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1 **Effects of operational parameters on dark fermentative hydrogen**
2 **production from biodegradable complex waste biomass**

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4 Anish Ghimire^{a,b,*}, Fabio Sposito^b, Luigi Frunzo^c, Eric Trably^d, Renaud Escudie^d, Francesco
5 Pirozzi^b, Piet N.L. Lens^e, Giovanni Esposito^a

6
7 ^aDepartment of Civil and Mechanical Engineering, University of Cassino and Southern
8 Lazio, via Di Biasio 43, 03043 Cassino (FR), Italy

9 ^bDepartment of Civil, Architectural and Environmental Engineering, University of Naples
10 Federico II, via Claudio 21, 80125 Naples, Italy

11 ^cDepartment of Mathematics and Applications Renato Caccioppoli, University of Naples
12 Federico II, via Cintia, Monte S. Angelo, I-80126 Naples, Italy

13 ^dINRA, UR0050 Laboratoire de Biotechnologie de l'Environnement, Avenue des Etangs, F-
14 11100 Narbonne, France

15 ^eUNESCO-IHE Institute for Water Education, Westvest 7, 2611 AX Delft, The Netherlands

16
17 * Corresponding author. Address: Department of Civil and Mechanical Engineering,
18 University of Cassino and Southern Lazio, via Di Biasio 43, 03043 Cassino (FR), Italy.
19 Tel. +39 0776 2994339 Fax +39 0776-2993939
20 *E-mail address:* a.ghimire@unicas.it, anishghimire@gmail.com (Anish Ghimire)

21 **Abstract**

22 This paper aimed to investigate the effect of the initial pH, combination of food to
23 microorganism ratio (F/M) and initial pH, substrate pre-treatment and different inoculum
24 sources on the dark fermentative biohydrogen (H₂) yields. Three model complex waste
25 biomasses (food waste, olive mill wastewater (OMWW) and rice straw) were used to assess
26 the effect of the aforementioned parameters. The effect of the initial pH between 4.5 - 7.0
27 was investigated in batch tests carried out with food waste. The highest H₂ yields were shown
28 at initial pH 4.5 (60.6 ± 9.0 mL H₂/g VS) and pH 5 (50.7 ± 0.8 mL H₂/g VS). Furthermore,
29 tests carried out with F/M ratios of 0.5, 1.0 and 1.5 at initial pH 5.0 and 6.5 revealed that a
30 lower F/M ratio (0.5 and 1.0) favored the H₂ production at an initial pH 5.0 compared to pH
31 6.5. Alkaline pre-treatment of raw rice straw using 4% and 8% NaOH at 55 °C for 24 hours,
32 increased the H₂ yield by 26 and 57 fold, respectively. In the dark fermentation of OMWW,
33 the H₂ yield was doubled when heat-shock pre-treated activated sludge was used as inoculum
34 in comparison to anaerobic sludge. Overall, this study shows that the application of different
35 operating parameters to maximize the H₂ yields strongly depends on the biodegradability of
36 the substrate.

37

38 **Keywords:** Biohydrogen; dark fermentation; waste biomass; biofuels; waste valorization

39

40 **Highlights**

- 41 • Combination of initial pH and F/M ratio affects H₂ yields from DF of food waste.
- 42 • Alkaline pre-treatment enhances the dark fermentative conversion of rice straw.
- 43 • Inoculum source and pre-treatment conditions influence H₂ yields in DF of OMWW.
- 44 • The selection of optimal operating parameters depends on substrate biodegradability.

45

46

47 **1 Introduction**

48 Dark fermentation (DF) of organic waste is one of the promising technologies for
49 biohydrogen (H₂) production. The DF processes are usually preferred over other light
50 dependent, photofermentation or biophotolysis processes because of the high bioreactor
51 productivities and the potential to utilize a wide range of organic wastes as feedstock
52 (Hallenbeck et al., 2009; Urbaniec and Bakker, 2015). In addition, the associated production
53 of organic acids and alcohols, among others, can be either used in sidestream processes like
54 anaerobic digestion for methane or photofermentative H₂ production for energy recovery, or
55 can be used for the production of platform molecules (Bastidas-Oyanedel et al., 2015; Sarma
56 et al., 2015).

57

58 Waste biomass is abundant and can sustain DF processes in scaled-up applications. An easily
59 degradable food waste (the organic fraction of municipal solid waste (OFMSW)), more
60 slowly degradable agricultural residues (i.e. rice straw) as well as agro-industrial waste such
61 as olive mill wastewaters (OMWW) can serve as sustainable feedstock sources for dark
62 fermentative H₂ production (Guo et al., 2010; Kapdan and Kargi, 2006; Ntaikou et al., 2010;
63 Show et al., 2012). A major bottleneck in the utilization of these low cost waste biomasses is
64 the rather low H₂ yields observed in the DF processes (Ghimire et al., 2015a; Urbaniec and
65 Bakker, 2015). Nevertheless, H₂ yields and process kinetics can be enhanced by optimizing
66 operating parameters, such as pre-treatment of inocula, food to microorganisms (F/M) ratio
67 (also substrate to inoculum ratio), pre-treatment of substrates, culture temperature and pH (De
68 Gioannis et al., 2013; Guo et al., 2010; Ntaikou et al., 2010; Wang and Wan, 2009). During
69 recent years, extensive experimental research has been devoted to the establish the optimal
70 operational conditions for maximizing H₂ production, with a special focus on operational pH,

71 temperature and substrate utilization (De Gioannis et al., 2013; Ghimire et al., 2015a; Wong
72 et al., 2014).

73

74 A wide range of optimal pH values have been reported for different substrates to enhance H₂
75 yields: an initial pH of 6.5 for food waste (Cappai et al., 2014), initial pH of 8.0 for food
76 waste (Kim et al., 2011), a controlled pH of 7.0 for vegetable kitchen waste (Lee et al., 2008),
77 an initial pH of 6.5 for rice straw (Chen et al., 2012), an initial pH of 6.0 for cheese whey (De
78 Gioannis et al., 2014) and an initial pH of 4.5 for sucrose and starch (Khanal et al., 2004).

79 This considerable variability in culture pH is mainly due to differences in temperature,
80 substrate type and concentration (F/M ratio), inoculum types and their pre-treatment methods.

81

82 H₂ yields in DF of organic waste are strongly affected by the operational temperature as it can
83 influence the rate of hydrolysis and the production of volatile fatty acids (VFAs) and thus the
84 final pH of the fermentation (De Gioannis et al., 2013; Ghimire et al., 2015a). A thermophilic
85 temperature has been reported to favor the dark fermentative H₂ production (Shin et al., 2004;
86 Valdez-vazquez et al., 2005). Likewise, the physico-chemical characteristics of the
87 substrates, and most importantly the biodegradability or bioavailability (can also be defined
88 as the fraction of easily accessible carbohydrates for fermentative conversion) crucially
89 affects the H₂ production (Monlau et al., 2013a). Therefore, several studies have established a
90 strong correlation between H₂ yields and the initial carbohydrate fraction (soluble sugars in
91 some cases) present in the substrates (Alibardi and Cossu, 2015; Guo et al., 2013; Monlau et
92 al., 2012).

93

94 In this context, alkaline pre-treatment methods have been popularly adopted for the
95 saccharification of lignocellulosic biomass (plant stalks, rice and wheat straw), which could

96 enhance the production of H₂ in DF and CH₄ in DF coupled to anaerobic digestion,
97 respectively and could thus give economic credentials (Monlau et al., 2015, 2013c; Sambusiti
98 et al., 2013). Alkaline pre-treatment of lignocellulosic biomass has been reported to be
99 carried out at different concentrations of alkaline agents (2 - 12% NaOH, weight basis),
100 temperature (40 - 190 °C) and treatment period (30 minutes - 24 hours), with varying level of
101 effectiveness in terms of increase in biogas yields (H₂ and CH₄) with consequent higher net
102 energy recovery and economic return (Monlau et al., 2015, 2013b; Sambusiti et al., 2013).
103 However, alkaline agents (i.e. Na⁺ from NaOH) might exert inhibitory effects on dark
104 fermentative microbial communities (Kim et al., 2009). Consequently, an investigation of
105 selected alkaline pre-treatment conditions for a particular substrate type becomes vital to
106 study the conditions that enhance the H₂ production.

107

108 H₂ production from organic waste is influenced by the presence of an effective hydrolyzing,
109 H₂ producing microbial community, which depends on the inoculum source and inoculum
110 pre-treatment method (Abreu et al., 2009; Bellucci et al., 2015; Chen et al., 2012; Pakarinen
111 et al., 2008). Abreu et al. (2009) and Chen et al. (2012) showed that the H₂ yields mainly
112 depend on the inoculum sources. However, the response of fermentative microorganisms
113 towards the presence of inhibiting substances present in a substrate can influence the DF
114 process. In a recent study, Bellucci et al. (2015) reported a varying response of fermentative
115 microbial communities for H₂ production, when the inhibitor 5-hydroxymethylfurfural
116 (HMF) was added. This was linked to the difference in inoculum pre-treatment methods
117 applied. Likewise, the presence of polyphenolic compounds in substrates such as OMWW
118 can exhibit inhibitory effects on fermentative microbial communities and H₂ yields (Hamdi,
119 1992; Ntaikou et al., 2009). Subsequently, investigating the effect of the inoculum source on

120 H₂ production performance from substrates like OMWW is fundamental to reach an optimum
121 in H₂ production.

122

123 Despite some studies attempted to establish the optimal operational conditions of initial pH,
124 F/M ratio, alkaline pre-treatment of substrate and inoculum selection, dissimilarities in H₂
125 production exist due to the differences between substrate types and experimental conditions.
126 Therefore, it becomes essential to investigate the optimum initial pH for food waste under
127 thermophilic DF conditions. So far, only few studies have considered the combined effects of
128 F/M ratio and initial pH on thermophilic DF of food waste (Ginkel et al., 2001; Pan et al.,
129 2008). Ginkel et al., (2001) revealed a profound impact of the concentration of substrate and
130 pH on the H₂ yields in sucrose DF of, with an optimum pH and substrate concentration at pH
131 of 5.5 and 7.5 g COD/L, respectively. In other study, Pan et al. (2008) established a F/M ratio
132 of 6.0 as optimum for thermophilic DF of food waste, without the consideration of initial pH.
133 Similarly, past studies on pre-treatment of substrates seemed more focused on maximizing
134 the methane yields in anaerobic digestion by adopting higher concentrations of alkaline
135 agents and treatment temperature (Monlau et al., 2013a). Therefore, optimum conditions of
136 alkaline pre-treatment for dark fermentative H₂ production need to be investigated for
137 lignocellulosic agricultural residues such as rice straw. Finally, different inoculum sources
138 can be explored to study the effect on H₂ production from a typical poorly biodegradable
139 feedstock such as OMWW, which contains polyphenolic compounds (Ntaikou et al., 2009).

140

141 The present study aims to investigate the effects of i) the initial pH and combined pH and
142 F/M ratio on food waste, ii) alkaline substrate pre-treatment on dark fermentative H₂
143 production from rice straw and iii) the effect of inoculum source and pre-treatment on H₂
144 production from OMWW. Cumulative H₂ production, H₂ yields, H₂ production rates, lag

145 phase and accumulation of DF metabolites (mainly organic acids and ethanol) were used to
146 evaluate the efficiency of these various strategies to improve the H₂ production performance
147 from these complex organic wastes.

148

149 **2 Materials and methods**

150 **2.1 Inoculum**

151 Two types of inoculum, i.e. anaerobic digested sludge (ADS) and waste activated sludge
152 (WAS) were used in the experiments. ADS was collected from the effluent of an anaerobic
153 digestion plant of a dairy farm located in Capaccio (Salerno, Italy). The plant features include
154 a 100 m³ CSTR operating at a hydraulic retention time of 24 days and operating within a pH
155 and temperature range of 7.4 - 7.5 and 52 - 56 °C, respectively. The plant is continuously fed
156 with buffalo manure, cheese whey of buffalo milk and sludge from an industrial wastewater
157 treatment plant. WAS was collected from a secondary clarifier unit at the Nola Municipal
158 Wastewater Treatment Plant located in Naples (Campania, Italy). The characteristics of the
159 ADS and WAS before pre-treatment are presented in Table 1. The inocula were stored at 4 °C
160 until used. The WAS and ADS underwent a heat shock treatment (HST) at 105 °C for 1.5 and
161 4 hours, respectively, in order to enrich spore forming *Clostridium* sp. and inhibit
162 methanogens (Ghimire et al., 2015b). WAS had a shorter time for HST than ADS because it
163 was obtained from an aerobic activated sludge process.

164

165 **2.2 Preparation of feedstock**

166 Three types of waste as reference models of complex waste biomass with different
167 characteristic biodegradability, were used in this study: i) food waste, representative of
168 moderately biodegradable organic waste was selected to study the effect of initial pH and
169 substrate concentration on H₂ yields, ii) rice straw as a representative of slowly degrading

170 lignocellulosic agricultural residues was used to study the technical feasibility of substrate
171 pre-treatment on biohydrogen production and iii) OMWW was used to study the effect of the
172 inoculum type and its adaptation to toxicants, as OMWW contains phenolic compounds and
173 long chain fatty acid that can affect microbial growth (Hamdi, 1992; Ntaikou et al., 2009).
174 Food waste was a mixed waste with a composition similar to the one reported by
175 VALORGAS (2010) for European countries as (% by weight): fruit and vegetables: 72%,
176 cooked pasta and rice: 10%, bread and bakery: 5%, dairy products (cheese): 2%, meat and
177 fish: 8% and snacks (biscuits): 3%. To prepare the food waste, food was bought fresh from
178 municipal markets in Naples (Italy), shredded with a blender (120 W Black and Decker,
179 Kitchen Blender) for 5 minutes without adding water and immediately stored at frozen
180 conditions (-20 °C) to avoid acidification. The rice straw was harvested from rice fields in
181 Pavia (Italy) in 2012 and stored inside an airtight plastic bag at room temperature. Rice straw
182 was reduced with the help of general paper scissors to a particle size of less than 2 mm
183 (sieved with sieve size of 2mm by 2mm). OMWW was collected from a pressure olive mill
184 of Frascati area (Lazio, Italy) in autumn 2013 and was stored at < 4 °C until use. The
185 characteristics of the feedstocks are presented in Table 1.

186

187 **2.3 Experimental set-up**

188 Batch tests were carried out in one-liter borosilicate glass bottles (Simax, Czech Republic)
189 maintained in thermophilic conditions ($55 \pm 2^\circ\text{C}$) with a thermostat in a water bath. The
190 operating reactor volume in all experiments was 600 mL. The batch reactors were sealed with
191 airtight caps having ports for sampling soluble metabolites and gas. The tests were carried out
192 in duplicates with 30 reactors in total. The different sets of experiments were carried out to
193 study the effect of the different operational parameters using the three selected model
194 substrates (Table 2).

195 **2.3.1 Effect of initial pH and F/M ratios on H₂ yield**

196 The effect of initial pH and F/M ratio on biohydrogen production was studied with food
197 waste and pretreated heat treated ADS as seed inoculum. The effect of the initial pH (4.5, 5,
198 5.5, 6.0, 6.5 and 7.0) was studied at a F/M ratio 0.5 and under thermophilic conditions (55 ± 2
199 °C). Another set of experiments was performed at F/M ratios 0.5, 1.0 and 1.5 with the two
200 initial pH values of 5.0 and 6.5. The F/M ratios and two initial pH values were selected due to
201 the fact that they are less affected by acidification at higher F/M ratios and the culture pH in
202 the tests was not buffered with external alkalinity source. In addition, pH 6.5 was previously
203 reported as optimal for food waste by Cappai et al. (2014), and thus considered for
204 investigation in this study. The F/M ratios 0.5, 1.0 and 1.5 were obtained by adding 10 g, 18 g
205 and 27 g food waste respectively, with a 190 g inoculum required to obtain the aimed F/M
206 ratio. The final volume of the mixture was made up to 600 mL by adding distilled water. The
207 initial pH was adjusted once, initially with 1 M HCl and 1 M NaOH prior to the start of the
208 tests.

209

210 **2.3.2 Effect of alkaline substrate pre-treatment on H₂ yield**

211 Direct conversion of lignocellulosic biomass to biohydrogen is often limited due to their low
212 biodegradability (Monlau et al., 2012; Pan et al., 2010). Biological hydrolysis is one of the
213 limiting factors in DF. The evaluation of the effect of alkaline pre-treatment on H₂ yields was
214 performed on rice straw. This study investigated an alkaline pre-treatment with 4 % NaOH (4
215 g/100g TS) and 8 % NaOH (8 g/100g TS) at a solid liquid ratio of 1:5 (w/v). This mixture
216 was kept at $55 (\pm 2)$ °C for 24 hours in a one-liter borosilicate glass bottle (Simax, Czech
217 Republic). The results were compared with untreated rice straw at thermophilic DF using 200
218 g of heat-treated WAS as inoculum. The concentration of rice straw was 45 gTS/L and the

219 initial pH was adjusted to 6.5 during the batch tests that gave the optimal dark fermentative
220 H₂ performance for rice straw as reported by Chen et al. (2012).

221 **2.3.3 Effect of inoculum sources on H₂ yield**

222 Heat shocked WAS and ADS was used as inoculum in a DF of OMWW carried out in batch
223 tests and operated under thermophilic conditions ($55 \pm 2^\circ\text{C}$). The F/M ratio was fixed at
224 approximately 1 gVS substrate/gVS inoculum in all sets of batch tests using 200 g of
225 OMWW and a respective volume of ADS and WAS. The initial pH was adjusted to pH 6.0 in
226 all experiments.

227

228 **2.4 Analytical methods**

229 Hydrogen was quantified with a gas chromatograph (VARIAN STAR 3400, USA) equipped
230 with a ShinCarbon ST 80/100 column and a thermal conductivity detector. Argon was used
231 as carrier gas with a front and rear end pressure of 20 psi. The duration of analysis was 14
232 minutes. The gas volume was measured with a volumetric displacement method. The biogas
233 was passed through acidic water (1.5 % HCl) and the volume was quantified by water
234 displacement (Ghimire et al., 2015c). The volume of hydrogen was calculated from the gas
235 composition. Fermentation end products (lactic, acetic, propionic and butyric acids) were
236 quantified by High Pressure Liquid Chromatography (HPLC) (Chromatography Oven LC 25
237 Model, Dionex, USA) equipped with a Synergi 4u Hydro RP 80A (size 250×4.60mm)
238 column and an UV detector (AD25 Model, Dionex, USA). Gradient elution consisted of 20%
239 methanol, 10% acetonitrile in 5 mM H₂SO₄ pumped at a rate of 0.9 mL/min by using a
240 gradient pump (GP 50 Model, Dionex, USA). The elution time was 18.5 minutes. Ethanol
241 and caproic acid were determined with an Aminex HPX-87H column (300 mm on 7,8 mm,
242 Bio-rad), using 5 mM H₂SO₄ as an eluent at a flow rate of 0.4 mL/min. pH was measured
243 with a pH meter (WTW, inolab, pH level 2). The COD of the food waste was measured as

244 reported by Noguerol-Arias et al. (2012). The total lipid content was measured by the Bligh
245 and Dyer chloroform/methanol total lipid extraction method (Bligh and Dyer, 1959). TS and
246 VS concentrations were determined by the Method 2540 (Part 2000), alkalinity by titration
247 (Method 2320, Part 2000) and TKN by macro-Kjeldahl (Method 4500-N_{org}, Part 4000) as
248 described in the Standard Methods (APHA, 2005).

249

250 **2.5 Measurements and data analysis**

251 The biogas accumulated in the reactors was measured daily, except at the starting period of
252 the experiments, i.e. 1-3 days, where it was measured twice a day, until the H₂ production
253 completely ceased. The biogas volumes were normalized at 0 °C and 1 atm (NmL) and
254 reported as a daily average. The average values were considered for the evaluations, while the
255 data range based on the duplicate samples is provided and indicated by “±”. H₂ yields were
256 calculated by dividing the final cumulative recovery of H₂ by the amount of VS added at the
257 start of the experiment.

258

259 De Gioannis et al. (2013) defined a parameter “t₉₅” as the time required to achieve 95% of the
260 maximum H₂ yield. This parameter was used to compare the kinetics associated to the
261 different batch tests, and to evaluate the effect of the experimental conditions.

$$t_{95} = \frac{H_o}{R \cdot e} (1 - \ln(-\ln 0.95)) + \lambda \quad (1)$$

$$H(t) = H_o \cdot \exp \left\{ -\exp \left[\frac{R \cdot e}{H_o} \right] (\lambda - t) + 1 \right\} \quad (2)$$

262 Equation (1) corresponds to a rearranged form of the modified Gompertz equation (2), that
263 has been widely used to model biohydrogen production kinetics (Gadhamshetty et al., 2010;
264 Wang and Wan, 2009). This empirical formula gives biohydrogen production trends and
265 includes five major parameters: i) cumulative biohydrogen production (or potential) (H_o,

266 mL/g VS), ii) biohydrogen production rate (R, mL/h), iii) e is 2.71828, iv) lag time (λ , hours)
267 and v) total cultivation time (t, hours). The cumulative biohydrogen production is a non-
268 linear curve and in the present study, the parameters H_0 , R and λ were estimated using the
269 Curve Fitting Toolbox in MATLAB[®] (Version MATLAB R2012b, Curve Fitting Toolbox
270 3.3) with an associated 95% confidence limit. The total cumulative production, hydrogen
271 production rates and lag phase time were used as parameters to compare the characteristics of
272 the biohydrogen production systems. R software (OSX version 3.1.3) with the package
273 Rcmdr (OSX version 2.1.7) was used for the statistical analysis of data obtained from the
274 experiments. The p value was set at 0.05 and the significance of the results tested with p
275 values: * < 0.05; ** < 0.01; *** < 0.001; while not significant results were with $p > 0.05$.

276

277 **3. Results**

278 **3.1 Effect of the initial pH and combined effect of F/M ratio and pH on H₂ yields**

279 The H₂ yields and the time required to achieve 95% of the maximum H₂ yield were plotted
280 against the initial pH values (Fig. 1). The H₂ yields showed a decreasing trend to the
281 increasing pH. Fig. 1 confirmed that H₂ production was favoured at the acidic pH range, i.e.
282 at initial pH 4.5 and 5.0 with H₂ yields of 60.6 (\pm 9.0) and 50.7 (\pm 1.0) N mL H₂/g VS,
283 respectively. This result is in agreement with the study reported by Khanal et al. (2004). The
284 fermentative H₂ production patterns at the various pH values investigated are described by a
285 modified Gompertz equation, as presented in Table 3 (Modeled plot is provided in
286 Supplementary information S1). The different initial pH values in the tests were characterized
287 by the differences shown in cumulative H₂ production, H₂ production rates and lag phase
288 (Table 3). H₂ production rates (R, mL/h) were high at initial pH 7.0, however, higher rates
289 were not co-related with higher H₂ yields (Fig. 1 and Table 3).

290

291 Unsurprisingly, the lag phase decreased when increasing the initial pH, which represents the
292 time required for spore forming H₂ producers present in heat-treated ADS to germinate or
293 adapt a sudden change of their environment (Ferchichi et al., 2005; Kim et al., 2011). Fig. 1
294 shows the time required to achieve 95% of the maximum H₂ yield decreased by increasing
295 the initial pH, while the rate of H₂ production was higher at initial pH 7.0 (Table 3). H₂
296 production started faster at higher pH and lasted for a short time while it continued for longer
297 time during the tests at lower pH. Thus, a decreasing lag phase did not correspond to an
298 increase in H₂ yields. This can be explained by the methanogenic activities which started at
299 higher initial pH, that was confirmed by the presence of methane in the biogas produced
300 when H₂ production ceased completely. The final pH at the end of the tests was mainly lower
301 than the initial pH (Table 3), which is mainly due to the production of VFAs (Table 3). As
302 exception, the final pH in the batch tests with initial pH 4.5 was higher than the initial pH
303 (Table 3), which could be due to the higher alkalinity of the inoculum (ADS) and the lower
304 substrate concentration (F/M 0.5) used to avoid the use of chemical buffer. The final pH in all
305 the tests were lower than 5.5, except for tests with initial pH 7.0 where the final pH was 6.6.
306 This can be due to the higher alkalinity (buffering capacity) of the ADS inoculum (Table 1).
307
308 The concentrations of the main accumulated metabolites at the end of the tests are
309 summarised in Table 3. Results confirm that different fermentation pathways occurred. The
310 presence of propionate and ethanol generally does not indicate H₂ favorable pathways (Kim
311 et al., 2011). The concentration of ethanol was comparatively higher in the tests with initial
312 pH range 6.0 – 7.0, that could be linked to the low H₂ yields. In particular, the butyric to
313 acetic acid ratio (B/A, mM:mM) co-related with the H₂ yields (Fig. 2). This observation is
314 consistent with a study by Kim et al. (2006), which reported a higher correlation between B/A
315 ratios (1.6 – 9.3) and H₂ yields. However, this ratio might not always give a good indication

316 of high H₂ production. Guo et al. (2013) reported that the homoacetogenic activities can
317 influence the concentration of end-metabolites due to acetate production from H₂ and CO₂.
318 The presence of acetate in higher concentrations between pH 5.5 – 7.0 might indicate the
319 prevalence of an homoacetogenic activity responsible of lower H₂ yields.

320

321 The results of the batch tests carried out at F/M ratios 0.5, 1.0 and 1.5 at two initial pH values
322 (5.0 and 6.5) are presented in Table 4. Table 4 shows the major metabolites accumulated at
323 the end of the tests. At the initial pH 5.0 and F/M ratios of 0.5, 1.0 and 1.5, H₂ yields were
324 50.7 (± 0.8), 60.3 (± 5.0) and 49.3 (± 12.2) mL H₂/g VS, respectively. Likewise, in tests
325 carried out with an initial pH 6.5, respective H₂ yields of 28.2 (± 4.2), 43.2 (± 2.0) and 54.1
326 (± 4.4) mL H₂/g VS were obtained. An ANOVA analysis confirmed the significance of
327 difference in H₂ yields at pH 5.0 and 6.5 for an F/M ratio of 0.5 (*p* value <0.05). However, it
328 was not significant for F/M ratios 1.0 and 1.5 at both initial pH values tested. Likewise, at
329 initial pH 5.0, the differences in H₂ yields were not significant for all the three tested F/M
330 ratios. Interestingly, the differences in H₂ yields were significant (*p* value <0.05) at an initial
331 pH of 6.5 for F/M ratios 0.5 and 1.5. This implies a combined influence of the F/M ratios and
332 initial pH on dark fermentative H₂ production. The result also suggests that the comparable
333 H₂ yields can be achieved through a combination of pH and F/M ratios by maximizing the
334 utilization of substrates.

335

336 The different metabolites yields measured at the end of the batch tests explain the differences
337 in H₂ yields (Table 4). The presence of different metabolites suggests a typical mixed type
338 fermentation that can occur in complex substrates like food waste. Acetate yields were higher
339 at initial pH 6.5 compared to pH 5.0, which was also confirmed in the tests carried out earlier
340 at different initial pH (Table 3). Similarly, higher ethanol yields were obtained at increasing

341 F/M ratios and initial pH. High levels of butyrate yield at pH 6.5 and F/M ratios 1.0 and 1.5
342 can be associated to higher H₂ yields obtained in respective tests, as the production of
343 butyrate is generally co-related to H₂ production (Kim et al., 2011).

344

345 **3.2 Effect of alkaline substrate pre-treatment on H₂ yields**

346 Fig. 3 shows the effects of alkaline substrate pre-treatment on biohydrogen production. The
347 results illustrate that biohydrogen production can be significantly improved with alkaline pre-
348 treatment of rice straw. As expected, the alkaline pre-treatment enhanced the saccharification
349 of sugars from rice straw, which increased along with the concentration of NaOH. The COD
350 values of hydrolysate after pre-treatment with 4% and 8% NaOH were 7.3 (\pm 0.8) and 8.3 (\pm
351 0.7) g/L respectively in comparison to the untreated rice straw with 3.8 (\pm 0.1) g/L soluble
352 COD (determined with solid liquid ratio of 1:5). The results of end-product accumulation
353 (Table 5) show that higher H₂ yields corresponded to higher B/A ratios (mM:mM),
354 irrespective of the concentration of acids accumulated at the end of the tests.

355

356 **3.3 Effect of inoculum sources on H₂ yields**

357 The cumulative H₂ yields and accumulation of end metabolites during the application of two
358 heat treated inoculum sources on biohydrogen production from OMWW is depicted in Fig. 4
359 and Table 6, respectively. The differences observed when using two inoculum types, i.e. ADS
360 and WAS, at thermophilic temperature gave an indication of the level of inhibition of the
361 polyphenols present in the OMWW on the microorganisms (Hamdi, 1992; Paraskeva and
362 Diamadopoulos, 2006). The initial lag phase observed in Fig. 4 can give evidence for the
363 adaptation of H₂ producing fermentative microbial communities to phenolic compounds
364 present in OMWW. The maximum H₂ yield from OMWW with WAS was almost 2 fold
365 higher than with ADS. In addition, WAS sludge required less heat-shock pre-treatment time

366 to inhibit hydrogen consuming methanogens and showed a shorter lag phase (Fig. 4, Table 6).
367 This shows that heat-shocked WAS is an appropriate inoculum for DF of OMWW for higher
368 H₂ recovery.

369

370 The lower H₂ yield obtained from OMWW in tests inoculated with ADS is further supported
371 by the analysis of the metabolic pathways (Table 6), which showed an accumulation of lactic
372 acid. Metabolic pathways leading to lactic acid are not favorable to H₂ production (Hawkes et
373 al., 2007), which explains the lower H₂ yields observed in the batch tests inoculated with
374 ADS. Likewise, the higher levels of acetate in the tests carried out with WAS than ADS can
375 explain the higher H₂ yields from OMWW, as acetate pathways generally yields to more H₂
376 per mole of glucose than the butyrate pathways (Hawkes et al., 2007).

377

378 **4. Discussion**

379 **4.1 Effect of the pH and F/M ratio on H₂ yield**

380 This study showed that higher H₂ yields can be achieved from easily biodegradable organic
381 waste like food waste, when compared to other complex substrates such as rice straw (Table
382 7). This is mainly a result of the high fraction of easily degradable carbohydrates contained in
383 food waste, as already suggested by Guo et al. (2013). The combination of initial pH and
384 substrate concentration is important to avoid inhibition of H₂ producers through elevated
385 VFA accumulation and consequent pH depletion, and high hydrogen partial pressure (Ginkel
386 et al., 2001). This is likely the case of substrates like food waste which generally show faster
387 hydrolysis kinetics compared to lignocellulosic biomass such as rice straw (Table 7), that
388 requires higher optimal substrate concentrations or F/M ratios compared to food waste.

389

390 Table 7 compares the results of the H₂ yields observed in this study with literature data
391 reported under similar conditions. The highest H₂ yields observed at initial pH 4.5 and 5.0
392 (60.6 ± 9 and 50.7 ± 1 mL H₂/ g VS food waste, respectively) in this study were in contrast
393 with Cappai et al. (2014), who obtained the highest H₂ yield (56.2 mL H₂/ g VS food waste)
394 at pH 6.5. This difference in optimum initial pH might be due to the higher substrate
395 concentrations used by Cappai et al. (2014) (Table 7). Furthermore, two possible
396 explanations can be given for the relationship between initial pH (4.5 and 5.0) and the higher
397 H₂ production: (i) a selection of hydrogen producers at pH range (4.5 – 5.0) and (ii) an
398 inhibition of H₂ consuming methanogens. In addition, the differences in metabolic products
399 accumulating at different initial pH ranges might support the growth of different microbial
400 communities which can influence the H₂ production as reported in the studies from Fang
401 and Liu (2002) and Lee et al. (2008). Khanal et al. (2004) reported that a microbial shift to
402 solventogenesis did not occur at a pH range 4.5 – 6.5, which provides further evidence of the
403 importance of the initial microbial community and pH to reach higher H₂ yields. In addition,
404 native microbial organisms present in the food waste might also influence the DF process in
405 real conditions (waste type and storing conditions). In this study, the storage of food waste at
406 freezing conditions might have impacted native microorganisms. Nevertheless, the
407 comparison of the results between the tests operated at different initial pH remains unaffected
408 as uniform substrates were used.

409

410 At lower F/M ratios (0.5 and 1.0), an initial of pH 5.0 favored the H₂ production whereas it
411 was the inverse at a F/M ratio 1.5 and initial pH 6.5. At the initial of pH 5.0 and F/M 1.5, a
412 lower H₂ yield was observed, which might be due to the shock load on the microbial systems.
413 This was also confirmed in the study of Ginkel et al. (2001), who reported an inhibition of H₂
414 production at higher substrate loading rates due to shock loads. The conversion of substrates

415 to metabolic products at pH 5.0 and F/M 1.5 is lower than at F/M ratios 0.5 and 1.0, which
416 can be due to an inhibition of the substrate conversion. In addition, a low final pH (4.5 ± 0.1)
417 at the end of the test at pH 5.0 and F/M 1.5 (Table 4) suggests that H₂ production might be
418 inhibited due to a 'load shock'. This can be supported by the time required to achieve 95% of
419 the maximum H₂ yield ($t_{95} = 47$ days) (Table 4). Pan et al. (2008) reported that a F/M ratio of
420 6.0 as appropriate for thermophilic (50 ± 2 °C) fermentation of food waste (Table 7).
421 However, the initial pH in their study varied from 6.2 to 6.7. Therefore, in the DF systems
422 where initial pH is not buffered, H₂ production is a combined function of suitable F/M ratio
423 and initial pH. Likewise, an optimal operational pH range could be maintained through
424 subsequent substrate feeding strategies which can guarantee higher H₂ production and avoid
425 the H₂ consuming activities i.e. methanogens and homoacetogens.

426

427 **4.2 Effect of alkaline substrate pre-treatment on H₂ yield**

428 The alkaline pre-treatment method applied in this study aimed at improving hydrolysis and
429 solubilization of the organic matter that limit the dark fermentative substrate conversion
430 (Monlau et al., 2015, 2013b). However, the level of effectiveness of the different pre-
431 treatment methods depends on the nature of the substrate (Ariunbaatar et al., 2014; Carlsson
432 et al., 2012). In the study of Monlau et al. (2013c), H₂ yields from sunflower stalks increased
433 from 2.3 (± 0.9) to 4.4 (± 2.6) mL H₂/g VS, while in our study an increase from 0.3 (± 0.1) to
434 6.6 (± 0.1) mL H₂/g VS from rice straw as the substrate was achieved under similar
435 conditions of thermo-alkaline pre-treatment (Fig. 3 and Table 7). Meanwhile, H₂ yields
436 further increased to 15.7 (± 1.0) mL H₂/g VS when 8 % w/w NaOH was applied (Fig. 3). This
437 H₂ yield is lower than the value reported by Chen et al. (2012) with untreated rice straw, i.e.
438 24.8 mL/g TS at a substrate concentration of 90 g TS/L, whereas, it is 2.2 fold higher when
439 the substrate concentration was 30 g TS/L (i.e. 7.1 mL H₂/g TS). This disagreement might be

440 due to physico-chemical properties of the lignocellulosic substrates, such as particle sizes,
441 soluble carbohydrates content and/or substrate concentration (Monlau et al., 2013a). Chen et
442 al. (2012) reported an increasing trend of H₂ yields, when the particle size of rice straw
443 decreased from 10 mm to < 0.297 mm. In their study, a maximum H₂ yield was obtained with
444 a particle size of < 0.297 mm (6.4 mL H₂/g TS) at a substrate concentration of 30 g TS/L.

445

446 The effects of the chemical agents applied (NaOH) and or by-products formed (furfural,
447 phenols) during the pre-treatment process and the response on the dark fermentative
448 microbial community should be taken into consideration while selecting appropriate pre-
449 treatment method. Kim et al. (2009) reported a decrease in H₂ yields when the Na⁺
450 concentration in a continuous DF reactor gradually increased from 0.27 to 21.00 g Na⁺/L
451 while the acclimatized fermentative community maintained their activity up to 6.00 g Na⁺/L.
452 Nonetheless, in this study, the H₂ yields increased when 8 % w/w NaOH was applied
453 compared to 4 % w/w NaOH (Fig. 3). Moreover, under similar pre-treatment conditions, 12
454 % w/w NaOH (i.e. 5.40 g Na⁺/L) might either enhance the H₂ yields or exert effect on
455 fermentative microbial community, depending on the inocula type and adaptation to Na⁺
456 concentration. However, the application of pre-treatment methods should be based on the
457 substrate type (biodegradability or bioavailability of easily fermentable carbohydrates), their
458 practicability and economy viability.

459

460 **4.3 Effect of inocula on H₂ yield**

461 The application of two different inoculum types for the DF of OMWW showed differences in
462 response of ADS and WAS in terms of dark fermentative conversion to H₂ and other
463 metabolites (Fig. 4 and Table 6). Comparatively, WAS exhibited better performances in
464 terms of H₂ production as shown by the H₂ production yields and kinetics in Table 6. The

465 difference in H₂ yields might be a result of the effect of polyphenolic substances present in
466 OMWW (total phenols in Table 1) on the fermentative communities present in ADS and
467 WAS (Hamdi, 1992; Ntaikou et al., 2009). Ntaikou et al. (2009) used diluted OMWW to
468 avoid growth inhibition, whereas, Hamdi (1992) observed an inhibition mainly on
469 methanogens. Nonetheless, the difference in response of the two inocula could be also due to
470 the difference in heat shock treatment time applied during the HST. ADS required a longer
471 HST time to inhibit the activity of methanogens (Ghimire et al., 2015b) compared to WAS
472 which has an aerobic origin. Therefore, the treatment time could have impacted the microbial
473 communities that could contribute to fermentative H₂ production.

474

475 The use of WAS as better inoculum is supported by the studies of Chen et al. (2012) and Kim
476 et al. (2011). Chen et al. (2012) achieved higher H₂ yields with a sludge originated from a
477 municipal wastewater treatment plant when compared with other inoculum sources like cow
478 dung, compost and paper mill sludge. The group attributed higher H₂ yields to the presence of
479 a potential hydrolytic and fermentative bacterial microbial community. Kim et al. (2011)
480 hypothesized that such increase in H₂ yields from sewage sludge addition was due to the
481 presence of iron (Fe), calcium (Ca) and phosphorous (P) at much higher concentrations (no
482 information on speciation was given). Further research on the nutrient and trace metal
483 content in inocula and how these affect the DF rates is thus required.

484

485 The selection and application of various optimum operational parameters depends highly on
486 the type of substrate, i.e. mainly its biodegradability. However, the improvement of dark
487 fermentative H₂ production should bear the cost of application of different optimal
488 operational parameters in terms of net energy and economy gain. It should be taken into
489 consideration that DF of waste biomass is not a complete conversion of organic waste, i.e.

490 organic acids and alcohols accumulate in the effluent, for which a subsequent treatment needs
491 to be provided. Valorization of these by-products can support the costs associated with the
492 optimization of the DF process. Several studies have suggested the integration of DF with
493 processes such as photofermentation (H_2), bioelectrochemical systems (H_2) and anaerobic
494 digestion (CH_4) for further energy recovery and production of platform molecules of
495 economic interest, such as biopolymers (Bastidas-Oyanedel et al., 2015; ElMekawy et al.,
496 2014; Ghimire et al., 2015c; Xia et al., 2013).

497

498 **5. Conclusion**

499 This study aimed to investigate the optimal operational parameters in the thermophilic DF of
500 three types of complex wastes biomass with varying biodegradability, i.e. food waste, rice
501 straw and OMWW. The DF applied to food waste was favored in the acidic pH range (4.5-
502 5.0), though an appropriate substrate concentration that must be considered while selecting an
503 acidic pH range. F/M ratios of 0.5 and 1.0 at an initial pH of 5.0 gave, respectively, 1.8 and
504 1.4 folds higher H_2 yields than at initial pH 6.5. Likewise, F/M ratios and pH can be
505 optimized to achieve higher substrate utilization and H_2 yields. During the tests, higher B/A
506 ratios (mM:mM) were associated with higher H_2 yields, a B/A ratio equivalent to 1.5 was
507 related to the optimal H_2 yield. Similarly, pre-treatment of rice straw with 4% NaOH and 8%
508 NaOH at 55 °C for 24 hours increased the H_2 yield by 26 and 57 fold, respectively.
509 Furthermore, WAS showed adaptability to OMWW containing phenols and gave a nearly 2
510 fold higher H_2 yield when compared to ADS. In conclusion, the selection and application of
511 optimal operational parameters for the optimization of H_2 production rely mainly on the
512 substrate biodegradability. Therefore, these parameters should be optimized for each
513 particular type of substrate prior to further application in scaled-up DF systems.

514

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522

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700 **Figure Captions**

701 Fig. 1 Effect of initial pH on H₂ yield and time required for H₂ production to achieve 95% of
702 the maximum yield during the DF of food waste at F/M ratio 0.5 and thermophilic
703 temperature (55±1 °C) using ADS

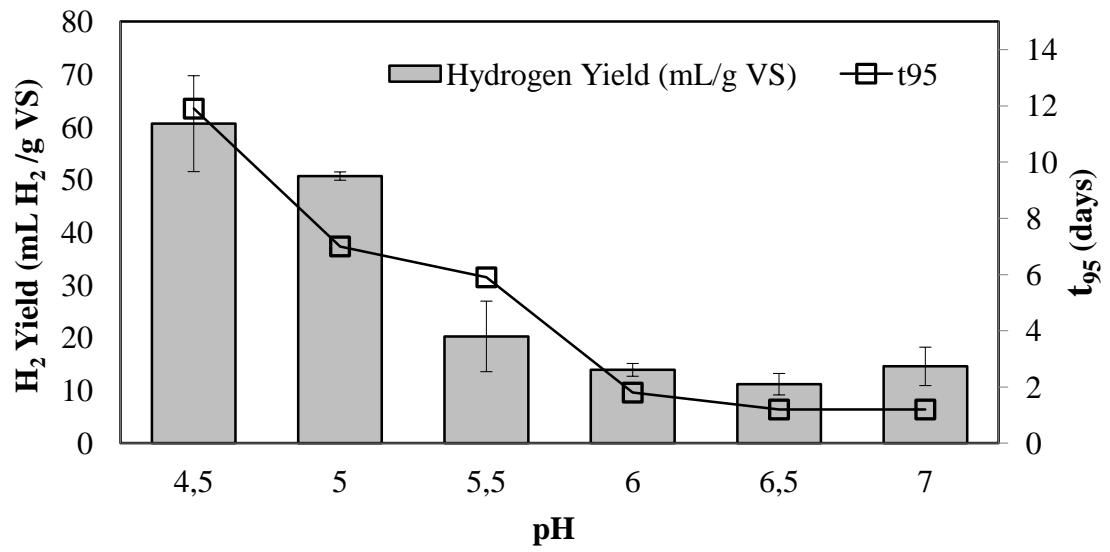
704 Fig. 2 H₂ yields and B/A ratio as a function of pH in the thermophilic DF of food waste at
705 F/M ratio 0.5

706 Fig. 3 Effect of alkaline pre-treatment of rice straw on H₂ yields

707 Fig. 4 Effect of inoculum source on cumulative H₂ production from the DF of OMWW using
708 ADS (anaerobic digested sludge) and WAS (waste activated sludge) as inoculum

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710 Fig. 1



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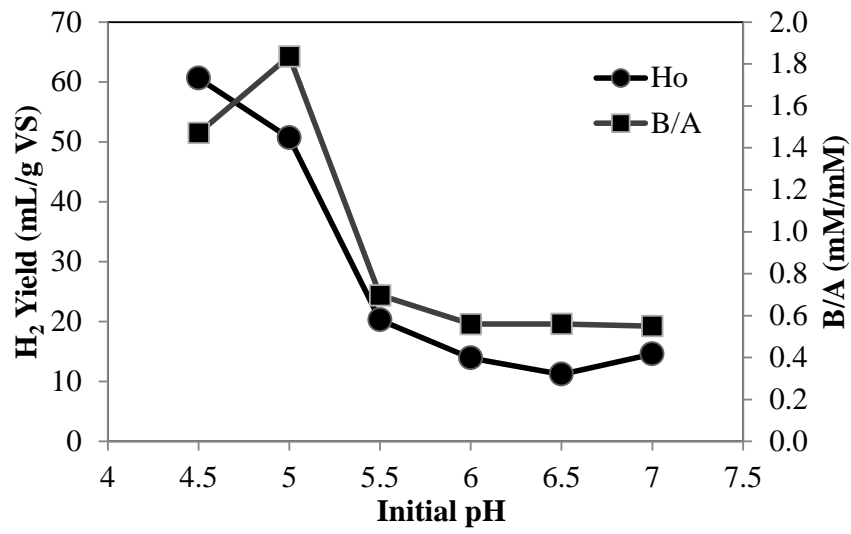
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716 Fig. 2



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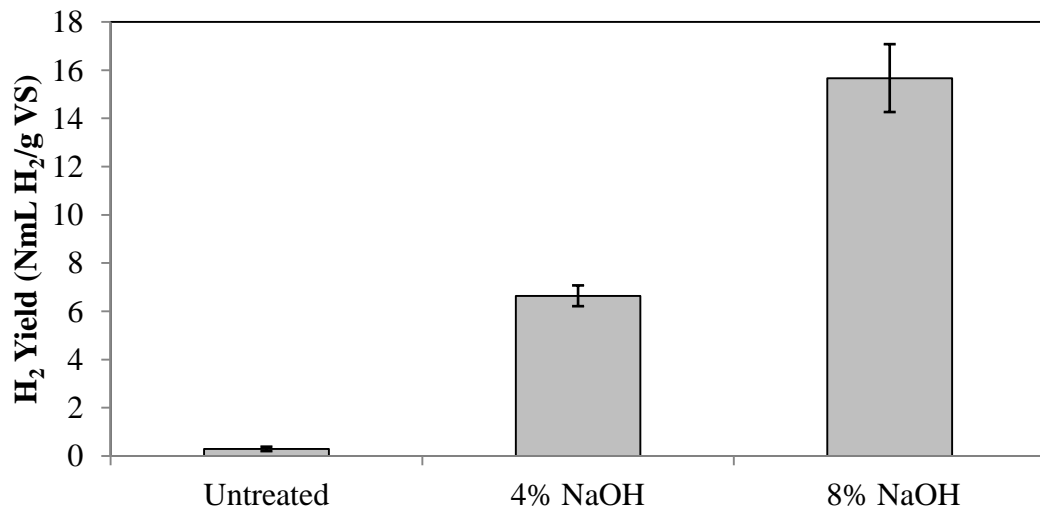
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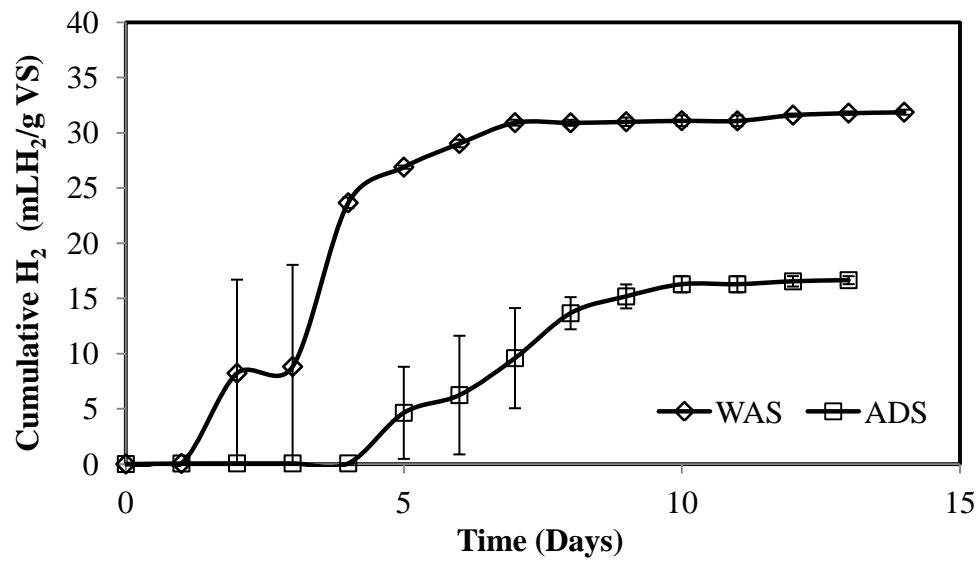
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732 **Table 1.** Characteristics of the substrates and inocula used in this study

Characteristics	Food waste	OMWW	Rice Straw	ADS	WAS
pH	4.4 ± 0.1	4.6 ± 0.1	NA	8.3 ± 0.1	7.0 ± 0.1
Chemical Oxygen Demand (COD)	347.6 ± 47.0 g/kg _{food waste}	141.5 ± 13.0 g/L _{OMWW}	NA	NA	NA
Total solids	21.0 ± 0.1 %	4.7 ± 0.1 %	92.3 ± 0.2 %	2.33 ± 0.4 %	2.9 ± 0.2%
Volatile solids	20.2 ± 0.1 %	3.1 ± 0.3 %	80.9 ± 0.6 %	1.93 ± 0.1 %	1.8 ± 0.1%
Carbohydrate content	105.8 ± 0.7 g/kg _{food waste}	12.9 ± 0.2 g/L _{OMWW}	NA	NA	NA
Lipids	17.5 ± 1.0 g/kg _{food waste}	45.3 ± 4.0 g/L _{OMWW}	NA	NA	NA
TKN	6.4 ± 0.2 g/kg _{food waste}	0.5 g/L _{OMWW}	NA	NA	NA
NH₄-N	NA	NA	NA	283.5 ± 11.0 mg NH ₄ -N/L	203.1 ± 3.0 mg NH ₄ -N/L
Alkalinity	NA	NA	NA	1437.2 ± 14 mg CaCO ₃ /L	2605.7 ± 70.0 mg CaCO ₃ /L
Total phenols	NA	1.16 ± 0.03 g/L _{OMWW}	NA	NA	NA

733 NA-Not Analyzed

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743 **Table 2.** Experimental conditions applied in the DF batch tests of the tested substrates

Investigation	Substrate	Inoculum	Initial pH	F/M
Effect of initial pH	Food waste	ADS	4.5, 5.0, 5.5, 6.0, 6.5 and 7.0	0.5
Combined effect of food waste and initial pH	Food waste	ADS	5.0 and 6.5	0.5, 1.0 and 1.5
Effect of pre-treatment of substrate	Rice straw	WAS	6.5	7.0
Effect of inoculum source and pre-treatment	OMWW	WAS and ADS	6.0	1.0

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747 **Table 3.** Effects of initial pH on H₂ production performance and characteristics of accumulated end products in DF of food waste at F/M 0.5

Initial pH	Parameters derived from modified Gompertz model					Characteristics of digestate at the end of DF				
	H ₀ (mL/gVS)	L (h)	R (mL/h)	R ²	Average final pH	H ₂ (mM/kg VS)	Acetate (mM/kg VS)	Propionate (mM/kg VS)	Butyrate (mM/kg VS)	Ethanol (mM/kg VS)
4.5	57.3	113.6	0.7	0.993	4.7 ± 0.1	1341.2 ± 201.3	1854.6 ± 114.0	964.5 ± 99.1	2728.7 ± 359.6	263.7 ± 16.1
5.0	50.9	68.1	1.0	0.999	4.9 ± 0.1	1121.3 ± 17.2	1611.8 ± 412	1686.7 ± 253.3	3018.7 ± 109.7	753.4 ± 290.6
5.5	20.3	41.2	0.4	0.995	5.2 ± 0.6	448.4 ± 148.2	2830.2 ± 381.0	1358.1 ± 392.1	1973.7 ± 374.9	623.7 ± 53.8
6.0	15.4	2.0	0.7	0.997	5.3 ± 0.1	308.0 ± 26.8	3558.9 ± 368.7	959.7 ± 6.4	1992.0 ± 238.1	2340.9 ± 263.7
6.5	11.2	3.3	0.8	0.995	5.5 ± 0.1	247.7 ± 45.3	3900.2 ± 838.5	260.0 ± 34.8	2185.5 ± 580.1	3056.7 ± 32.3
7.0	14.6	25.3	6.7	1.000	6.6 ± 0.1	322.6 ± 80.7	5922.4 ± 43.9	877.2 ± 41.4	3255.6 ± 308.1	1673.6 ± 48.4

748 R² represents the regression coefficient

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752 **Table 4.** Effects of initial pH and F/M ratio on H₂ production performance and characteristics of accumulated end products in DF of food waste

pH	F/M	Parameters derived from modified Gompertz model						Characteristics of digestate at the end of DF						
		H ₀ (mL/g VS)	L (h)	R (mL/h)	t ₉₅ (day)	R ²	Average final pH	H ₂ (mM/kg VS)	Lactate (mM/kg VS)	Acetate (mM/kg VS)	Propionate (mM/kg VS)	Butyrate (mM/kg VS)	Ethanol (mM/kg VS)	Caproate (mM/kg VS)
5.0	0.5	50.9	68.1	1.0	7.0	0.949	4.9 ± 0.1	2264.9 ± 34.8	17.5 ± 8.1	1610.7 ± 411.8	1687.0 ± 253.3	3018.7 ± 109.7	753.4 ± 290.6	0.0 ± 0.0
	1.0	58.5	81.9	1.4	9.7	0.997	4.7 ± 0.1	2690.9 ± 206.5	18.1 ± 2.2	1264.0 ± 27.1	3135.4 ± 245.7	2959.9 ± 35.2	1876.5 ± 5.9	0.0 ± 0.0
	1.5	54.2	87.9	0.3	46.5	0.991	4.5 ± 0.1	2202.1 ± 545.2	98 ± 10.3	420.3 ± 119.7	842.8 ± 59.2	2638.1 ± 202.9	1402.9 ± 325.6	0.0 ± 0.0
6.5	0.5	11.2	3.4	0.8	1.2	0.995	5.5 ± 0.1	1259.7 ± 188.4	0.0 ± 0.0	6043.0 ± 357.2	830.3 ± 38.9	2344.0 ± 73.3	3056.7 ± 32.3	0.0 ± 0.0
	1.0	42.6	17.0	1.6	4.6	0.938	5.7 ± 0.1	1928.7 ± 89.3	126.3 ± 124.2	1700.0 ± 305.8	775.8 ± 91.1	2062.9 ± 169.1	3602.1 ± 20.7	70.3 ± 9.4
	1.5	56.9	2.3	1.8	7.0	0.944	5.3 ± 0.1	2413.4 ± 197.0	0.0 ± 0.0	2364.5 ± 216.1	655.5 ± 166.3	2410.5 ± 47.5	2206.0 ± 63.1	263.3 ± 23.1

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755 **Table 5.** Effect of substrate pre-treatment on H₂ production performance measured by the modified Gompertz model and characteristics of accumulated end products in DF
 756 of rice straw

Pre-treatment method	Parameters derived from modified Gompertz model				Characteristics of digestate at the end of DF						
	H ₀ (mL/g VS)	L (h)	R (mL/h)	R ²	Average final pH	H ₂ (mM/kg VS)	Acetate (mM/kg VS)	Propionate (mM/kg VS)	Butyrate (mM/kg VS)	Ethanol (mM/kg VS)	B/A (mM:mM)
Without treatment	0.3	37.3	0.1	0.958	4.7 ± 0.1	12.8 ± 4.1	462.6 ± 42.7	50.8 ± 15.8	46.4 ± 13.7	41.0 ± 7.2	0.10
4% NaOH	6.7	23.9	2.9	0.999	4.9 ± 0.0	296.3 ± 19.2	775.0 ± 13.5	189.4 ± 18.5	227.7 ± 38.5	129.4 ± 44.8	0.29
8% NaOH	15.4	11.3	3.6	0.965	5.2 ± 0.6	699.4 ± 62.8	468.6 ± 84.4	55.6 ± 15.4	614.1 ± 105.8	148.9 ± 11.8	1.31

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760 **Table 6.** Effects of inoculum source on H₂ production performance measured by the modified Gompertz model and characteristics of accumulated end products in DF of
 761 OMWW

Inoculum type	Parameters derived from modified Gompertz model					Characteristics of digestate at the end of DF						
	H ₀ (mL/g VS)	L (h)	R (mL/h)	R ²	Average final pH	H ₂ (mM/kg VS)	Lactate (mM/kg VS)	Acetate (mM/kg VS)	Propionate (mM/kg VS)	Butyrate (mM/kg VS)	Ethanol (mM/kg VS)	B/A (mM:mM)
ADS	106.1	101.0	1.0	0.996	5.6 ± 0.1	751.2 ± 15.2	1651.8 ± 573.4	1752.2 ± 510.9	269.5 ± 183.3	4293.5 ± 93.1	3423.2 ± 1104.2	1.95
WAS	204.1	34.4	2.2	0.984	5.5 ± 0.2	1479.7 ± 46.3	0.0 ± 0.0	6823.0 ± 904.1	282.0 ± 217.1	5062.5 ± 131.0	3022.6 ± 0.8	0.44

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763 **Table 7.** Summary of various strategies to improve the H₂ yields from substrates with different biodegradability

Substrates	Optimization parameters	Optimal conditions	Substrate concentration (g VS/L)	Culture system	H ₂ Yield (NmL/g VS _{added})	Reference
Food waste	Initial pH (4.5-8.5)	pH 6.5	53.1 ± 0.9	Activated sludge, 39 °C, batch	56.2	(Cappai et al., 2014)
Food waste	Initial pH (4.5-7)	pH 4.5 – 5.0	3.4	Anaerobic sludge, 55 ± 2 °C, batch	61.0 ± 9.0 at pH 4.5 51.0 ± 1.0 at pH 5.0	This study
Food waste	F/M ratio (1-10)	F/M ratio of 6.0	18.5	Anaerobic sludge, thermophilic (50 °C), batch	39.0	(Pan et al., 2008)
Food waste	F/M ratio (0.5, 1, 1.5) at pH 5 & 6.5	F/M ratio of 1 at pH 5.0	6.1	Anaerobic sludge, 55 ± 2 °C, batch	60.3 ± 5.0	This study
Sun flower stalks	Substrate pre-treatment (thermo-alkaline)	4% NaOH at 55 °C, 24 hour	5.0	Anaerobic sludge, 35 °C, pH 5.5	4.4 ± 2.6	(Monlau et al., 2013b)
Rice straw	Thermal alkaline pre-treatment	8% NaOH at 55 °C, 24 hour	43.0	Activated sludge, thermophilic (55 °C), initial pH 6.0, batch	15.7 ± 1.0	This study
Rice straw	Inoculum source (MWWS ^b , PMS ^c & CDC ^d)	MWWS	30.0 g TS/L	55 °C, initial pH 6.5, batch	7.1 ^e	(Chen et al., 2012)
OMWW	Inoculum source (activated sludge & anaerobic digestate)	Activated sludge	10.5	55 °C, initial pH 6.0, batch	33.1 ± 1.0	This study

764 ^aN L H₂/kg total organic carbon

765 ^bMWWS: Municipal wastewater plant sludge

766 ^cPMS: Paper Mill Sludge

767 ^dCDS: Cow Dung Compost

768 ^emL H₂/g TS

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Supplementary Information on
Effects of operational parameters on dark fermentative hydrogen
production from biodegradable complex waste biomass

Anish Ghimire^{a,b,*}, Fabio Sposito^b, Luigi Frunzo^c, Eric Trably^d, Renaud Escudie^d, Francesco Pirozzi^b, Piet N.L. Lens^e, Giovanni Esposito^a

^aDepartment of Civil and Mechanical Engineering, University of Cassino and Southern Lazio, via Di Biasio 43, 03043 Cassino (FR), Italy

^bDepartment of Civil, Architectural and Environmental Engineering, University of Naples Federico II, via Claudio 21, 80125 Naples, Italy

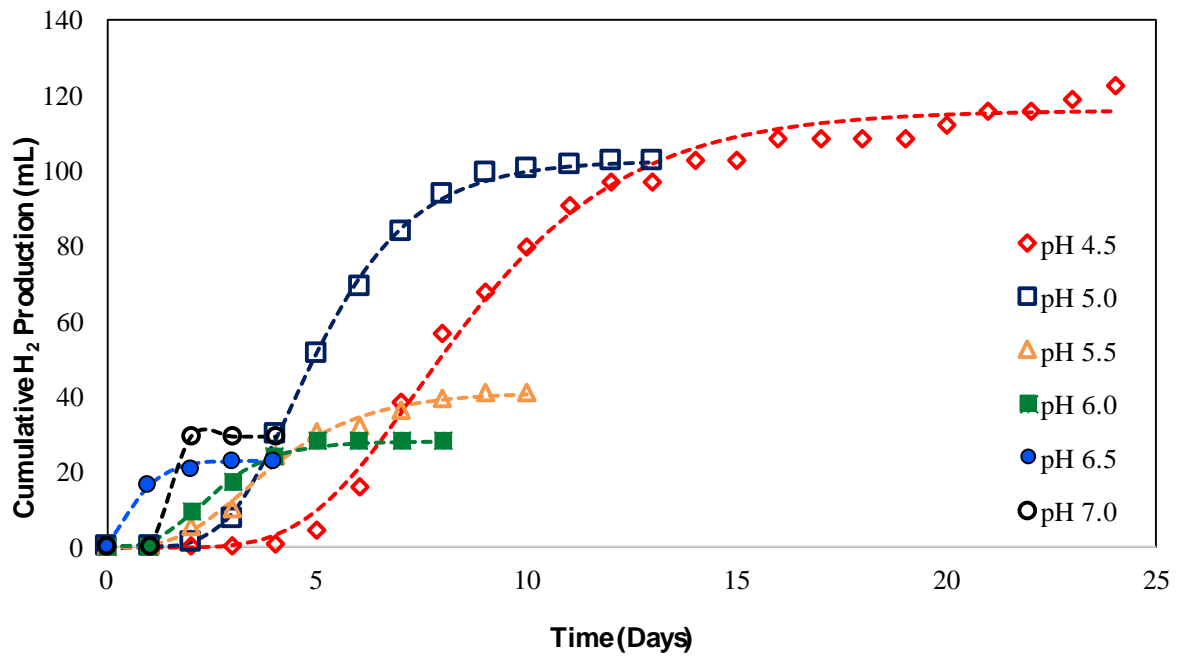
^cDepartment of Mathematics and Applications Renato Caccioppoli, University of Naples Federico II, via Cintia, Monte S. Angelo, I-80126 Naples, Italy

^dINRA, UR0050, Laboratoire de Biotechnologie de l'Environnement, Avenue des Etangs, F-11100 Narbonne, France

^eUNESCO-IHE Institute for Water Education, Westvest 7, 2611 AX Delft, The Netherlands

* Corresponding author. Address: Department of Civil and Mechanical Engineering, University of Cassino and Southern Lazio, via Di Biasio 43, 03043 Cassino (FR), Italy. Tel. +39 0776 2994339 Fax +39 0776-2993939

E-mail address: a.ghimire@unicas.it, anishghimire@gmail.com (Anish Ghimire)



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794 Fig. S1. Cumulative H₂ production at different initial pH values using food waste at a F/M
 795 ratio 0.5 and ADS as inoculum (dotted lines represents the results from a modified Gompertz
 796 model)