1 **Dark fermentative hydrogen production from lignocellulosic hydrolysates – A review**

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10

11 **Abstract**

12

13 The demand for renewable energy is increasing due to increasing energy consumption and global

14 warming associated with increasing use of fossil fuels. Hydrogen gas is considered a good energy

15 carrier due to its high energy content. Biomass (e.g. agricultural and forestry residues, food industry

16 wastes, and energy crops) is amenable to dark fermentative hydrogen production. However,

17 lignocellulosic materials require pretreatment and/or hydrolysis prior to dark fermentation. This

18 paper reviews potential biomass sources for hydrogen fermentation as well as the effects of

19 different pretreatment and hydrolysis methods on sugar yields as well as hydrogen yields from

20 hydrolysates. The effects of process parameters on dark fermentative hydrogen production from

21 lignocellulosic hydrolysates are also discussed.

22

23 Keywords: pretreatment, hydrolysis, dark fermentation, hydrogen, lignocellulose, renewable

24 energy, biomass

25

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46 **1. Introduction**

47

48 At present, most of the global energy is produced from fossil fuels resulting in CO_2 emissions
49 associated with climate change [1]. However, fossil fuels are diminishing [2], while energy
50 requirements are increasing due to population growth [3]. The world energy production can be
51 increased and the problems related to fossil fuels reduced by increasing the share of renewable
52 energy, such as hydro, wind, solar or biomass energy. Biomass can be converted to energy through
53 i) thermochemical processes, such as combustion (heat/electricity), gasification (syngas), pyrolysis
54 or liquefaction (bio-oils), ii) physicochemical processes (biodiesel), or iii) biochemical processes,
55 including anaerobic digestion (methane) or ethanol, butanol or hydrogen fermentation (for a review,
56 see [4]). Advantages of biomass-based energy include the local availability of biomass, its
57 renewability, feasibility of biomass conversion without high capital investments, reduction of
58 greenhouse gas emissions and creation of new jobs [5].
59 Hydrogen is considered as a good energy carrier for the future due to its high energy content
60 (lower heating value of 122 MJ kg^{-1}) [6] and clean usage for electricity production in fuel cells or
61 for combustion with air [7,8]. At present, hydrogen is produced from fossil fuels by reforming,
62 pyrolysis, biomass gasification, or electrolysis (for a review, see [9]). Hydrogen can also be
63 produced biologically through photolysis, photofermentation, dark fermentation, or with microbial
64 electrolysis cells (MEC). Dark fermentative hydrogen production has many advantages; It does not
65 require light energy, has wide substrate versatility and high hydrogen production rates, and the
66 production can be maintained at non-aseptic conditions and in simple reactors [10,11,12].
67 Cellulosic materials are composed of cellulose and hemicellulose, whilst lignocellulose
68 contains also lignin that binds to cellulose and hemicellulose limiting their hydrolysis (for reviews,
69 see [13,14]). Cellulose is a linear polysaccharide composed of thousands of glucose molecules
70 connected by β-glycosidic bonds. Crystalline cellulose molecules are tightly packed together with

71 hydrogen bonds (for reviews, see [15,16]), while amorphous cellulose contains large gaps and
72 irregularities and hydrolyzes much faster (for reviews, see [14,17]). Hemicellulose binds cellulose
73 molecules and consists of pentoses, hexoses and sugar acids [18].

74 Lignin can be degraded biologically by some aerobic fungal species (for reviews, see [13,14]).
75 Cellulose can be degraded by anaerobic microorganisms, but the process is slow [19,20]. Thus,
76 lignocellulosic biomass may require pretreatment prior to biological hydrogen fermentation to break
77 the lignin seal, decrease cellulose crystallinity and increase cellulose surface area [21]. Pretreatment
78 is usually done with physical (milling or grinding), chemical (acid, alkali or ionic liquid) or
79 physicochemical (steam) methods. Pretreated substrate can be further hydrolyzed to fermentable
80 sugars chemically (acid, alkaline or ionic liquid) or biologically (enzymes, fungi or bacteria).

81 Several studies compare the effects of pretreatment and hydrolysis methods on bioethanol
82 production (e.g. [13, 22]). The requirements for pretreatment and hydrolysis are different for
83 bioethanol or biohydrogen production. This is due to different operational conditions and biological
84 processes. Bioethanol is produced using pure cultures and thus, the hydrolysate should contain
85 hexose and pentose sugars directly amenable to pure cultures. In large scale, biohydrogen is
86 produced using mixed microbial communities. More complex substrates than hexoses and pentoses
87 can be utilized by mixed cultures, i.e., the hydrolysis does not have to be complete for the
88 hydrolysates to be amenable for H_2 fermentation. Further, competition and other bacterial
89 interactions in mixed culture fermentation affect the metabolic patterns setting certain prerequisites
90 for the hydrolysates. For example, sulfate remaining in the hydrolysates after acid hydrolysis may
91 support sulfate reducing bacteria that compete with hydrogen producers and consume the produced
92 H_2 [23]. Due to different bioethanol and biohydrogen production processes, the pretreatment and
93 hydrolysis requirements are also different.

94 This paper reviews potential biomass sources for dark fermentative hydrogen production.
95 Furthermore, the effects of different pretreatment and hydrolysis methods on subsequent hydrogen

96 fermentation from the hydrolysates are critically reviewed. The sugar titers and hydrogen yields
97 after different pretreatments are summarized and the effects of process parameters on hydrogen
98 fermentation from lignocellulosic hydrolysates are evaluated.
99

100 ## 2. Biomass sources

101

102 The annual, worldwide production of lignocellulosic material is about 220 Pg (dry weight) [24]
103 consisting of agricultural, forestry and food processing residues, energy crops, aquatic plants and
104 algae [25,26]. The selection of biomass for dark fermentative hydrogen production depends on the
105 cost, availability, carbohydrate content and biodegradability of the material [27]. The compositions
106 of different lignocellulosic and cellulosic materials have been reviewed, e.g., by Hamelinck et al.
107 [22], Mosier et al. [28], Chandra et al. [29] and Saratale et al. [30]. Depending on the biomass
108 composition, it may require pretreatment and/or hydrolysis prior to use for hydrogen fermentation.
109 Pretreated lignocellulosic biomass studied for dark fermentative hydrogen production include,
110 e.g., sugarcane bagasse [31,32,33], corncob [34], wheat straw [35,36], corn stalks [37,38], energy
111 crops [39], grass [40,41], silage [42], and oil palm trunk [43].
112

113 ## 3. Methods for pretreatment and hydrolysis

114

115 Pretreatment breaks the lignin seal of the lignocellulosic material and modifies the size, structure
116 and chemical composition of the substrate [28]. Furthermore, it hydrolyzes some of the
117 hemicellulose, decreases cellulose crystallinity and increases cellulose surface area [21].
118 Pretreatment of biomass can be done with physical procedures, such as milling [32,44], grinding
119 [45,46] or comminution [40], chemical procedures, e.g. acid [33,47,48,49], alkaline [33,50] or ionic
120 liquid [51], and with physicochemical procedures, including hydrothermal [36,52] and steam

121 explosion [53]. Mechanical pretreatments are most often used for lignocellulosic materials, such as
122 straws, bagasse, cornstalk, or wheat wastes [32,45,47,54]. However, they are considered too costly
123 for large-scale applications [17]. According to Agbor et al. [55] their use before hydrolysis should
124 be limited, although they are most likely required prior to treating lignocellulosic materials, such as
125 straws.

126 Hydrothermal treatment and steam explosion are energy-intensive pretreatment methods and
127 may not be economically feasible [52]. Furthermore, they may produce toxic compounds, such as
128 furfural, phenolics and 5-hydroxymethylfurfural (HMF) [36,52,53] that can inhibit subsequent
129 hydrogen fermentation [56,57] Chemical treatments can be used as pretreatment or hydrolysis step.
130 Diluted acid treatment results in high sugar titers [31,46,48,58]. However, they can produce
131 inhibitory compounds [32,40] and the acid residues may also inhibit H_2 fermentation [34,59]. The
132 use of concentrated acids may not feasible due to production of inhibitors [27] and demand for
133 recovery of acids and neutralization of the hydrolysates [60]. Alkaline treatment may also produce
134 inhibitors [32,61]. In general, higher H_2 yields have been obtained after acid than alkaline
135 treatments [32,34,40,59,62]. The main advantage of ionic liquids is that they can be reused [63].
136 However, they are expensive [17] and their use before H_2 fermentation has not been widely studied.

137 Hydrolysis can be used after pretreatment to increase the sugar yield from cellulose and
138 hemicellulose. For example, steam explosion and hydrothermal treatments result in cellulose-rich
139 solid fraction that can be further hydrolyzed into sugars [53]. Hydrolysis should fulfill the following
140 requirements: (i) increase sugar yield, (ii) avoid degradation or loss of sugars, (iii) minimize the
141 formation of inhibitory by-products, (iv) be cost-effective, and (v) recover lignin that can be further
142 converted to co-products (for reviews, see [17,29]). Selection of pretreatment/hydrolysis method
143 depends on the type of raw material and operating conditions [14,18]. Hydrolysis can be done with
144 chemical treatments (described above) or with biological methods. Biological hydrolysis can be
145 performed with cellulolytic enzymes, fungi or bacteria that secrete enzymes to the growth

environment (for reviews, see [13,14,15,64]). Biological hydrolysis can be performed after acid or alkaline pretreatments [33,50,62,65] or directly from the biomass [61,66,67]. The advantages of biological treatments include moderate operational conditions and low energy requirements (for a review, see [47]). However, their use for hydrolysis complicates the overall process resulting in separate optimization and monitoring of two biological processes, i.e. hydrolysis and the H_2 fermentation.

4. Hydrogen fermentation of hydrolysates

4.1 Effects of pretreatment and hydrolysis methods on H_2 production

4.1.1 Sugar yields

High sugar yields after pretreatment and hydrolysis are required to increase the biomass amenability to hydrogen fermentation. The sugar yields after different pretreatment and hydrolysis procedures are summarized in Table 1. Fungal hydrolysis resulted in high sugar yield of 480 g kg^{-1} of dry substrate, whilst the sugar yields after diluted acid hydrolysis and diluted acid followed by enzymatic hydrolysis varied between 270 and 560 g kg^{-1} of dry substrate. The results show a large variation in the sugar titres after bacterial hydrolysis due to simultaneous bacterial oxidation of produced sugars (Table 1). Many studies do not report the highest theoretical sugar yields and thus, the relative yield (=actual yield/theoretical yield) is unknown. We recommend that in future studies the yield reporting should be standardized and given as a fraction of the theoretical value based on the analysis of the composition of the substrates used.

Table 1

172 ### *4.1.2 Hydrogen yields*

173 The hydrogen yields from different hydrolysates are summarized in Table 2 and in Figures 1
174 and 2. In addition, Figure 1 compares H_2 yields from hydrolysates to those obtained from direct
175 fermentation of biomass to H_2. The highest theoretical hydrogen yields on hexose with acetate or
176 butyrate as the sole soluble metabolite were 4 or 2 mol mol^{-1}, respectively. The highest reported
177 hydrogen yield on hexose from hydrolysates was 3.00 mol mol^{-1} from corn stover pretreated
178 simultaneously with steam explosion and diluted sulfuric acid (Figure 2, [53]). High H_2 yields on
179 hexose were also reported after diluted acid or hydrothermal pretreatments of wheat straw, 2.84 and
180 2.56 mol mol^{-1}, respectively [36,68]. These yields are high even as compared to the H_2 yields
181 obtained with pure sugars. For example, the H_2 yields on hexose from glucose with mixed cultures
182 of digester sludge and cow manure and with a pure culture *Caldicellulosiruptor saccharolyticus*
183 were 2.88 [11], 2.56 [69], and 3.60 mol mol^{-1} [70], respectively.

184

185 Table 2, Figures 1 and 2

186

187 Figure 1 demonstrates that pretreatment and/or hydrolysis of biomass is required for high H_2
188 fermentation yields. Eggeman and Elander [71] made similar conclusions in their process and
189 economic analysis of different pretreatment methods prior to bioethanol fermentation. They
190 suggested that the total capital costs of bioethanol production would be at least 4-times higher
191 without pretreatment. Further, the sugar yields in enzymatic hydrolysis could be significantly
192 increased by using a pretreatment step [71]. Economic analysis is also required to compare the
193 overall costs of the two-step hydrolysis and H_2 fermentation processes that have different
194 pretreatment, hydrolysis and H_2 recovery steps. Pilot-scale experimentations using continuous-flow
195 subprocesses are needed to provide data for the economic analysis.

196 Low and variable hydrogen yields from biomass treated with either ionic liquid, alkaline,
197 concentrated acid or bacterial hydrolysis indicate their unsuitability for H_2 production from

198 lignocellulosic materials (Figure 1). Low H_2 yields after alkaline and concentrated acid hydrolyses
199 are likely associated with production of inhibitory compounds [27,72]. Only a few reports on
200 hydrogen fermentation from ionic liquid hydrolysates exist and further optimization of this
201 hydrolysis process is required to untangle the potential H_2 yields. Hydrolytic bacteria may grow on
202 their hydrolysis products decreasing available sugars for H_2 fermentation and the subsequent H_2
203 yield [33,66].

204 High H_2 yields have been reported from hydrothermal and steam explosion hydrolysates (Table
205 2), although only a few studies have been published. These methods have high energy demands [52]
206 that are likely not met with the increases in hydrogen yields. Furthermore, hydrothermal and steam
207 explosion hydrolyse efficiently only the hemicellulose part of the lignocellulosic biomass [36,53].
208 Thus, these pretreatments should be carefully designed and followed by a further hydrolysis of the
209 cellulose fraction prior to H_2 fermentation [35]. Also, lignin fraction should be recovered and
210 converted to valuable co-products [17] to make the overall process economic. Enzymatic and fungal
211 hydrolyses are also promising pretreatments as they are followed by high H_2 yields (Table 2),
212 moderate operation conditions, production of no or small amounts of inhibitory compounds, and
213 ease of operation. Another benefit of fungal hydrolysis is the ability to degrade lignin. However,
214 their use requires rather long treatment time and careful optimization of growth conditions [53].

215 The number of studies on the effects of different pretreatment and hydrolysis methods on dark
216 fermentative hydrogen production is significantly smaller as compared to, e.g., those prior to
217 bioethanol production. Thus, further studies on optimization of pretreatment and/or hydrolyses steps
218 for H_2 fermentation is required for further increases in sugar yields and H_2 yields.

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223 **4.2 Effects of process parameters on H_2 fermentation**

224

225 In direct fermentation of biomass to H_2, hydrogen production is often limited by the hydrolysis by
226 cellulolytic microorganisms [73]. In addition, optimal conditions for cellulose hydrolysis and
227 hydrogen fermentation are different. For example, efficient cellulose hydrolysis has been reported
228 near neutral pH [74,75], while H_2 yields from sugars are often the highest at lower pH values
229 ranging from 5.0 to 5.5 [76,77]. Table 3 lists the effects of process parameters on H_2 production
230 from sugars and from cellulosic materials. The effects of process conditions on hydrogen
231 fermentation from hydrolysates are discussed in detail.

232

233 Table 3

234

235 ***4.2.1 Temperature***

236 Hydrogen fermentation of sugars has been widely studied with mesophilic (20-40°C), thermophilic
237 (50-65°C) and hyperthermophilic (≥70°C) cultures. Change in operational conditions from
238 mesophilic to thermophilic has resulted in increased H_2 yields and rates and decreased lag time
239 from acid hydrolyzed wheat powder [78] and from heat- and enzyme-pretreated bagasse [65]. With
240 mesophiles, the highest hydrogen yield from pulp hydrolyzed with concentrated acid was reported
241 at 28°C (temperature range of 25-43°C). Temperature affected the soluble metabolite distribution,
242 and lactate production dominated at other temperatures than 28°C [79]. However, temperature
243 effect studies with hydrolysates are scarce and further research is required to optimize the H_2 yields.

244

245 ***4.2.2 pH***

246 According to Li and Fang [80], the optimal pH for hydrogen production from carbohydrates is in
247 the range of 5.2-7.0. The optimal initial pH for H_2 production from hydrolysates has varied in
248 similar range of 5.5 and 8.0 (Figure 3). Lower yields have been reported at initial pH values 5 and

249 9, and initial pH below 5 has often inhibited hydrogen production [34,37]. The optimal initial pH is
250 determined by the H_2 producing bacterial community. However, most studies on pH effects have
251 been conducted under conditions without pH control. Optimal initial pH for H_2 production from
252 hydrolysates has been between 6.5 and 7 with enrichment cultures from cow dung compost [45,59],
253 5.5 with *Clostridium butyricum* [31], and 8.0 with dairy manure bacteria [34]. These studies give
254 only an indication of suitable initial pH, but not the optimal H_2 production condition. In further
255 research, on-line pH control should be used.
256
257 Figure 3
258
259 ***4.2.3 Inhibitory compounds***
260 Inhibitory compounds, such as furfural, HMF and carboxylic acids, are likely produced in steam
261 explosion, acid and alkaline pretreatments. HMF and furfural are oxidation products of glucose and
262 xylose, respectively, while other phenolic compounds result from the partial degradation of lignin
263 [56,81]. These compounds may inhibit dark fermentative hydrogen production [52,57]. Furfurals
264 inhibit dark fermentation by decreasing the enzyme activities, inhibiting protein and RNA synthesis
265 and breaking down DNA [82], while phenolic compounds may damage the microbial membranes
266 [57]. Acetic acid is released from the acetylxylan of hemicellulose [56,83]. Non-ionized acetic acid
267 diffuses through the membrane decreasing the intracellular pH inhibiting dark fermentative
268 hydrogen production [84].
269 Cao et al. [56] studied hydrogen production from xylose with *Thermoanaerobacterium*
270 *thermosaccharolyticum* W16 in the presence of inhibitors. They concluded that furfural and HMF
271 inhibited H_2 production at concentrations of 1.5-2.0 g L^{-1}, while syringaldehyde severely inhibited
272 already at 1.0 g L^{-1}. However, acetic acid (10 g L^{-1}) and vanillin (2.0 g L^{-1}), a phenolic compound,
273 did not affect the growth and H_2 production of *T. thermosaccharolyticum* [56]. Quéménéur et al.
274 [57] reported that furfural compounds (1.0 g L^{-1}) inhibited H_2 production from xylose the most with

275 a heat-treated anaerobic sludge (H_2 yield on hexose 0.51 mol mol^{-1} compared to 1.67 mol mol^{-1}),
276 while inhibition by phenolic compounds (1.0 g L^{-1}) had less impact on H_2 production (H_2 yield on
277 hexose 1.28 mol mol^{-1} compared to 1.67 mol mol^{-1}). Monlau et al. [85] produced hydrogen from
278 glucose and different volumes (volume fraction of 4-35%) of diluted acid hydrolysate containing
279 1.2 g L^{-1} furfural, 0.1 g L^{-1} 5-HMF and 0.02 g L^{-1} phenolic compounds. They concluded that the H_2
280 yields on hexose decreased from 2.04 to 1.83 and 0.45 mol mol^{-1} with increased hydrolysate
281 volumes from volume fraction of 0% to volume fractions of 3.75 and 7.5%, respectively, and that
282 hydrolysates volume fraction of 15% inhibited hydrogen production completely.

283 Inhibitors can be removed from hydrolysates by detoxification using chemical, physical or
284 biological methods (for reviews, see [83,86]). For example, Chang et al. [46] reported that no H_2
285 was produced directly from the acid hydrolysate of rice straw, whilst detoxification with $Ca(OH)_2$
286 (overliming) removed furfural and parts of VFAs increasing the H_2 yield. Inhibitory compounds
287 have been removed before dark fermentation with, e.g. charcoal, cation exchange resin, activated
288 carbon, overliming [87,88], or with yeasts [89]. Optimizing detoxification conditions is important
289 and has resulted in 30% increase in H_2 yield [60].

290

291 ***4.2.4 Concentration of hydrolysate***

292 Hydrogen yields and production rates increase with increasing hydrolysate concentrations up to a
293 certain level (Figure 4), after which volatile fatty acids accumulate inhibiting H_2 producers [90] or
294 decreasing the pH below appropriate range for H_2 producers [91]. Furthermore, at high
295 concentrations hydrolysates may contain inhibitory compounds [36,85]. High substrate
296 concentrations may also increase the lag times for H_2 production [92,93], cause substrate inhibition
297 [34], and increase the partial pressure of hydrogen [59] changing the metabolism from acid to
298 solvent production. Effects of substrate concentrations on hydrogen production have been mainly
299 studied in batch assays. In these experiments, volatile fatty acids (VFAs) accumulate, H_2 partial
300 pressure increases and pH decreases resulting in continuously changing conditions. Therefore,

301 hydrogen production potentials with different hydrolysate concentrations should also be revealed in
302 continuous processes, where the operational conditions and the accumulation of inhibitory
303 compounds can be controlled.
304
305 Figure 4
306
307 **4.3 Continuous hydrogen production from hydrolysates**
308
309 Only a few continuous hydrogen fermentation studies from hydrolysates have been reported (Table
310 4). The highest H_2 yields on hexose (2.38 and 2.00 mol mol^{-1}) in continuous bioreactors have been
311 reported with starch hydrolyzed with *Caldimonas taiwanensis* [94] and with acid hydrolyzed oat
312 straw [44], respectively. Kongjan et al. [36] produced H_2 continuously from volume fraction of 20%
313 wheat straw hydrolysates and concluded that inhibitory compounds decreased during operation. Liu
314 et al. [95] obtained 1.5 times higher H_2 yields at continuous than batch mode. Optimization of
315 process parameters on hydrogen fermentation from hydrolysates, including pH, temperature and
316 hydrolysates concentration, as well as the fate of inhibitory compounds requires continuous-flow
317 reactor studies.
318
319 Table 4
320
321 **4.4 Microbial communities producing H_2 from hydrolysates**
322
323 Only a limited number of reports coexist on microbial communities producing hydrogen from
324 hydrolysates. These studies demonstrate the effects of hydrolysates on the composition of microbial
325 communities. Hydrogen production from hydrolyzed sugarcane bagasse with elephant dung culture
326 at 37°C enriched for H_2 producing *Clostridium acetobutyricum* and a lactate producing

327 *Sporolactobacillus* sp. that decreased H_2 yields [32]. From hot spring culture growing on oil palm
328 trunk hydrolysate at 55°C also a H_2 producing *Clostridium* sp. and a lactate producer *Lactobacillus*
329 sp. became enriched [43]. Lactate production competes with H_2 production. In addition, lactic acid
330 bacteria excrete proteins called bacteriocins that have bactericidal activity against Gram-positive
331 bacteria and may inhibit H_2 production [96]. Thus, selection of lactate-producing bacteria on
332 hydrolysates should be avoided, e.g., with optimizing process conditions.

333 Enrichment of hot spring cultures on oil palm trunk hydrolysates resulted in decreased
334 microbial community diversity when compared to cultures enriched on mixed sugars [43]. Different
335 diversities of microbial communities growing on hydrothermally pretreated wheat straw in batch or
336 continuous mode have also been reported [36]. In batch cultures, only one or two H_2 producing
337 bacterial species, *Caldanaerobacter subteraneus*, *Thermoanaerobacter subteraneus* and/or
338 *Thermoanaerobacterium thermosaccharolyticum*, were detected depending on the hydrolysate
339 concentration. In CSTR, the same three bacteria were detected and enriched during reactor
340 operation, but in the beginning also two *Lactobacillus* sp. and other bacterial strains were reported
341 [36]. Due to the possible inhibitory effects of hydrolysates on H_2 producing bacteria the changes in
342 the bacterial communities should be monitored both in batch and continuous mode experiments.
343

344 **4.5 Kinetic models used in H_2 fermentation studies from hydrolysates**

345

346 Modified Gompertz equation (Equation 1) has been widely used to describe hydrogen fermentation
347 in batch (for a review, see [97]) and hydrolysates H_2 fermentation studies. The variables in the
348 equation include H = cumulative H_2 production (mL) at time t (h), P = maximum potential H_2
349 production (mL), R_m = maximum rate of H_2 formation (mL h^{-1}), λ = duration of lag phase (h), and e
350 = 2.71828. Cumulative H_2 production, maximum H_2 production rate, and lag time in batch
351 fermentation studies are thus obtained. These kinetic constants can be used for design of reactor

352 studies [98]. The variables can be calculated also based on the liquid volume [65] or the amount of
353 substrate as g sugars [36], g VSS [37], or g TVS [34]. Modified Gompertz equation has also been
354 used to calculate the kinetics of enzymatic hydrolysis, where the obtained variables were rate and
355 yield of reducing sugar production [99].
356

357
$$H(t) = P * exp\left\{-\exp\left[\frac{R_m * e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

358

359 The fitted curves obtained with modified Gompertz equation often match well with the
360 experimental points, which is determined with the regression coefficient (R^2). Good correlation has
361 been reported with hydrogen production from hydrolysates obtained with different enzyme [100],
362 NaOH [40] and HCl [61] concentrations, or with different pretreatments [72,101]. Further, the
363 correlation has been good with different initial pH values [58] or hydrolysate concentration [91].
364 Kongjan et al [36] reported good correlation between calculated and experimental data up to
365 hydrolysate volume fractions of 25 %, while with higher hydrolysate concentrations the correlation
366 decreased. Further, the Gompertz equation has been used after thermal pretreatment at different
367 conditions (temperature, time) [52], after treatment with diluted acid at different time points [102],
368 and after steam explosion with or without acid [53]. Thus, Gompertz equation is a useful tool when
369 proceeding from batch to reactor experiments.
370

371
5. Conclusions

372

373 Dark fermentative hydrogen production from lignocellulosic hydrolysates is an appealing
374 method for renewable energy. A significant quantity of research on hydrogen fermentation from
375 hydrolysates has been conducted. Unfortunately, many of the studies report H_2 production results
376 from batch experimentations characterized by continuous changes of multiple conditions and often

377 using units that do now allow comparisons between articles. Batch study reporting should always
378 provide the sugar yields as a fraction of the theoretical value based on the analysis of the
379 composition of the substrates used. In addition, the hydrogen yields should always be reported as H_2
380 on hexose (mol mol^{-1}) or on substrate (L kg^{-1}).

381 For lignocellulosic biomass to become amenable to H_2 fermentation pretreatment and/or
382 hydrolysis is required. The highest H_2 yields are obtained after hydrothermal and steam explosion
383 pretreatments. However, these processes and utilization of their side streams (i.e. cellulose and
384 lignin fractions) have to be carefully designed to become economically feasible. Fungal and
385 enzymatic hydrolyses also result in high H_2 yields but are less energy-intensive due to moderate
386 operational conditions. In addition, their use does not form inhibitory compounds. Pilot-scale tests
387 using continuous processes is crucial to compare and optimize the overall costs of the sequential
388 pretreatment/hydrolysis and subsequent H_2 fermentation and to select the optimal treatment method
389 for given biomasses.

390 In addition to the pretreatment/hydrolysis step, dark fermentative H_2 production from
391 hydrolysates has to be optimized. At present, most of the studies on H_2 fermentation from
392 lignocellulosic hydrolysates have been conducted in batch mode. Based on these results, the optimal
393 pH and hydrolysates concentration for H_2 fermentation of lignocellulosic hydrolysates are between
394 5.5-7 and 10-20 g L^{-1}, respectively. However, batch mode provides incomplete and misleading
395 information for the process design. Thus, continuous reactor studies on H_2 fermentation from
396 hydrolysates are required for utilization of on-line pH control, optimization of hydrolysate
397 concentration, and minimization of inhibitory compounds in continuous system. To support the
398 process optimization, kinetic models should be included when designing reactor studies. Main
399 hydrogen producing and consuming organisms together with those who compete with hydrogen
400 producers should be delineated.

401

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Figure captions

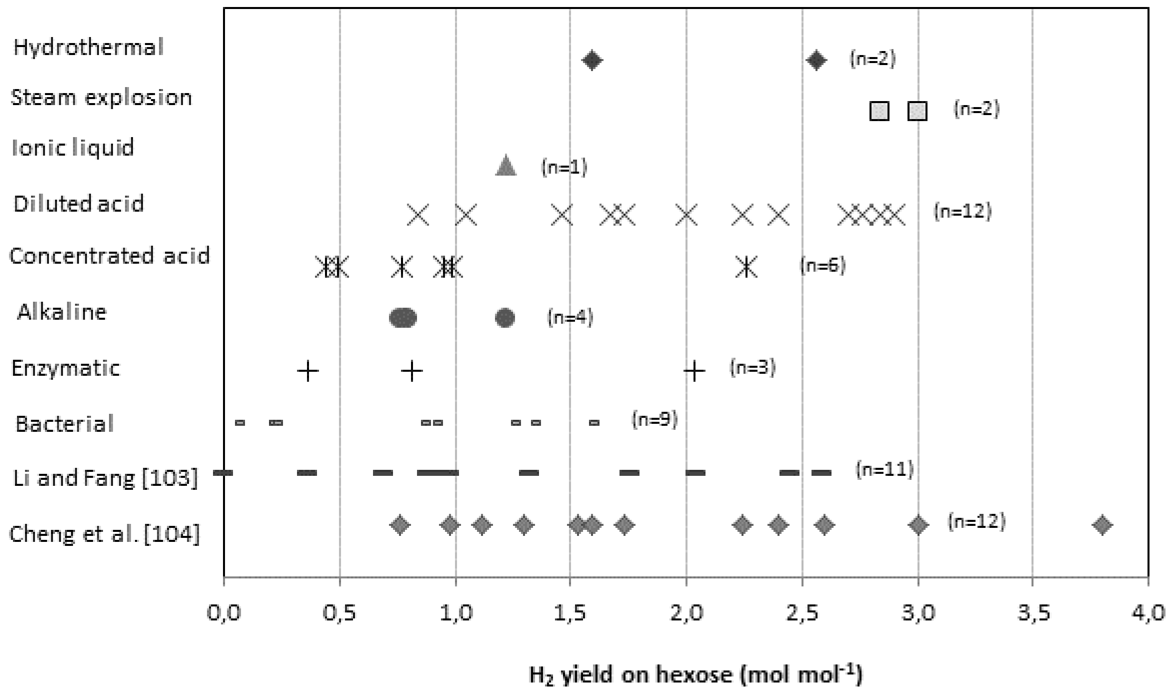
Figure 1. Comparison on hydrogen yields on hexose obtained after different pretreatments (Table 2) and in simultaneous saccharification and fermentation ([103,104], circled with dark grey), n: sample size (A). Hydrogen yields on hexose (B), volatile solids (C) and dry substrate (D) from lignocellulosic biomass with and without pretreatment.

Figure 2. Highest hydrogen yields on hexose obtained after different pretreatments.

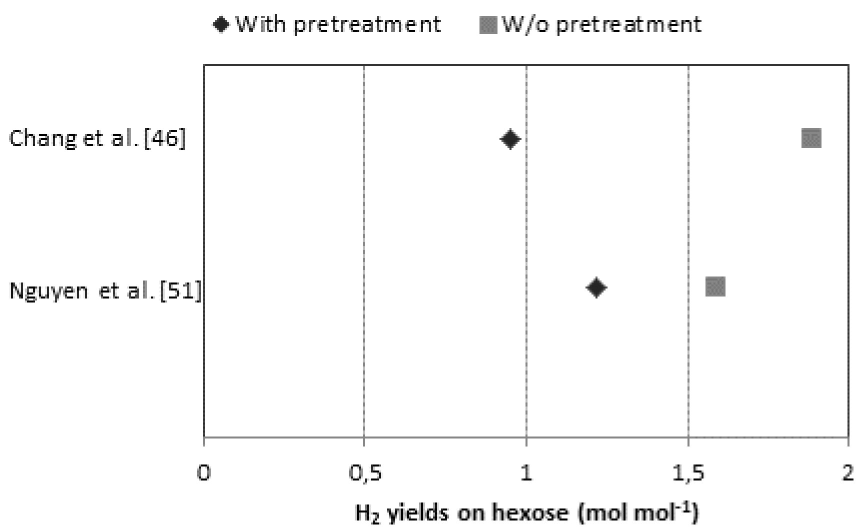
Figure 3. Effects of different initial pH values on H_2 yield on hexose as mol mol^{-1} (A) or on total volatile solids (TVS) as L kg^{-1} (B) from hydrolysates. Symbols: ●: Average, ✳: [31], +: [92], -: [79], --: [93], ×: [32], Δ: [63], □: [34], ♦ [59], ○: [96], ■: [107], ◊: [95], n: sample size.

Figure 4. Effects of substrate concentrations on H_2 yield on hexose as mol mol^{-1} (A) or on total volatile solids (TVS) as L kg^{-1} (B) from hydrolysates. Symbols: ●: Average, ✳: [32], +: [92], -: [93], ×: [31], Δ: [90], □: [34], ♦: [63], ○: [59], ▲: [96], ◊: [95], n: sample size.

A



B



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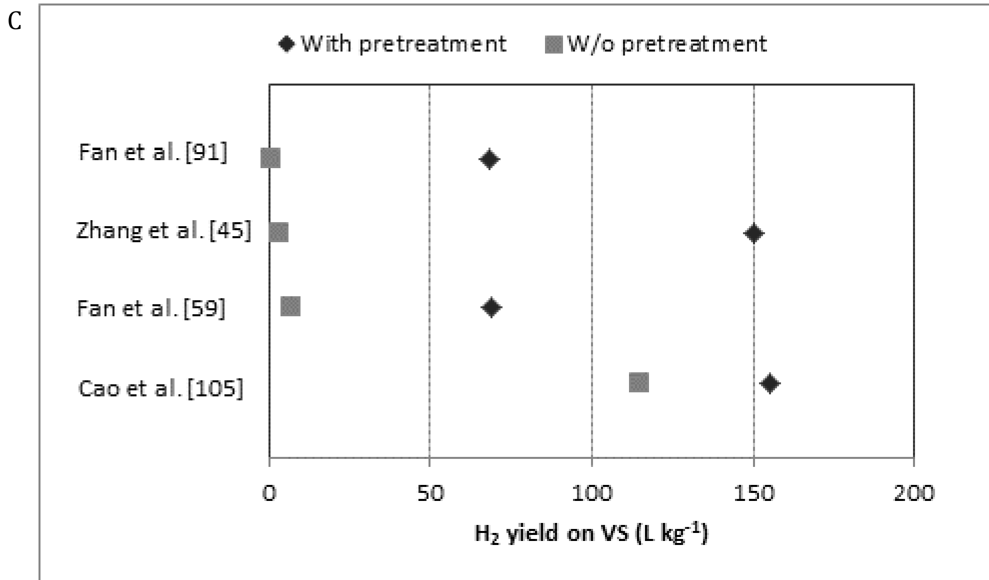

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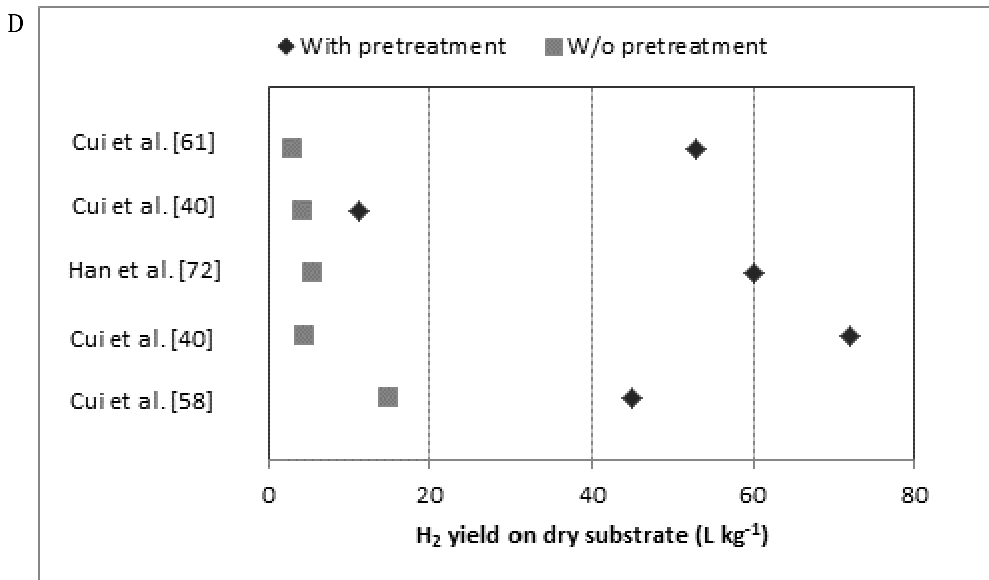

Figure 1

PRETREATMENT	HIGHEST H_2 YIELD ON HEXOSE (mol mol^{-1})	
Hydrothermal	2.56	[36]
Steam explosion + acid	3.00	[53]
Ionic liquid	1.22	[51]
Diluted acid	2.84	[68]
Concentrated acid	2.26	[60]
Alkaline	1.22	[51]
Enzymatic	2.03	[106]
Bacterial	1.58	[33]

Figure 2

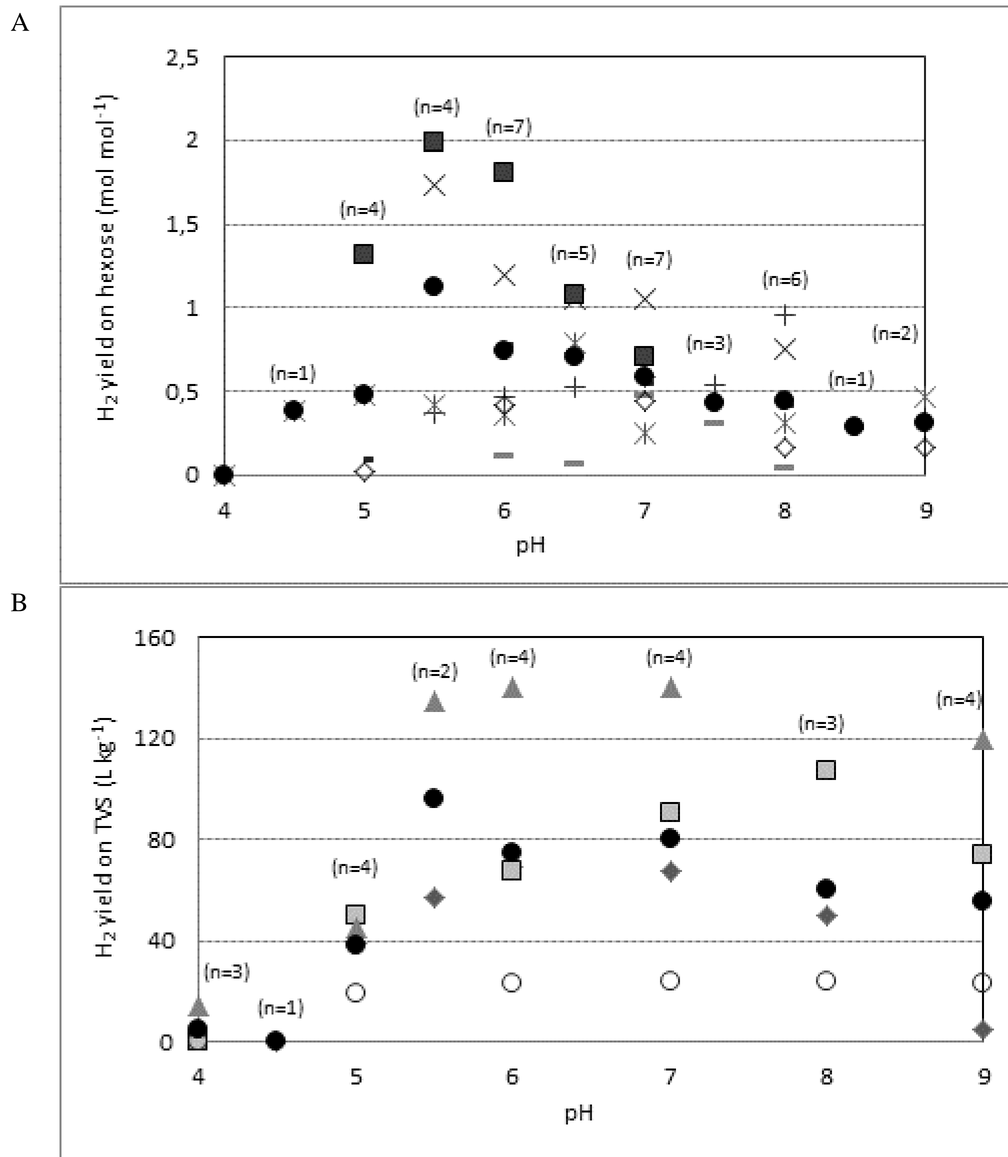

Figure 3

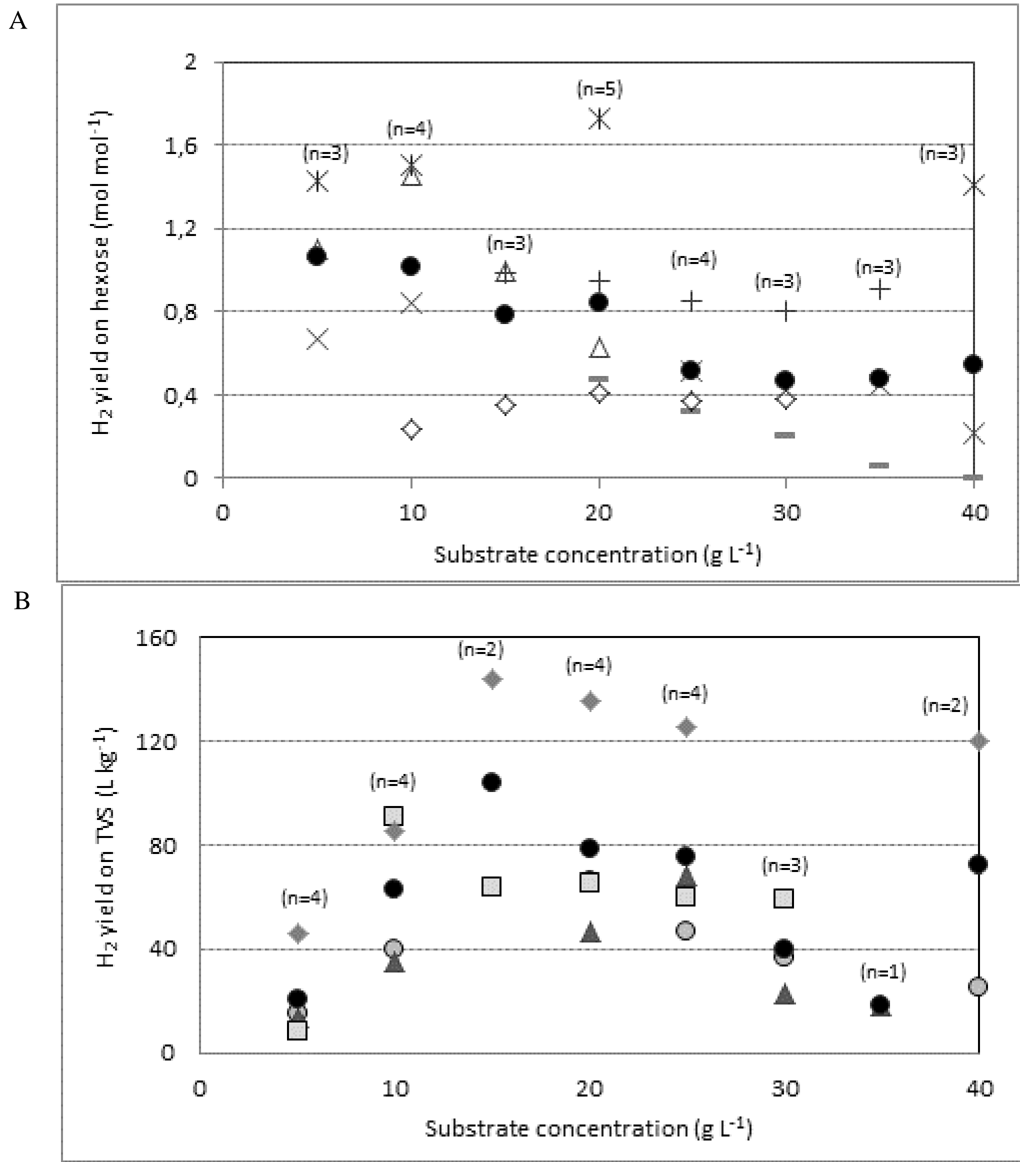

Figure 4

Table 1. Sugar titres in hydrolysates after different pretreatment methods.

Pretreatment	Pretreatment conditions	Substrate	Sugar titre ($g\ L^{-1}$)[a]	Sugars (%)[b]					Reference
				Glu	**Xyl**	**Arab**	**Cellob**	**Others**	
Diluted acid	1% ($L\ L^{-1}$) H_2SO_4	Sugarcane bagasse	11.3	16.5	80.5	6.4	-	-	[32]
	0.5% ($L\ L^{-1}$) H_2SO_4	Sugarcane bagasse	24.5	44.9	46.1	9.1	-	-	[31]
	2% ($L\ L^{-1}$) HCl	Oat straw	16.0	5.6	7.5	5.0	-	12.7	[44]
			37.3	5.4	15.5	4.3		5.1	
	0.9% ($kg\ kg^{-1}$) H_2SO_4	Rice straw	33.2	6.1	34.4	13.2	5.5	-	[46]
	0.9% ($kg\ kg^{-1}$) HCl		64.0	0.6	58.2	8.8	18.3		
	0.9% ($kg\ kg^{-1}$) HNO_3		65.7	3.3	50.6	10.1	17.5		
	6% ($kg\ L^{-1}$) H_2SO_4, 120°C, 15 min	Oil palm empty fruit bunch	18.8 (56%)	10.4	89.4	-	-	3.6	[107]
Concentrated acid	55% ($L\ L^{-1}$) H_2SO_4	Dry conifer pulp	4.83	37.7	51.1	-	-	11.2[c]	[60]
Acid + bacterial	H_3PO_4 + *C. uda*	Sugarcane bagasse	1.30	24	-	-	65	11	[33]
Acid + enzymatic	1.8% ($kg\ kg^{-1}$) H_2SO_4	Barley straw	16.5 (27%)	71.5	28.5	-	-	-	[47]
		Corn stalk	21.7 (37%)	78.8	21.2				
	1% ($kg\ kg^{-1}$) H_2SO_4 + cellulase	Barley straw	24.7 (30%)	67.7	27.5	4.9	-	-	[48]
	1% ($kg\ kg^{-1}$) HCl + cellulase		25.1 (32%)	67.3	27.9	4.8			
	1% ($kg\ kg^{-1}$) HNO_3 + cellulase		22.3 (29%)	70.0	25.6	4.5			
	1% ($kg\ kg^{-1}$) H_3PO_4 + cellulase		23.3 (29%)	69.1	26.2	4.7			
Acid + microwave	1.6% ($kg\ L^{-1}$) H_2SO_4, 450 W	Oil palm trunk	21.8	41.1	38.0	21.0	-	-	[43]
Alkaline	5% ($kg\ L^{-1}$) NaOH	Sugarcane bagasse	1.98	42.4	7.5	50.5	-	-	[32]
Alkaline + bacterial	1.5% ($L\ L^{-1}$) NaOH, 2 $g\ L^{-1}$ H_2O_2 + *C. uda*	Sugarcane bagasse	1.34	15	5	4	42	32	[33]
Bacterial	*Cellulomonas uda*	CMC	2.88 (14%)	13	-	-	40	47	[33]
		Xylan	10.4 (40%)	-	8		-	92	
	C. taiwanensis On1	Starch	23.0	27.4	-	-	-	41.8	[94]
			13.9	45.3				68.9	
	Clostridium TCW1	Cellulose	2.08	43.3	-	-	19.7	-	[67]
		Napier grass	0.74	16.2	17.6		13.5		
		Bagasse	0.71	22.5	22.5		1.4		
Fungal + enzymatic	*Phanerochaete chrysosporium* + cellulase (*T. viride*)	Cornstalk	- (48%)	77.2	16.3	3.3	-	-	[66]
Enzymatic	Cellulase	Paper and pulp industry effluent	22.9	78.6	15.3	6.1	-	-	[106]
	α-amylase + glucoamylase	Barley grains	97.0	97.1	-	-	-	2.9	[54]
		Corn grains	108	96.3				3.8	

[a] Sugar yield as the fraction of theoretical yield is given in parenthesis ($kg\ kg^{-1}$), [b] fraction of individual sugars per total sugars ($kg\ kg^{-1}$), [c] mainly sucrose, Glu: glucose, Xyl: xylose, Arab: arabinose, Cellob: cellobiose

Table 2. Hydrogen yields from hydrolysates.

Pretreatment method	Pretreatment conditions	Substrate	Culture	T (°C)	pH	H_2 yield on hexose (mol mol^{-1})	Reference
Hydrothermal	180°C	Wheat straw	Enrichment culture	70	nr	1.59	[35]
	nr	Wheat straw	Enrichment culture	70	nr	2.56	[36]
	170°C	Marine algae (*Laminaria japonica*)	Anaerobically digested sludge	35	nr	110 L kg^{-1} [b]	[52]
Steam explosion	H_2O, 220°C 1.2% (L L^{-1}) H_2SO_4, 190°C	Corn stover	Digested sludge	35	5.5	2.84 3.00	[53]
	1% (kg L^{-1}) H_2SO_4, 121°C	Corn stalks	*Clostridium acetobutylicum*	37	nr	82 L kg^{-1} [c]	[38]
	1.5 Mpa	Corn stalks	*Clostridium butyricum*	35	nr	68 L kg^{-1} [c]	[37]
Ionic liquid	10% (kg kg^{-1}) [C_4mim]Cl	Cellulose	*Thermotoga neapolitana*	80	7.5	1.22	[51]
Diluted acid	0.2% (L L^{-1}) HCl	Beer lees waste	Cow dung compost	36	6.5	69 L kg^{-1} [d]	[59]
	4% (kg L^{-1}) HCl	Beer lees	Cracked cereals	35	7.0	53 L kg^{-1} [e]	[58]
	0.5% (L L^{-1}) H_2SO_4	Cassava pulp	*Clostridium butyricum, Enterobacter aerogenes*	36	5.5	2.76[f]	[108]
	1% (kg kg^{-1}) HCl	Corncob	Dairy manure	36	8.0	110 L kg^{-1} [d]	[34]
	0.2% (L L^{-1}) HCl	Cornstalk waste	Cow dung compost	36	7.0	150 L kg^{-1} [d]	[45]
	1.7% (L L^{-1}) H_2SO_4	Corn stover	*Thermoanaerobacterium thermosaccharolyticum*	60	7.0	2.24	[109]
	1.08% (kg kg^{-1}) H_2SO_4	Corn stover	*Clostridium thermocellum*	55	6.8	1.67	[68]
	4% (kg L^{-1}) HCl	Grass	Cracked cereal	35	7.0	72 L kg^{-1} [e]	[40]
	H_2SO_4 (pH 2.5)	Ground wheat	Anaerobic sludge	55	5.9	2.40	[54]
	2% (L L^{-1}) HCl	Oat straw	Anaerobic sludge	30	5.5	2.90[f]	[44]
	6% (kg L^{-1}) H_2SO_4, 120°C, 15 min	Oil palm empty fruit branch	Palm oil mill waste sludge	35	5.5	2.38	[107]
	0.5% (kg kg^{-1}) H_2SO_4, 161-164°C	Red algal biomass	Anaerobic sludge	35	>5.3	37 L kg^{-1}[e]	[110]
	3% (L L^{-1}) HCl	Reed canary grass	Enrichment culture	35	nr	30 L kg^{-1} [c]	[41]
	0.9% (kg kg^{-1}) H_2SO_4	Rice straw	Sewage treatment plant	40	6.5	0.95	[46]
	4% (kg L^{-1}) HCl	Soybean straw	Cracked cereals	35	7.0	60 L kg^{-1} [c]	[72]
	0.5% (L L^{-1}) H_2SO_4	Sugarcane bagasse	*Clostridium butyricum*	37	5.5	1.73	[31]
	1% (L L^{-1}) H_2SO_4	Sugarcane bagasse	Elephant dung	37	6.5	0.84	[32]
	H_2SO_4 (pH 3)	Waste ground wheat	Anaerobic sludge	37	6.8	1.46	[90]
	H_2SO_4 (pH 2.5)	Waste ground wheat	Anaerobic sludge	55	7.0	2.70	[111]
	H_2SO_4 (pH 3)	Wheat starch	Anaerobic sludge	37	6.8	2.84	[68]

	HCl	Wheat straw	Compost	36	nr	68 L kg^{-1} [g]	[91]
Microwave and acid	1.6% (kg L^{-1}) H_2SO_4 + 450 W	Oil palm trunk	Hot spring	55	6.0	0.71	[43]
Concentrated acid	10% (kg L^{-1}) H_2SO_4	Cellulose	*Thermotoga neapolitana*	80	7.5	0.95	[51]
	55% (L L^{-1}) H_2SO_4	Cotton cellulose	Seed sludge	37	8.2	0.99	[92]
	55% (L L^{-1}) H_2SO_4	Dry conifer pulp	Enrichment culture	37	6.0	0.77	[79]
	55% (L L^{-1}) H_2SO_4	Dry conifer pulp	Enrichment culture	37	7.0	2.26	[60]
	55% (L L^{-1}) H_2SO_4	Mushroom farm waste	Anaerobic sludge	37	7.0	0.49	[93]
	55% (L L^{-1}) H_2SO_4, 40°C	Rice straw	Sludge	37	7.0	0.44	[95]
Alkaline	1% (kg kg^{-1}) $Ca(OH)_2$	Cornstalk waste	Rottled wood crump	60	7.0	155 L kg^{-1} [g]	[105]
	NaOH (pH 12.5)	Fruits and vegetables waste	Wastewater sludge	35	5.6	0.73	[101]
	0.5% (kg L^{-1}) NaOH	Poplar leaves	Cracked cereal	35	7.0	11.3 L kg^{-1} [c]	[40]
	2% (kg kg^{-1}) NaOH	Corn cob	Dairy manure	36	7.0	14.2 L kg^{-1} [d]	[34]
	NaOH (pH 12)	Beet-pulp	Anaerobic sludge	35	nr	0.79	[112]
	1% (L L^{-1}) NaOH	Beer lees waste	Cow dung compost	36	6.5	11.5 L kg^{-1} [d]	[59]
	4% (kg kg^{-1}) NaOH	Grass silage	Anaerobic digester	55	6.0	6.5 L kg^{-1} [g]	[39]
	15% (kg L^{-1}) NaOH	Cellulose	*Thermotoga neapolitana*	80	7.5	1.22	[51]
Acid and alkaline	0.1% (L L^{-1}) H_2SO_4 + for solids 0.1% (L L^{-1}) NaOH[a]	Oil palm trunk	Geothermal spring	60	6.2	2.24[f]	[49]
Acid and bacterial	H_3PO_4 + *C. uda*	Sugarcane bagasse	*C. butyricum*	35	7.5	1.08	[33]
Alkaline and enzymatic	15 g L^{-1} NaOH, 2 g L^{-1} H_2O_2 +Cellulase (*Pseudomonas sp.*)	Bagasse	*Clostridium pasteurianum* CH4	37	nr	0.96	[50]
Enzymatic	Cellulase (*T.viride*)	Cornstalk	*T. thermosaccharolyticum*	60	6.5	90.6 L kg^{-1} [c]	[113]
	Cellulase (*T. viride*)	Cornstalk waste	Enrichment culture	36	6.5	122 L kg^{-1} [g]	[73]
	Celluclast 1.5 L®	Oat straw	Anaerobic sludge	35	4.5	0.81[f]	[114]
	Cellulase (*T. reesei*)	Paper and pulp industry effluent	*Enterobacter aerogenes*	35	7.0	2.03	[106]
	Viscozyme L[c]	Poplar leaves	Cracked cereals	35	7.0	45 L kg^{-1} [e]	[61]
	OPTIMASH 86®	POME	Anaerobic sludge	44	7	0.36	[115]
Fungal and enzymatic	*Phanerochaete chrysosporium* + cellulase (*T. viride*)	Cornstalk	*Thermoanaerobacterium thermosaccharolyticum*	60	7.0	80.3 L kg^{-1} [c]	[66]
Fungal	*Trichoderma reesei* Rut C-30	Cornstalk	Thermophilic anaerobic digester	55	nr	48.7 L kg^{-1} [c]	[116]

Bacterial	Soil sample	CMC	*Clostridium pasteurianum*	35	7.0	0.20	[117]
	Clostridium TCW1	Cellulose	*Clostridium butyricum*	37	7.5	0.50	[74]
		Napier grass				1.33	
		Bagasse				1.25	
	C. uda	CMC	*Clostridium butyricum*	35	7.5	1.58	[33]
		Xylan				0.91	
	Cellulomonas sp.	Cellulose	*Clostridium pasteurianum*	37	7.5	0.22	[117]
	Cellulomonas uda	Cellulose	*Clostridium butyricum*	37	7.5	0.86	[67]
		Xylan				0.05	
	Caldimonas taiwanensis	Starch	*Clostridium butyricum*	37	6.5	13 L kg^{-1} [h]	[94]

[a] 120°C, 1 bar, 25 min, [b] H_2 yield on chemical oxygen demand (COD), [c] H_2 yield on substrate, [d] H_2 yield on total volatile solids (TVS), [e] H_2 yield on dry substrate, [f] per mol removed substrate, [g] H_2 yield on volatile solids (VS), [h] H_2 yield on total solids (TS), CMC: carboxymethyl cellulose, nr: not reported

Table 3. Effects of process parameters on hydrogen production from hydrolysates (on H_2 production) and directly from cellulosic materials (on H_2 production from cellulosic materials).

Parameter	Effects on	Reference(s)

Temperature	***H_2 production***
	- Solubility of gases, effect of pH_2
	- Chemical and enzymatic reaction rates, stability of enzymes
	- H_2 production rate and yield, lag time
	- Metabolic pathways
	- Microbial community composition
	- High temperature (> 50°C) results in
	    - Treatment of pathogens, absence of most H_2 consuming bacteria
	    - Increased H_2 yields
	    - Increased energy demand
	H_2 production from cellulosic materials
	- Chemical and enzymatic reaction rates, stability of enzymes
	- Cellulase adsorption, hydrolysis efficiency
	- High temperature (> 50°C) results in simultaneous biomass hydrolysis
		[54,69,73,77,79,116, 118], for reviews, see [15,119]

pH	***H_2 production*** - H_2 production rate and yield, lag time - Metabolic pathways - Microbial community composition ***H_2 production from cellulosic materials*** - Production and release of cellulases - Hydrolysis efficiency	[14,120,121,122,123]
Alkalinity	***H_2 production*** - Low alkalinity leads to decrease in pH - H_2 content and production rate, lag time	[124,125]
Redox potential	***H_2 production*** - H_2 production rate and yield - Use of reducing agents increases production costs ***H_2 production from cellulosic materials*** - Rate and efficiency of cellulose utilization	for reviews, see [19,26]
H_2 partial pressure (pH_2)	***H_2 production*** - H_2 production rate and yield (temperature dependent) - Metabolic pathways - Redox potential of $H+/H_2$ and electron flow from ferredoxin to H_2	[96,118,125]
Carbon source	***H_2 production*** - H_2 production rate and yield, lag time - Metabolic pathways - Microbial community composition - High substrate concentration may cause substrate inhibition on H_2 production ***H_2 production from cellulosic materials*** - Crystallinity and available surface area affects hydrolysis rate - Substrate concentration affects cellulase production and hydrolysis efficiency	[31,34,59,92,126], for a review, see [14]
Hydraulic retention time (HRT)	***H_2 production*** - H_2 production rate and yield - Metabolic pathway - Biomass content and H_2 consuming microorganisms - Oxidation-reduction potential - Low HRT: wash out of granular bacterial biomass - High HRT: product inhibition due to accumulation of VFAs ***H_2 production from cellulosic materials*** - Substrate conversion: larger cellulose particles require longer HRT	[44,127,128,129]

Table 4. Hydrogen yields from hydrolysates in continuous mode bioreactors.

Pretreatment method	**Substrate**	**Reactor type**	**HRT (h)**	**H_2 yield on hexose ($mol\,mol^{-1}$)**	**Reference**
Hydrothermal	Wheat straw	CSTR	72	1.43	[36]
	Wheat straw	UASB	24	1.59	[35]
Diluted acid	Ground wheat starch	nr	24	0.97	[130]
	Oat straw	Biotrickling filter	12	2.00	[44]
Concentrated acid	Rice straw	CSTR	4	0.69	[95]
Bacterial	Starch	CSTR	12	2.38	[94]

nr: not reported, CSTR: continuous stirred tank reactor, UASB: upflow anaerobic sludge blanket reactor