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1 **Biohydrogen production from food waste by coupling semi-continuous**
2 **dark-photofermentation and post treatment of residues by anaerobic**
3 **digestion: a synergy for energy recovery**

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20 **Abstract**

21 The study aimed at maximizing the energy yields from food waste in a three-step conversion
22 scheme coupling dark fermentation (DF), photofermentation (PF) and anaerobic digestion
23 (AD). Continuous H₂ production was investigated over a period of nearly 200 days in a
24 thermophilic semi-continuous DF process with no pH control. The highest H₂ yield of 121.45
25 ± 44.55 N L H₂/kg VS was obtained at an organic loading rate of 2.5 kgVS/m³·d and a
26 hydraulic retention time of 4 days. The DF effluents mainly contained volatile fatty acids
27 (VFAs) and alcohols as metabolites and un-hydrolyzed solid residues. The supernatant, after
28 separation, was used to recover H₂ in a PF using *Rhodobacter sphaeroides*. The solid residual
29 fraction along with PF effluent was converted into methane by anaerobic digestion. By
30 combining DF and PF, the H₂ yield from the food waste increased 1.75 fold. Moreover, by
31 adding AD as a post treatment, the total energy yield was substantially increased to reach
32 5.51 MJ/kg VS_{food waste} added, versus 3.55 MJ/kg VS_{food waste} when applying solely AD.

33 **Keywords:** Biohydrogen, food waste, dark fermentation, photofermentation, anaerobic
34 digestion

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36

37 **1 Introduction**

38 The inherent characteristics of hydrogen (H₂), such as higher energy content (142 MJ per kg),
39 energy and water as the only by-products generated from its combustion, application in fuel
40 cells for electricity generation and the ability to be produced biologically, makes H₂ a very
41 interesting alternative future sustainable energy carrier [1]. Among several biological
42 technologies proposed for H₂ production, dark fermentation (DF) is emerging as one of the
43 prominent options, shown by the increasing research interests in this technology [2]. The
44 advantages such as the flexibility to operate under different conditions of temperature and
45 pressure, higher production rates, possibility to use renewable waste biomass as feedstock
46 and its treatment capability makes the DF process attractive. Waste biomass such as
47 agricultural residues, the organic fraction of municipal solid waste (OFMSW) and agro-
48 industrial wastes are economically competitive when considering a supply of sustainable
49 feedstock, aiming at the industrial development of DF systems for biological treatment of
50 waste [3–5].

51 OFMSW which is mainly composed of food waste (FW) has been receiving a lot of attention
52 because of its potential to be used for the production of biofuels and other value added
53 products [6]. Especially, about 1.3 billion tonnes of food per year get wasted, which is
54 approximately one-third of the food produced for human consumption [7]. FW is generated
55 from agricultural production, industrial manufacturing processes and final consumption in
56 households. In the European Union, the total annual generation of FW is estimated around
57 89.3 million tonnes, comprising 37.7 million tonnes generated from household consumption
58 alone [8]. The volatile solids content in FW ranges from 21 to 27% which shows its high
59 content of organic carbon that can be further valorized [9], and in particular for H₂ production
60 by DF as demonstrated in the literature [10–15]. Some studies have reported the operational
61 feasibility of continuous H₂ production using these food or kitchen wastes as a feed in DF
62 processes [10,14,16].

63 With the advantage of a stable operation, continuous DF processes are usually preferred and
64 scaling-up is more viable in comparison to batch processes which involve regular downtime
65 periods of maintenance [17]. However, stable operation of continuous DF of FW is highly
66 influenced by bioreactor operating parameters such as pH, temperature, organic loading rates
67 (OLRs) and hydraulic retention times (HRTs) [4,5,18]. These factors also influence the
68 microbial communities and thus the biochemical pathways that can affect the total H₂ yields

69 in mixed cultures [19]. In addition, there is growing interest in coupling DF either with
70 photofermentation (PF) [20,21] or bioelectrochemical systems (BES) [22] to obtain higher
71 overall H₂ yields or with anaerobic digestion (AD) for methane production [23–25], due to
72 the post-treatment requirement of DF effluents (DFEs) and net positive energy gain from
73 coupling these bioprocesses [26].

74 H₂ production rates and total H₂ yields are mainly a function of substrate types and OLRs
75 applied [2]. A varying range of optimal OLR values has been reported for dark fermentative
76 H₂ conversion from FW carried out in thermophilic DF processes [2]. Shin et al. [27] found
77 an optimal H₂ yield of 126.25 L H₂/kg VS at an OLR of 8 kg VS/m³/d while the H₂
78 production decreased when the OLR was increased to 10 kg VS/m³/d. The authors reported 8
79 kg VS/m³/d, 5 days and pH of 5.5, respectively, as optimal OLR, HRT and culture pH. In a
80 study coupling DF and AD, Cavinato et al. [10] reported 66.7 L H₂/kg VS added at an
81 optimum OLR of 16.3 VS/m³/d, a HRT of 3.3 days and for a pH maintained in the range of 5-
82 6 through the recirculation of AD effluent. Generally, HRTs in a range of 2-6 days have been
83 reported as optimum for DF of organic FW in a CSTR process [2]. This range of HRTs is
84 similar to the first stage of two-stage AD process [28]. Moreover, the HRT is also a function
85 of the substrate types and bioreactor operational parameters.

86 It has been well documented that dark fermentative H₂ production is generally due to the
87 conversion of the initial soluble fraction of carbohydrates present in the complex organic
88 biomass, that will lead to accumulation of volatile fatty acids (VFAs) and alcohols in DFEs
89 [29,30]. Some recent studies have shown the potential of these DFEs to be utilized in PF
90 processes for H₂ production [20,21]. Combining DF with PF, Su et al. [31] achieved an
91 increase in H₂ yield from 76.7 to 596.1 L H₂/kg VS from water hyacinth. Meanwhile, Rai et
92 al. [20] achieved 43% higher volumetric H₂ yields from acid hydrolyzed sugarcane bagasse in
93 two step DF-PF systems. However, during the conversion of complex organic biomass like
94 FW, a part of the unhydrolyzed solid residues will remain that can be further valorized in AD
95 systems producing methane (CH₄) in three steps conversion scheme (Fig. 1). Xia et al.
96 [32,33] reported that a three-step conversion of algal biomass combining DF-PF-AD can
97 achieve 1.7 and 1.3 times higher energy yields in comparison to a two-stage DF-AD and one
98 stage AD process, respectively.

99

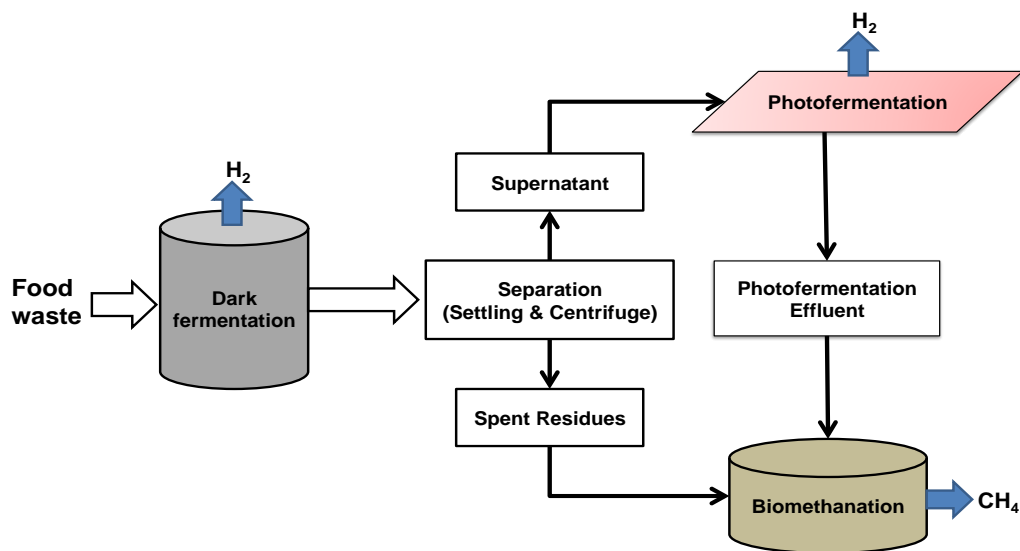


Fig. 1. Schematic of three-stage conversion of FW to hydrogen and methane

Furthermore, high OLRs are often responsible for a decrease in culture pH due to the accumulation of VFAs present in DFE. Thus, most of the continuous DF systems utilizing acidic substrates such as food waste require constant addition of external alkalinity sources such as alkaline chemicals (NaOH or KOH) or buffering agents (bicarbonate or phosphate buffers) [14,27,34]. A long-term study of continuous H₂ production at varying operating conditions of OLR and HRT to establish a long-term operability for continuous H₂ production in relation with the production of metabolites could provide further insights for the development of scaled-up DF systems. Similarly, a three step conversion process (DF, PF and AD) might contribute to an increase in overall energy yields and could provide the biological treatment to the by-products generated from DF systems.

This study aims to demonstrate the long-term operational feasibility of continuous H₂ production from FW using a semi-continuous thermophilic DF reactor at various low OLRs and HRTs without pH control. The experiment also aimed at reducing the dependency on chemical buffering agents that are used to maintain the culture pH at working conditions. H₂ production through different possible biochemical pathways was discussed in relation to major metabolites present in DFEs, obtained during the varying experimental conditions. The potential of coupling DF with photofermentative H₂ production was investigated in batch PF processes by using the liquid fraction of the DFE after physical separation. Further, the waste streams generated from the coupling of DF-PF were utilized in AD to maximize the energy yields and provide waste treatment solutions.

122 2 Materials and methods

123 2.1 Preparation of feedstock

124 An average composition of waste, as found in European countries, was prepared as cited
125 elsewhere [9]. The waste mixture was prepared at the laboratory and was composed of (in %
126 by weight): fruit and vegetables 72%, cooked pasta and rice 10%, bread and bakery 5%, dairy
127 products (cheese) 2%, meat and fish 8% and snacks (biscuits) 3%. The FW ingredients were
128 freshly bought at municipal markets in Naples (Italy), shredded with a blender and
129 immediately stored at $-20\text{ }^{\circ}\text{C}$ to avoid acidification. The FW characteristics were (in g/kg
130 FW): chemical oxygen demand (COD), 347.6 ± 47.4 ; carbohydrate content, 105.80 ± 0.7 ;
131 total Kjeldahl nitrogen (TKN), 6.4 ± 0.18 ; lipids, 17.50 ± 1.19 ; total solids (TS), $23.79 \pm$
132 0.44% ; volatile solids (VS), $22.8 \pm 0.42\%$ and the pH was 4.4 ± 0.1 .

133 DFE were collected from the outlet of the fermenter and had a pH of 4.5 ± 0.1 . After
134 undergoing settling for 30 minutes and centrifugation at 4500 rpm for 20 minutes, the
135 supernatant was collected. The DFE characteristics are presented in Table 1. The DFE was
136 supplemented with KH_2PO_4 , 3 g/L; NaHCO_3 , 0.7 g/L; ferric citrate 24.5 mg/L and 10 mL of a
137 trace metals solution (for composition, see below). pH was adjusted to 6.5 and then the DFE
138 medium was autoclaved at $121\text{ }^{\circ}\text{C}$ for 20 minutes.

139 **Table 1.** Characteristics of the DFE used in PF experiments

Parameters	Values (mg/L)
Chemical Oxygen Demand (COD)	3561.8 ± 131.1
TOC	2447.7 ± 7
TKN	208.0 ± 7
NH_4^+	1.14 ± 0.3
Phosphate	130.5 ± 1
Total iron (Total-Fe)	≤ 0.7
Lactic Acid	33.0
Acetic Acid	465.9
Propionic Acid	449.6
Butyric Acid	1075.4
Ethanol	323.0

140

141 The solid residues left after settling and centrifugation of DFE along with the PF effluents
142 mainly containing photofermentative biomass were used as feed for AD. The characteristics
143 of the solid residues generated from solid-liquid separation was comprised of undigested FW
144 which had a pH of 4.5 ± 0.1 and solid DF residue with content of: COD 2.64 ± 0.4 g/kg

145 residue; TS $2.42 \pm 0.02\%$ and VS $2.31 \pm 0.02\%$. The PF effluent had a pH of 7.26 ± 0.01 ; and
 146 contained a soluble COD of 1407.69 ± 109 mg/L; with 0.71 ± 0.01 % TS and 0.28 ± 0.01 %
 147 VS contents.

148 2.2 Experimental setup and operational conditions

149 2.2.1 Dark fermentation bioreactor

150 Anaerobic digested sludge was collected from an anaerobic digestion plant of the farm "La
 151 Perla del Mediterraneo" (Campania, Italy). The sludge was used as start-up seed inoculum
 152 after thermal pretreatment at 105 °C for 4 hours to enrich the microbial consortia of H_2
 153 producers, like spore forming *Clostridia*, and to inhibit the methanogens [35]. The inoculum
 154 had (in g/L): TS 29.54 ± 0.22 ; VS 18.36 ± 0.14 ; ammonium (NH_4^+), 0.28 ± 0.011 ; total
 155 alkalinity (as $CaCO_3$), 1.44 ± 0.014 and had a pH of 8.3 ± 0.1 .

156 A continuously stirred serum bottle of 1500 ml working volume was used as DF bioreactor,
 157 which was maintained at a constant thermophilic temperature (55 ± 2 °C). The reactor was
 158 started with initial S/X ratio (substrate to inoculum ratio, as gVS substrate/gVS inoculum) of
 159 0.5 and operated in semi-continuous mode with three different HRTs and four OLRs in six
 160 different operational conditions (Table 2). The pH of the initial feed (4.5 ± 0.1) was adjusted
 161 manually to an initial pH of 7.0 with 1 M NaOH. The culture pH in the reactor was not
 162 adjusted allowing the digesting mixture to reach indigenous chemical equilibrium.

163 Effluent and gas samples from the reactor were analyzed daily for determining the major
 164 metabolic intermediates, i.e. acetate, propionate, butyrate, lactate, ethanol and the gas
 165 composition (H_2 and CO_2). The total gas volume was measured by volumetric water
 166 displacement. The gas was passed through acidic water (1.5 % HCl) and the volume of water
 167 displaced corresponded to the volume of total gas. The volume of hydrogen produced was
 168 calculated by considering this volume and the gas composition and was then normalized for
 169 standard conditions.

170 **Table 2.** Experimental design used for the operation of semi-continuous reactor

Experimental periods	I	II	III	IV	V	VI
OLR (kg VS/m ³ /d)	1	1	1.5	2	2	2.5
HRT (d)	12	6	6	6	4	4
Concentration (kg VS/m ³)	12	6	9	12	8	10

171 **2.2.2 Photofermentation bioreactor**

172 *Rhodobacter sphaeroides* AV1b (provided by professor Roberto De Philippis, University of
173 Florence, Italy) was previously isolated from the Averno lake in Naples (Italy) cited
174 elsewhere in Bianchi et al. [36] and was used as inoculum for PF. *R. sphaeroides* AV1b was
175 first grown in a medium as previously described by Bianchi et al. [36], which was composed
176 of (in g/L): DL-malic acid, 2; sodium glutamate, 1.7; K₂HPO₄, 0.5; KH₂PO₄, 0.3;
177 MgSO₄·7H₂O, 0.4; NaCl, 0.4; CaCl₂·2H₂O, 0.075; ferric citrate, 0.005; yeast extract, 0.4 and
178 10 mL of trace metals solution containing (in mg/L) ZnSO₄·7H₂O, 10; MnCl₂·4H₂O, 3;
179 H₃BO₃, 30; CoCl₂·6H₂O, 20; CuCl₂·2H₂O, 1; NiCl₂·6H₂O, 2 and Na₂MoO₄·2H₂O, 30.

180 The *R. sphaeroides* AV1b pre-culture was grown again in a DFE supplemented with
181 appropriated chemicals and autoclaved, as explained in section 2.1. It was mainly composed
182 of (in mg/L): acetic acid, 848; propionic acid, 457; butyric acid, 1184; NH₄⁺, 6; phosphate (as
183 PO₄³⁻), 35.8 and total Fe 0.045. Ten mL of the culture (1.52 g TSS/L) that represents 2.5 %
184 V/V of the reactor working volume was used as inoculum in the PF experiments with DFE
185 (Table 1).

186 Transparent 500 mL borosilicate serum glass bottles (Simax, Czech Republic) with 400 mL
187 working volume were used as photofermentative batch reactor. The batch reactors were
188 maintained at room temperature (24 ± 2 °C, April-May) under a luminance of about 4000
189 Lux and positioned on the top of the stirrers. Caps of the reactors presented two separate
190 ports for biogas and culture medium sampling. The bottles were sealed with silica and
191 flushed with argon to ensure anaerobic conditions and eliminate the nitrogen from the
192 headspace since nitrogen can inhibit the activity of the nitrogenase enzyme responsible for
193 photofermentative H₂ production [37]. The H₂ production was quantified as described in
194 section 2.2.1.

195 **2.2.3 AD of residues from DF-PF process**

196 A batch test was carried out in 1 liter transparent borosilicate serum glass bottles (Simax,
197 Czech Republic) and was maintained at 34 ± 1°C in a water bath. The working volume of the
198 reactor was 600 mL with an initial S/X ratio of 0.5 with a substrate concentration of 4.5 g
199 VS/L. A low S/X ratio 0.5 was selected to assess the biomethane potential of the feed used.
200 Based on the substrate type, a range of S/X ratio 0.5 - 2.3 gVS substrate/gVS inoculum is
201 suggested to prevent the acidification of the AD reactor [38]. The source of inoculum used in

202 the tests was the same as the start up inoculum used in the semi-continuous DF reactor. The
203 characteristics of the inoculum were (in g/L): TS, 23.71 ± 0.17 ; VS, 14.55 ± 0.11 ; ammonium
204 (NH_4^+), 0.46 ± 0.02 ; and had a pH 8.2 ± 0.1 . The tests were carried out in duplicates.

205 **2.3 Analytical methods**

206 Hydrogen was quantified with a Varian Star 3400 gas chromatograph equipped with a
207 ShinCarbon ST 80/100 column and a thermal conductivity detector. Argon was used as the
208 carrier gas with a front and rear end pressure of 20 psi. The duration of analysis was 14
209 minutes. The fermentation products (lactic, acetic, propionic and butyric acids) were
210 quantified by High Pressure Liquid Chromatography (HPLC) (Dionex LC 25
211 Chromatography Oven) equipped with a Synergi 4u Hydro RP 80A (size 250×4.60mm)
212 column and UV detector (Dionex AD25 Absorbance Detector). Gradient elution consisted of
213 20% methanol and 10% acetonitrile in 5 mM H_2SO_4 pumped at a rate of 0.9 mL/min, using a
214 Dionex GP 50 Gradient pump. The elution time was 18.5 minutes. Ethanol was quantified by
215 HPLC (Aminex HPX-87H column (300 mm on 7,8 mm, Bio-rad) using 5 mM H_2SO_4 as an
216 eluent. The COD of the FW was measured described elsewhere [39]. The carbohydrate
217 content was determined according to the Dubois method [40]. Total lipids were measured
218 following a Bligh and Dyer chloroform/methanol total lipid extraction method [41]. The light
219 intensity was measured with a light meter (Lutron-LX-107). The TS and VS of the seed
220 sludge and TKN were determined according to the Standard Methods [42].

221 **2.4 Data analysis**

222 Hydrogen production rates (HPR) were expressed in $\text{L H}_2/\text{m}^3/\text{d}$ while the H_2 yields (HY)
223 were determined considering the total daily organic load fed to the reactor and expressed as L
224 $\text{H}_2/\text{kg VS}$ added. Average and deviations for daily production were determined during the
225 steady state reached after 3-4 days of operation. The H_2 Production Stability Index (HPSI)
226 was evaluated by considering the ratio of standard deviation and average HPR as reported by
227 Tenca et al. [16]:

$$228 \quad HPSI = 1 - \frac{SD.(HPR)}{Avg.HPR} \quad (1)$$

229 A HPSI index closer to 1 represents a stable hydrogen production.

230 FactoMineR, an extension on R software, was used for multivariate analysis of the
231 metabolites distribution from the different experimental periods in relation to the hydrogen
232 yields and co-relation circles of the major metabolites were generated.

233 **3 Results and discussion**

234 **3.1 Continuous dark fermentative biohydrogen production**

235 **3.1.1 Effect of operational parameters on H₂ production rate and yields**

236 The results in terms of H₂ yields (HY), hydrogen production rates (HPR) and H₂ Production
237 Stability Index (HPSI) during the different OLRs and HRTs investigated in the six operation
238 periods (Table 2) are summarized in Table 3. Fig. 2 shows the HPR and pH trends over the
239 operation period of 193 days. The results show an increase in HPR when OLRs were
240 increased. During the operating periods II, III and IV at a constant HRT of 6 days, the HPR
241 increased from 54.1 ± 41 , to 109.5 ± 33 and 210.2 ± 30 N L/m³/d, when the OLR was
242 increased from 1 to 1.5 and 2 g VS/m³/d, respectively (Tables 2 and 3). Meanwhile, the
243 overall HY increased from 54.1 ± 41.3 N L/kg VS_{added} to 105.1 ± 14.9 N L H₂/kg VS_{added}.
244 During the experimental period IV, the H₂ production had a comparatively better stability as
245 shown by a HPSI of 0.86. However, no significant effect was observed on the total HY and
246 HPR when the HRT changed to 4 days during operational period V (Table 3). When the OLR
247 was changed from 2 to 2.5 g VS/L/m³/d during period VI, both HY and HPR increased.
248 However, the H₂ production was not stable, supported by a lower value of HPSI of 0.63. This
249 instability could be explained by the accumulation of acids and a subsequent decrease in pH
250 to 4.4 ± 0.1 , which might have affected the microbial community.

251 During a short operation period (at the end of period IV), the culture pH inside the reactor
252 was regulated manually to an initial culture pH 5.5 with 1 M NaOH, during feeding, with the
253 objective to assess the influence of pH on the H₂ production performance (Fig. 2(b)).

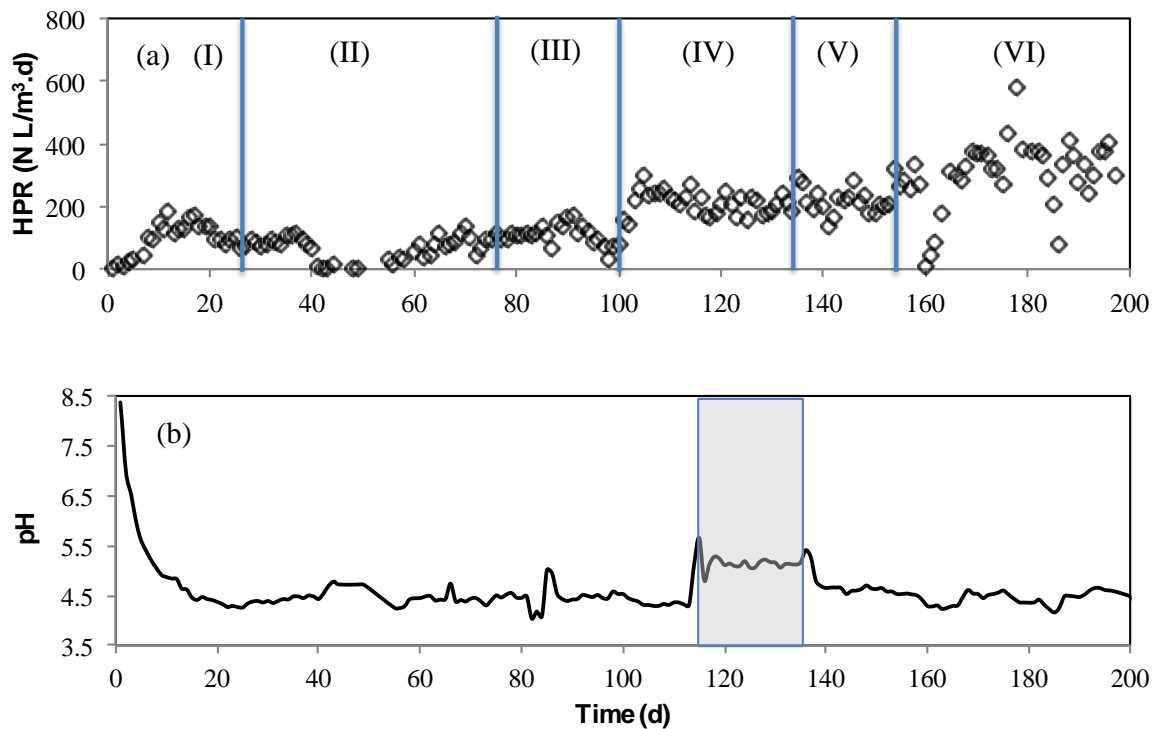
254 However, pH regulation did not show any effect on the HPR (Fig. 2(a)). Nevertheless, the
255 increased HPSI (Table 3) showed that H₂ production was stable during that period in
256 comparison to the experimental period when the culture pH was uncontrolled. The percentage
257 of H₂ and CO₂ in the gas averaged $59 \pm 6\%$ and $39 \pm 6\%$, respectively, when the H₂
258 production stabilized. However, the H₂ production performances in experimental period IV
259 (HPR: 210.2 ± 29.8 N L/ m³-d and HY: 105.1 ± 14.9 N L/kg VS_{added} at HRT of 6 days and
260 OLR 2 g VS/L/m³/d) were comparable to experimental period V (HPR: 208.0 ± 34.8 N L/

261 m^3/d and HY: $104.0 \pm 17.4 \text{ N L/kg VS}_{\text{added}}$ at a HRT of 4 days and OLR of $2 \text{ g VS/L/m}^3/\text{d}$.
 262 Thus, the operational conditions of period V were considered as ideal for the DF of FW in
 263 thermophilic semi-continuous reactors with lower HRT are generally more economically
 264 efficient in terms of bioreactor design and operation.

265 Table 3. H_2 production rate, yields and production stability from FW by mixed anaerobic
 266 cultures

Exp. Period	HPR ($\text{N L/m}^3/\text{d}$)	HY ($\text{N L/kg VS}_{\text{added}}$)	H_2 in biogas (%)	HPSI
I	116.9 ± 40.1	116.9 ± 40.1	$52.8 \pm 1\%$	0.66
II	54.1 ± 41.3	54.1 ± 41.3	$31.2 \pm 1\%$	0.24
III	109.5 ± 32.8	73.0 ± 21.9	$43.8 \pm 20\%$	0.70
IV	210.2 ± 29.8	105.1 ± 14.9	$59.4 \pm 6\%$	0.86
V	208.0 ± 34.8	104.0 ± 17.4	$57.2 \pm 6\%$	0.83
VI	303.6 ± 111.4	121.4 ± 44.5	$55.8 \pm 10\%$	0.63

267



268

269 Fig. 2 (a) HPR ($\text{L H}_2/\text{m}^3/\text{d}$) and (b) pH trends in semi-continuous thermophilic reactor.
 270 Shaded region represents the experimental period when the culture pH inside the reactor was
 271 adjusted daily at pH 5.5 during the feeding operation.

272

273 A comparison of previous studies on dark fermentative H_2 production from FW with the
 274 results from this study (Table 4) suggests that comparable results in terms of H_2 production

275 can be achieved even at low OLRs and without pH control. Nonetheless, the characteristics of
 276 FW can also affect the overall HY as H₂ production is mainly function of the soluble fraction
 277 of carbohydrates present in the substrate [30]. The OLRs reported in the past studies were
 278 higher than in this study, and thus a source of alkalinity to balance the pH conditions at
 279 optimum was required. Valdez-Vazquez et al. [14] used NaHCO₃ and K₂HPO₄ to maintain
 280 the optimum pH at 6.4, while Lee et al. [43] used NaOH and H₃PO₄ to maintain the culture
 281 pH at 6. Thus, this pH decrease resulting from the production of acids can be minimized by
 282 the use of lower OLRs. Higher OLRs can exert detrimental effects on the microbial
 283 community, and thus H₂ production, by decreasing the pH due to the accumulation of
 284 metabolites [44].

285

286 **Table 4.** Comparison of dark fermentative H₂ production using FW by anaerobic mixed
 287 cultures

Substrate type	Reactor type	T (°C)	pH	OLR (kg VS/m ³ ·d)	Maximum assessed H ₂ yield (N L H ₂ /kg VS _{added})	H ₂ in biogas (%)	Reference
FW	Batch	55	4.5 (initial)	6	46.3	23	[45]
Vegetable kitchen waste	Intermittent-CSTR	55	6.0	28 ^a	38.1 ^b	40	[43]
FW and sewage sludge	Batch	35	5.0-6.0	-	122.9 ^c	-	[46]
OFMSW (FW+paper)	Semi-continuous CSTR	55	6.4	11 ^d	360	58	[14]
OFMSW	Packed bed reactor	38±2	5.6±0.2	16 ^e	99	47	[47]
FW	Semi-continuous CSTR	55±2	4.7±0.2	2	104.0±17.4	57.2(±6)	This study

288 ^agCOD/L·d, ^b mL H₂/g COD, ^c mL H₂/g carbohydrate COD, ^dg VS/kg wet mass reactor·d, ^eg
 289 VS/kg·d, FW=food waste, OFMSW= organic fraction of municipal solid waste

290 3.1.2 Metabolic intermediates

291 Lactate, acetate, propionate, butyrate and ethanol were the main metabolic intermediates
 292 observed during the different experimental periods. Such a mixture of intermediates is
 293 characteristic of mixed fermentation pathways occurring with complex substrates [30].

294 Average concentrations of the main metabolites during the six different experimental periods

295 are summarized in Table 5. There can be a number of possible H₂ production pathways
 296 during mixed type fermentation, as represented by equations 2 – 5 (Table 6), whereas H₂
 297 consuming or unfavorable pathways presented in equations 6 – 9 might exist at the same time
 298 [17,19]. The presence of ethanol, acetate and butyrate are evidences for the presence of an
 299 ethanol-acetate or butyrate-acetate pathway for H₂ production in the DF of the FW
 300 investigated. On the other hand, the presence of lactate or propionate can be attributed to
 301 fluctuations in H₂ production resulting in low H₂ yields.

302 **Table 5.** Characteristics of influent and effluents from DF of FW during different
 303 experimental periods

Exp. Period	pH_IN	pH_OUT	Lactate (mM)	Ethanol (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)
I	7.00	4.7±0.3	0.1±0.2	4.8±0.2	13.1±3.6	3.85±2.21	10.4±2.8
II	7.00	4.5±0.1	0.6±1.4	5.4±3.5	3.2±2.0	3.44±2.33	6.2±4.2
III	7.00	4.5±0.2	4.0±9.1	8.7±2.7	4.9±0.6	5.97±2.16	11.0±1.6
IV	7.00	4.9±0.4	0.0±0.0	17.2±8.6	8.5±1.8	9.65±2.91	12.0±2.9
V	7.00	4.7±0.2	0.0±0.0	17.1±6.6	6.7±1.9	5.70±2.15	9.9±3.2
VI	7.00	4.4±0.1	0.5±0.9	9.4±5.3	5.7±2.8	5.89±2.70	11.1±7.5

304

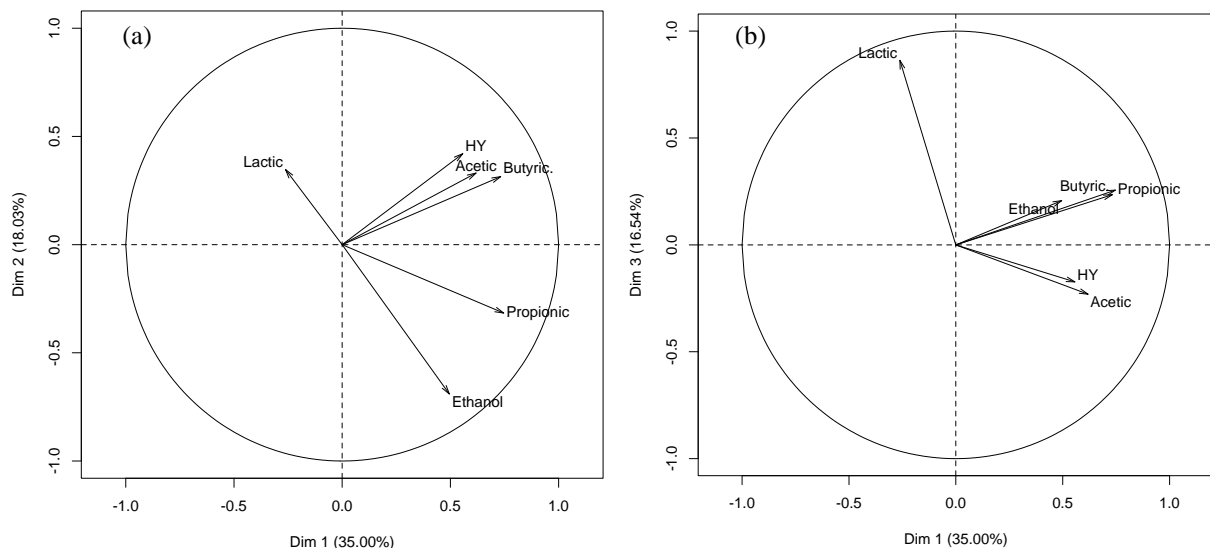
305 **Table 6.** Reaction stoichiometry in DF of glucose

Possible H ₂ producing pathways	Metabolic pathway	ΔG ^o _a (kJ/mol)	Eqn
$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$	Acetate	-206.3	(2)
$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$	Butyrate	-254.8	(3)
$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2CO_2 + 2H_2$	Ethanol & acetate	-215.7	(4)
$4C_6H_{12}O_6 + 2H_2O \rightarrow 3CH_3CH_2CH_2COOH + 2CH_3COOH + 8CO_2 + 10H_2$	Butyrate & acetate	-254.0	(5)
Unfavorable and H ₂ consuming pathways			
$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$	Propionate	-359.6	(6)
$1.5 C_6H_{12}O_6 \rightarrow 2C_2H_5COOH + CH_3COOH + CO_2 + H_2O$	Propionate & acetate	-310	(7)
$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$	Ethanol	-235.0	(8)
$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$	Lactate	-198.1	(9)

306 ^a ΔG^o values are adapted from [48,49]

307 Fig. 3 shows the plot of correlation circles of the five major metabolites and the HY. Fig. 3(a)
 308 shows that the butyrate and acetate concentration is well correlated with the HY values. Not

309 surprisingly, propionate, lactate and ethanol are in the Dim 2 and are not correlated with the
 310 HY, which is supported by the Equations 6 - 9 (Table 6) in a DF with glucose as model
 311 substrate. However, the pathways leading to ethanol-acetate also yield H₂, as shown in
 312 Equation 4 [50,51]. Nonetheless, from Fig. 3, it can be seen that the ethanol is not correlated
 313 with acetate. Therefore, most of the H₂ yields can be attributed from the butyrate-acetate
 314 pathways, which showed a good correlation and is explained in Dim 1. The variable Dim 3 is
 315 mostly explained by lactate concentrations (Fig. 3 (b)), which correlated oppositely with HY
 316 and is an orthogonal and independent variable. The proximity of butyrate, ethanol and
 317 propionate suggests that these metabolites can be expected from DF by mixed microbial
 318 consortia. This is also supported in a study by Hwang et al. [50] who obtained butyrate,
 319 ethanol and propionate as the major metabolites during the DF at pH range 4-4.5, 4.5-5.0,
 320 5.0-6, respectively.

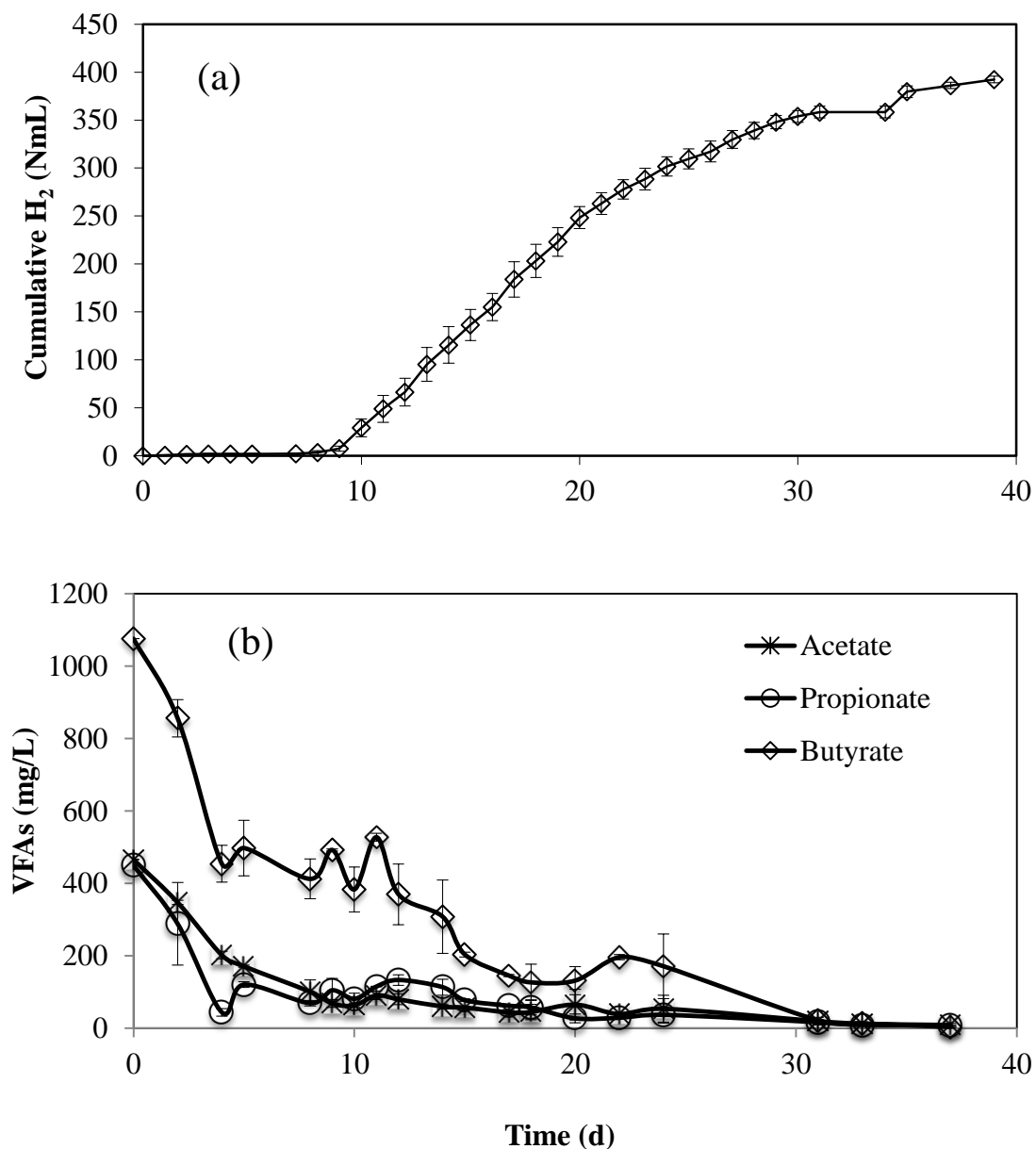


321 Fig. 3 Correlation circle of five metabolites and HY formed by the first three principle
 322 components Dim1, Dim 2 and Dim 3 representing 35.00, 18.03 and 16.54 % of the total
 323 variance, respectively. (a) Projections according to the first two factors (Dim 1 and Dim 2).
 324 (b) Projects according to the first and third factors (Dim 1 and Dim 3)

325 3.2 Photofermentative H₂ production from the liquid fraction of DF

326 The DFE from the semi-continuous DF reactor obtained during experimental period VI was
 327 further converted to H₂ by *R. sphaeroides* AV1b in a PF process. Cumulative H₂ production
 328 and VFA consumption trends during the PF experiments are shown in the Fig. 4 (a) and 4 (b),
 329 respectively. VFA and ammonium concentrations in the DFE medium (shown in Table 2)

330 were both in non-inhibiting levels for photofermentative H₂ production. Han et al. [52]
 331 reported that concentrations equal to 9.8 mM, 10.9 mM and 4.2 mM, respectively, for acetate,
 332 butyrate and propionate gave the optimum H₂ yield using *R. sphaeroides*. However,
 333 concentrations up to 30 mM of acetate have been reported in a study by Hustede et al. [53].
 334 Similarly, the ammonium concentration was at non-inhibitory levels, as only a concentration
 335 higher than 2 - 5 mM of NH₄⁺ has been reported to inhibit the photofermentative production
 336 of H₂ [54,55].



337 Fig. 4. (a) Cumulative hydrogen production and (b) depletion of major VFAs (acetate,
 338 propionate and butyrate) in a PF tests using DFE and *R. sphaeroides* AV1b

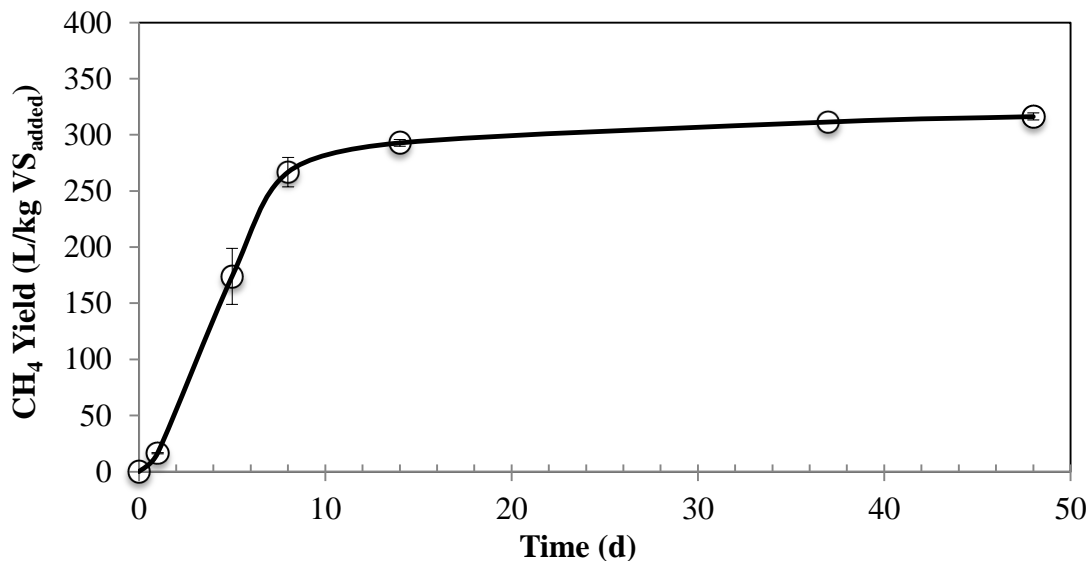
339 The PF of spent DFE yielded a cumulative production of 365.6 ± 3.2 NmL H₂, corresponding
340 to a volumetric yield of 914 ± 8 N L H₂/m³ and a substrate yield of 427 ± 6 N L H₂/kg COD
341 consumed. The batch experiments were carried out for 40 days until the H₂ production
342 completely ceased (Fig. 4 (a)). This is longer than any H₂ production time reported elsewhere
343 [20,33]. The long lag phase (9 days) can partly explain this result. The final effluents were
344 analyzed for COD, VFAs and biomass concentration which showed a COD reduction of
345 60.1%, while more than $98 \pm 1\%$ of VFAs were removed to reach a final biomass
346 concentration of 1.6 g TSS/L. Theoretical COD removal calculated from the VFA
347 concentration in final effluents showed a COD removal efficiency of 99.2%. However, the
348 production of biomass and other bacterial carotenoids increased the final total COD of the PF
349 effluent and thus reduced the total COD removal efficiency. This was evident by the reddish
350 brown color of the effluent. The maximum percentage of H₂ in the biogas was 89% with
351 8.9% of CO₂.

352 The volumetric H₂ production obtained in this study (914 ± 8 N L H₂/m³) is higher than the
353 study of Rai et al. [20] using *Rhodopseudomonas* BHU 01 with a volumetric H₂ yield of 755
354 L H₂/ m³. In another study by Uyar et al. [56] using *Rhodobacter capsulatus* (DSM 155) as
355 biomass and DFE of *Miscanthus* hydrolysate as substrates, a volumetric yield of 1000 L H₂/
356 m³ was obtained, which is slightly higher than in this study. The present study showed the
357 potential of an integrated DF-PF system to achieve higher H₂ yields. Thus, the combined DF-
358 PF processes can help in the industrial development of DF processes using FW. The residues
359 generated from the downstream of these processes can nevertheless still be treated with
360 anaerobic digestion in order to provide additional conversion of organic matter to further
361 recover energy.

362 **3.3 AD of DF-PF waste stream**

363 The solid residues generated by the coupled DF-PF process can be ideal for AD as the
364 undigested FW residues from the DF process and the PF effluent containing biomass
365 generated from the PF can be converted to methane in a biorefinery model (Fig. 1). The result
366 of the average cumulative methane production trends during the biomethane potential test
367 using the waste stream generated from the DF-PF process is presented in Fig. 5. The
368 cumulative CH₄ production stabilized after 50 days and the average cumulative CH₄
369 production was 871 ± 16 mL, corresponding to the total average yield of 324 ± 6 N L CH₄/g
370 VS added (feed) and 0.9 kg COD/kg VS removed (calculated from CH₄ produced), evaluated

371 after subtracting the endogenous methane produced in the controls. The initial and final
372 average pH in the BMP tests was 7.0 and 7.7, respectively, while the pH of the dark
373 fermentation and photofermentation residues were respectively, 4.33 and 7.26. The pH was
374 not adjusted with a buffering agent because the alkalinity of the inoculum was sufficient to
375 maintain the pH, this further adds the practicability of the AD as a post-treatment option.
376



377

378 **Fig 5.** Methane yields from mesophilic AD of waste stream generated in the coupled DF-PF
379 processes

380 **3.4 Energy yields from gas biofuels produced from food waste**

381 When considering the conversion of the initial VS added at the beginning of the DF process,
382 the overall average H₂ yield from coupling of the DF-PF process was increased from 105.1 N
383 L H₂/kg VS_{initial} to 184.3 N L H₂/kg VS_{initial}, with an additional 79.2 N L H₂/kg VS_{initial} from
384 PF and 99.3 N L CH₄/kg VS_{initial} from AD. The increase in energy yields obtained in his
385 study was compared with energy yields from the coupled process previously reported in the
386 literature (Table 7). The energy yields of hydrogen and methane from the stand alone DF as
387 well as the two stage DF-PF and DF-AD was calculated based on the heating values of H₂
388 (242 kJ/mol) and methane (801 kJ/mol). These calculated energy yields represent the energy
389 gain from the conversion of substrates by biological processes. However, the net energy gain
390 can be estimated by considering the energy input in the processes, which is not representative
391 in lab scale reactors and thus not calculated in this study.

392
393

Table 7. Comparison of energy yields from gaseous biofuels produced out of FW as feedstock using stand alone or coupling of different technologies

Feedstock	Process/ type ^e	H ₂ yield from DF / DF+PF (N L H ₂ /kg VS)	^a Energy yield from H ₂ (MJ/kg VS)	CH ₄ yield from AD (L CH ₄ /kg VS)	^a Total energy yield (MJ/kg VS)	Reference
FW+paper	Semi- continuous DF	360	3.89	-	3.89	[45]
FW	DF+PF (batch)	671 ^b	7.25	-	7.25	[57]
Vinegar residue treated by HCl	DF+AD (batch)	53.2	0.57	192	7.4	[58]
FW	DF+AD (batch)	55	0.60	94	3.96	[25]
<i>N. oceanica</i> ^c	DF+PF+AD (batch)	183.9	1.98	161.3	7.74	[33]
<i>C. pyrenoidosa</i> ^d	DF+PF+AD (batch)	198.3	2.14	186.2	6.66	[32]
FW	Semi- continuous DF + PF (batch) +AD (batch)	184	1.99	99.3	5.55	This study

394 ^a The energy yield was calculated from the yield of biogas based on the heating values of
395 hydrogen (242 kJ/mol and methane (801kJ/mol)

396 ^b L H₂/kg food waste

397 ^c Algal biomass pre-treatment by microwave heating with dilute H₂SO₄

398 ^d Algal biomass pre-treatment by steam heating with dilute H₂SO₄

399 By coupling DF with PF and AD processes, an additional 4.4 MJ/kg VS of energy yield can
400 be achieved from food waste, which is higher than the coupled DF - AD process or stand
401 alone DF processes (Table 7). Out of the overall energy recovered from the three-stage
402 conversion (DF-PF-AD) of food waste, H₂ contributes only 35.8% out of 5.55 MJ/kg VS.
403 However, this may be a positive add-on to the overall economic return compared to CH₄
404 productivity only. Therefore, the three-step process can definitely increase the recovered
405 energy yield. Moreover, it is a very good solution for waste treatment as a higher FW
406 conversion was accomplished. Table 7 shows that the energy yield of DF and PF from the
407 study of Zong et al. [57] is higher than the energy yield reported in this study. This is likely
408 because of the difference in H₂ yield achieved in these studies. In other studies by Xia et al.
409 [32,33] and Wang et al. [58], although the overall energy yields obtained from the respective

410 three and two step conversion were high, the pre-treatment of the substrate required some
411 energy input. Therefore, the overall energy yields obtained from the coupling of various
412 processes depends on the H₂ and CH₄ yields and production rates in individual processes,
413 which are mainly a function of process operational conditions such as pH, temperature, HRT
414 and OLR as well as carbohydrate content and nature of the feedstock. However, the coupling
415 of the PF and AD processes in the downstream process is not only advantageous from the
416 energy point of view, but it also provides biological treatment of waste stream generated from
417 DF processes by COD and pathogen removal [59].

418 **4. Conclusion**

419 This study has shown the long-term feasibility of continuous H₂ production as well as the
420 possibility to further recover energy through integration of PF and AD using FW as the
421 substrate. In addition, the viability of H₂ production at low OLRs without the culture pH
422 control can minimize the excessive use of chemical buffering agents for pH control. The
423 integration of DF with PF can increase the overall H₂ yield 1.75 folds. On the other hand,
424 coupling AD for the post treatment of waste streams generated by the coupling of the DF-PF
425 processes can further increase the overall energy yield by 4.83 MJ/kg VS of food waste,
426 adding a synergistic effect on the overall energy recovery during the conversion of food
427 waste.

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