

1 **Hydrogen production from agricultural waste by dark** 2 **fermentation: a review**

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8 **Abstract**

9 The degradation of the natural environment and the energy crisis are two vital issues for
10 sustainable development worldwide. Hydrogen is considered as one of the most promising
11 candidates as a substitute for fossil fuels. In this context, biological processes are considered
12 as the most environmentally-friendly alternatives for satisfying future hydrogen demands. In
13 particular, biohydrogen production from agricultural waste is very advantageous since agri-
14 wastes are abundant, cheap, renewable and highly biodegradable. Considering that such
15 wastes are complex substrates and can be degraded biologically by complex microbial
16 ecosystems, the present paper focuses on dark fermentation as a key technology for producing
17 hydrogen from crop residues, livestock waste and food waste. In this review, recent findings
18 on biohydrogen production from agricultural wastes by dark fermentation are reported. Key
19 operational parameters such as pH, partial pressure, temperature and microbial actors are
20 discussed to facilitate further research in this domain.

21

22 **Keywords**

23 Agricultural waste, Anaerobic digestion, Biohydrogen production, Biological processes, Dark
24 fermentation

25

26

27 **Abbreviations**

28 ASBR: Anaerobic Sequencing Batch Reactor

29 CSTR: Continuous Stirred Tank Reactor

30 COD: Chemical Oxygen Demand

31 HRT: Hydraulic Retention Time

32 HAB: Homo Acetogenic Bacteria

33 LCFA: Long Chain Fatty Acids
34 MPB: Methane-Producing Bacteria
35 SRB: Sulfate-Reducing Bacteria
36 UASB: Upflow Anaerobic Sludge Blanket
37 VS: Volatile solids
38 VFA: Volatile Fatty Acids

39
40

41 **1. Introduction**

42

43 The energy crisis and environmental degradation are currently two vital issues for global
44 sustainable development. It is now accepted that the dependence on fossil fuels - over 80% of
45 energy consumption - contributes not only to climate change and global warming, but also to
46 a rapid exhaustion of natural energy sources [1]. Almost all countries worldwide are interested
47 in the search for new, clean and renewable energy supplies. Over the last decades, research
48 efforts have focused mainly on bioethanol and biodiesel production. These first generation
49 biofuels made from food crops such as corn, sugar cane, and palm oil, have been seen as
50 possible alternatives to ease the world's dependence on gasoline or diesel. However, they have
51 indirectly caused an increase in food prices and thus contributed to the recent global food
52 crisis. Hence, the production of second generation biofuels by the conversion to biofuels of
53 whole plants, including agricultural residues, is now essential in the move towards renewable
54 energy.

55 The original concept of “environmental biorefinery” consists of installations designed to
56 produce a wide range of products to optimize the conversion of biomass. Alternative energy
57 sources such as biogas from waste and especially biohydrogen need to be considered [2].
58 Biohydrogen can be used directly in combustion engines for transportation or, after
59 purification, in fuel cells for producing electricity. Its high energy content per unit of weight
60 (142 kJ.g^{-1}) and since water is the only by-product generated by oxidative combustion, makes
61 hydrogen the ideal and most environmentally friendly alternative to fossil fuels [3]. To date,
62 hydrogen is not commercialized as an energy source but it is widely used as a chemical
63 reactant in the production of fertilizers, for refining diesel and for the industrial synthesis of
64 ammonia. Schemes for the use of the hydrogen as energy resource have been restricted in
65 large part by high production costs, technical storage requirements and distribution methods
66 [4]. At present, 88% of commercial hydrogen derives from fossil fuels (natural gas, heavy oils
67 or coal) [5]. Water electrolysis has extensively developed in recent years, and is now more
68 widely used, supplying up to 4% of current total hydrogen production. However, all such
69 techniques are highly energy-consuming and are unsustainable processes. One promising
70 alternative is hydrogen produced biologically which requires much less energy. Regardless of
71 the great interest in biohydrogen production from biomass at a laboratory research level,

72 substantial technical advances in the biological processes involved are still required if the
73 biohydrogen market is to become economically viable. The most promising sources of
74 biohydrogen involve direct water biophotolysis by green algae, indirect water biophotolysis
75 by cyanobacteria, the photo-fermentation by photosynthetic bacteria, and dark-fermentation
76 by strict or facultative anaerobic bacteria. Considering that agri-waste is made up of complex
77 substrates and can be degraded biologically by complex microbial ecosystems, dark
78 fermentation is a key technology for the production of hydrogen from crop residues, livestock
79 waste and food waste.

80 The purpose of this paper is to present an up-to-date overview of current knowledge
81 about biological dark fermentation processes producing hydrogen from agricultural and food
82 waste.

83

84 **2. Feedstock and hydrogen potential**

85

86 Many studies investigating hydrogen production by dark fermentation have used simple
87 sugars such as glucose or sucrose as model substrates. In contrast, fewer studies have looked
88 into solid substrate conversion. For organic materials to be potentially useful as substrates for
89 sustainable biohydrogen production, they must be not only abundant and readily available
90 but, also, cheap and highly biodegradable. Agri-waste and food waste meet all these
91 requirements. As to their abundance, about 0.7 billion tons of agricultural and forestry waste
92 were generated in Western Europe between 1998 and 2001 [6]. In France, a survey of the
93 years 1995 to 2006 showed that total annual waste production had increased to about 849
94 million tons by 2006, of which agricultural and forestry waste represented around 43%, *i.e.*
95 374 million tons [7]. In Germany, the second biggest agricultural country in Europe, agri-
96 waste represented more than 175 million tons per year in 2000, including 25 million tons per
97 year of agricultural biomass. By way of comparison, German municipal waste represented
98 only 16 million tons per year and industrial waste 9 million tons [8].

99 Three categories of agricultural residues can be distinguished: (i) the waste generated
100 from direct agricultural production, *i.e.* crop residues; (ii) livestock waste, *i.e.* animal manure,
101 and (iii) food waste.

102

103 **2.1. Crop Residues**

104

105 Agricultural residues from harvested crops are the most abundant, cheapest and most
106 readily available organic waste to be biologically transformed; they include straw, stover,
107 peelings, cobs, stalks, bagasse, and other lignocellulosic residues [9]. The annual
108 lignocellulosic biomass generated by the primary agricultural sector has been evaluated at
109 approximately 200 billion tons worldwide [10]. All agricultural crops are biodegradable and,
110 to varying degrees, may be converted biologically in anaerobic digestion processes to

111 biohydrogen and biomethane.

112 Hydrogen yields from various crop substrates, as recorded in the literature, are presented
113 in Table 1. The origins of the organic substrates are quite similar, nevertheless, untreated raw
114 material presents generally lower yields, ranging from 0.5 to 16 mL_{H₂}·g_{VS}⁻¹. Under
115 mesophilic conditions the lowest yield was reported from the conversion of wheat straw to
116 hydrogen in a batch reactor [11], while the highest was obtained using cornstalks [12]. The
117 yield of fermentative hydrogen from crop residues in thermophilic conditions at 70°C was
118 higher than that in mesophilic conditions indicating that temperature favors hydrolysis [13].
119 Indeed, the “cornstalks” category in Table 1 shows variable hydrogen yields, likely because of
120 the varied composition of the carbohydrates, which include cellulose, hemicellulose and
121 lignin [12][14]. Moreover, as reported in anaerobic digesters producing methane from
122 agricultural waste, the crop species, the harvesting time and the variable silage period must all
123 be considered as main factors impacting on biogas fermentation [15]. A recent review of the
124 literature summarized the composition of different crops residues, *e.g.* wheat straw, corn
125 stover and rice straw as containing cellulose, hemicelluloses and lignin in a range of approx.
126 32-47 %, 19-27% and 5-24%, respectively [16]. Although no trend was observed in the
127 reported data, a reasonable hypothesis is that biohydrogen yields may be inversely correlated
128 to the cellulose and lignin contents of the waste, as observed by Buffiere *et al.* [17] for
129 methane production.

130 The production of biohydrogen from crop waste biomass is limited by the hydrolytic
131 activity of the microorganisms involved in the biological attack of the heterogeneous and
132 microcrystalline structure of lignocellulosic component, and in the decomposition of
133 cellulose-like compounds to soluble sugars. Appropriate pretreatment steps for the raw
134 material are often required in order to favor hydrolysis. The main pretreatments are based on
135 mechanical, physical, chemical and biological techniques [9]. A mechanical shredding step is
136 essential to reduce particle size and increase the surface area of the organic waste prior to
137 fermentation. As a consequence, solubility and fermentation efficiency are both favored in the
138 acidogenic fermentation process (Figure 1). In all studies reported in Table 1, the crop residues
139 were mechanically treated prior to the experiments and this technique should be further
140 investigated to determine the influence of such pretreatment on overall performances.
141 Chemical pretreatments methods using oxidizing agents, alkali, acids and salts are most
142 frequently investigated because they require no direct energy input [9]. The biohydrogen yield
143 from cornstalks treated by NaOH (0.5%) reached 57 mL_{H₂}·g_{VS}⁻¹, *i.e.* 19-fold the initial value
144 of raw material (3 mL_{H₂}·g_{VS}⁻¹)[14]. Zhang *et al.* [14] also investigated biohydrogen production
145 from cornstalk waste after an acidification pretreatment coupled to heat pretreatment. A
146 maximum cumulative H₂ yield of 150 mL_{H₂}·g_{VS}⁻¹ was obtained after a 0.2% HCl treatment, *i.e.*
147 50 times the initial value, thus proving the efficiency of the acidification pretreatment step
148 [14]. Although this value is remarkable in the light of the average values reported in Table 1,
149 such performances are within the range of the theoretical biohydrogen yield in mixed

150 cultures, *i.e.* $311 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{Hexose}}^{-1}$, calculated from $2.5 \text{ mol}_{\text{H}_2} \cdot \text{g}_{\text{Hexose}}^{-1}$ according to Hawkes *et al.*
151 [18]. Fan *et al.* [11] demonstrated that an acidic pretreatment of 2% HCl coupled to
152 microwave heating led to the increase of soluble sugar content of wheat straw from 0.2% to
153 9.6% and to the decrease of cellulose and hemicellulose content from, respectively, 22% to
154 15% and 21% to 13%. The maximum hydrogen yield observed in this case was $68 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$,
155 which is 136 times the initial value ($0.5 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$) observed on untreated material [11].
156 Similar results were observed with steam explosion as pretreatment, with a yield increasing
157 from $9 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{raw corn straw}}^{-1}$ to $68 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{treated corn straw}}^{-1}$ [12]. Given the present state of
158 knowledge, further experimentation is required to better understand the impact on
159 biohydrogen production performances of the compositions and characteristics of organic
160 substrates. Pretreatment processes for crop residues also require specific investigation since
161 the origins and compositions of the organic substrates determine which specific pretreatment
162 is the most suitable.

163

164 **2.2 Animal manure – livestock waste**

165

166 Three main types of animal manure have been distinguished: urinary waste *i.e.* slurry or
167 liquid manure from livestock or poultry; solid manure or farm yard manure; and wastewater
168 which is a collection of process water in farms, feedlot runoff, silage juices, bedding,
169 disinfectants and liquid manure [19]. More than 1500 million tons of animal manure is
170 produced yearly, including 1284 million tons of cattle manure and 295 million tons of pig
171 manure across the 27 member states of the European Union [20]. Where manure is not
172 managed or treated, it represents a major risk of air and water pollution. On the one hand,
173 nutrient leaching (primarily nitrogen and phosphorous) and pathogen contamination can lead
174 to direct surface water damage and, on the other hand, manure can release up to 18% CO_2
175 equivalent and 37% CH_4 , contributing to the green house effect [20].

176 On European farms, animal manure is usually treated in storage tanks, and then the liquid
177 fraction is separated by centrifugation and finally spread on farmland. The solid fraction is
178 subsequently treated by anaerobic digestion to be further used as fertilizer in agriculture [21].
179 Since agricultural biogas facilities have been extensively used to co-digest manure and other
180 residues suitable for methane production, these large-scale farm installations provide the
181 necessary equipment to readily implement biohydrogen bioprocesses [22].

182 Biohydrogen yields from livestock waste are presented in Table 1. Mainly, they are much
183 lower than those observed from crop residues, with values ranging from 4 to $29 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$. In
184 most studies, either chemical or thermal pretreatment associated to thermophilic conditions
185 are required to avoid methanogenic activity. Indeed, the indigenous methanogenic microflora
186 will rapidly convert hydrogen to methane, as shown by Yokoyama *et al.* [23]. The highest
187 yield (*i.e.* $65 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$) was reported in a study investigating the potential for hydrogen
188 production of cattle manure thermally pretreated (Table 1). This high yield was likely the

189 result of using fresh manure sampled directly at the cattle feedlot prior to the experiment. This
190 assumption is supported by the study of Bonmati *et al.* [24] who observed a 3.5-fold decrease
191 in methane production when the pig slurry was stored for several months. Meanwhile, the
192 ammonium concentration increased 3-fold over the initial value because of the decomposition
193 of organic matter [24]. A similar inhibition has been observed for biohydrogen production
194 from animal slurry. Indeed, Kotsopoulos *et al.* [25] concluded that the low production yield of
195 $4 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$ from pig slurry was due to ammonium inhibition. Livestock manure from pork
196 and poultry have been reported to contain up to $4 \text{ g N} \cdot \text{L}^{-1}$ and cattle manure about $1.5 \text{ g N} \cdot \text{L}^{-1}$
197 [26]. Because of the high nitrogen content, shock loading of slurry can cause severe inhibition
198 of the whole biological anaerobic and hydrogen fermentation processes [27] [28].
199 Additionally, it has also been observed that high sulfate concentrations in swine manure act as
200 a strong inhibitor of biohydrogen production through the growth of highly competitive
201 hydrogen-consuming sulfate-reducing bacteria [29]. With the aim of avoiding nitrogen
202 inhibition, another study on liquid swine manure showed a high yield of $209 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$ after
203 the addition of glucose as an additional substrate in a semi-continuously-fed reactor [30]. This
204 observation suggests the potential use of the co-digestion of animal manure and carbohydrate-
205 rich feed to produce biohydrogen. In this case, the co-digestion process should even be
206 envisaged locally, in the light of agricultural facilities to directly use local crop materials, in
207 order to optimize the loading ratio C/N by dilution of other inhibiting factors. This should,
208 consequently, increase the stability of the biological process. A recent study investigating the
209 anaerobic co-digestion of cattle slurry with vegetable/fruit wastes and chicken manure
210 showed a substantial 2-fold increase in the methane yield [31].

211

212 **2.3. Food waste**

213

214 Food waste has high energy content and is highly biodegradable, *e.g.* it contains 85-95%
215 of volatile solids and 75-85% moisture, favoring microbial development [32]. Food waste is
216 usually disposed as landfill which can lead to problems of putrid smells and leachates
217 polluting underground water if not handled properly [22]. Anaerobic digestion is
218 recommended for treating food wastes [33]. Over the last decades food waste has been the
219 most studied feedstock for hydrogen production, including kitchen refuse [34], a part of
220 municipal waste [36], food industry co-products such as oil mill [36] [37], cheese whey [38],
221 and starch-manufacturing waste [39]. In Table1, several maximal biohydrogen production
222 yields observed in anaerobic reactors are reported. As in the results obtained with crop
223 residues and livestock waste, the performances display great variation, from $3 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$ to
224 more than $290 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$, due to the different composition of the matter involved. The
225 average production is substantially higher than the values obtained from crop residues and
226 livestock. About ten years ago, individual food substrates *i.e.* rice, carrot, cabbage, chicken
227 skin, egg and lean meat began to be sorted out from municipal waste for assessment [40]. In

228 the latter study, biohydrogen production was assessed from a range of relatively simple
229 substrates for further assessment of the production potential with mixtures made up of such
230 simple constituents. Later, other studies using food waste from institutional catering were
231 carried out in batch tests and showed yields of $60 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$ to $196 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$ [32][41].
232 Studies of continuous fermentation systems have been reported more recently, showing no
233 significantly higher yield, but they have proved the feasibility of using food waste in future
234 continuous pilot or industrial-scale applications [13] [42]. Again, more recently, many studies
235 have focused on agri-food industry waste as a source of substrates for producing biohydrogen
236 [36] [37] [38] [43] [44]. Among them, carbohydrate-rich waste shows great promise for the
237 intensive production of biohydrogen. For instance, biohydrogen yields from molasses and
238 cheese whey approached a value of $2.5 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{hexose}}^{-1}$, which corresponds to the maximal
239 expected yield in mixed culture [38] [44].

240 In addition, thermophilic conditions also favor biohydrogen production. Indeed, food
241 waste from institutional catering generated around $81 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$ under thermophilic
242 conditions, compared to $63 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$ under mesophilic conditions [45]. Other studies
243 reported increasing yields from $13 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$ to $65 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$, respectively under mesophilic
244 and thermophilic conditions [13] [42]. For the lowest values, *i.e.* $12.6 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$, a mixture of
245 slaughterhouse waste, food waste and manure was utilized as substrate. It included much
246 protein and fat [13], which might well explain of the low hydrogen yield. Although
247 thermophilic conditions are recommended, they are energy consuming. If the energy for
248 heating the fermentation system could be generated through a biogas/thermal exchange
249 system, thermophilic continuous processes could then be considered as sustainable.

250

251 In conclusion, crop residues, livestock, and food waste are potentially suitable substrates
252 for hydrogen production by dark fermentation. Food waste gives the highest yield of
253 hydrogen, followed by crop residues and animal manure. It is recommended that waste
254 generated by agricultural activities such as crop residues, should be co-digested with animal
255 manure using already existing biogas plants by implementing a dedicated biohydrogen
256 production stage. By coupling with methane bioprocesses, the treated effluent could be finally
257 used as fertilizer. In this scheme, the production of biohydrogen and biomethane might be
258 used for heating and electricity generation or, in the case of biohydrogen, also as a chemical
259 reactant. Although food waste offers great potential as a hydrogen resource, the performances
260 of the biological processes are related not only to the operating conditions, but also, to the
261 composition of the organic waste. Future research is recommended to better understand the
262 influence of feedstock composition, to predict bioreactor performances and optimize the co-
263 digestion system.

264

265 **3. Biological reactor operation**

266

267 The major limitation of biohydrogen production at an industrial scale concerns the low
268 productivity and the low conversion yields of the fermentative biological processes. Based on
269 current hydrogen productivity, industrial processes would require very large-volume reactors.
270 Levin *et al.* [46] reported that the minimum size of a bioreactor required to power a small
271 proton exchange membrane fuel cell installation of 1 kW was 198 L, when considering H₂
272 productivity of 2.7 L.L⁻¹.h⁻¹ using dark fermentation and mesophilic conditions [46]. The
273 productivity of hydrogen-producing bioreactors treating agri-waste is substantially lower than
274 the result cited above because of the use of complex and polymeric organic substrates and
275 also the mixed cultures as inoculum. However, the optimization of the operating conditions of
276 biological reactors remains a key parameter for the improvement of biohydrogen production.
277 Specifically-optimized bioreactors could help to determine whether the use of agricultural
278 waste *in situ* would be technically feasible and economically viable. To develop practical
279 independent biohydrogen practical applications on farms, likely coupled with methane
280 production, it is vital to consider concomitantly advances in biotechnology to enhance
281 biohydrogen yield and biogas quality along with fuel cell development [46]. In order to meet
282 these requirements, the following operating conditions must be considered.

283 3.1 Operating conditions

284 3.1.1 pH

285 pH is one of the most important factors to be regulated in anaerobic digestion processes
286 [47][48]. Indeed pH affects not only the yields of hydrogen production in mixed cultures, but
287 can also modify by-product spectrum and impacts the structure of the microbial communities
288 [49][50][51]. Table 2 summarizes the operating parameters in reactors treating agricultural
289 residues inoculated with naturally mixed microbial cultures. Optimal H₂ production appears to
290 take place with a pH of 5.0 - 6.0 for food wastes [41][52][53], whereas a neutral pH is
291 recommended for crop residues and animal manure [12][14][25][23]. Two different types of
292 experimentation have been performed to determine the optimal pH : one involved adjusting
293 different initial pHs in a series of batch tests while the other maintained the same pH in
294 continuous reactors during the fermentation process [13] [54] [23]. Li *et al.* [12] investigated a
295 large range of initial pHs, from 4 to 8, in batch tests. They showed that a pH of 7-7.5 as
296 optimal for the conversion of corn straw to biohydrogen [12]. As the accumulation of by
297 products, *i.e.* acetate and butyrate, lowered the pH of the medium, higher pH (*i.e.* around
298 neutrality) led to better hydrogen yields. As suggested by Wang *et al.*[55], who reported that
299 batch reactors with not regulated pH and treating sucrose are the systems most commonly
300 studied, further investigations should focus rather on pH-controlled systems and on more
301 complex organic wastes as substrates. In continuous reactors, in contrast, pH is usually
302 controlled. A varied pH ranging from 4.5 to 6.5 was tested on tequila's vinasses in a semi-
303 continuous CSTR reactor [48]. It was concluded that a pH of 5.5 was optimal for hydrogen

304 production. A similar value was proposed in another study devoted to brewery waste in a
305 CSTR with a pH ranging from 5.0-6.5 [56]. As a general rule, the optimal pH in terms of
306 biohydrogen production is within a range of 5.0 - 7.0 which probably favors the activity of the
307 hydrogenases and is also suitable for microbial development in dark fermentation [57].

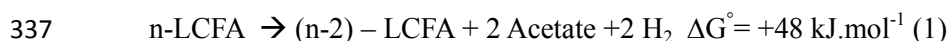
308 In addition, the pattern of intermediate VFAs is different under variable pH conditions.
309 Butyrate and acetate are the two main products, but at low pHs butyrate is preferentially
310 produced. Hydrogen-producing butyrate-acetate pathways are favored at pH 4.5-6.0 while at
311 neutral or higher pH conditions, ethanol and propionate accumulate [18][41][58][59]. When
312 using brewery waste as a substrate, Fan *et al.* [56] observed that, at pH 6.0 or below, acetate
313 and butyrate were the major by-products whereas solventogenesis (propanol, butanol and
314 ethanol) occurred at pHs higher than 6.5 [56]. This was confirmed by Fang *et al.* [60] in a
315 study investigating the effect of pH from 4.0-7.0 on by-product formation. At low pH,
316 butyrate and acetate were dominant products while ethanol, lactate, propionate and caproate
317 appeared at higher pHs [60]. Temudo *et al.* [61] studied the impact of the pH on metabolic
318 activity and microbial diversity in fermentation processes with glucose, xylose, and glycerol
319 at 30°C. They showed that a low pH conditions (< 6), the product spectrum consisted mainly
320 of butyrate and acetate while at high pH, the spectrum shifted to acetate and ethanol. It is
321 noteworthy that under both high and low pH conditions, the fermentation pattern was clearly
322 associated with the dominance of *Clostridium* species, whereas at intermediate pHs,
323 metabolic shifts involved higher microbial diversity [61]. This suggests that pH effects result
324 not only from a shift in metabolic pathways but also in major changes in microbial
325 communities.

326

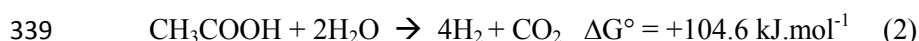
327 **3.1.2 Biohydrogen Partial Pressure**

328 Many studies have already reported that partial pressure of hydrogen is a restrictive factor
329 in the course of the fermentation of organic waste. The oxidation of reduced components such
330 as Long-Chain Fatty Acids to VFAs, concomitantly with hydrogen production, is the
331 consequence of a low biohydrogen concentration in the medium because reactions are
332 thermodynamically unfavorable [62]. The positive Gibbs energy of LCFA degradation ($\Delta G^0 =$
333 $+48$ mJ/mol) shows that the degradation of fat through the β -oxidation pathway is
334 thermodynamically unfavorable and therefore requires an extremely low level of hydrogen
335 partial pressure (see Equation 1) [62]

336



338



340

341 Additional formation of hydrogen could also derive from the degradation of acetate (see

342 Equation 2) [63]. This conversion is thermodynamically unfavorable at moderate
343 temperatures and the reaction is therefore extremely sensitive to biohydrogen concentration.
344 Furthermore, the inverse reaction, called homoacetogenesis, is rather favored in the
345 fermentation process and partly reduces the performance of bioreactors through the
346 accumulation of acetate in the medium. By the increase of the hydrogen concentration in the
347 medium due to microbial metabolism, not only biohydrogen production may be affected but
348 also a shift of metabolic pathways towards solventogenesis has been observed, *i.e.* the
349 accumulation of lactate, ethanol, acetone and butanol [46]. Recent research indicates,
350 however, that the main factor leading to solventogenesis is the accumulation of volatile fatty
351 acids rather than hydrogen partial pressure [64]. Especially when feeding with a high glucose
352 concentration, the intermediate acids produced, particularly butyric acid, initiate
353 solventogenesis [65].

354 To decrease p_{H_2} in the medium, especially in highly concentrated bioprocesses treating
355 organic waste, agitation is the most usual technique. Chou *et al.* [66] studied the conversion of
356 brewery grains to hydrogen in a 100 L pilot bioreactor. Experiments showed that the rate as
357 well as the yield of biohydrogen production increased from $1.8 \text{ mL} \cdot \text{L}_{\text{reactor}}^{-1}$ to $6.1 \text{ mL} \cdot \text{L}_{\text{reactor}}^{-1}$
358 while the stirring was speeded up from 20 to 100 rpm [66]. Several other alternatives exist to
359 improve gas extraction, including gas sparging and biohydrogen stripping from reactor
360 headspace by membrane absorption. Mizuno *et al.* [67] showed that sparging nitrogen gas into
361 a fermentor fed with simple sugars led to double the biohydrogen yield from $86.76 \text{ mL}_{H_2} \cdot \text{g VS}^{-1}$
362 to $187.86 \text{ mL}_{H_2} \cdot \text{g VS}^{-1}$. Other gases such as argon or a mixture of recirculation gases have
363 also been used [67] [68]. The main disadvantage of these techniques is that, regardless of the
364 significant biohydrogen removal, the sparging gas dilutes the biohydrogen content and creates
365 a further reduction in separation efficiency. In the event of upscaling to an industrial level, the
366 high energy consumption in sparging processes and H_2 purification would raise the
367 production costs, and the fluctuation in gas prices would impact directly on the economic
368 viability of the process. Membrane-absorption techniques offer other energy-effective
369 alternatives for hydrogen removal from a gas mixture. Liang *et al.* [69] reported a reduced
370 biogas partial pressure by introducing a submerged hollow-fiber silicone membrane into the
371 reactor. A Pd-Ag membrane reactor [70] and a synthetic polyvinyltrimethyl silane membrane
372 reactor [71] exhibited the highest hydrogen selectivity. The main disadvantage of using
373 membrane-absorption techniques is the presence and the development of a biofilm over time
374 which may favor the emergence of methanogenic bacteria.

375 Despite the different techniques available for reducing the partial hydrogen pressure,
376 more research is still required to develop efficient and low cost gas purification systems
377 aiming at the direct use of hydrogen from biogas to fuel cells at industrial scale.

378

379 **3.1.3 Temperature**

380 Temperature is often considered as one of the most important parameters affecting both
381 biohydrogen production yields and microbial metabolisms in mixed cultures [57]. Because of
382 the complexity of the agri-waste and the variable operating conditions, no optimal
383 temperature for hydrogen fermentation can be assessed from the data in the literature. Most
384 studies on fermentative hydrogen production have been based on mesophilic temperatures. Li
385 *et al.* [57] reported that 73 of 101 case studies were carried out at mesophilic temperatures.
386 Crop residues usually present higher yields at thermophilic temperatures due to a better
387 hydrolysis of the lignocellulosic compounds. For instance, the highest amounts of hydrogen
388 from grass were obtained at 70°C using a heat-treated inoculum from a dairy farm digester,
389 *i.e.* 16 mL_{H₂}.g_{VS}⁻¹ [58]. Regarding food waste, thermophilic temperatures seem more suitable
390 to hydrogen production despite significantly different observations reported in the literature.
391 These differences might be due to the origin of the inoculum, the quantity of readily-
392 biodegradable compounds as well as the operating conditions. At 55°C, acetate was the
393 dominant by-product while a propionate production pathway was favored at 20°C [13]. To
394 examine the effect of the fermentation temperature on biohydrogen production, dairy cow
395 waste slurry was cultured at 37°C, 50°C, 55°C, 60°C, 67°C, 75°C and 85°C [23]. Although
396 two optima of production were observed at 60°C and 75°C, with yields of 29.25 mL_{H₂}.g_{VS}⁻¹
397 and 18.5 mL_{H₂}.g_{VS}⁻¹, the increase in hydrogen production globally correlated with higher
398 operating temperatures. Performances were also influenced by changes in the microbial
399 community structure. The structure of the microflora was significantly different at the two
400 optimal fermentation temperatures. At 60°C, the predominant bacteria were affiliated to
401 *Bacteroides xyloxyticus*, *Clostridium stercorarium*, and *Clostridium thermocellum*, while at
402 75°C three strains of the extremophilic thermophilic bacterium *Caldanaerobacter*
403 *subterraneus* were dominant [23]. Without pretreatment of the initial inoculum, temperatures
404 higher than 60°C are recommended in order to reduce hydrogen-consuming activity [59]. In
405 any event, the main disadvantage of thermophilic anaerobic fermentation processes is the
406 energy requirement for heating and maintenance.

407

408 **3.2 Bioreactor configuration**

409 At laboratory-scale, most studies dealing with dark fermentation from solid substrates
410 have been performed in batch reactors [58] [72]. Batch-mode reactors possess the advantage
411 of being easily operated and flexible. This has resulted in the wide utilization of batch reactors
412 for determining the biohydrogen potential of organic substrates. However, in an industrial
413 context, for practical reasons of waste stock management and for economic considerations,
414 continuous bioprocesses are recommended. To date, no biohydrogen industrial-scale reactor
415 has been set up, but it is expected that bioreactor design and system configuration will be

416 similar to methane biogas plants: only the operational parameters may vary between these two
417 anaerobic applications. In view of the extensive the experience acquired in biogas plants
418 treating agricultural organic waste, especially in Germany, the most probable reactor for
419 biohydrogen production would be a vertical, continuously-stirred tank reactor with different
420 types of mixers [73]. More than half of this type of reactor is covered with a single or double-
421 membrane roof to store the biogas (see Figure 2) [73]. Within the one-stage fermentation
422 concept at laboratory-scale, continuous stirred tank reactors (CSTR) are the most common
423 continuous system used for anaerobic digestion [74][25] in hydrogen production research on
424 substrates such as pig slurry [25], swine manure [30] for food waste [42][75](see Table 1).
425 Other studies have reported successful use of ASBR, rather than CSTR, for food waste
426 conversion [76]. Only a few studies have concerned the processes for treating high-solid-
427 content agricultural waste [57]. The reasons could well be the instability of such systems in
428 the course of hydrogen fermentation due to the highly variable composition of the feed and
429 the metabolic instability of the microbial consortia. A remarkable reactor design was set up by
430 Jayalakshmi *et al.* [34] to investigate kitchen waste in hydrogen conversion. This was a pilot-
431 scale, inclined, plug-flow reactor, cylindrical in shape and kept at a 20° angle to the horizontal
432 to facilitate movement of the waste. A screw arrangement inside the reactor, serving to push
433 the material from the inlet at the bottom to the outlet at the top was designed with 14 leads to
434 maintain seven days retention time, which was important for the solid waste to have sufficient
435 hydrolysis time [34]. Additionally, a start-up in batch mode favored the formation of stable
436 microflora granule, and consequently enhanced seed source activity [34] [66].

437 In order to complete the degradation of organic substrates, a two-stage systems coupling
438 hydrogen fermentation with methane production is recommended for treating substrates such
439 as livestock waste and food waste [38] [42] [77]. Such a two-phase anaerobic digestion
440 system was first proposed by Pohland and Ghosh in 1971 [78]. In this system, only fast-
441 growing acidogens are dominant in the first step and produce mainly VFAs, whereas slow-
442 growing acetogens and methanogens are the main microorganisms present in the second step
443 in which VFAs are converted to methane and carbon dioxide. This combination of
444 fermentation systems greatly enhances the energy conversion compared to the one-stage
445 process. A study estimated that only 5.78% of the influent COD was converted to hydrogen in
446 the first stage, compared to 82.18% of COD converted to methane in the second stage [42].
447 Nevertheless, a maximum hydrogen yield of $65 \text{ mL}_{\text{H}_2} \cdot \text{g}_{\text{VS}}^{-1}$ and a H_2 production rate of 22.65
448 $\text{kg}_{\text{VS}} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ were observed using food waste and with an inoculum derived from the
449 indigenous microbial cultures contained in this substrate [42]. Chu *et al.* [47] reported the
450 successful association of reactors for hydrogen and methane production from food waste,
451 under specific conditions of fermentation for each: respectively, 55°C, pH 5.5, 31h HRT and
452 35°C, neutral pH, 120h HRT. They demonstrated that a short HRT and acidic pH prevent
453 methanogenic activity in the acidogenic stage. After optimization of the reactor association
454 system, higher biogas yield ($464 \text{ mL}_{\text{CH}_4} \cdot \text{g}_{\text{VS}}^{-1}$, 70%-80%) was observed thanks to the

455 hydrolytic activity in the first step; but treatment time was also reduced. An HRT of 5 days
456 was already enough for the methane stage instead of a more usual HRT of 10 - 15 days in
457 thermophilic and mesophilic conditions, respectively [79].

458 Another suggested two-stage system consists of the combination of dark and photo-
459 fermentation. Nath *et al.* [86] described one sort of process associating dark and photo-
460 fermentation in a sequential batch reactor. A glucose-based media was inoculated with
461 *Enterobacter cloacae* DM11 to produce H₂, CO₂ and VFAs in dark fermentation. Then, in a
462 second reactor, acetate was subsequently used by *Rhodobacter sphaeroides* O.U.001 to form
463 hydrogen. The yield of hydrogen in the first stage was about 3.31 molH₂.mol glucose⁻¹ and in the
464 second stage in the range of 1.5-1.72 molH₂.molacetic acid⁻¹, equivalent to 3-3.4 molH₂.mol glucose⁻¹
465 ¹. Thus, the overall yield exceeded 6 molH₂.mol glucose⁻¹, which is higher than of the maximum
466 4 molH₂.mol glucose⁻¹ obtained with the dark fermentation process alone. The use of agri-waste
467 as a substrate in these types of association remains to be tested.

468

469

470 **4. Microbiology of biohydrogen production from agricultural waste**

471

472 Anaerobic digestion (AD) is a ubiquitous phenomenon found in nature under anaerobic
473 conditions. The first stages in AD are hydrolysis and acidogenesis, in which dark fermentation
474 is involved, with hydrogen-producers. Then, hydrogen as a key intermediate can be rapidly
475 consumed by others microorganisms in mixed culture, mainly by homoacetogens,
476 methanogens, and sulfate-reducing bacteria (Figure 1) [81] [29] [82]. The metabolic network
477 of carbohydrates has been the most widely investigated. Among the large range of end
478 products generated by the various microbial metabolisms, acetic acid accumulates from acetic
479 fermentation as sole end product with a theoretical production of 4 molH₂.mol hexose⁻¹,
480 equivalent to 498 molH₂.mol hexose⁻¹ (0°C, 1atm.); while in the butyrate pathway, a lower molar
481 hydrogen yield is observed with 2 molH₂.mol hexose⁻¹, equivalent to 249 molH₂.mol hexose⁻¹ (0°C,
482 1atm.) (Eqs. (3) and (4) below) [18].

483



485



487

488 However, the accumulation of acetate in the medium does not necessarily imply higher
489 biohydrogen production since several microbial species can convert hydrogen and carbon
490 dioxide to acetate (Eqs. (5)) [83].

491



493

494 In mixed cultures, a ratio of 3:2 of butyrate / acetate is usually observed, resulting in a
495 theoretical average hydrogen yield of 2.5 mol_{H₂}.mol_{hexose}⁻¹ [18]. In mixed cultures,
496 propionate, ethanol, and lactic acid may also accumulate. Propionate is a metabolite of a
497 hydrogen-consuming pathway, while ethanol and lactic acid are involved in a zero-hydrogen-
498 balance pathway (Eqs. (6), (7) and (8)).

499



501



503



505

506 In a previous review paper, Nandi and Sengupta [84] listed the major hydrogen-
507 producing bacteria related to strict anaerobic genera (*Clostridia*, methylootrophs, rumen
508 bacteria, methanogenic bacteria, archaea), to facultative anaerobic genera (*Escherichia coli*,
509 *Enterobacter*, *Citrobacter*) and to aerobic genera (*Alcaligenes*, *Bacillus*). In relation to
510 biohydrogen production from agricultural waste, *i.e.* in mixed cultures, three classes of
511 microorganisms could be distinguished: hydrogen producers, hydrogen consumers and
512 metabolic competitors.

513

514 **4.1. The biohydrogen producers**

515

516 Although pure cultures have been intensively investigated over the past years, involving
517 amongst of others *Bacillus coagulans*[85], *Thermoanaerobacterium* spp.[86], *Enterobacter*
518 *aerogenes* [87], *Clostridium butyricum* [88], few studies refer to the characterization of mixed
519 cultures. A large range of microbial sources has been used to obtain inocula for biohydrogen
520 production, including anaerobic sludge from municipal wastewater plants and cow dung
521 composts [47] [86] [42] [89], cattle or dairy residue composts [90] [11], sludge from palm oil
522 mill effluent [91] [92], soil, rice straw compost, fermented soy bean meal [93] as well as
523 landfill lixivates [13] [32]. Akutsu *et al.* [94] showed that the origin of the inoculum affects
524 the overall performance of the bioreactor. In another study, four natural mixed-microflora
525 seed sources (sludge from sewage treatment; cow dung compost; chicken manure compost;
526 and river sludge) were tested for fermentation in a hydrogen reactor treating cattle
527 wastewater, and sewage sludge showed the highest hydrogen-producing potential [89].

528

529 Another investigation of the effect on grass silage fermentation of the inoculum source,
529 *i.e.* sludge from a dairy farm digester and from a wastewater treatment plant, showed only
530 significant biohydrogen production for bioreactors inoculated with the dairy farm digester
531 sludge [58]. This suggests that acclimation of the seed source is a major parameter that needs
532 to be taken into account for biohydrogen fermentation.

533 From hydrogen-producing mixed cultures, a wide range of species have been isolated,
534 more specifically from the genera *Clostridium* (*Clos. pasteurianum*, *Clos. saccharobutylicum*,
535 *Clos. butyricum*), *Enterobacter* (*Ent. aerogenes*) and *Bacillus* under mesophilic conditions;
536 and from the genera *Thermoanaerobacterium* (*Thermoanaerobacterium*
537 *thermosaccharolyticum*) *Caldicellulosiruptor* (*C. saccharolyticus*), *Clostridium thermocellum*,
538 *Bacillus thermozeamaize* under thermophilic or extremophilic temperatures
539 [95][96][97][98][99]. Under mesophilic conditions, mainly sporulating bacteria of the
540 *Clostridium* genus have been found in mixed mixtures, in all likelihood because of the
541 systematic use of heat shock treatment on the inoculum. In thermophilic conditions,
542 *Thermoanaerobacterium spp.* is preferentially selected by the operating conditions in mixed
543 cultures [99].

544 As to microbial performances, a biohydrogen yield of $3.8 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{glucose}}^{-1}$, at 70°C very
545 close to the theoretical maximum, was reported for *Caldicellulosiruptor saccharolyticus* [98].
546 Maximum hydrogen production of $2.53 \text{ mol}_{\text{H}_2} \cdot \text{mol}_{\text{hexose}}^{-1}$ was observed for
547 *Thermoanaerobacterium thermosaccharolyticum* at a temperature of 60°C [99]. Other
548 thermophilic hydrogen producers reach maximum hydrogen yields ranging from 1.5 to 3.3
549 $\text{mol}_{\text{H}_2} \cdot \text{mol}_{\text{hexose}}^{-1}$ for *Thermotoga elfii*, *Caldicellulosiruptor saccharolyticus*, *Clostridium*
550 *thermocellum*, *Clostridium thermolacticum*, *Clostridium thermobutyricum*, and *Clostridium*
551 *thermosaccharolyticum* [100] [101][102][103][104][105]. Higher conversion yields were
552 observed at high temperature for such microbes. This may partly explain the higher
553 performances observed in bioreactors treating organic waste as well as the fact that hydrolysis
554 is favored at thermophilic temperatures.

555

556 **4.2. H₂ consumers and metabolic competitors**

557

558 Three groups of bacteria are known to interfere directly or indirectly, by diversion of the
559 biohydrogen potential from carbohydrates, *i.e.* the Sulfate-reducing bacteria (SRB), the
560 Methane-producing Bacteria (MPB), and the Homoacetogenic Bacteria (HAB) (Figure 1).

561

562 **4.2.1 Homoacetogenic bacteria**

563

564 Homoacetogenic bacteria are strictly anaerobic microorganisms which catalyze the
565 formation of acetate from H_2 and CO_2 . They were first observed by Fischer *et al.* (1932)
566 [108]. *Clostridium acetium* and *Clostridium thermoaceticum* were the model species used to
567 elucidate the metabolic pathway [106] [107]. They possess special enzymes which catalyze
568 the formation of acetyl-CoA that is converted either to acetate in catabolism or to cell carbon
569 in anabolism. The homoacetogens are very versatile anaerobes, which convert a variety of
570 different substrates to acetate as the major end product [108]. This implies, therefore, that in
571 experimental studies the biohydrogen production measured might be lower than the expected

572 value calculated from the accumulation of acetate [83]. Thomas *et al.* [25] used pig slurry as
573 substrate in a CSTR and observed that the actual production of hydrogen was substantially
574 lower than the value expected from VFA accumulation. As no methane was detected in the
575 biogas and the propionate mass balance did not explain hydrogen losses, hydrogen was
576 assumed to be consumed by acetogenic bacteria [25]. Siriwongrungson *et al.* [109] reported
577 that considerable homoacetogenesis occurred in CSTR reactors using digested dairy manure
578 as inoculum and operated under thermophilic temperatures [109]. It was shown that the
579 biohydrogen produced from butyrate oxidation reacted rapidly with CO₂ to form acetate by
580 homoacetogenesis [109]. Unfortunately, the pretreatment of the inoculum by heating to select
581 spore-forming bacteria is not suitable for inhibiting of homoacetogenic bacteria since some of
582 them belong to the same genus *Clostridium* [110]. Thus, only operating parameters could
583 favor biohydrogen production, *e.g.* by removing CO₂ from the headspace [111].

584

585 **4.2.2 Sulfate-Reducing Bacteria**

586

587 According to theoretical thermodynamics, the most efficient biochemical reaction using
588 hydrogen involves the sulfate/nitrate-reducing microorganisms ($\Delta G^0 = -165 \text{ kJ.mol}^{-1}$), even at
589 a low hydrogen concentration of only 0.02 ppm in the presence of sulfate or nitrate [112]. It
590 has been shown that SRB have a thermodynamic advantage over MPB and HAB [82]. Some
591 waste especially from pulp/paper industry, sea-food processing, distilleries, edible oil and wet
592 corn milling, contains high sulfate concentrations which perturb hydrogen anaerobic digestion
593 as well as produce sulfide gas which is hazardous for fuel cells [113] [114]. Short HRTs are
594 not sufficient to inhibit these microorganisms. Even at a HRT of 2h, the interspecies transfer
595 metabolites such as hydrogen, carbon dioxide and VFA, are immediately consumed by SRB
596 under sulfate-rich conditions [82]. At longer HRT, hydrogen is converted either to methane
597 with carbon dioxide by MPB under sulfate-limited conditions, or to sulfidic acid by SRB if
598 sulfate is abundant in the substrate [115]. Along with the concentration of sulfate and HRT,
599 pH is a key factor in sulfate reduction. pH values lower than 6 significantly inhibit the activity
600 of SRB [115] [113].

601

602 **4.2.3 Methanogens**

603

604 Methanogens are considered as the main hydrogen-consuming microorganisms in
605 anaerobic environments [116] [117] [118]. Many options exist for inhibiting methanogenesis:
606 chemical inhibition, low pH control, heat treatment of the inoculum, short hydraulic retention
607 times.

608 The most commonly used chemical inhibitors are Bromoethanesulfonate (BES), acetylene
609 and chloroform [57]. BES is specific against methanogens and acts as an analog of the
610 coenzyme M in the respiratory chain. However, treating with effective concentrations of BES

611 is not environmentally friendly and too costly for large-scale operations [57]. pH is also a
612 factor in preventing methanogenic activity since most methanogens can only grow at a narrow
613 pH range from 6 to 8 [119]. In absence of pH control during a batch process, an acidic initial
614 pH is strongly recommended [120] [121]. The most common treatment of inoculum is heating
615 the medium to around 100 degrees for approximately ten minutes to select spore-forming,
616 hydrogen-producing bacteria. Methanogens do not sporulate and do not survive such
617 conditions [122] [123]. Because methanogens present low growth rates (approx. 0.2 h^{-1}), the
618 application of short HRT ($< 8 \text{ h}$) quickly leads to a washout of methanogens from the reactor,
619 when no biofilm is formed. To obtain stable hydrogen production in a methane-free biogas,
620 the optimal HRT observed were 3-6 h, 9h, 18h up to 48h for respectively, molasses, bean curd
621 waste, brewery waste and food waste [44] [95] [56] [75]. In a kinetic study of hydrogen
622 production in an anaerobic system, Chen *et al.* [124] calculated a maximum specific growth
623 rate for methanogenic microflora of 0.172 h^{-1} . They concluded that HRT of less than 6h are
624 recommended to selectively wash out the methanogens in continuous reactors [124] [82].

625

626 **4.2.4 Lactic Acid Bacteria**

627

628 Noike *et al.* [125] studied the inhibition of hydrogen production by lactic acid bacteria
629 (LAB). They observed the replacement of hydrogen fermentation by lactic acid fermentation
630 when two lactic acid bacteria (LAB) strains, *i.e.* *Lactobacillus paracasei* and *Enterococcus*
631 *durans*, were cultivated with two hydrogen-producing strains, *Clostridium acetobutylicum*
632 and *Clostridium butyricum*. Secretion of bacteriocins was recognized as the inhibitory effect
633 and temperatures above 50°C were proposed to prevent LAB influence [125]. In mesophilic
634 systems, LAB growth could not be limited by temperature, and the accumulation of lactic
635 acid led to the instability of the mixed culture processes. Indeed, Wang *et al.* [42] showed that
636 lactic acid inhibited hydrogen fermentation in a two-stage continuous system using food
637 waste as substrate [42]. The hydrogen yield dropped from 71 to $49 \text{ mL}_{\text{H}_2}\cdot\text{g}_{\text{VS}}^{-1}$ when the lactic
638 acid increased from 2.3 to $4.4 \text{ g}\cdot\text{L}^{-1}$. Increasing the organic loading rate resulted in an increase
639 in lactic acid concentration and in the microflora indigenous in food waste, *i.e.* lactic acid
640 bacteria, and then led to the perturbation of the system if no pretreatment had been previously
641 carried out [42]

642

643

644 **5. Conclusion**

645

646 The present review reports recent findings on biohydrogen production from agricultural
647 waste by dark fermentation. Three categories of agricultural residue have been considered in
648 the present review: (i) the waste directly generated from agricultural production (ii) animal
649 manure and (iii) food waste. It is shown that all three possess great potential as a substrate for

650 hydrogen production by dark fermentation, in decreasing order: food waste, crop residues and
651 livestock waste. But further research is necessary to better understand the impact of the
652 composition of the substrate on biohydrogen performances. Moreover, the biological
653 processes involved are not only restricted by the composition of the organic waste, but also
654 they are highly dependent of the operating conditions. Key operational parameters such as
655 low pH, low partial pressure, high temperature and acclimated microbial communities are
656 recommended. These operating parameters affect not only the yields of biohydrogen in mixed
657 culture, but also redirect by-product spectrum and impact the structure of the microbial
658 communities. Since a pattern of metabolites are concomitantly produced, the association of a
659 hydrogen fermentor with a methanogenic reactor is strongly recommended to achieve the
660 conversion of biodegradable organic matter to bioenergy. Finally, we suggest it is important to
661 distinguish three classes of microorganisms that require further characterization in mixed
662 cultures: hydrogen producers, hydrogen consumers and metabolic competitors. The presence
663 of various hydrogen consumers and the control of the occurrence of H₂ consuming pathways
664 in mixed cultures constitute the main challenge to improving the stability of bioreactors
665 treating agricultural waste.

666

667

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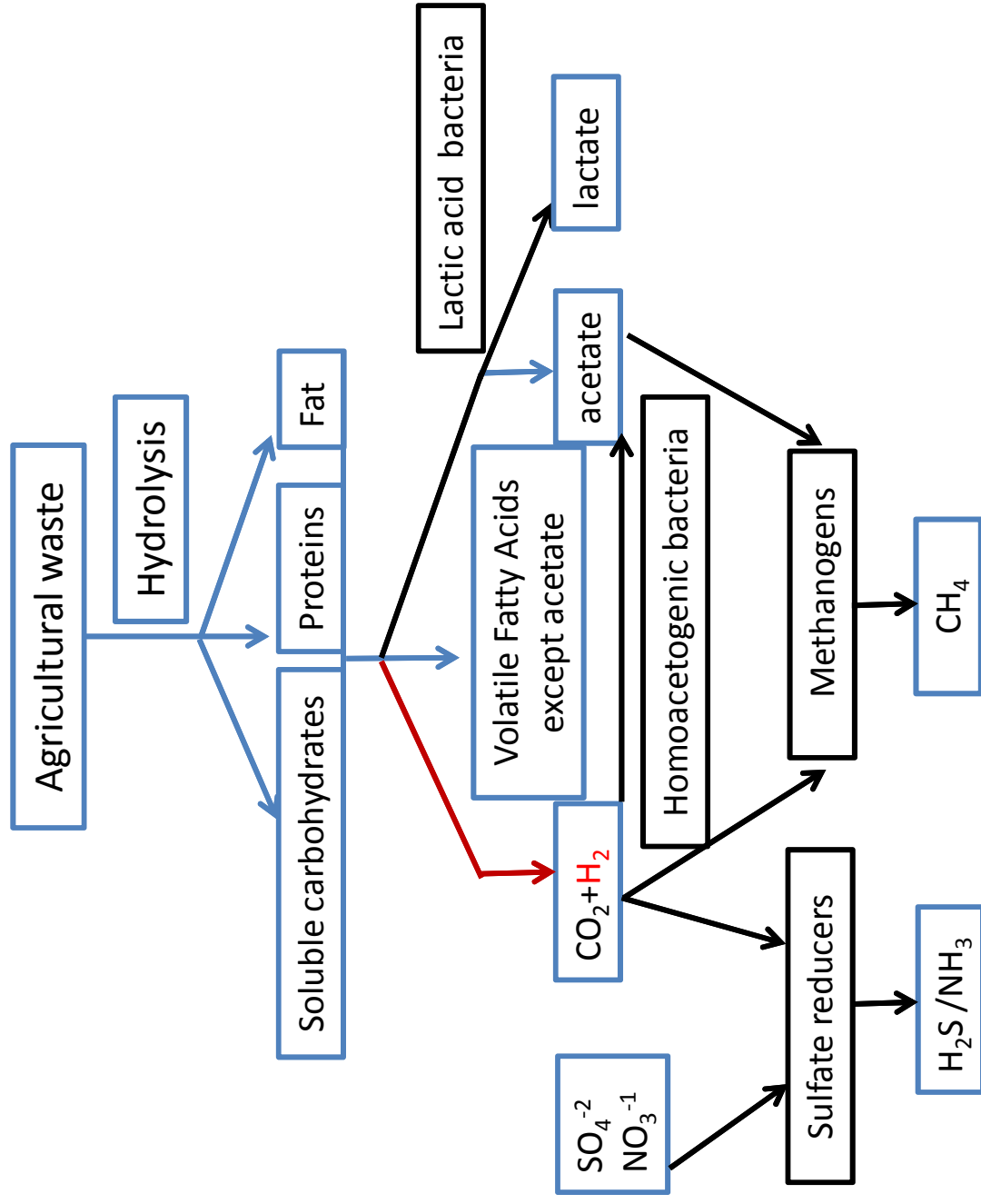
Figure 1: Microbial pathways in an ecosystem degrading agricultural waste, in which red arrows indicate hydrogen producers and black arrows hydrogen consumers.

Figure 2: Different types of anaerobic digestion plant, adapted from Weiland 2006 [73].
a/b/c: Vertical, completely-stirred tank reactor (a/b: mechanical stirring; c: biogas mixing),
d/e: Horizontal plug-flow reactor (mechanical stirring)

Table 1: Estimated H₂ production yields of anaerobic reactors treating agricultural waste
(*calculated from literature data, - no pretreatment of feedstock, n.d. not determined)

Table 2: Optimal pH for biohydrogen production according to the organic substrate.

Figure



Figure

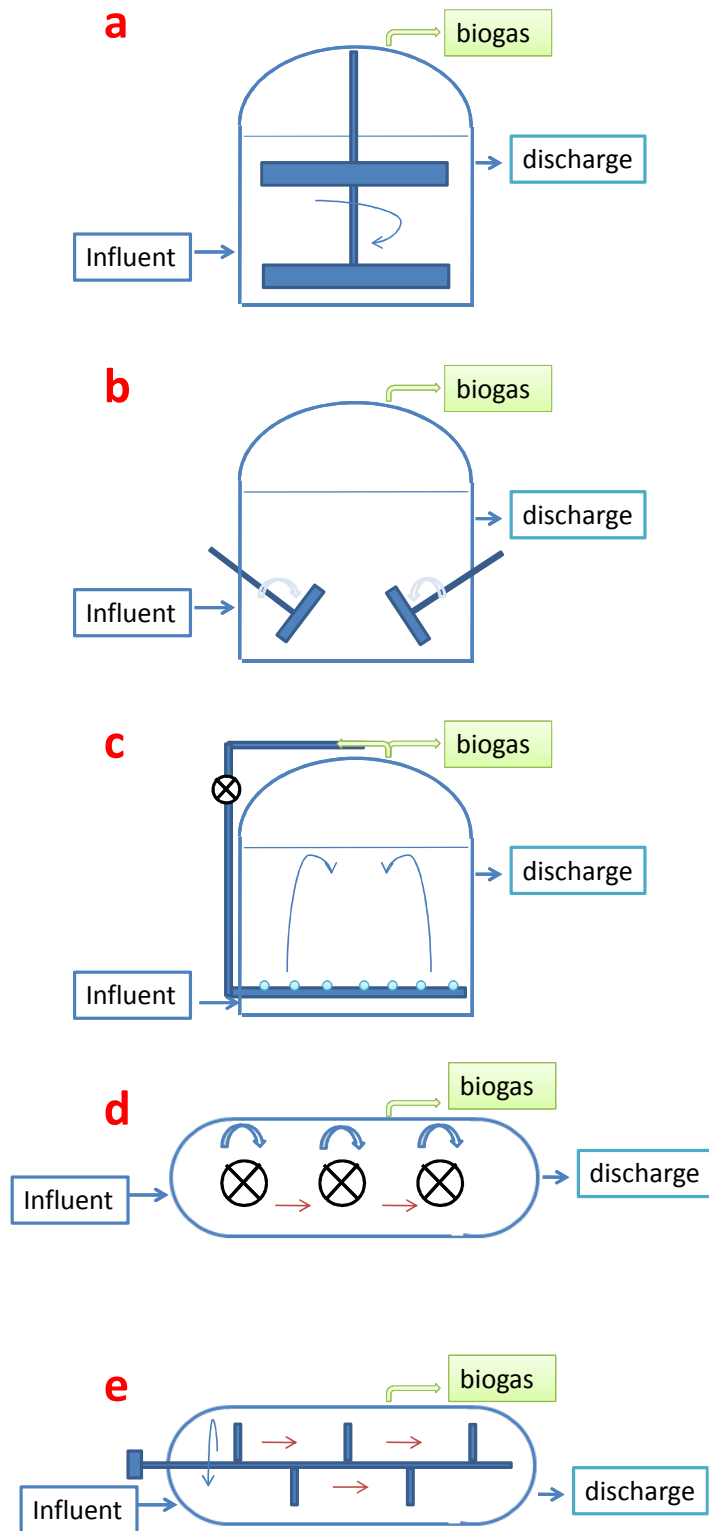


Table 1: Estimated H₂ production yields of anaerobic reactors treating agricultural waste

(*calculated from literature data, - no pretreatment of feedstock, n.d. not determined)

Substrate	Maximum assessed production yield (mlH ₂ ·gvs ⁻¹)	Pretreatment*	Temperature (°C)	Reactor operation mode	Reference
Corn straw	9	-	35	Batch	[12]
Corn straw	68*	1.5MPa10min	35	Batch	[12]
Corn stover	49*	220°C 3min	35	Batch	[132]
Corn stover	66*	1.2% HCl+200°C 1min	35	Batch	[132]
Cornstalk	3	-	36	Batch	[14]
Cornstalk	57	0.5% NaOH	36	Batch	[14]
Cornstalk	150	0.2%HCl boiled 30min	36	Batch	[14]
Grass silage	6	-	35	Batch	[13]
Grass silage	16	-	70	Batch	[13]
Maize leaves	18	-	70	Batch	[98]
Maize leaves	42	130°C 30min	70	Batch	[98]
Rice bran	61	n.d.	35	Batch	[93]
Sweet sorghum plant	32.4*	130°C 30min	70	Batch	[98]
Sugarcane bagasse	19.6*	130°C 30min	70	Batch	[98]
<i>Silphium trifoliatum</i> leaves	10.3*	130°C 30min	70	Batch	[98]
Wheat straw	1	-	36	Batch	[11]
Wheat straw	68	HCl 2%+microwave heating	36	Batch	[11]
Wheat straw	49*	130°C 30min	70	Batch	[98]
Wheat bran	43	n.d.	35	Batch	[93]
Cow feces and urine	18*	-	75	Batch	[23]
Cow feces and urine	29*	-	60	Batch	[23]
Cow feces and urine	0.7*	-	37	Batch	[23]
Cattle manure	65	90°C 3h	52	Batch	
Cattle wastewater	53*	-	45	Batch	[89]
Dairy manure	18	0.2%HCl boiled 30min	36	Batch	[133]
Dairy manure	14	0.2%NaOHboiled30min	36	Batch	[133]
Dairy manure	14	infrared radiation 2h	36	Batch	[133]
Pig slurry	4	-	70	CSTR	[25]
Swine liquid manure	209*	-	35	Semi-continuously -fed fermenter	[30]
Rice	96	-	35	Batch	[40]
Carrot	71	-	35	Batch	[40]
Cabbage	62	-	35	Batch	[40]
Chicken skin	10	-	35	Batch	[40]
Egg	7	-	35	Batch	[40]
Lean meat	8	-	35	Batch	[40]
Foodwaste	196	160°C 2h	36	Batch	[32]
Foodwaste	60*	n.d.	35	Batch	[41]
Foodwaste	77	-	35	Batch	[122]
Foodwaste	125*	-	35	CSTR	[75]
Foodwaste	63	pH12.5 1day	35	ASBR	[45]
Foodwaste	65	-	40	Demi-continuous rotating drum	[42]
Foodwaste	13	-	20	CSTR	[13]
Foodwaste	3	-	37	CSTR	[13]
Foodwaste	16.5	-	55	CSTR	[13]
Kitchen waste	72	-	n.d.	Inclined plug flow reactor	[34]
Molasses	2.5 molH ₂ /molsucrose	-	37	CSTR	[44]
Molasses	2.1molH ₂ /molhexose	-	35	CSTR	[95]
Sweet lime peelings extracts	76.4ml/g COD*	121°C pH=7 40min	32	Batch	[43]
Bean curd manufacturing waste	21	n.d.	35	CSTR	[93]
Cheese whey	290*	NaHCO ₃ 20g/L	35	CSTR	[38]
Palm oil mil effluent	84.4*	-	60	Batch	[37]

Table 2: Optimal pH for biohydrogen production according to the organic substrate.

Substrate	Reactor	pH range	pH optimum	Reference
Corn straw	Batch	4-8 each 0.5 unit	7.0-7.5	[12]
Grass silage	Batch	4; 5; 6	6	[13]
Rice bran	Batch	7 initial	-	[93]
Wheat bran	Batch	7.0 initial	-	[93]
Wheat straw	Batch	4-9	7	[11]
Cow waste slurry	Batch	6-7.5	7.0	[23]
Cattle wastewater	Batch	4.5-7.5	5.5	[89]
Foodwaste	Batch	6 initial		[41]
Foodwaste	CSTR	5.0-6.0	5.5	[75]
Foodwaste	ASBR	5.3 constant	-	[45]
Foodwaste	CSTR	5.5-6.0 constant	-	[13]
Foodwaste	CSTR	5.5 constant	-	[47]
Vegetable kitchen waste	Batch	5.5-7 constant test	6.0-7.0	[54]