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FACTOR-BASED ASSESSMENT OF CONTINUOUS BIO-H_2 PRODUCTION FROM CHEESE WHEY

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ABSTRACT

Despite having been widely investigated, dark fermentative H_2 production from organic residues is still limited by process-related issues which may hamper the perspectives of full-scale process implementation. Such constraints are mainly due to the process complexity, which is largely affected by multiple and often mutually interacting factors. In the present work, the results of continuous fermentative H_2 production experiments using synthetic cheese whey as the input substrate were processed to gain detailed knowledge of the process features and identify suitable and critical operating conditions. Specifically, process interpretation involved a combination of analytical characterization of the fermentation broth, mass balance calculations and statistical methods (correlation and principal component analyses). The metabolic products mainly included acetate and butyrate, which however were likely to derive (in different proportions depending on the operating conditions) from both hydrogenogenic and competing pathways. For some tests, lactate and succinate were also found to have been formed. It was observed that the main features of the process (H_2 yield and rate, stability condition) were correlated with the operational and analytical parameters. The first three principal components identified by the statistical analysis were able to account for: 1) the effect of retention time and total metabolites produced; 2) biogas (H_2 and CO_2) generation, butyrate production and stability condition; and 3) organic loading rate and propionate production. The results suggested that the main features of hydrogenogenic fermentation can be described by a reduced set of factors that may be usefully adopted for both process monitoring and prediction purposes.

Keywords*: dark fermentation; metabolic products; carbon mass balance; principal components; process stability*

1. INTRODUCTION

Among dairy industry residues, cheese whey (CW) is regarded as an attractive substrate for H_2 production through dark fermentation (DF) due to its organic content, spanning the ranges 55–117 g COD/L and 27–46 g BOD_5/L. The most abundant components are lactose (39–60 g/L), proteins (1.4–18 g/L), lipids (1–21 g/L), and mineral salts (5–10 g/L). Further details on CW composition derived from individual studies is reported in Table 1. Although the high concentrations of biodegradable organic matter in CW may pose potential environmental hazards and may also lead to overloading wastewater treatment plants, the exploitation of its energy content may open up large challenges for recovery.

The latest available statistical data on annual cheese production report values of ~24.7 million tons worldwide (OECD-FAO, 2021) and ~10.1 million tons in the EU-27 (Eurostat, 2020), with a constant increase over the last decades. It is evident that this also gives rise to large amounts of dairy residues being generated, with CW production being estimated to reach ~9–10 L per kg of cheese manufactured (Carvalho et al., 2013).

DF of organic residues has been widely investigated over the past decades, but some big challenges still exist along the way to full-scale implementation. These include mainly the wide variability of H_2 production along with the poor stability of the biochemical process, and the thermodynamic and biochemical limitations to the H_2 yield attainable. Therefore, one of the main goals of the several studies on DF has involved investigating the influence of the key factors on the H_2 yield and the feasibility of promoting hydrogenogenic pathways by adjusting the operating conditions. Considerable advance has been made to relate the process parameters and the metabolic routes, though most of the studies have been conducted in a batch mode (Akhlaghi et al., 2017; Dareioti et al., 2014; Davila-Vazquez et al., 2008; Kargi et al., 2012; Lopez-Hidalgo et al., 2018; Muñoz-Páez et al., 2014; Patel et al., 2016; Vasmara et al., 2018; Ziara et al., 2019). In recent years, therefore, the need has arisen to verify the performance and stability of the process, and the effects on the same of the operating parameters adopted, under continuous feeding conditions, in turn better approximating the application on a real scale. In this respect, recent studies have been performed evaluating

50 continuous H_2 production from a range of residual organic substrates (dairy effluents, agricultural
51 products, sugar and ethanol industry effluents, industrial wastewater, food waste, organic fraction of
52 municipal waste and sewage sludge) and using a variety of reactor configurations and operating
53 conditions (see e.g. Lopez-Hidalgo et al. (2022) and references therein). Despite such efforts made
54 for an improved understanding of the real-scale performance of fermentative H_2 production, deeper
55 understanding of some unresolved issues is mandatory to assess the process viability under
56 continuous operation for industrial scale-up purposes,. More specifically, the main open questions for
57 the implementation of continuous biohydrogen production from real residual substrates include:

58 • defining strategies to limit substrate-competitive microbial pathways, which typically
59 encompass solventogenesis (Antonopoulou et al., 2012), lactate fermentation (Castillo
60 Martinez et al., 2013; Sikora et al., 2013) and heterotrophic homoacetogenesis (Ljungdahl et
61 al., 1989)
62 • defining strategies to limit the competitive influence of hydrogenotrophic microbial pathways,
63 the most common of which comprising autotrophic homoacetogenesis (Saady, 2013),
64 propionate fermentation (Antonopoulou et al., 2008a) and methanogenesis
65 • optimizing the operating variables to drive the metabolic pathways towards the desired
66 direction (Kisielewska et al., 2014; Perna et al., 2013)
67 • preventing process inhibition by either internal or external factors (such as excessive buildup
68 of metabolic products, presence of toxic substances, etc. (Castelló et al., 2020)).

69 Properly addressing such issues is essential to ensure at the same time adequate stability of the
70 biochemical reactions involved and satisfactory H_2 production yields. Different parameters, mainly
71 involving biogas volume and composition, are commonly used to monitor the performance of DF,
72 while other indicators of the process evolution include measurements of metabolite production and
73 characterization of the microbial community (Antonopoulou et al., 2008b; Azbar et al., 2009; Castelló
74 et al., 2018, 2009; Davila-Vazquez et al., 2009; Venetsaneas et al., 2009; Yang et al., 2007). Such
75 indicators are however mainly useful to monitor potential fluctuations in the process evolution, but,

due to the numerous variables involved in the process and their potential mutual interactions, it is often hard to univocally identify trends that correlate the main features of the process (H_2 yield and rate, stability conditions) with the analytical and operational parameters of the fermentation system. In this regard, it may be useful, in order to describe the profile of the fermentation process, to derive suitable indicators to be adopted to explain and assess process performance. Ideally, it would be desirable to develop aggregate indicators capable of accounting for the complexity of the system and grasping the relevant characteristics of the process. To the best of the authors' knowledge, while attempts have been made in this regard, no such criteria are yet available for use in the interpretation of the fermentation process. This deficiency represents one of the main obstacles on the road to full-scale application, despite the general conspicuous availability of experimental data and the recent increase in studies carried out under continuous conditions. The absence of tools capable of extrapolating from the experimental data reliable indications about the expected performances and the main operating parameters to be adopted, has so far contributed to discouraging the investments from companies in the process.

To fill this gap, the present work aims at proposing an approach for the comprehensive chemometric and statistical analysis of the interrelations among the process operating variables and the monitoring indicators. The approach was tested on the results of an extensive and specially designed experimental campaign consisting of 17 continuous fermentation tests performed adopting different values for some of the main operating parameters. The nature of the underlying phenomena that lead to different H_2 production patterns was investigated by means of statistical processing. This is believed to contribute to clarify the main features of hydrogenogenic fermentation and, in turn, to foster the transition to full-scale implementation.

Table 1. Physical and chemical characteristics of cheese whey derived from various literature sources. Note: when available, either the observed range or the standard deviation (in parentheses) of values are provided.

pH	**Lactose**	**Proteins**	**Fats and oils**	**Mineral salts**	**BOD_5**	**COD**	**TS**	**TSS**	**TN**	**TKN**	**Reference**
---	g/L	g/L	g/L	g/L	g/L	g/L	g/L	g/L	g/L	g/L	
3.44	---	---	---	---	---	55.2 −74.5	---	9.4 (0.5)	---	0.15	(Ergüder et al., 2001)
4.9	50	---	---	---	---	74.2	66.8	22.1	---	1.49	(Ghaly & Kamal, 2004)
4.5−5	---	---	---	---	---	73−86	---	20−22	0.9−1.2	---	(Farizoglu et al., 2004, 2007)
---	39	7.2	7.9	4.6	---	---	58.7	---	---	---	(Rektor & Vatai, 2004)
4.2	49.2	---	---	---	---	102.5	70.9	---	1.76	---	(Ferchichi et al., 2005)
3.8−6.3	---	---	---	---	35.5−46.0	60.3−66.7	---	4.1−10	---	---	(Blonskaja & Vaalu, 2006)
4.9 (0.27)	45.9 (0.88)	2.71 (0.05)	9.44 (1.14)	---	37.7 (2.8)	68.6 (3.3)	59.3 (3.8)	1.3 (0.06)	---	1.12 (0.01)	(Saddoud et al., 2007)
---	43.9	1.42	---	6.1	---	100	---	---	---	---	(Yorgun et al., 2008)
5.8	---	---	0.99	---	29.5	73.4	---	7.2	---	---	(Janczukowicz et al., 2008)
4.46 (0.3)	---	---	0.9 (0.5)	---	40 (2.55)	60 (10)	59 (0.5)	1.5 (0.23)	---	---	(Gannoun et al., 2008)
4.7	42.6	---	---	---	---	86.3	---	---	0.2	---	(Azbar et al., 2009)
6.0−6.5	50−60	---	---	---	27−36	50−70	55−65	10−15	---	0.01−0.02	(Ebrahimi et al., 2010)
6.23 (0.1)	---	---	1.0 (0.1)	---	---	60.5 (2.8)	---	8.7 (1.34)	---	0.37 (0.05)	(Venetsaneas et al., 2009)
4.88 (0.01)	---	---	---	---	---	67.02 (6)	60.6 (0.3)	---	---	0.86	(Ghimire et al., 2017)
---	44−46	6.0−10.0	---	---	---	---	63.0−70.0	---	---	---	(Jelen, 2011)
---	---	---	---	---	---	60 (5)	---	---	1.33	---	(Castelló et al., 2018)
6.0 (0.1)	---	4.7	1.0 (0.1)	---	---	61 (1.5)	---	6.8		0.83	(Antonopoulou et al., 2008)
6.34	50.2	6.3	8.4	5.7	---	---	70.7	---	---	---	(Sanmartín et al., 2012)
---	---	8.9−14.1	7.8−14.5	5−10.1	---	---	69.2−76	---	---	---	(Henriques et al., 2011)
5.6	---	17.7	20.8	---	---	---	108.3	---	2.7	---	(Macedo et al., 2011)
4.2−4.5	---	---	---	6−8	---	90.6−117	---	---	---	---	(Mostafa Imeni et al., 2019)

--- = not reported

2. MATERIALS AND METHODS

2.1 Feedstock, inoculum and analytical methods

The substrate used was a synthetic cheese whey (SCW), which was then diluted to pre-set concentrations in order to adjust the organic loading rate (OLR). The inoculum consisted of activated sludge (AS) from the aerobic unit of a municipal wastewater treatment plant and was used for the start-up phase of the continuous hydrogenogenic experiments (see below) after a heat-shock pre-treatment at 105 °C for 30 min. For further details about sample storage and preparation for the tests, please refer to previous work (Polettini et al., 2022).

The substrate and the inoculum were characterized for the content of total solids (TS), volatile solids (VS), total organic carbon (TOC) and soluble carbohydrates. For the determination of TS and VS the Standard Methods for the Examination of Water and Wastewater (APHA; AWWA;WEF, 2017) were adopted. TOC was measured using a Shimadzu TOC analyser (TOC-VCHS and SSM-5000 module, Shimadzu, Japan). The analysis of soluble carbohydrates was performed through the colorimetric phenol–sulfuric acid method using glucose as the standard (Dubois et al., 1956).

The results of the analytical characterization for SCW and AS are shown in Table 2.

Table 2. Characterization parameters for SCW and AS

Parameter	Unit of measure	SCW	AS
Total Solids, TS	g/L	1109 ± 10.4	20.5 ± 1.8
Volatile Solids, VS	g/L	1076 ± 6.8	16.0 ± 1.4
Total suspended solids, TSS	g/L	441 ±26.3	18.2 ± 0.1
Total Organic Carbon (TOC)	g C/L	487 ± 8.3	9.0 ± 0.7
Soluble carbohydrates	g hexose/L	637 ± 2.5	0.7 ± 0.08

2.2 Continuous fermentation experiments

Seventeen continuous fermentation runs were performed in 1-L (working volume = 0.5 L) magnetically stirred lab-scale CSTRs operated in the mesophilic regime (T = 39 ± 1 °C). The system

123 pH was automatically controlled at 6.5, as indicated by previous batch optimization experiments
124 (Akhlaghi et al., 2017), by means of 1 M NaOH addition.
125 The conditions for the process start-up were derived from previous work (Akhlaghi et al., 2017). In
126 particular, the reactors were initially fed with diluted SCW and pre-treated (see above) AS at
127 concentrations of 10.8 g TOC/L and 16±1.4 g VS/L, respectively, flushed with N_2 to draw off oxygen
128 from the reactor headspace and maintained without further feeding (batch mode) for 40 h to allow for
129 the growth of H_2-producing biomass. The final fermentation broth was then thickened upon settling
130 for 24 h and retained after the discharge of the supernatant for the rest of the experimental run,
131 switching to continuous operation conditions, with no further inoculum feeding. Under this regime,
132 the feed was prepared by SCW dilution with deionized water to the desired substrate concentration,
133 S_f, that was determined based on the condition HRT = S_f/OLR.
134 The continuous fermentation tests were operated at different HRTs (6–20 h) and OLRs (16−129
135 g TOC/(L·d)) (see Table 3) in order to explore the features of fermentative H_2 production under a
136 range of operating conditions and identify key factors of the process that may be used to explain
137 differences in the observed yields.
138 The experimental design was arranged starting from the test condition corresponding to 95% of the
139 plateau of volumetric H_2 production as derived from preliminary batch experiments on the same
140 substrate (HRT = 16 h). The lower and upper HRT values (6 h and 20 h) were fixed, again on the
141 basis of batch results, as the residence times corresponding to the maximum H_2 production rate and
142 to a residual incremental H_2 production of less than 1%. The investigated OLR values were set to
143 span the widest practicable range based on the typical concentrations of real CW substrates. On the
144 basis of the results obtained at HRTs of 6, 16 and 20 h, further exploration of intermediate HRT
145 conditions at 8 h and 12 h and selected OLR values was made.
146 The feed solution was kept at 4 °C in a refrigerator to prevent degradation before testing. The reactors
147 were discharged and fed intermittently every hour through a peristaltic pump controlled by an
148 automatic control system. The flowrate was adjusted so as to keep low feeding and discharge times

149 compared to the HRT (between 6 and 9 minutes depending on the experimental conditions adopted)
150 while avoiding system perturbations.
151 Instantaneous samples of the fermentation broth were taken at selected time intervals and kept in a
152 refrigerator at −15 °C until analytical characterization.
153 Table 3. Experimental conditions adopted in the continuous tests

Run no.	Run code	HRT (h)	OLR (g TOC/(L·d))
1	R-6-32.5	6	32.5
2	R-6-65	6	65.0
3	R-6-97.5	6	97.5
4	R-6-129	6	129.0
5	R-8-32.5	8	32.5
6	R-8-65	8	65.0
7	R-8-129	8	129.0
8	R-12-65	12	65.0
9	R-12-97.5	12	97.5
10	R-16-16	16	16.0
11	R-16-33	16	33.0
12	R-16-52	16	52.0
13	R-16-65	16	65.0
14	R-16-129	16	129.0
15	R-20-32.5	20	32.5
16	R-20-52	20	52.0
17	R-20-65	20	65.0

154

155 **2.3 Analytical methods**

156 The fermentation process was studied through analytical characterization of both the fermentation
157 broth and the biogas.
158 The volumetric amount of biogas produced was measured by means of a eudiometer connected to the
159 fermentation reactor using the volume displacement principle. To this end, an electronic load cell
160 connected to the control system automatically recorded the weight of the displaced aqueous solution
161 (NaCl-saturated deionised water acidified with H_2SO_4 to pH = 2 to prevent gas dissolution)
162 discharged into a collection tank. The weight of the solution was converted to the corresponding
163 biogas volume by accounting for the liquid density and the temperature and pressure conditions
164 during the tests. All gas volumes were converted to standard thermodynamic conditions
165 ($T = 273.15$ K, $P = 10^5$ Pa).

166 The analytical characterization of biogas involved sampling with a gastight syringe and subsequent
167 gas-chromatographic analysis. The gas chromatograph was equipped with a thermal conductivity
168 detector and a 2-m stainless-steel packed column with an inner diameter of 1 mm. The operating
169 temperatures of the injector and detector were 100 and 130 °C, respectively, with He as the carrier
170 gas. The oven temperature was initially set at 80 °C and subsequently increased to 100 °C at 2 °C/min.
171 The fermentation broth was subjected to solid/liquid separation through centrifugation followed by
172 0.2 μm filtration, and subsequently analysed for TS, VS, TOC, dissolved organic and inorganic
173 carbon (DOC and DIC) as well as soluble carbohydrates according to the analytical methods
174 mentioned in section 2.1. In addition, the concentration of soluble metabolic products (SMPs),
175 including volatile fatty acids (VFAs: acetic [HAc], propionic [HPr], butyric + iso-butyric [HBu],
176 valeric + isovaleric [HVal], hexanoic + isohexanoic [HHex], heptanoic [HHep]) and ethanol [EtOH])
177 was determined in order to derive information about the prevalent metabolic pathways. The SMPs
178 were analysed in HCl-acidified (pH = 2) samples with a gas chromatograph equipped with a flame
179 ionization detector (FID) and a 30 m capillary column with an inner diameter of 0.53 mm. The
180 temperatures of the detector and the injector were 270 and 250 °C, respectively. The oven temperature
181 was initially set at 60 °C, held for 3 min at this value, subsequently increased to 180 °C at a rate of
182 10 °C/min and finally increased to 220 °C at a rate of 30 °C/min and held for 2 min.
183 For some samples additional metabolic products including lactic acid (HLa) and succinic acid (HSu)
184 were also analysed through high-performance liquid chromatography (HPLC) using an UV-vis
185 detector (λ = 220 nm) and a refractive index detector. The column had sizes of 300 × 7.8 mm. The
186 analysis was performed at 40 °C under isocratic conditions using 0.008 N H_2SO_4 as the eluent with
187 an elution rate of 0.6 mL/min.
188 All the analytical determinations were performed in duplicate.
189

190 **2.4 Statistical analyses**

191 The analytical data were processed to derive a set of variables that were considered to be relevant as
192 process monitoring parameters. Three different types of variables were accounted for in the analysis:
193 1) independent operating variables (HRT and OLR); 2) process performance parameters (H_2 and CO_2
194 production yield); and 3) process monitoring variables (individual and total amounts of metabolites
195 produced and the fractions of gasified, dissolved and particulate carbon). A supplementary variable
196 was further added for the statistical analysis to account for the stability characteristics of the tests.
197 The list and definition of the 14 variables adopted is reported in Table 4.
198
199 Table 4**.** Process variables adopted for the statistical analyses

Type of variable	Definition	Symbol	Unit of measure
Operating variables	Hydraulic residence time	HRT	h
(independent variables)	Organic loading rate	OLR	g TOC/(L·d)
Process performance indicators	H_2 production yield	HPY	L H_2/kg TOC_{feed}
(response variables)	CO_2 production yield	CPY	L CO_2/kg TOC_{feed}
Process monitoring variables	Metabolite production:		
	ethanol production	EtOH	% TOC_{feed}
	acetate production	HAc	% TOC_{feed}
	propionate production	HPr	% TOC_{feed}
	butyrate production	HBu	% TOC_{feed}
	total SMPs production	SMP	% TOC_{feed}
	Fraction of gasified carbon	GasC	% TOC_{feed}
	Fraction of dissolved inorganic carbon	DIC	% TOC_{feed}
	Fraction of residual soluble carbon	Res_sol	% TOC_{feed}
	Fraction of residual particulate carbon	Res_part	% TOC_{feed}
Supplementary variable	Stability index	SI	---

200
201 The dataset consisted of a ($m \times p$) matrix, with m = number of data points (63, corresponding to
202 samples taken at different times during the 17 experimental runs – see Supplementary Information
203 document) and p = number of variables (14). The data points (individuals) were processed through
204 Principal Component Analysis (PCA), which is a statistical technique that allows extracting the
205 relevant information from a certain dataset in the presence of multiple inter-correlated variables. The
206 extracted information is expressed as a function of a reduced number of new mutually uncorrelated
207 variables (the so-called principal components, PCs), that are obtained from a linear combination of
208 the original variables by minimizing the loss of information associated to the reduction of the dataset
209 dimensionality. PCA is a useful tool to group data on the basis of similarities (finding common

patterns among the individuals in each group) and isolate data that display statistically significant differences. For the fermentation tests, the PCA was applied in order to explain the observed H_2 production performance and relate it to key factors of the process. The statistical analyses were carried out using the *FactoMineR* package (Lê et al., 2008) available in the R environment (R Core Team, 2021).

3. RESULTS AND DISCUSSION

3.1 H_2 production and organic matter degradation

Previous studies of the research group on continuous H_2 production from SCW (Boni et al., 2021; Polettini et al., 2022) showed that the operating conditions adopted (HRT/OLR combination) significantly affected both the yield and rate of conversion of the organic substrate into H_2. Due to the high sensitivity of the hydrogenogenic biomass to the fermentation environment, the process performance also displayed a large variability over time. In some cases, the fluctuations of the H_2 volume produced tended to progressively dampen with the operation time reaching steady conditions. Conversely, in other tests the observed oscillations were as large as the average value (or higher), or the measured H_2 production gradually declined to almost zero as a result of progressive washout of the hydrogenogenic species. A discriminating criterion between stable and unstable operation was based on a dynamic stability index, derived as:

$$SI = 1 - \frac{\text{st.dev. } (\text{H}_2 \text{ production})}{\text{average } (\text{H}_2 \text{ production})} \quad (1)$$

using the moving average and standard deviation of the volumetric H_2 production measurements. The period for the calculation of the statistical parameters of the analytical data (recorded on an hourly basis) was assumed to be 1 HRT. Stability was considered to be achieved when SI was ≥ 0.75 for at least 3 consecutive periods until the end of the test (for further details please refer to (Boni et al., 2021; Polettini et al., 2022)).

While a detailed discussion of the criteria to identify the stability condition for H_2 production is

provided in the above mentioned companion study, it is worth stating here that only under specific operating conditions could steady-state operation be achieved. Furthermore, in several cases, prolonged timespans were required to attain the stability condition, pointing out at the existence of long transient periods for either biomass acclimation or competition among the different microbial species involved. Figure 1 reports a summary of the results obtained in terms of HPY and hydrogen production rate (HPR), showing the main statistical values of the distribution of hourly H_2 production data. For the stable runs, such parameters are reported separately for the initial transient period and the stable operation regime, while for the remaining tests the box plots refer to the whole duration of the experiments. Concerning the stable period, the lowest performance in terms of HPY was observed for the R-12-97.5 (16.2 L H_2/kg TOC) and R-8-129 (18.6 L H_2/kg TOC) runs and in terms of HPR for the R-20-32.5 run (0.8 L H_2/(L_{react}·d)). It was however evident that properly adjusting the operating conditions was able to produce a more than threefold increase in H_2 production. As a matter of fact, the best results, in terms of both HPY and HPR, were displayed by run R-6-97.5 (HPY = 49.5 L H_2/kg TOC; 4.8 L H_2/(L_{react}·d)), followed by run R-8-65 (HPY = 42.1 L H_2/kg TOC; 2.7 L H_2/(L_{react}·d)). The fact that short HRTs were generally associated to the region of higher HPY confirms the results of previous literature studies, which indicated that HRTs below 12 h are required to enhance the activity of hydrogenogenic microbial species (Davila-Vazquez et al., 2009; García-Depraect et al., 2020; Palomo-Briones et al., 2017).

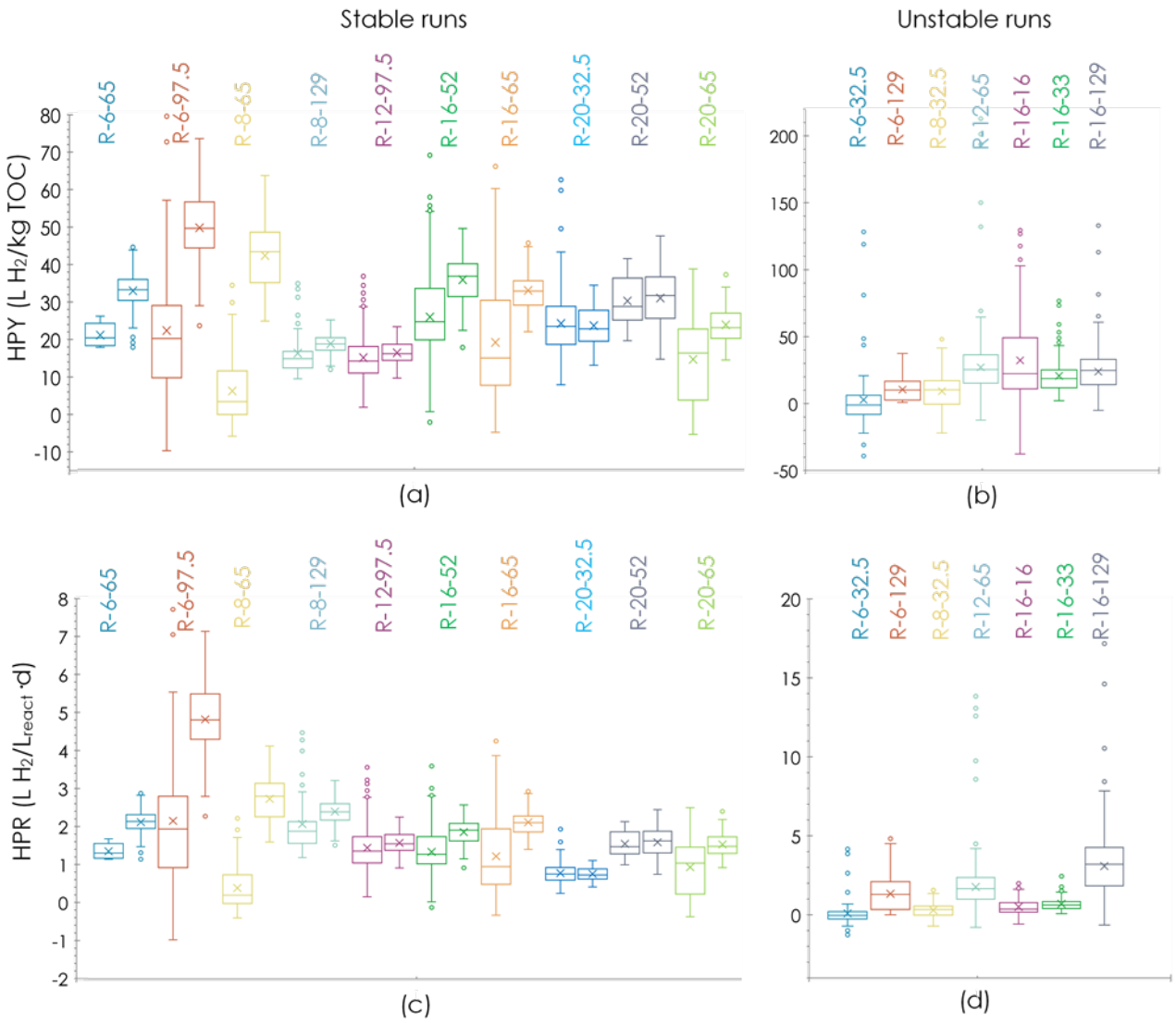


Figure 1. Average values (×) and range of variation (quartiles and min-max range) of the HPY (a, b) and HPR (c, d) for the individual experimental runs. Note: each box pair of the stable runs refers to the initial transient period (1st box) and the stable period (2nd box). Dots represent the outliers.

To explain the H_2 production performance observed in the different tests, the fate of organic matter during the process was studied through the analysis of carbon partitioning among the various products of the metabolic reactions. Assessing either the carbon mass balance (see e.g. (Akhlaghi et al., 2019; Cai et al., 2010; Cooney et al., 2007; Hamilton et al., 2010; Sarma et al., 2017)) or the COD/electron balances (see e.g. (Cota-Navarro et al., 2011; Lee and Rittmann, 2009; Moat et al., 2002)) during fermentation can assist the interpretation of substrate conversion into the end products and help

explain the observed H_2 yield on the basis of the products obtained with a view to process optimization. Considering that in DF the amount of electrons directed to H_2 production is a comparatively low portion of the total amount of mobilized electrons, in the present study the carbon mass balance was preferred for use to describe the fate of organic matter. The carbon balance was derived from the measured concentrations of the analysed species in the fermentation broth and the gaseous phase; the only term that was derived indirectly was the mass of dissolved inorganic C (DIC) in the liquid phase, that was estimated from the CO_2 partial pressure in the gas phase and the pH and temperature of the liquid phase by applying the Henry's law. The analysis of organic matter partitioning included the following terms: 1) SMPs; 2) residual organic C, present as both soluble and particulate species (i.e., non-degraded compounds, other metabolic products not directly analysed, and C associated to biomass cells), which were in turn calculated from the difference between TOC (or DOC) and (SMP + carbohydrate) concentrations; 3) DIC; 4) CO_2 in the biogas. A conceptual scheme showing the meaning of the different terms of the mass balance and the partitioning of the C species between the solid and gaseous phases is depicted in Figure 2. The term "balance" that appears in the figure is the amount of C required, due to experimental errors/uncertainties, to meet the mass conservation principle.

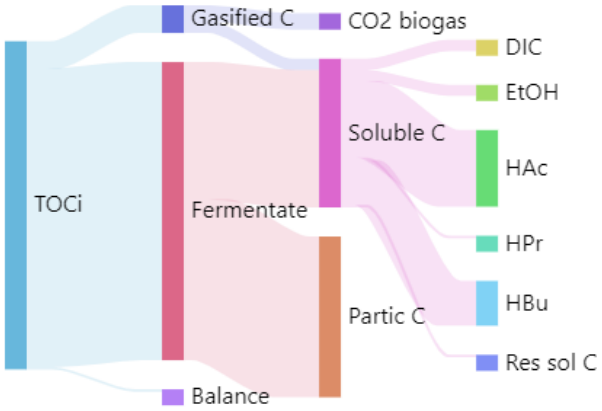


Figure 2. Conceptual layout of C partitioning among the different forms

286 The results of the C mass balance for a number of example runs are reported in Figure 3. In the plots,
287 the percent distribution of the input TOC among the different forms (left) and its partitioning among
288 the individual SMPs (right) are depicted. A number of distinguishing features can be derived that
289 relate C speciation to the observed HPY. In general terms, it can be noticed that, as expected, a major
290 portion (88±14%) of the input C was retained in the liquid phase during the fermentation process.
291 Moreover, for this fraction of C, the proportions associated to the particulate and soluble forms were
292 found to be of the same order of magnitude, although with some fluctuations across the tests. As
293 shown in Figure 3, the amount of soluble carbon in the fermentation broth was likely mainly
294 associated to the SMPs (see below for further details) rather than to non-degraded soluble molecules.
295 It is also worth mentioning that for most of the fermentate samples the analysed metabolites explained
296 more than 85% of DOC (as shown by the low values of the residual soluble C term in the plots),
297 indicating that the pool of fermentation products was mainly made of HAc and HBu, followed by
298 lower contributions of HPr and EtOH; on the other hand, the cumulative amount of HVal, HHex and
299 HHep always accounted for less than 10% of TOC and in some cases was even undetectable. In some
300 selected samples, additional metabolites were identified which included HLa and HSu, although this
301 could not be systematically assessed for all tests due to analytical constraints.
302 As far as the fraction of particulate C was concerned, this could in principle be present in different
303 forms, including either non-hydrolysed molecules from the original substrate or microbial cells. It
304 has been shown in previous studies (Jung et al., 2010; Lay et al., 2010; Palomo-Briones et al., 2017)
305 that in DF the biomass growth yield may range from ~0.2 up to 0.5 (on a COD basis), therefore the
306 values obtained in the present work for the amount of particulate C are in line with other literature
307 references and may be interpreted as being predominantly related to the contribution of biomass (Kim
308 et al., 2006).
309 In more specific terms, comparing the two tests that displayed the highest H_2 production performance,
310 it was noted that during the stability period run R-8-65 (with a HPY of 52–56 L H_2/kg TOC) displayed

311 notably higher associated HBu (21.1–25.8% TOC) and HAc (17.1–23.5% TOC) concentrations
312 compared to run R-6-97.5 (HPY = 53–64 L H_2/kg TOC, HBu = 9.5–13.2% TOC, HAc = 11.7–14.8%
313 TOC). Based on the results of a metabolic model that was described in a companion study (Polettini
314 et al., 2022), for run R-8-65 the analysed samples were found to display slightly higher HAc and HBu
315 concentrations net of homoacetogenesis and other interfering reactions, indicating some larger
316 contribution of hydrogenogenic pathways compared to the other experiments. Conversely, the initial
317 transient phase for run R-8-65 was characterised by remarkably lower SMPs concentrations (HAc =
318 14.0–17.8% TOC; HBu = 1.4–7.6% TOC) which explain the poor H_2 production performance
319 observed in this period (HPY = 2–12 L H_2/kg TOC).
320 When looking at the metabolic products detected for run R-6-65 compared to R-8-65, HAc production
321 during the stable period was much higher (average = 26.9% TOC vs. 18.1% TOC) while HBu was
322 only slightly lower; in addition, some occurrence of alcoholic fermentation was observed (EtOH =
323 2.2–5.1% TOC). The lower H_2 production measured for run R-6-65 (HPY = 39–45 L H_2/kg TOC)
324 was explained by the considerable contribution of homoacetogenic consumption (300–490
325 L H_2/kg TOC) that was estimated by the above mentioned metabolic model. Therefore, although
326 having a higher gross H_2 production from the hydrogenogenic acetic and butyric pathways, the
327 performance of run R-6-65 was negatively affected by the significant competitive role played by
328 homoacetogenic microorganisms. It was also interesting to note that the contribution of the residual
329 soluble C for run R-6-65 was negligible, indicating that HAc, HBu and to a much lesser extent EtOH
330 were the only metabolic products produced at appreciable levels. Conversely, for the two tests R-8-
331 65 and R-6-97.5 the estimated values of the residual soluble C (5.4–29.2% TOC and 14.8–26.5%
332 TOC, respectively) pointed out at a presumably more complex set of microbial reactions with the
333 formation of an appreciable amount of additional metabolic products. Unfortunately, in these cases it
334 was not possible to determine the nature of the products formed, however the results obtained for the
335 other samples (see below) may suggest that lactic and succinic pathways were also taking place

concomitantly with the previously mentioned reactions.

Among the tests in which additional metabolites were measured, run R-20-65 revealed a significant presence of HLa during the initial stages of the process, as already noted by previous studies for HRTs ≥ 12 h (Palomo-Briones et al., 2017) that clearly indicated that long HRTs are favourable towards the selection of lactic acid bacteria. Other studies (Park et al., 2015) also suggested that HLa is formed at high concentrations during the perturbation phases and tend to disappear when these are recovered. In agreement with this observation, during run R-20-65 the whole transient phase was dominated basically by HLa and HAc (concentrations of 5.8–22.2% TOC and 6.9–9.6 % TOC, respectively), but the former was found to progressively decrease during this phase as the operating conditions approached stability. The lower HPY observed for this test compared to, for example, run R-8-65 was clearly related to a lower total amount of SMPs measured in the fermentation broth. Similar considerations also apply for other tests with high HRTs such as run R-16-65.

Compared to R-16-65, run R-16-33 was classified as unstable and displayed a gradual decline in the production of both HAc and HBu, with an accompanying reduction in HPY. Among the other unstable runs, R-6-129 can be taken as an example of the evidence of the progressive selection of competitive microbial species over the testing time. In this case, the only metabolic products measured at detectable concentrations were HAc and HBu, and the decline of H_2 production over the testing time was likely due to the gradual decrease in the relative proportion between the hydrogenogenic and competitive biomass species. As a matter of fact, while HAc production was approximately constant during the testing period and so was the ratio between the contributions of the hydrogenogenic and homoacetogenic pathways, HBu production was found to progressively decrease with time, from 14.0 to 6.7 % TOC, mirroring the declining trend of HPY.

Overall, taking into account the results shown in Figure 3 and the findings from the metabolic model detailed in previous paper (Polettini et al., 2022), it was concluded that the relative contribution of the H_2-producing pathways decreased at longer HRTs (see Section 3.2 for further details). This confirms that operation at low HRTs is an effective strategy to promote H_2 production. Among the

362 reactions recognized as either H_2-neutral or competitive with hydrogenogenesis, alcohols were
363 always found to be produced at much lower levels than acetate, butyrate and (when detected) lactate
364 and succinate; nevertheless, the contribution of alcoholic fermentation was observed to increase at
365 longer HRTs and lower OLRs. Some butyrate production that originated from a chain elongation
366 reaction starting from acetate and ethanol (Spirito et al., 2014) and therefore not associated to H_2
367 production, was also observed, although with a varying contribution depending on the operating
368 conditions adopted. Among the H_2-consuming pathways, propionic fermentation had little relevance
369 in all the investigated conditions, while homoacetogenesis was found to play a very important role in
370 H_2 consumption as well as to be correlated inversely with HRT and positively with OLR.
371 The considerations above therefore point out at two contrasting factors that play a role at low HRTs:
372 as a matter of fact, while decreasing the HRT is expected to favour the growth of hydrogenogenic
373 biomass, it would also lead to increased H_2 consumption through homoacetogenesis. This suggests
374 that, in order to maximize H_2 production, biokinetic control should be coupled with other
375 biotechnological strategies to inhibit homoacetogenic microorganisms.
376 On the basis of the simulations of the above mentioned metabolic model, the two best conditions in
377 terms of H_2 production were governed by the following contributions of the main metabolic
378 pathways: 38.5–52.2% (R-6-97.5) and 33.9–38.7% (R-8-65) for the acetate pathway; 12.4–16.5%
379 (R-6-97.5) and 12.2–20.9% (R-8-65) for the butyrate pathway; 34.5–45.4% (R-6-97.5) and
380 45.2–50.1% (R-8-65) for homoacetogenesis.
381
382

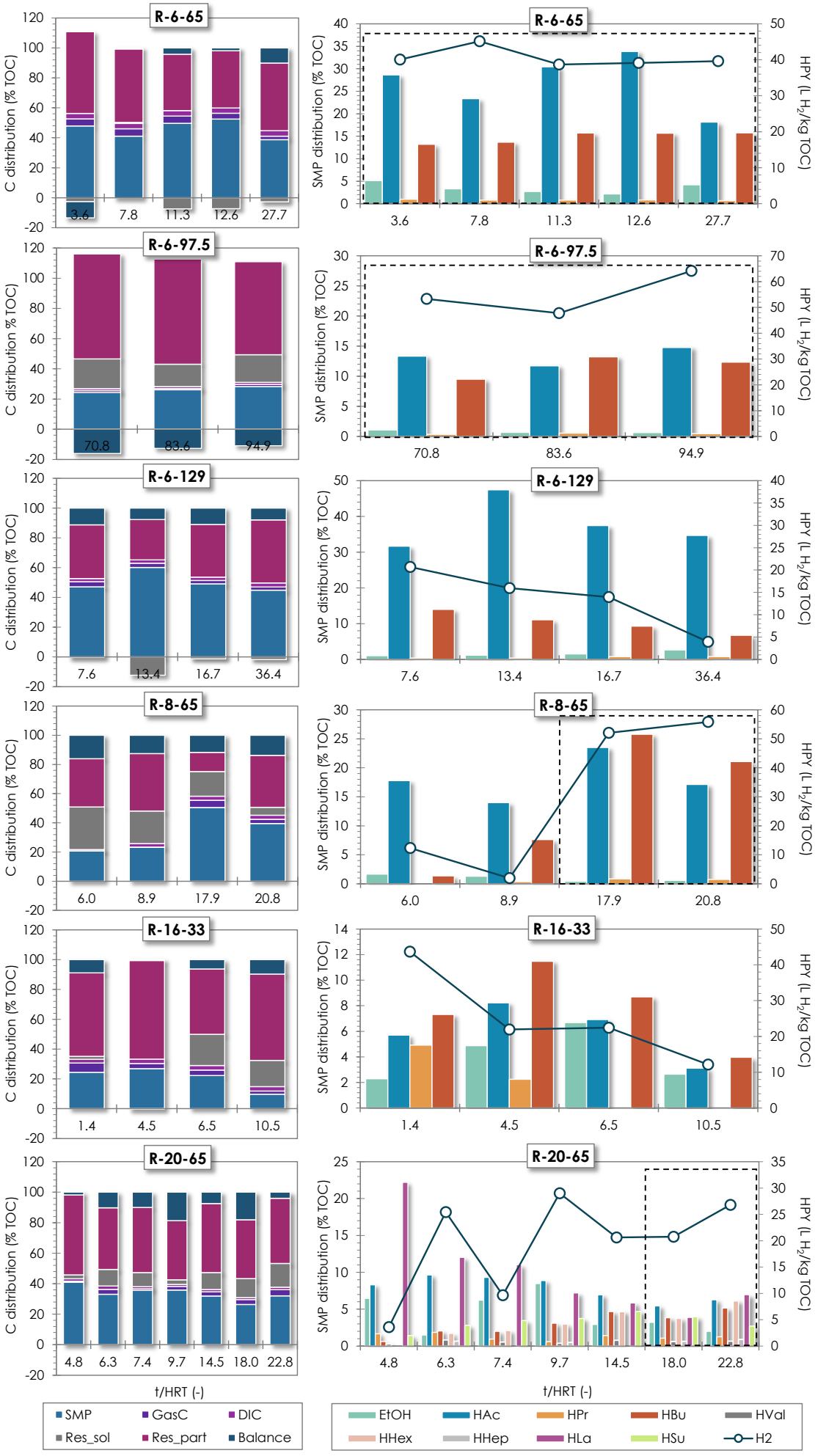

R-6-65
C distribution (% TOC)
SMP distribution (% TOC)
HPY (L H2/kg TOC)
3.6
7.8
11.3
12.6
27.7
R-6-97.5
70.8
83.6
94.9
R-6-129
7.6
13.4
16.7
36.4
R-8-65
6.0
8.9
17.9
20.8
R-16-33
1.4
4.5
6.5
10.5
R-20-65
4.8
6.3
7.4
9.7
14.5
18.0
22.8
t/HRT (-)
SMP
GasC
DIC
Res_sol
Res_part
Balance
EtOH
HAc
HPr
HBu
HVal
HHex
HHep
HLa
HSu
H2

Figure 3. Carbon mass balance (left) and partitioning of the feed TOC among the individual SMPs (right) for selected experimental runs at specified sampling times (Notes: the HPY values indicated refer to the sampling times reported and may differ from the average values shown in Figure 1. The dashed areas indicate the stable period for each test)

3.2 Effects of the process variables

The PCA of the experimental data revealed that the first 5 PCs were required to explain ~81% of the overall variance. Nevertheless, the first 3 PCs were found to explain most of the variance, with a cumulative contribution of 65%.

The individual PCs allowed grouping the different variables related to the evolution of fermentation into a smaller set of parameters explaining specific features of the metabolic process while retaining most of the information associated to the original data. It is worth mentioning that the variables that were used for the statistical analyses were selected following a first screening based on Spearman's correlations (results not shown here) that allowed to exclude factors that were found to be mutually correlated.

The analysis of the correlations between the PCs and the original variables is reported in Figure 4 in terms of loading plots depicted in the planes of PCs couples. In loading plots both the distance of a given variable from the origin and the relative position of variables in the plot convey important information. Loadings that are close to −1 or 1 (variable lying close to the circle) indicate a strong correlation with the specific PC. Furthermore, variables grouped together in the plot are positively correlated, variables lying on opposed quadrants display a negative correlation, while orthogonal variables are non-correlated. The inspection of the loading plots reveals that PC_1 was correlated with total SMPs (r = 0.96), HAc (r = 0.94), HRT (r = –0.80) and the fraction of residual soluble C (r = –0.74). The observed correlation among the HRT and the amount of acetate and total metabolites confirms the findings of previous studies (Palomo-Briones et al., 2017). The second factor, PC_2, was

correlated mainly with CO_2 production and the fraction of gasified TOC (r = 0.92, r = 0.80), HBu (r = 0.70) and H_2 production (r = 0.60). Although not specifically included in the PCA, it is emphasised here that HPR was found to be highly correlated (r = 0.89) with HPY, and similar considerations also apply for CO_2 production (r = 0.80). As a result, the considerations provided below regarding HPY and CPY are also valid for the corresponding rates. The third component, PC_3, displayed appreciable correlations with OLR (r = –0.79) and HPr (r = 0.62), while PC_4 was correlated with DIC (r = 0.63) and PC_5 with the fraction of particulate C (r = 0.78). On the basis of the calculated correlations, it was therefore inferred that the two independent parameters HRT and OLR were significantly associated to PC_1 and PC_3. Furthermore, PC_1 mainly accounted for the overall substrate conversion into SMPs (particularly HAc, which in most cases was the prevalent species detected), while PC_2 retained most of the information on biogas (H_2 and CO_2) production along with butyrate generation, which can be justified by the fact that the measured amount of HBu mainly derived from the hydrogenogenic butyrate pathway (see e.g. (Castelló et al., 2018; Ghimire et al., 2017; Kim et al., 2006; Lee et al., 2008)). In this regard, some authors have shown that butyrate production from hydrogenogenic fermentation cultures is thermodynamically favoured (Lee et al., 2008).

In order to relate the PCA results to the stability features of the fermentation process, a supplementary variable was added to the analysis. In particular, stability was measured through the dynamic stability index (SI) defined by Eq. (1), which was calculated – depending on the specific test considered and the operating phase – during the transient period, the stable period or the whole test length as described for Figure 1. Since the SI was defined over 1-HRT periods while the sampling interval was lower than 1 HRT, all data points for each test were assigned the same SI value on the basis of the operating regime. As visible in Figure 4, the SI was found to be correlated with PC_2 and in turn with biogas and butyrate production. As a result, the PCA conducted on the experimental data was capable of recognizing the stability characteristics of the analysed samples and associating them to one of the identified factors.

The individual contribution of the original variables to the PCs is shown in Figure 5, where the dashed

460 lines indicate the value that would be expected if each variable were assumed to contribute uniformly
461 to a specific PC (in this case = 100%/13 = 7.7%). Variables displaying contribution levels beyond
462 this cut-off are interpreted as important variables for the PC of concern, since they contribute to it by
463 more than the average. Specifically, the results of the analysis indicated that the most relevant
464 contribution was provided by the following variables:

465 • for PC_1: total SMPs, HAc, HRT and residual soluble C
466 • for PC_2: CO_2 production + fraction of gasified C, HBu and HPY
467 • for PC_3: OLR, HPr, residual soluble C and residual particulate C
468 • for PC_4: DIC, HPY, HPr and EtOH
469 • for PC_5: residual particulate C and HPY

470 The predictions of the PCA model using the above mentioned five PCs as the relevant factors are
471 reported in Figure 6 for the original variables that showed higher correlations with the PCs. It was
472 found that the reduced set of factors was capable of providing a reasonably close description of the
473 original data for most of the variables of concern. However, for HAc and total SMPs production, the
474 estimations of the PCA model were found to be less accurate in the extreme regions of values,
475 underestimating low concentrations and overestimating high concentrations of such metabolic
476 products.

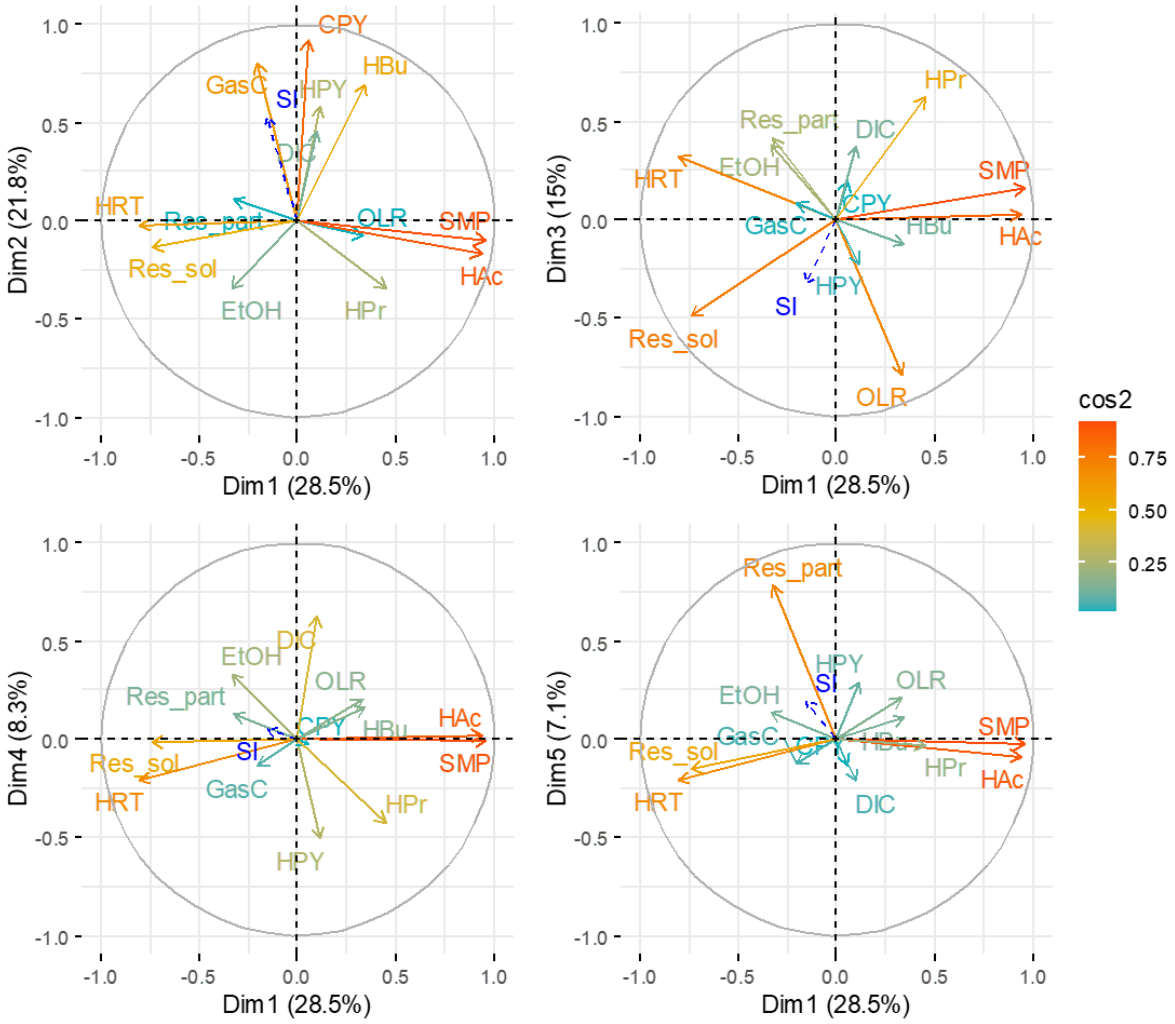
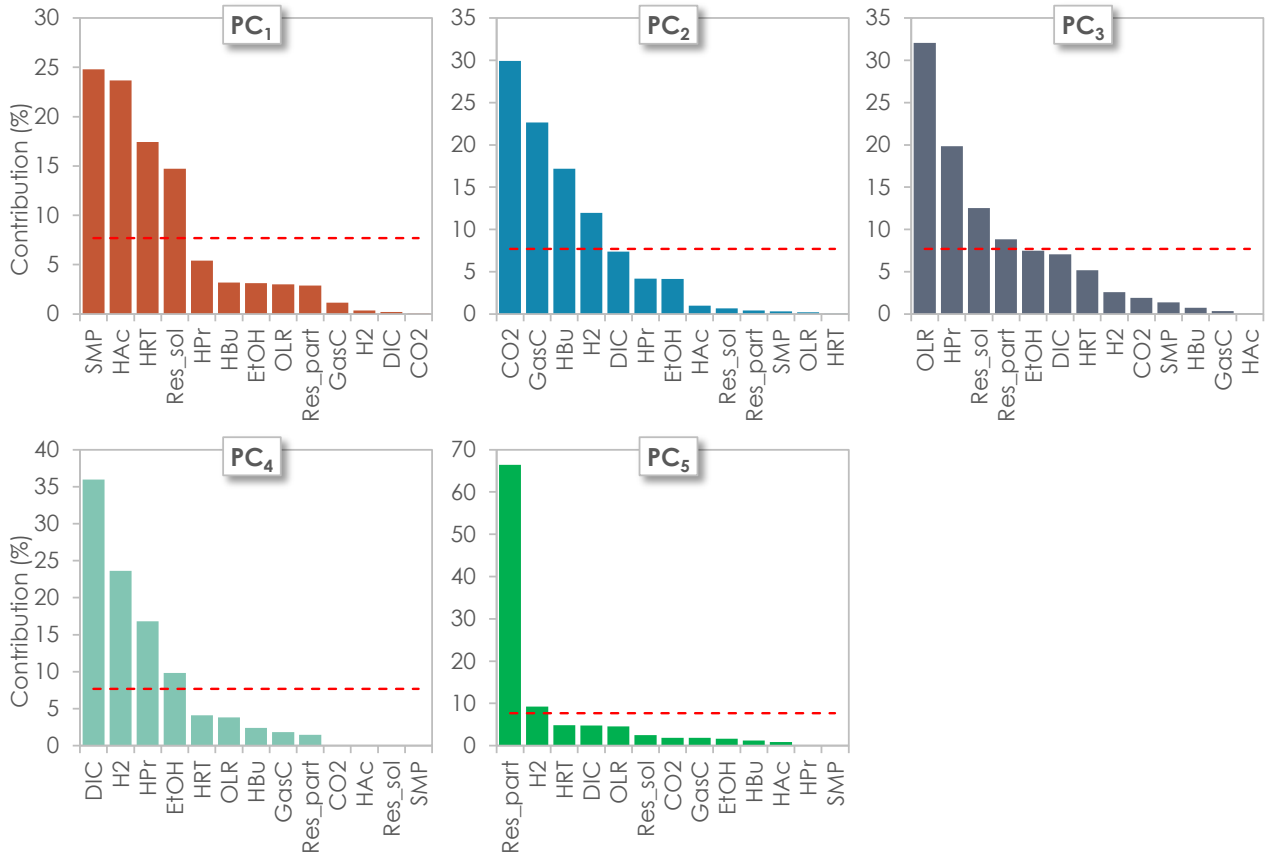


Figure 4. Loading plots in the first 5 PCs space



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494 Figure 5. Contribution of the original variables to the first 5 PCs

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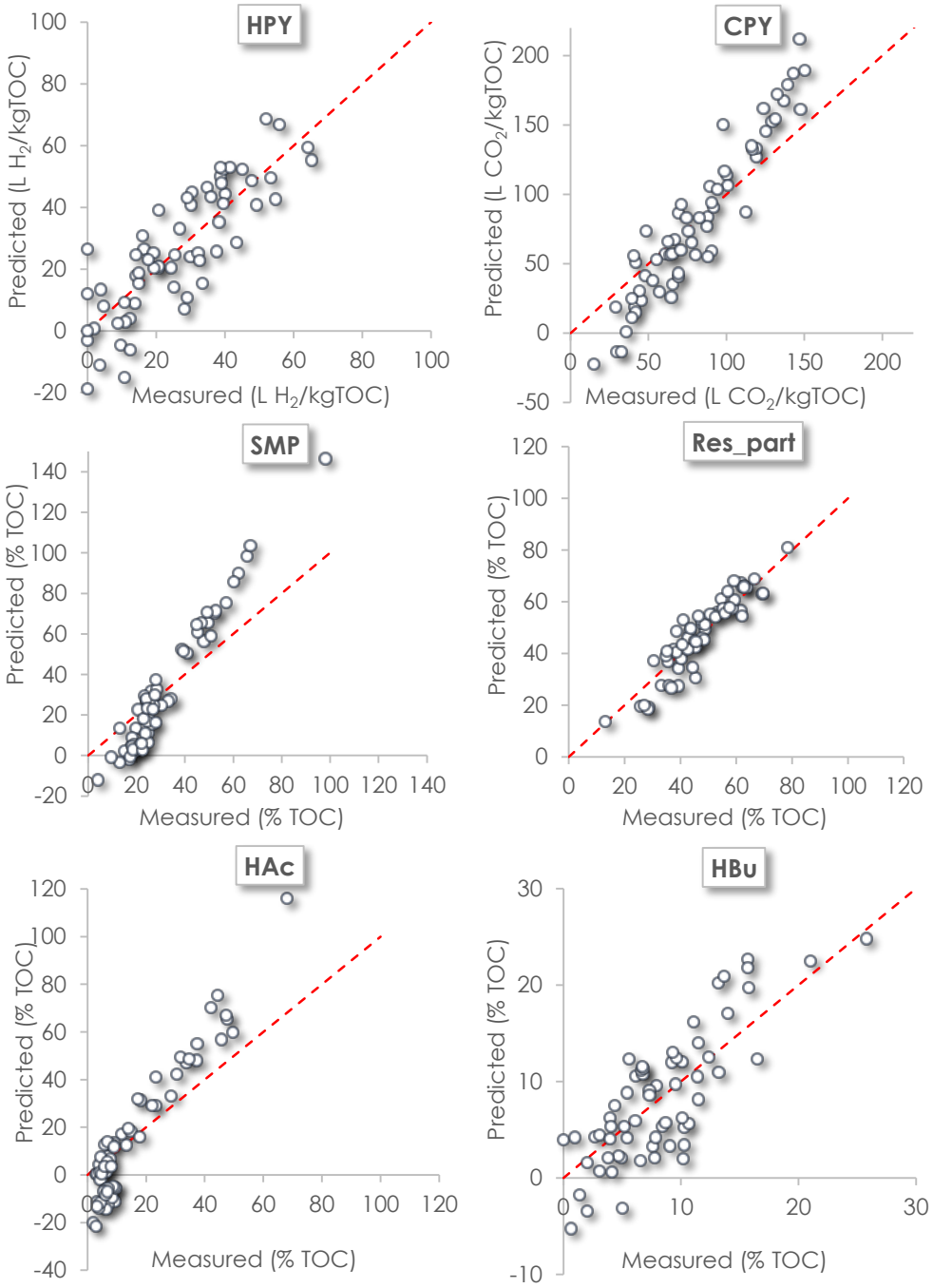

Figure 6. Predicted-vs.-fitted values of selected parameters (model: 5-PCs PCA)

Conclusions

In the present work, the results of continuous fermentation experiments for biohydrogen production from SCW were analysed based on carbon mass balance considerations and statistical processing. The H_2 yield was found to vary largely depending on the specific operating conditions adopted in the

503 experiments, being related to both the amount and the relative distribution of the individual
504 metabolites generated. In most cases, the pool of metabolic products was dominated by acetate and
505 butyrate; however, they were found to derive not only from hydrogenogenic pathways but also from
506 competing reactions and the contribution of the different metabolic routes was in turn a function of
507 the specific operating conditions. For some experimental runs, lactate and – to a lesser extent –
508 succinate were also detected in the fermentation broth, indicating the occurrence of a more complex
509 set of metabolic reactions that had negative implications in terms of substrate conversion into H_2.
510 H_2-neutral pathways (that however compete for substrate utilization with other metabolic reactions)
511 included ethanol fermentation and chain elongation of acetate and ethanol to butyrate, while H_2-
512 consuming reactions involved propionate fermentation and homoacetogenesis. Among these,
513 homoacetogenesis was found by far to be the major contributor to the set of biochemical reactions;
514 the order of magnitude of H_2 consumption by homoacetogenic biomass was comparable to that of H_2
515 production from acetic and butyric fermentation. While the H_2-producing pathways were favoured
516 by short HRTs, homoacetogenesis displayed a negative correlation with HRT and a positive
517 correlation with OLR.
518 The statistical analysis of the experimental results also showed that ~80% of the information
519 associated to the set of process parameters analysed could be described by five factors that
520 individually explained specific features of the process itself. Among these, the first three components
521 were able to explain cumulatively the largest share (65%) of the sample variance. In particular, the
522 first principal component grasped the effect of retention time and total metabolic products formed,
523 with shorter retention times being accompanied by higher total metabolites (and acetate) production
524 and in turn decreased fractions of residual soluble carbon. The second factor mainly accounted for
525 biogas (H_2, CO_2) generation and butyrate production, showing a correlation between the two despite
526 the fact that H_2-neutral butyrate production from chain elongation reactions was also likely to play a
527 role during the experiments. The third principal component mainly explained the effect of the organic
528 loading rate and that of propionate production, which showed a mutual negative correlation.

529 The statistical analysis was also capable of grasping the stability features of the process, which were
530 mainly explained by the second factor.
531 In summary, processing of the experimental data proved useful to group the performance and
532 monitoring indicators of fermentation into a reduced set of variables, each representing a specific
533 feature of the process. This result is worthy of interest as it contributes to define indications regarding
534 pivotal operating parameters, such as the HRT and OLR, and the metabolic pathways that are suitable
535 for ensuring the stability of the process.
536 Low TRL processes go through development phases characterized by a huge number of studies that
537 are performed considering different substrates and applying a great variability of operating conditions
538 (number of parameters considered and range of values adopted), the latter often far from real-scale
539 application. Depending on the complexity of the process under concern, these phases can last so long
540 as to determine the stasis of development or even its abandonment. Studies such as the present are
541 innovative as they give momentum to overcome a critical step in the development path.The
542 production of hydrogen through DF is no exception. The process is very sensitive to a wide array of
543 factors, which are also mutually interacting. Such a complexity, although expected for transitional
544 biochemical processes, represents an obstacle to full-scale implementation, which is still a long way
545 off despite the large number of studies conducted so far on various substrates. Systematic analysis,
546 processing and understanding of data from reduced-scale experimental tests are intended to validate
547 the research outcomes from previous studies and consolidate the knowledge on the fermentation
548 process, in turn providing reliable indicators that may form a solid basis for the transition to full-scale
549 application of the process itself.
550

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