for enzymatic activities and biomass growth, which affects the H₂
676 production. Biomass rich in carbohydrates such wheat wastes and palm oil effluents may be
677 deficient in nutrients (such as nitrogen, phosphorous) or minerals (such as trace metals).
678 Therefore, nutrients or micro nutrients must be provided as supplement for optimum microbial
679 activities for biohydrogen conversion from carbohydrate rich substrates [185–188].

680 *Nitrogen and phosphorous*

681 Nitrogen has great significance for hydrogen producers as it is an important component of
682 proteins, nucleic acids and enzymes. Similarly, besides being an important nutrient, phosphate
683 also serves in buffering the biochemical reactions [30]. In the thermophilic DF of palm oil mill
684 effluents (POME), O-Thong et al. [78,81] showed that supplementing iron (257 mg Fe²⁺/L),
685 adjusting the C/N ratio from 95 to 74 (using peptone as nitrogen source) and the C/P ratio from
686 650 to 559 (using Na₂HPO₄·2H₂O) could enhance H₂ production. In these studies, the hydrogen
687 production rate increased by 60% [78] and COD removal improved from 35.5±9.8 to 62.2±2.8%
688 [81] compared to raw POME without nutrient supplementation.

689 Likewise, Argun et al. [185] studied the effects of the C/N and C/P ratio on the hydrogen yield
690 and specific H₂ production rate in DF of wheat powder solution by supplementing nitrogen and
691 phosphorous. The results of the study showed the highest H₂ yield of 281 NmL H₂/g starch were
692 obtained at a C/N ratio of 200 and C/P ratio of 1000. However, there are some disagreements in
693 the carbon to nitrogen and phosphorous ratios. Lin and Lay [188] achieved a 500% and 80%
694 increased hydrogen yield and hydrogen production rate at a C/N ratio of 47 compared with the
695 blank. Similarly, O-Thong et al. [78,81] attained an optimum hydrogen production and COD
696 removal at a C/N ratio of 74 and a C/P ratio of 559. Several studies have used integration of co-
697 substrates as a strategy to maintain the appropriate C/N ratio; examples includes use of swine
698 manure as a source of nitrogen in co-fermentation with vegetable waste [189] and use of cassava
699 starch in co-fermentation with microalgae *Chlorella pyrenoidosa* [190].

700 *Metal ions and micronutrients*

701 Higher concentrations of metal ions exert inhibitory effects on the hydrogen producers
702 [8,191,192]. However, trace amounts of some metal ions enhance the reactor performance [193].
703 Karadag & Puhakka [193] found that iron and nickel improved the reactor performance and H₂
704 production was enhanced by 71%. O-Thong et al. [78] obtained the optimal hydrogen production
705 when the substrate contained 257 mg Fe²⁺/L during the thermophilic DF of POME.

706 **4.3.2 Inhibition due to heavy metals**

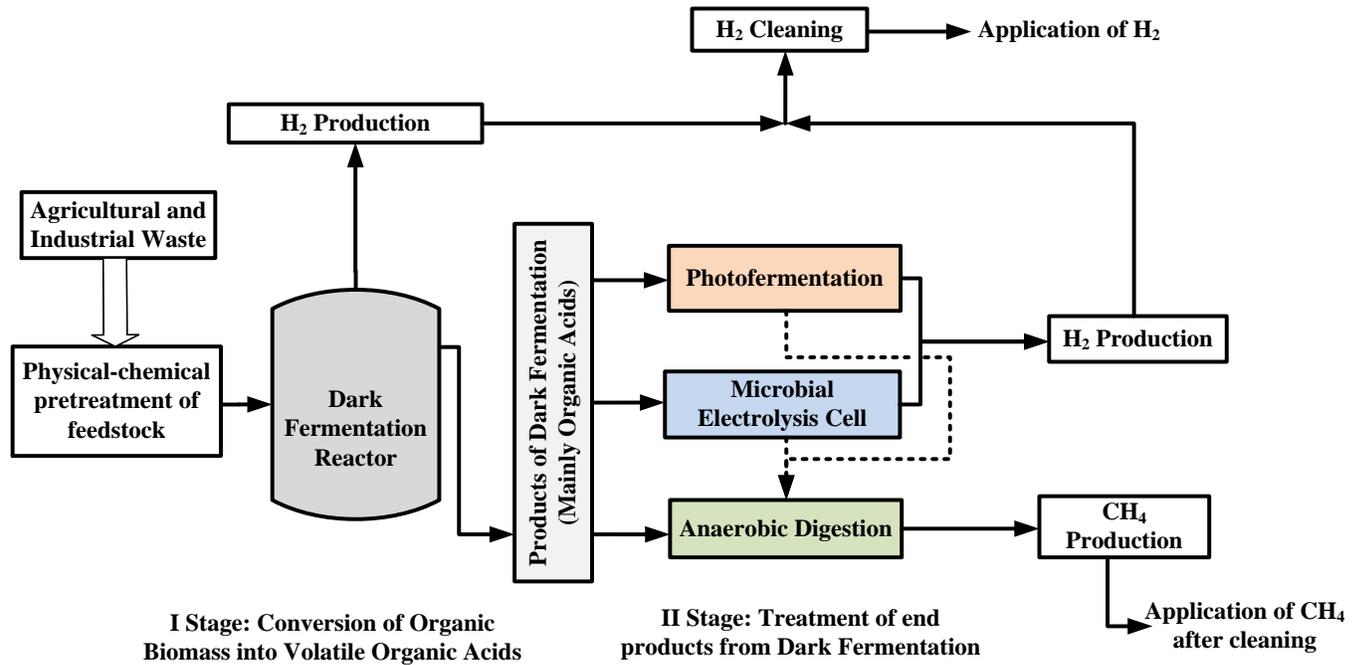
707 Toxic heavy metals such as cadmium (Cd), chromium (Cr), zinc (Zn), copper (Cu), nickel (Ni),
708 and lead (Pb) which may be present in industrial and municipal solid waste may lead to upset or
709 ultimately failure of anaerobic reactors [8]. Altaş et al. [194] studied the inhibitory effect of
710 heavy metals on methane-producing anaerobic granular sludge. The order of toxicity for the
711 individual heavy metals in decreasing order was: Zn (most toxic, 7.5 mg/L) >Cr (27 mg/L) >Ni
712 (35 mg/L) ≈ Cd (least toxic, 36 mg/L).

713 Lin and Shei [191] showed the relative toxicity of the heavy metals to fermentative hydrogen
714 production was in the order of Zn>Cu>Cr. The maximum concentration of these metals that
715 reduced the hydrogen producing activity by 50% was 4.5 mg Zn/L, 6.5 mg Cu/L and 60 mg Cr/L
716 [191]. However, Li and Fang [192] reported the relative toxicity to H₂ production in the
717 following order: Cu (most toxic)>>Ni~ Zn > Cr > Cd > Pb (least toxic). The bioactivity of the
718 sludge was reduced to 50% of the control at 30 mg Cu/L, 1600 mg Ni and Zn/L, 3000 mg Cr/L,
719 3500 mg Cd/L and >5000 mg Pb/L.

720 **5. Use of by-products**

721 The low process yield and the incomplete conversion of organic biomass are two major
722 bottlenecks for commercial dark fermentative biohydrogen production [12,27]. As overviewed in
723 Section 4, dark fermentative biohydrogen can be enhanced by suitable substrate selection,
724 inoculum enrichment strategies, and optimal operation of bioreactor or substrate pre-treatment.
725 However, a single DF system cannot achieve beyond the highest yield of 4 moles H₂ per mole
726 hexose, as DF has a maximum yield of 33% (on sugars) [27]. Besides, DF residues mainly
727 contain volatile acids, major by-products of the DF process, which need to be utilized to achieve
728 complete conversion of the organic biomass. The dual systems are integrated by the conversion
729 of carbohydrates to organic acids in the first stage (DF) and the use of conversion by-products in

730 the second stage, either to H₂ (photofermentation, bioelectrochemical cells) or CH₄ (AD). Also,
 731 AD can be considered as the final stabilization stage to stabilize the residues of DF,
 732 photofermentation and bioelectrochemical cells. Figure 5 shows an example of different
 733 possibilities of integrating DF to other post treatment processes.



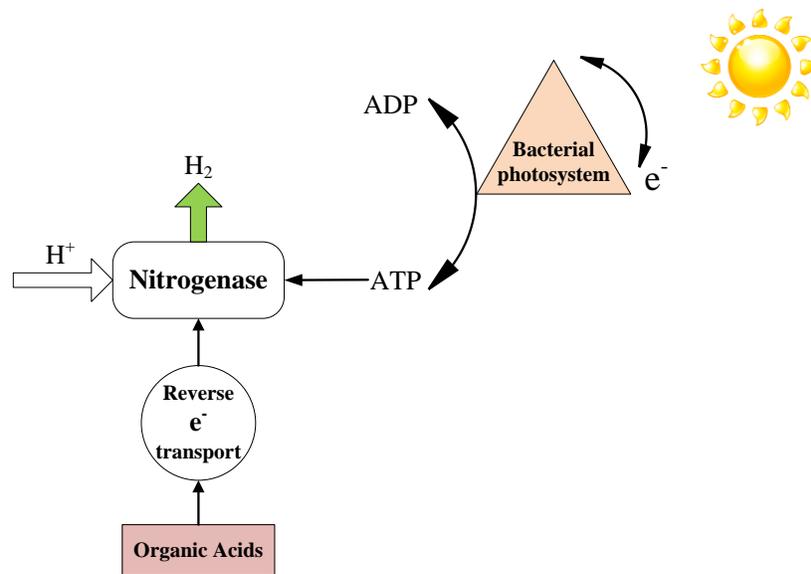
734
 735 Fig. 5. Different integration strategies for integrating DF with post treatment processes for
 736 improved biofuel production
 737

738 A number of studies have been carried out combining DF either with photofermentation [195–
 739 202] or/and using bio-electrochemically assisted microbial reactor [142,203–206] for improving
 740 the biohydrogen yield or with the AD process for improving the economic viability [89,106,207–
 741 210]. Light dependent fermentative processes could be a good option for a second stage H₂
 742 production, because of its higher substrate conversion efficiency, and being less energy intensive
 743 and environmental friendly [211]. On the other hand, bio-electrochemically assisted microbial
 744 fuel cells are also an option to treat the effluents from DF and increase the H₂ yield [212].
 745 Likewise, the economic viability of the DF process could be enhanced by AD as a final

746 step. Some examples of integrated processes of DF combined with post treatments are presented
747 in Table 10.

748 5.1 Photofermentation

749 Under anaerobic conditions, purple non sulfur (PNS) photosynthetic bacteria carry out anaerobic
750 photosynthesis using light as energy source for synthesizing hydrogen [213,214]. The PNS
751 bacteria use the captured light energy to produce ATP and high energy electrons through reverse
752 electrons flow which reduces ferredoxin (Fig. 6). Then, the ATP and reduced ferredoxin drives
753 the proton reduction to hydrogen by nitrogenase [215]. The research attention in these organisms
754 is increasing because of their higher biohydrogen yield potential and better light utilization
755 proficiency as they are able to absorb and utilize both visible (400 – 700 nm) and near infrared
756 (700 – 900 nm) light. Moreover, they are able to use a wide variety of substrates [213].

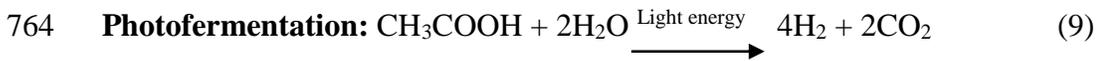


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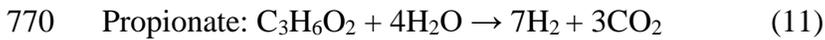
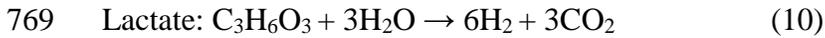
758 Fig. 6. Schematic presentation of photofermentation (adapted and modified from [215])

759 The ability of PNS organisms to convert the organic acids to biohydrogen makes
760 photofermentation a good post treatment for biohydrogen production from DF effluents. An
761 example of integrated dark and photofermentative conversion of acetic acid to biohydrogen is the
762 following:





765 Depending on the operating conditions of the bioreactors and other parameters described earlier
766 (See section 4), DF might follow different pathways rather than only the acetic acid pathway,
767 therefore, a theoretical biohydrogen potential of DF effluents containing acetate, propionate and
768 butyrate can be written as follows [216,217]:



772 PNS species such as *Rhodospirillum rubrum*, *Rhodopseudomonas palustris*, *Rhodobacter*
773 *sphaeroides* and *Rhodobacter capsulatus*, *Rhodopseudomonas faecalis* have been widely used in
774 photofermentation studies for H_2 production [213,214], while some studies have been done with
775 mixed cultures isolated from wastewater sludge [218–220] Yangling et al. [221] evaluated the
776 microbial community dynamics in a mixed photofermentative culture enriched from a digestate
777 from AD of pig dung and found the prevalence of mostly *Rhodopseudomonas palustris*.

778 Redwood et al. [21] presented different integration strategies for combining two step dark and
779 photofermentation processes. Nath et al. [201] studied the combined dark and photo fermentation
780 for biohydrogen production using glucose as the substrate. DF was carried by *Enterobacter*
781 *cloacae* strain DM11, followed by photofermentation by *Rhodobacter sphaeroides* strain
782 O.U.001 using the spent medium from the DF which mainly contained acetic acid. The
783 combined hydrogen yield was higher than a single biohydrogen system, i.e. 1.86 mol H_2 /mol
784 glucose in DF and 1.5–1.72 mol H_2 /mol acetic acid in the photofermentation. Similarly,
785 combining the two fermentation processes, Chen et al. [211,222] and Tao et al. [222] attained a
786 total yield of 10.25 mol H_2 /mol sucrose and 6.63 mol H_2 /mol sucrose respectively. In a study by
787 Su et al. [195], a yield of 4.16 mol H_2 mol/ mol glucose was obtained from photo fermentation of
788 DF effluents using glucose as the substrate, which increased the total yield to 5.48 mol H_2 /mol
789 glucose.

790 Other researchers have used effluents from DF of diverse substrate types in photofermentative
791 biohydrogen production. Argun et al. [202] use the DF effluent of ground wheat solution with

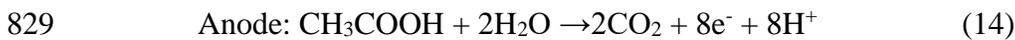
792 the H₂ yield of 781 ml/g total VFA. In another studies by Su et al. [196,197], cassava starch and
793 water hyacinth were used as the substrates in DF and its effluent was utilized successfully for
794 photofermentative biohydrogen production. The studies reported the increase in total H₂ yield
795 from 240.4 mL H₂/g starch to 402.3 mL H₂/g starch [196] and 76.7 to 596.1 mL H₂/VS [197]
796 using *Rhodopseudomonas palustris*. These studies have shown that combined dark and
797 photofermentation is a potential technology for biohydrogen production using diverse substrates.
798 Some drawbacks of photofermentative systems include the inherent high energy demand
799 associated with the nitrogenase enzyme, lower solar conversion efficiencies and economic issues
800 of anaerobic photobioreactors covering large areas [33]. However, these inefficiencies can be
801 overcome by developing an efficient photobioreactor [223,224]. Besides the presence of light
802 conditions, the culture medium of photofermentation should be under ammonia limitation and
803 the absence of oxygen, as both inhibit the nitrogenase activity [225–227] . Higher ammonia
804 concentrations (in excess of 2-5 mmol) can be detrimental to hydrogen production [225,228].
805 Thus, the effective removal of ammonia from DF residues can be a bottleneck in coupling
806 photofermentation with DF processes. Therefore, the substrates with a high C/N ratio seem more
807 suitable for H₂ conversion in these systems. Nonetheless, several ammonia removal strategies
808 such as stripping, natural zeolites and selective membranes can be applied which could facilitate
809 the coupling of these two processes. [229,230].

810 In addition to biohydrogen production, accumulation of poly-hydroxybutyrate (PHB) could raise
811 future interests, as it possess economic value as a biodegradable polymer [231]. Thus, energy
812 recovery and economic sustainability of the commercial development of DF depends also on the
813 development of the post-treatment processes like photofermentation.

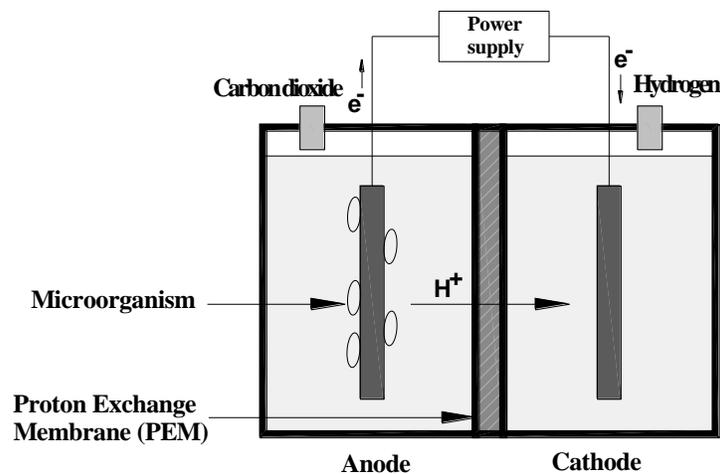
814 **5.2 Microbial Electrolysis Cells**

815 Biohydrogen production from DF residues is also possible through an emerging technology
816 known as electrohydrogenesis or biocatalyzed electrolysis or microbial electrolysis
817 [24,25,27,29,203,232]. Electrochemically assisted Microbial Fuel Cells (MFCs) or Microbial
818 Electrolysis Cell (MECs) or Bioelectrochemical Systems (BES) use microorganisms to catalyze
819 the biochemical reactions at the anode and/or cathode, producing protons and electrons from the
820 oxidation of organic matter [142,206,212]. MECs should not be confused with MFCs, the former

821 is an electrolysis reactor which produces hydrogen, while a MFC is a fuel cell that produces
 822 electricity [212]. In MECs, hydrogen can be produced at the cathode by applying a small circuit
 823 voltage, theoretically 0.14 V [212] (Fig. 7). Some exoelectrogenic microorganisms which are
 824 capable of electron transfer to an electrode (anode) include genera *Geobacter*, *Shewanell* and
 825 *Pseudomonas sp.* [24,206,212], while the function and the community of microorganisms at the
 826 cathode is not known [212]. The evolution of hydrogen in BESs can be represented in the
 827 following reactions:



831 A minimum theoretical voltage required to produce hydrogen at pH 7 is - 410 mV (Normal
 832 Hydrogen Electrode). However, the anode potential produced by the oxidation of organic matter
 833 is approximately 300 mV. Thus, hydrogen can theoretically be produced at the cathode by
 834 applying a circuit voltage higher than 110 mV (i.e. $V_{\text{applied}} = V_{\text{anode}} - V_{\text{cathode}} = 410-300$ mV),
 835 though it has been found that a minimum applied voltage of more than 250 mV is needed due to
 836 ohmic resistance and electrode over potential [7,206]. This applied voltage required is
 837 considerably lower than 1210 mV, a theoretical voltage needed for hydrogen production via
 838 electrolysis of water at neutral pH conditions [206].



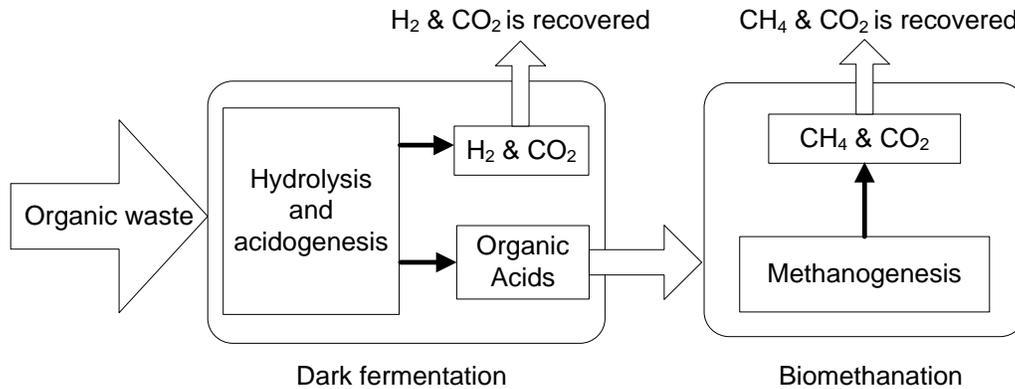
839
 840 Fig. 7. Schematic diagram of two chambered MEC separated by a proton exchange membrane
 841 and power supply (adapted and modified from [206])

842 Lalaurette et al. [233] tested a two stage process, combining DF using cellulose as a substrate
843 and MEC systems for hydrogen production. This improved the total hydrogen yield to 9.95 mol
844 H₂/mol glucose from the fermentative hydrogen yield of 1.64 mol H₂/mol glucose using
845 cellulose. Similarly, Liu et al. [203] used the volatile acids accumulated in fermentation of waste
846 activated sludge as a carbon source for biohydrogen production in a MEC with a H₂ yield and
847 production rate of 1.2 mL H₂/mg COD and 120 mL H₂/g VSS/d, respectively. The results from
848 the analysis of the electrohydrogenesis end products showed that more than 90% of the acetate
849 and propionate were converted to hydrogen, but with lower conversion of n-butyrate and n-
850 valerate (<20%). Likewise, Moreno et al. [24] obtained 94.2 L H₂/kgVS from the two stage DF-
851 MEC systems using cheese whey wastewater.

852 The MECs are still under research and development. One of the challenges to MECs is to
853 suppress the methanogenic activity during the electrohydrogenesis with mixed cultures as it
854 negatively affects the H₂ production rate. Hu et al. [234] has proposed to inhibit the
855 methanogenic activity by exposing the cathodes to air. They studied a single chambered MEC to
856 investigate the hydrogen production using mixed and pure culture (*Shewanella oneidensis* MR-
857 1). The major objective was to reduce the potential losses associated with the membrane and
858 increase the energy recovery of the process. Studies of the long term performance of MEC
859 systems are needed to further develop and achieve the technical and economic edge of this
860 technology.

861 **5.3 Anaerobic digestion**

862 Anaerobic Digestion (AD) is a proven biological waste treatment method for volume reduction,
863 waste stabilization and biogas recovery (CH₄) from organic waste [92]. The AD process can be
864 combined with DF to achieve further conversion of end products of DF and the residues from
865 photofermentation and MECs systems (Fig. 5). Photofermentation requires a clear medium for
866 efficient light utilization. Thus, the residue from the filtration of DF effluents, microbial biomass
867 produced in photofermentation and the residues from MECs (if any) can be utilized in AD for the
868 final stabilization. The two stage processes combining biohydrogen production in the first stage
869 and AD in the second stage not only increase the sustainability of the process, but also guarantee
870 the complete treatment of the organic waste [27].



871

872

Fig. 8. Two-stage process for hydrogen and methane production

873

874 A number of studies have been done on dual systems [89,106,207–210,235]. DF followed by AD

875 (Fig. 8) has shown technical and economic feasibility of the integrated process up to pilot scale

876 [77,94]. Wang and Zhao [236] ran a successful pilot scale unit consisting of hydrolysis-

877 acetogenesis for H₂ generation in a rotating drum of 200 liters, followed by a methanogenesis

878 stage in 800 liters reactor. Likewise, Cavinato et al. [207] established successful two stage

879 conversion of hydrogen and methane from organic waste. Similarly, Antonopoulou et al. [237]

880 investigated two stage hydrogen and methane production using sweet sorghum with a H₂ yield of

881 10.41 L H₂/kg sweet sorghum and methane yield of 29 L CH₄/kg sweet sorghum utilizing DF

882 residues as a sole substrate in AD, while Kvesitadze et al. [238] got the cumulative H₂ yield of

883 104 L H₂/g VS and methane yield of 520 L CH₄/g VS using OFMSW. Similarly, Antonopoulou

884 et al. [237] showed the feasibility of two stage hydrogen-methane process using cheese whey.

885 Jung et al. [239] showed that two stage H₂-CH₄ conversion from molasses is economically

886 feasible. Ruggeri et al. [209] used energy balance as a tool to determine the sustainability of

887 integrated DF and AD, which showed the positive energy gain. Similarly, Schievano et al. [240]

888 reported 8%-43% increment of energy in two stage system in comparison to a single stage AD.

889 Thus, in light of recent popularity of two stage AD processes for treating high strength

890 wastewater or concentrated solids, the former stage can be modified to be used for hydrogen

891 production [43]. Also, the DF process can be seen as a pre-treatment stage if the organic waste of

892 interest is subjected to complete stabilization [236]. Thus, in order to improve the economic

893 competence of commercial DF, AD could provide an attractive solution [241].

894 **5.4 Other applications**

895 Besides conversion of volatile fatty acids and other reduced carbon sources to biomethane or
896 biohydrogen in biological processes, VFAs can be used in various applications: biological
897 nutrient removal from wastewater [242,243], sulfur and sulfate reduction [244,245], biopolymer
898 (such as polyhydroxybutyrate) production [83] and microbial lipids production [55,246]. Lim et
899 al. [242] studied the use of volatile fatty acids produced from food waste as carbon sources in the
900 removal of nitrogen and phosphorous from municipal wastewater in a sequential batch reactor
901 (SBR) [242]. Similarly, Elefsiniotis et al. [243] studied the denitrification process, removal of
902 nitrate-nitrogen (20 to 200 mg/L) using VFA generated from the AD of starch rich industrial and
903 municipal wastewater as a carbon source in batch reactors.

904 Similarly, a “carboxylate platform” or third biorefinery platform has been introduced to generate
905 a mixture of carboxylates as intermediate for the production of complex fuels utilizing waste
906 biomass [247]. Ntaikou et al. [83] investigated the combined production of biohydrogen and
907 biopolymers from the DF of olive mill wastewater and the use of DF effluents which mostly
908 contained VFAs in SBR using polyhydroxyalkanoates (PHAs) producing bacteria culture.

909 Tuna et al. [248] uses the volatile fatty acids produced in DF processes for hydrogen production
910 by electrohydrolysis. Hydrogen was generated by applying a low voltage in the range of 1-3 V
911 DC current to DF effluents of wheat powder containing different VFAs concentrations. The
912 applied voltage of 2 V and 10.85 g/L of total VFA gave the highest energy efficiency of 56%.

913 **Table 10**

914 Examples of operational conditions and system performances of integrated DF systems

First Stage: DF							Second Stage: Photo fermentation							Reference
Substrate type	Microbial Inoculum	pH	T (°C)	HRT days	Max. H ₂ Prod. Rate	Max. H ₂ Yield	Dominant end products	Process & Microbial Inoculum	pH	T (°C)	HRT days	Max. Biogas Prod. Rate	Max. H ₂ Yield	
Sucrose	<i>Clostridium pasteurianum</i>	7	37	-	-	3.85 mol H ₂ /mol sucrose	Butyrate and acetate	<i>Rhodopseudomonas palustris</i> WP3-5	7.1	32	96 hours	25.2 mL H ₂ /L/h	4.03 mol H ₂ /mol sucrose	[211]
Sucrose	Heat treated Cattle dung and sludge from biogas plant	6	38	Batch	360 mL H ₂ /L/h	3.67 mol H ₂ /mol sucrose	Butyrate and acetate	<i>Rhodobacter sphaeroids</i> SH2C	7	30	Batch	-	4.06 mol H ₂ /mol sucrose	[222]
Acid hydrolyzed sugarcane bagasse	<i>Enterobacter aerogenes</i> MTCC 2822	6.8	38	Batch	1000 mL H ₂ /L	-	Butyrate and acetate	<i>Rhodopseudomonas</i> BHU 01	6.8	34	Batch	755 ml/L	-	[22]
First Stage: DF							Second Stage: AD							Reference
Substrate type	Microbial Inoculum	pH	T (°C)	SRT days	Max. H ₂ Prod. Rate	Max. H ₂ Yield	Dominant end products	Process & Microbial Inoculum	pH	T (°C)	SRT days	Max. CH ₄ Prod. Rate	Max. CH ₄ Yield	
OFMSW	Heat treated sludge from biogas plant	5.2	37	2	640 mL H ₂ /d	43 mL H ₂ /g VS added	Acetate and butyrate	Sludge from biogas plant	7.5	37	15	7475 mL CH ₄ /g VS added	500 mL CH ₄ /g VS added	[106]
Food waste	Indigenous microbial cultures from food waste	5.2–5.8	40	6.66	-	65 mL H ₂ /g VS	Acetate and butyrate	Anaerobic granular sludge from UASB	6.8	40	26.67	-	546 mL CH ₄ /g VS	[236]

Micro algae (<i>Chlorella vulgaris</i>)	<i>Clostridium thermocellum</i>	-	55	Batch	-	53.4 mL H ₂ /g VS	Acetate and butyrate	Anaerobic granular sludge from ASBR	-	55	batch	22.38 mL CH ₄ /g VS·d	320.6 mLCH ₄ /g VS	[249]
First Stage: DF								Second Stage: Bioelectrochemical systems						
Substrate type	Microbial Inoculum	pH	T (°C)	HRT days	Max. H ₂ Prod. Rate	Max. H ₂ Yield	Dominant end products	Process & Microbial Inoculum	pH	T (°C)	HRT days	Max. H ₂ Prod. Rate	Max. H ₂ Yield	Reference
Molasses	-	-	-	Batch	700 mL H ₂ /L/d	0.27 mol H ₂ /mol COD	Ethanol, acetic and butyric acid	Domestic wastewater	6.7- 7.0	25	Batch	1410 mL H ₂ /L/d	-	[250]
Corn Stover	<i>Clostridium thermocellum</i>	6.8	50	Batch	0.25 L H ₂ /L/h	1.67 mol H ₂ /mol- glucose	Acetic acid and ethanol	Inoculum from microbial fuel cell Wastewater	7.3	-	Batch	1 L/L/d	750 mL/g COD	[233]
Corn stalk	<i>Microwave irradiation pre- treated cow dung</i>	7.0	36	Batch	1.73 m ³ H ₂ /m ³ d	129.8 mL H ₂ /g corn stalk	Acetate, butyrate, propionate, ethanol	Spent dark fermentation medium (Single chambered cell)	7.0	36	Batch	3.43 ±0.12 m ³ H ₂ /m ³ d	257.3 mL H ₂ /g corn stalk	[232]

916 **6. Pilot scale applications**

917 Most DF studies have been carried out in laboratory scale batch, semi-semi-continuous or
918 continuous reactors. To the best of our knowledge, no studies have reported on the industrial
919 or full scale application of DF processes. Limited studies have been done on pilot-scale
920 applications of DF processes [77,88,94,167,251,252]. Ren et al. [88] studied a 1.48 m³
921 continuous flow anaerobic reactor for 200 days at an OLR of 3.11-85.57 kg COD/m³/d fed
922 with molasses. The maximum hydrogen yield was 26.13 mols H₂/kgCOD removed in the
923 OLR range of 35–55 kg COD/m³/d and a maximum production rate of 5.57m³ H₂/m³
924 reactor/d was reached. Jayalakshmi et al. [167] worked with a plug-flow inclined DF reactor
925 of volume 0.15 m³ with kitchen waste as the substrate. The reactor gave a H₂ yield of 72 mL
926 H₂/gVS added.

927 Another reported long term pilot-scale study was carried out at Fen Chia University
928 (Taiwan), comprising of two feedstock storage tanks (0.75m³ each), a nutrient storage tank
929 (0.75m³), a mixing tank (0.6 m³), an agitated granular sludge bed fermenter (working volume
930 0.4 m³), a gas-liquid-solid separator (0.4 m³) and a control panel. A pilot-scale high-rate
931 reactor was operated for a period of 67 days under mesophilic conditions (35 °C) at an OLR
932 of 40-240 kg COD/m³/d with sucrose as the substrate. An OLR of 240 kg COD/m³/d gave a
933 hydrogen production rate of 15.59 m³/m³/d and a hydrogen yield of 1.04 mol H₂/mol sucrose.
934 In another study, Cavinato et al. [77] carried out a two-stage pilot-scale thermophilic DF and
935 AD of food waste for the production of, respectively, biohydrogen and methane with
936 recirculation of AD effluents to DF to control the pH (5-6). The organic loading rate of 16.3
937 kgTVS/m³d was maintained with a HRT of 3.3 days in the DF stage, yielding 66.7 L H₂/kg
938 TVS.

939

940 **7. Challenges and future prospects**

941 **7.1 Modeling and simulation**

942 Several researches have been proposed to integrate DF processes with AD, photofermentation
943 or bioelectrochemical systems to utilize the VFAs produced to increase its viability.

944 Modeling of kinetic parameters and biohydrogen production becomes important for the
945 design, analysis and operation of the fermentative processes. Also, the predictive capacity of
946 the model for end products helps to design the downstream processes. Several models have

947 been proposed to describe the biohydrogen production, growth of hydrogen fermenters,
948 substrate consumption and intermediate biochemical processes [253–255]. With increasing
949 research on DF, the modeling of the biohydrogen production process could be of primary
950 interest to achieve a better understanding of the DF pathways and control of the process.

951 Parameters such as substrate concentration, pH, temperature, and HRT affect the H₂ yield and
952 production rate and the nature of the end products (See section 4). Wang and Wan [254]
953 reviewed existing mathematical models such as the Modified Gompertz model for product
954 formation (H₂ production), the Logistic model for biomass growth [256], substrate utilization
955 based on Monod Kinetics, the Arrhenius model for temperature effects, pH inhibition models
956 based on the IWA Anaerobic Digestion Model no. 1 (ADM1) [257] and the Modified
957 Luedeking-Piret models for the formation of by-products [256].

958 There is, however, a need to upgrade the different kinetic models, including complex
959 biochemical processes, which involve the fermentative biohydrogen production such as
960 hydrolysis, acidogenesis and H₂ production from complex substrates (Fig. 2). The IWA
961 ADM1 has been used extensively to model AD processes [173,174,257,258]. ADM1 is a
962 structured mathematical model based on the COD balance of composite substrates and
963 includes a number of biochemical processes involving disintegration of substrates such as
964 hydrolysis, acidogenesis, acetogenesis and methanogenesis, biomass growth and decay
965 processes and the physical interaction of the gas-liquid phases. Because of the similarity of
966 some initial biochemical and physical processes, a modified ADM1 has been proposed to
967 model dark fermentative biohydrogen production processes [31,253,255]. Nonetheless, a
968 model that can simulate the process and predict the formation of all the major intermediates
969 and biohydrogen considering all influencing parameters is a present necessity.

970 **7.2 Energy balance and COD conversion**

971 The net energy gain in DF processes is an important issue that has been addressed by few
972 researchers [209,259,260]. The energy balance is an important factor for the process
973 sustainability. Higher culture temperatures have been suggested in the literature
974 [40,41,45,133,261] for maximizing H₂ yield, without considering the net energy gain [259].
975 Some studies [209,259] suggested that DF processes have to be operated at ambient
976 temperature in order to obtain a positive net energy. After evaluation of literature data on DF
977 of different substrates, Perera et al. [259] reported the net energy gain in dark fermentative
978 processes is positive when the process temperature is below 25 °C. In another study by

979 Ruggeri et al. [209], the optimum working temperature of 20 °C has been recommended
980 which offers 20% of the available energy. However, these studies have suggested to couple
981 DF processes with AD, microbial fuel cells, bioelectrochemical systems or photofermentation
982 to obtain a more positive net energy balance from the recovery of energy from the DF end-
983 products and residues.

984 Perera et al. [259] reported that DF combined with BES or DF with AD can result in positive
985 energy yield. Similarly, Ruggeri et al. [209] found that the AD step after DF can deliver a
986 positive net energy with 40-90% available energy. Su et al. [196] obtained a higher
987 conversion efficiency of the heat value in DF from 13.3% to 46.0% when combined with
988 photofermentation. This was due to an increase of the H₂ yield from 1.59 to 5.48 mol H₂/mol
989 glucose.

990 Lower rates of COD reduction efficiencies could be a concern if the DF process aims to treat
991 the waste biomass. The conversion of COD to hydrogen is low; theoretically 16 g of COD
992 reduction is achieved per mole of H₂ obtained. However, the COD is remained in the by-
993 products as VFAs and alcohols. Mohammadi et al. [79] obtained 0.41 mmol H₂/g COD from
994 mesophilic DF of POME with a COD removal efficiency of 86%. In another study, O-Thong
995 et al. [78] obtained a COD removal efficiency of 55% with H₂ yield of 0.142 LH₂/L POME.
996 Nonetheless, it has been suggested from the studies (presented in Table 10), that combining
997 DF processes with AD, BES or PF, will not only improve the energy recovery, but give
998 higher COD reduction efficiencies and provide complete treatment of organic waste
999 biomasses.

1000 **7.3 pH control**

1001 Unlike AD processes where the production of acidity from VFAs generation is balanced by
1002 alkalinity of the systems, DF processes are unstable because of the continuous production of
1003 acidity (VFAs production). As discussed in earlier (See Section 4.2.3), a very low pH can
1004 inhibit the hydrogen production, while the acidic range (5-6) favors H₂ production depending
1005 on the type of substrate. The use of an excessive amount of buffers, acids or base to maintain
1006 the pH acidic can decrease the sustainability of the process and increase the salt concentration
1007 of the DF effluents.

1008 One of the sustainable solutions could be to explore substrates with a higher pH or alkalinity
1009 to equilibrate the system. Choi et al. [56] suggested the use of substrates with a high pH to
1010 replace the use of buffers. Life Cycle Analysis (LCA) of two-step thermophilic DF followed

1011 by photofermentation of potato peels, showed that most of the impact was generated by the
1012 use of phosphate buffer during the process [262]. To provide natural buffering, Cavinato et
1013 al. [261] recycled the reject water (effluent) from the AD step in the two-step DF and AD.
1014 The AD reject water provided the alkalinity to maintain the pH in the DF step around 5.5,
1015 giving a H₂ yield of 51 L/kgVS of food waste fed with a H₂ content of 37% in the biogas.
1016 However, the major concern with the recirculation of the AD reject water is the activity of
1017 methanogens present in the reject water, which could affect the purity of the biohydrogen
1018 produced in the DF step. In addition, inhibition of H₂ production due to higher levels of
1019 ammonia present in the reject water could be another concern as reported in the study by
1020 Cavinato et al. [77]. Thus, long term studies to assess the effect of reject water recirculation
1021 from the AD step on the H₂ content in biogas produced from DF could open further doors to
1022 ensure the sustainability of DF systems.

1023 **7.4 Solid State Dark Fermentation (SSDF)**

1024 Anaerobic reactors are generally categorized into wet (<10% TS), semi-dry (10–20% TS) and
1025 dry (>20% TS) processes [263]. However, some categorized wet digestion for low (<15%
1026 TS) substrates and dry digestion of high solids (>15% TS) processes [134]. By increasing the
1027 TS content, dry fermentation processes can be operated at a high OLR with little water
1028 addition, which offers advantages such as smaller reactor volume, easy handling of the
1029 digestate residues and technical simplicity [134,263]. This could be attractive for
1030 commercialization of these processes. However, the drawbacks of SSDF are the low H₂
1031 yields due to mass and energy transfer limitations, which affects the product formation [137].
1032 Using agro-industrial wastes (70% sugarcane bagasse, 15% of pineapple peelings and 15% of
1033 waste activated sludge) under mesophilic conditions, Robledo-Narváez [137] found a
1034 decrease in H₂ yield (3 mmol H₂/g TS) at a TS content higher than 18 %TS in a tested TS
1035 content range from 15-35%. Similar results were obtained by Valdez-vazquez and Poggi-
1036 Varaldo [138], where highest H₂ productivity and yield (463.7 NmL/kg.d and 54.8 N mL/g
1037 VS removed, respectively) was obtained at a TS of 20.9 % using organic solid waste (paper
1038 (40%) and food (60%) wastes) for the tested TS range from 20.9 – 35.1 %TS. Likewise,
1039 Motte et al. [136] also reported 19 %TS as the limit to achieve higher H₂ production
1040 performance during the DF of wheat straw, as metabolic pathways shifted towards lactic acid
1041 formation at higher TS content. Further research is required on SSDF in order to elucidate the

1042 mechanisms involved during dark fermentation at high TS contents. This research could
1043 provide practical solutions for biohydrogen production from organic solid waste.

1044 **8. Conclusions**

1045 DF technology has an excellent future potential for biohydrogen production as renewable
1046 biomass can be used as a feedstock and the integration with other systems could foster a
1047 higher H₂ yield and economic feasibility. The economic considerations and production at
1048 industrial scale recommend a continuous bioprocess. Thus, more research on continuous DF
1049 processes needs to be carried out to demonstrate the long term operational feasibility of
1050 continuous process. Microbial community of hydrogen producers and innovative substrates
1051 needs to be explored. The use of spent dark fermentation residues in photofermentation and
1052 or electrochemical systems as a secondary step could pave the way towards sustainable
1053 biohydrogen production in up-scaled systems. Finally, anaerobic digestion is required to
1054 further stabilize the residues generated from the upstream processes. The future design and
1055 configuration of industrial scale dark fermentative processes is expected to be similar as of
1056 anaerobic digestion processes, with some modifications in process parameters [43]. The
1057 available two stage methane producing plants can be modified for dark fermentation [264],
1058 while solid state dark fermentation opens new opportunities for biohydrogen production from
1059 renewable biomass.

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