CONTINUOUS BIO-H_2 PRODUCTION FROM CHEESE WHEY. PART 2: FACTOR-BASED ASSESSMENT

A. Polettini[(1)], R. Pomi[(1)], A. Rossi[(1)], T. Zonfa[(1)], G. De Gioannis[(2)], A. Muntoni[(2)]

[(1)] Department of Civil and Environmental Engineering, University of Rome "La Sapienza" (Italy)
[(2)] Department of Civil and Environmental Engineering and Architecture, University of Cagliari (Italy)

ABSTRACT

***Keywords**: dark fermentation, continuous H_2 production, process stability, statistical analysis*

1. INTRODUCTION

Dairy industry residues are regarded as attractive substrates for H_2 production through dark fermentation (DF) due to their organic content, ranging largely between 0.8 and 102 g COD/L (Carvalho et al., 2013). While high concentrations of biodegradable organic matter in such residues may pose potential environmental hazards and may overload 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 cheese whey (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 recent 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. While considerable advance has been made to relate the process parameters and the metabolic routes, 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). On the other hand, in order to assess the process viability under continuous operation for industrial scale-up purposes, deeper understanding of some unresolved issues is mandatory. The main open questions in this regard include:

- defining strategies to limit substrate-competitive microbial pathways, which typically encompass solventogenesis (Antonopoulou et al., 2012), lactate fermentation (Castillo Martinez et al., 2013; Sikora et al., 2013) and heterotrophic homoacetogenesis (Ljungdahl et al., 1989)
- defining strategies to limit the competitive influence of hydrogenotrophic microbial pathways, the most common of which comprising autotrophic homoacetogenesis (Saady, 2013), propionate fermentation (Antonopoulou et al., 2008a) and methanogenesis
- optimizing the operating variables to drive the metabolic pathways towards the desired direction

- preventing process inhibition by either internal or external factors (such as excessive buildup of metabolic products, presence of toxic substances, etc. (Castelló et al., 2020)).

Properly addressing such issues is essential to ensure at the same time adequate stability of the biochemical reactions involved and satisfactory H_2 production yields. It is worth highlighting that different parameters, mainly involving analyzing the biogas volume and composition, are commonly used to monitor the performance of DF, while other indicators of the process evolution include measuring the production of metabolites and characterizing the microbial community in the fermentation system (Antonopoulou et al., 2008b; Azbar et al., 2009; Castelló et al., 2018, 2009; Davila-Vazquez et al., 2009; Venetsaneas et al., 2009; Yang et al., 2007). Such 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 characteristics 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. In the present work, the results of an experimental campaign including 17 continuous fermentation tests are presented, and the nature of the underlying phenomena that led to different H_2 production patterns is investigated by means of statistical processing of the experimental data.

2. MATERIALS AND METHODS

2.1 Feedstock, inoculum and analytical methods

The substrate used was a synthetic cheese whey (SCW), which was diluted to preset 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 et al., 2017) were adopted. TOC was measured using a Shimadzu TOC analyzer (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 1.

Table 1. 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 pH was automatically controlled at 6.5 by means of 1 M NaOH addition.
The conditions for the process start-up were derived from previous work (Akhlaghi et al., 2017). In particular, the reactors were initially fed with SCW and pre-treated (see above) AS at concentrations of 10.8 g TOC/L and 16±1.4 g VS/L, respectively, flushed with N_2 to draw off oxygen from the reactor headspace and maintained without further feeding (batch mode) for 40 h to allow for the growth of H_2-producing biomass. The final fermentate was then thickened upon settling for 24 h and retained after the discharge of the supernatant for the rest of the experimental run, switching to continuous operation conditions, with no further inoculum feeding.
The continuous fermentation tests were operated at different HRTs (6−20 h) and OLRs (16−129 g TOC/(L·d)) (see Table 2) in order to explore the features of fermentative H_2 production under a range of operating conditions and identify key factors of the process that may be used to explain differences in the observed yields.
The feed solution was kept at 4 °C in a refrigerator to prevent degradation before testing. The reactors were discharged and fed intermittently every hour through a peristaltic pump controlled by an automatic control system. The flowrate was adjusted so as to keep low feeding and discharge times compared to the HRT (between 6 and 9 minutes depending on the experimental conditions adopted) while avoiding system perturbations.
Instantaneous samples of fermentate were taken at selected time intervals and kept in a refrigerator at −15 °C until analytical characterization.

Table 2. Experimental conditions adopted in the semi-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

2.2 Analytical methods

The fermentation process was studied through analytical characterization of both the fermentate and the biogas.
The volumetric amount of biogas produced was measured by means of a eudiometer connected to the fermentation reactor using the volume displacement principle, automatically recording the weight of the displaced aqueous solution (NaCl-saturated deionised water acidified with H_2SO_4 to pH = 2 to prevent gas dissolution) through an electronic load cell connected to the control system. The weight of the solution was converted to the corresponding biogas volume by accounting for the liquid and gas densities and the temperature and pressure conditions during the tests. All gas

volumes were converted to standard thermodynamic conditions (T = 273.15 K, P = 10^5 Pa).
The analytical characterization of biogas involved sampling with a gastight syringe and subsequent gas-chromatographic analysis. The gas chromatograph was equipped with a thermal conductivity detector and a 2-m stainless-steel packed column with an inner diameter of 1 mm. The operating temperatures of the injector and detector were 100 and 130 °C, respectively, with He as the carrier gas. The oven temperature was initially set at 80 °C and subsequently increased to 100 °C at 2 °C/min.
The fermentate was subjected to solid/liquid separation through centrifugation followed by 0.2 μm filtration, and subsequently analysed for TS, VS, TOC, dissolved organic and inorganic carbon (DOC and DIC) as well as soluble carbohydrates according to the analytical methods mentioned in section 2.1. In addition, the concentration of soluble metabolic products (SMPs), including volatile fatty acids (VFAs: acetic [HAc], propionic [HPr], butyric + iso-butyric [HBu], valeric + isovaleric [HVal], hexanoic + isohexanoic [HHex], heptanoic [HHep]) and ethanol [EtOH]) was determined in order to derive information about the prevalent metabolic pathways. The SMPs were analysed in HCl-acidified (pH = 2) samples with a gas chromatograph equipped with a flame ionization detector (FID) and a 30 m capillary column with an inner diameter of 0.53 mm. The temperatures of the detector and the injector were 270 and 250 °C, respectively. The oven temperature was initially set at 60 °C, held for 3 min at this value, subsequently increased to 180 °C at a rate of 10 °C/min and finally increased to 220 °C at a rate of 30 °C/min and held for 2 min.
For some samples additional metabolic products including lactic acid (HLa) and succinic acid (HSu) were also analysed through high-performance liquid chromatography (HPLC) using an UV-vis detector (λ = 220 nm) and a refractive index detector. The column had sizes of 300 × 7.8 mm. The analysis was performed at 40 °C under isocratic conditions using 0.008 N H_2SO_4 as the eluent with an elution rate of 0.6 mL/min.
All the analytical determinations were performed in duplicate.

2.3 Statistical analyses

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

Table 3. 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	---

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

3. RESULTS AND DISCUSSION

3.1 H_2 production and organic matter degradation

Our previous work 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:

$$SI = 1 - \frac{st.dev.(H_2\ production)}{average(H_2\ production)} \quad (1)$$

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 our previous work, it is worth mentioning 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 $H_2/(L_{react} \cdot d)$). 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 $H_2/(L_{react} \cdot d)$), followed by run R-8-65 (HPY = 42.1 L H_2/kg TOC; 2.7 L $H_2/(L_{react} \cdot d)$). 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)

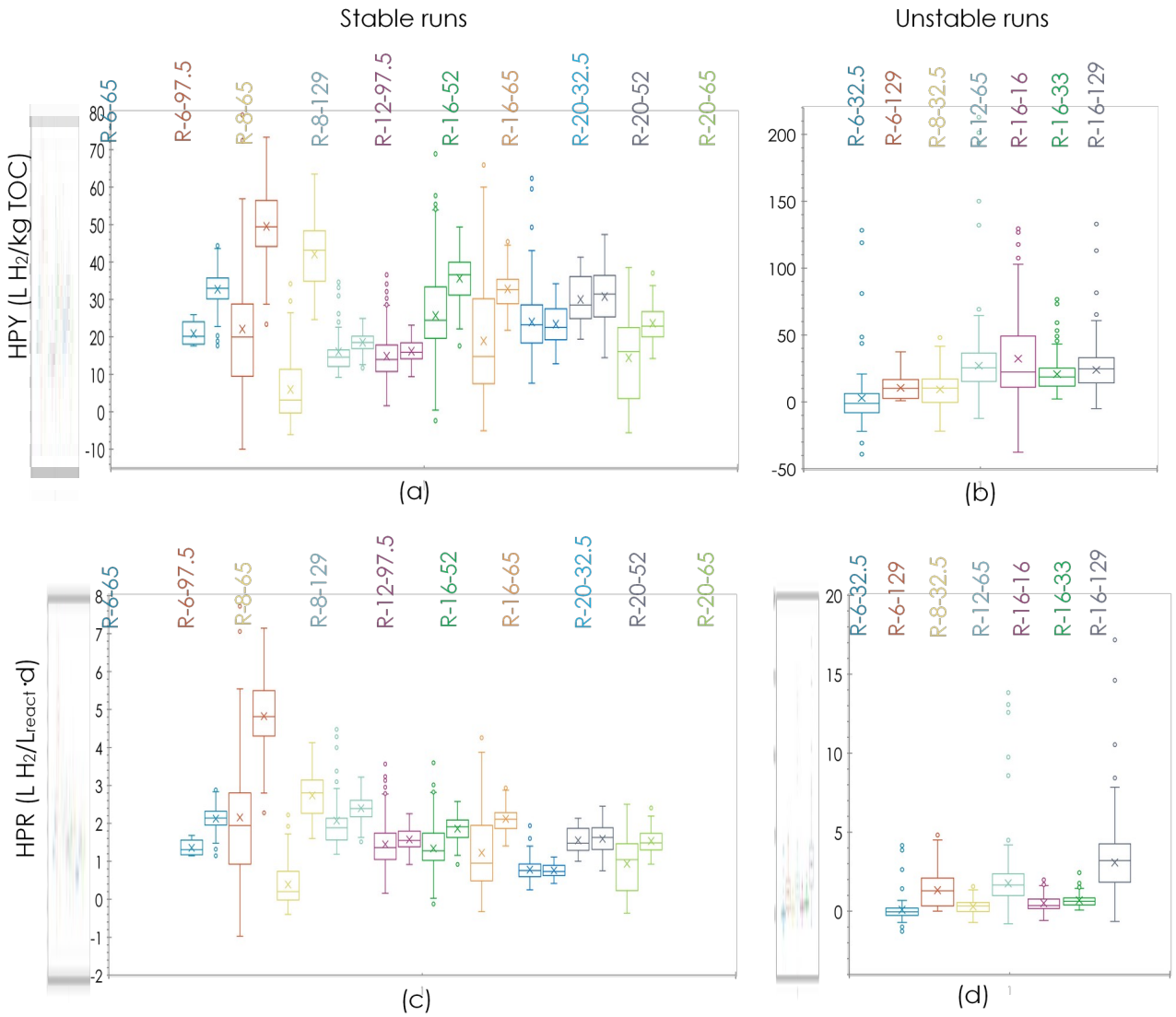

Figure 1. Average values (×) and range of variation (quartiles and min-max range) of the HPY (a, b) and HPR (c, d) for the individual experimental runs. Note: each box pair of the stable runs refers to the initial transient period (1st box) and the stable period (2nd box). Dots represent the outliers.

To explain the H_2 production performance observed in the different tests, the fate of carbon during the process was studied through the analysis of organic matter partitioning among the various products of the metabolic reactions. Assessing either the mass balance of carbon (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 the pathways 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 dark fermentation 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 we used the carbon mass balance to describe the transformation of organic matter. The carbon balance was drawn from the analytical measurements of the species of concern in both the liquid (fermentate) 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); 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.

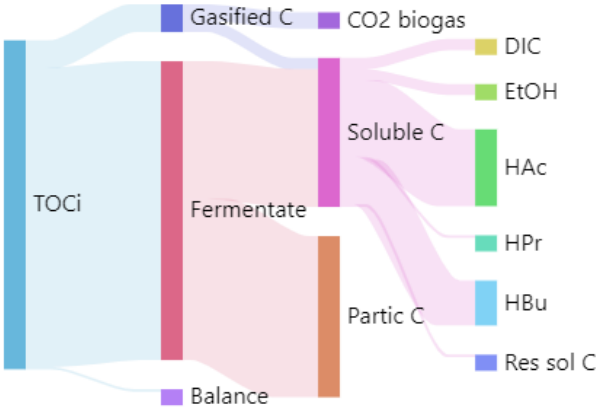

Figure 2. Conceptual layout of C partitioning among the different forms

The results of the C mass balance for a number of example runs are reported in Figure 3 in terms of percent distribution among the above mentioned forms relative to the input TOC and its partitioning among the individual SMPs. A number of distinguishing features can be derived that relate C speciation to the observed HPY. In general terms, it can be noticed that, as expected, a major portion (88±14%) of the input C was retained in the liquid phase during the fermentation process. Moreover, for this fraction of C, the proportions associated to the particulate and soluble forms were found to be of the same order of magnitude, although with some fluctuations across the tests. As shown in Figure 3, the amount of soluble carbon in the fermentate was likely mainly associated to the SMPs (see below for further details) rather than to non-degraded soluble molecules. It is also worth mentioning that for most of the fermentate samples the analysed metabolites explained more than 85% of DOC (as shown by the low values of the residual soluble C term in the plots), indicating that the pool of fermentation products was mainly made of HAc and HBu, followed by lower contributions of HPr and EtOH; on the other hand, the cumulative amount of HVal, HHex and HHep always accounted for less than 10% of TOC and in some cases was even undetectable. In some selected samples, additional metabolites were identified which included HLa and HSu, although this could not be systematically assessed for all tests due to analytical constraints.

As far as the fraction of particulate C was concerned, this could in principle be present in different forms, including either non-hydrolysed molecules from the original substrate or microbial cells. It has been shown in previous studies (Jung et al., 2010; Lay et al., 2010; Palomo-Briones et al., 2017) that in dark fermentation the biomass growth yield may range from ~0.2 up to 0.5 (on a COD basis), therefore the values obtained in the present work for the amount of particulate C are in line with other literature references and may be interpreted as being predominantly related to the contribution of biomass (Kim et al., 2006).

In more specific terms, comparing the two tests that displayed the highest H_2 production performance, it was noted that during the stability period run R-8-65 (with a HPY of 52–56 L H_2/kg TOC) displayed notably higher associated HBu (21.1–25.8% TOC) and HAc (17.1–23.5% TOC) concentrations 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% TOC). Based on the results of a metabolic model that was described in our previous study (Polettini et al., 2022), for run R-8-65 the analysed samples were found to display slightly higher HAc and HBu concentrations net of homoacetogenesis and other interfering

reactions, indicating some larger contribution of hydrogenogenic pathways compared to the other experiments. Conversely, the initial transient phase for run R-8-65 was characterised by remarkably lower SMPs concentrations (HAc = 14.0–17.8% TOC; HBu = 1.4–7.6% TOC) which explain the poor H_2 production performance observed in this period (HPY = 2–12 L H_2/kg TOC).

When looking at the metabolic products detected for run R-6-65 compared to R-8-65, HAc production during the stable period was much higher (average = 26.9% TOC vs. 18.1% TOC) while HBu was only slightly lower; in addition, some occurrence of alcoholic fermentation was observed (EtOH = 2.2–5.1% TOC). The lower H_2 production measured for run R-6-65 (HPY = 39–45 L H_2/kg TOC) was explained by the considerable contribution of homoacetogenic consumption (300–490 L H_2/kg TOC) that was estimated by the above mentioned metabolic model. Therefore, although having a higher gross H_2 production from the hydrogenogenic acetic and butyric pathways, the performance of run R-6-65 was negatively affected by the significant competitive role played by homoacetogenic microorganisms. It was also interesting to note that the contribution of the residual soluble C for run R-6-65 was negligible, indicating that HAc, HBu and to a much lesser extent EtOH were the only SMPs produced at appreciable levels. Conversely, for the two tests R-8-65 and R-6-97.5 the estimated values of the residual soluble C (5.4–29.2% TOC and 14.8–26.5% TOC, respectively) pointed out at a presumably more complex set of microbial reactions with the formation of an appreciable amount of additional metabolic products. Unfortunately, in these cases it was not possible to determine the nature of the products formed, however the results obtained for the other samples (see below) may suggest that lactic and succinic pathways were also taking place concomitantly with the previously mentioned reactions.

With regard to 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 fermentate. 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.

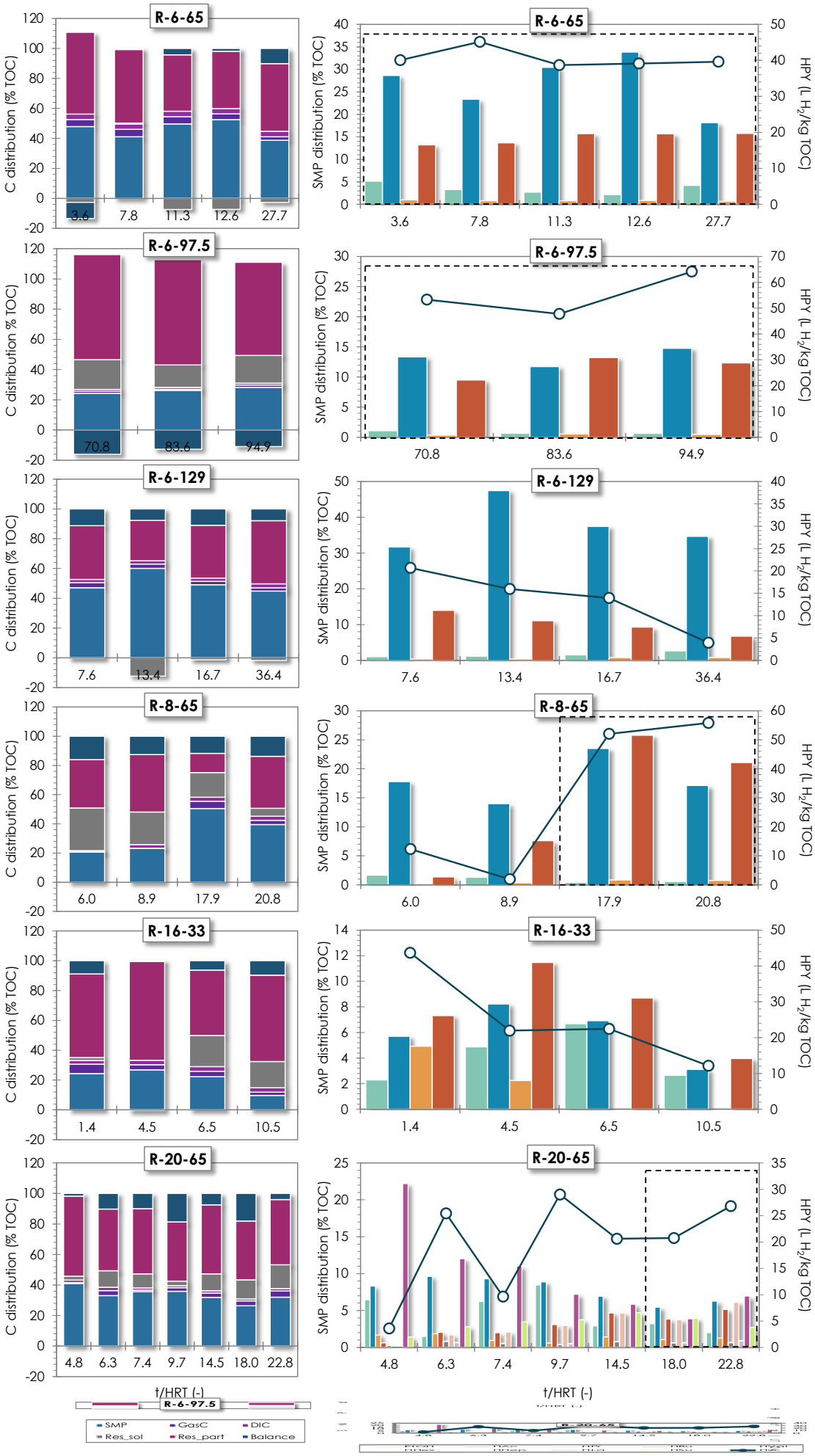
R-6-65
C distribution (% TOC)
SMP distribution (% TOC)
HPY (L H2/kg TOC)
3.6
7.8
11.3
12.6
27.7
R-6-97.5
C distribution % TOC)
70.8
83.6
94.9
R-6-129
7.6
13.4
16.7
36.4
R-8-65
6.0
8.9
17.9
20.8
R-16-33
1.4
4.5
6.5
10.5
R-20-65
4.8
6.3
7.4
9.7
14.5
18.0
22.8
t/HRT (-)
SMP
GasC
DIC
Res_sol
Res_part
Balance

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). However, other authors (Palomo-Briones et al., 2018), who worked on continuous fermentation of CW powder, found surprisingly no correlation between HPY and butyrate generation and, even more unexpectedly, a negative correlation between the latter and HPR.

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

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

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

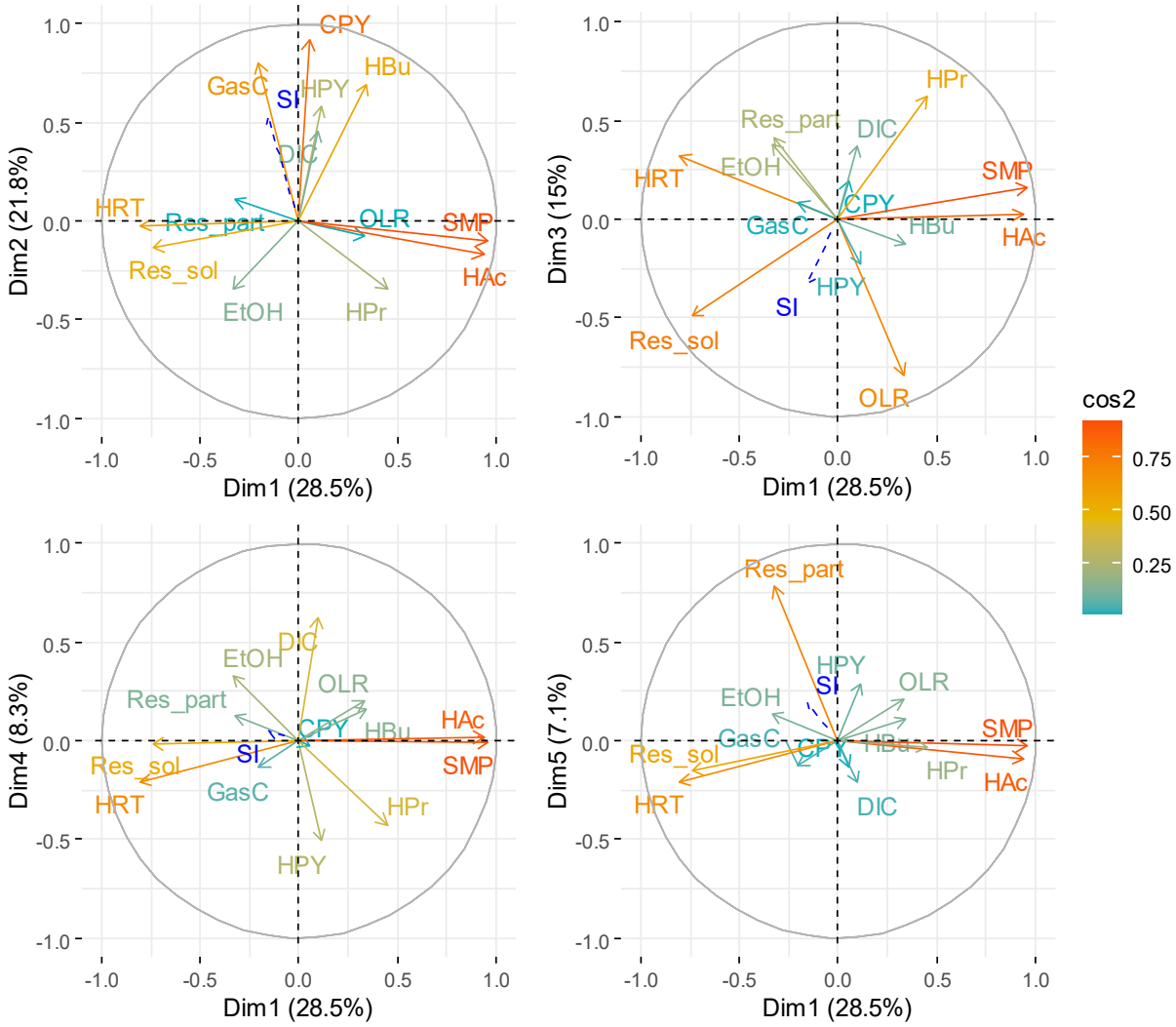

Figure 4. Loading plots in the first 5 PCs space

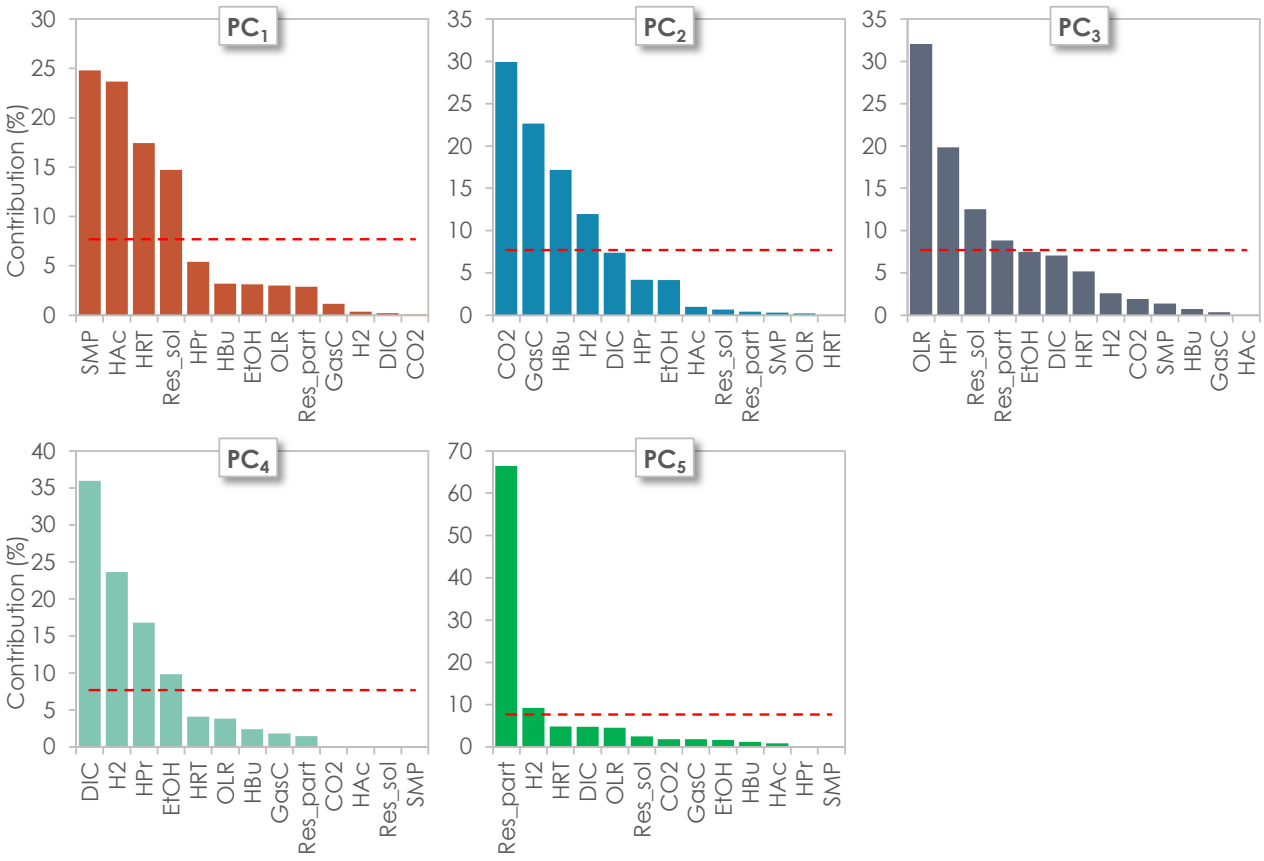

Figure 5. Contribution of the original variables to the first 5 PCs

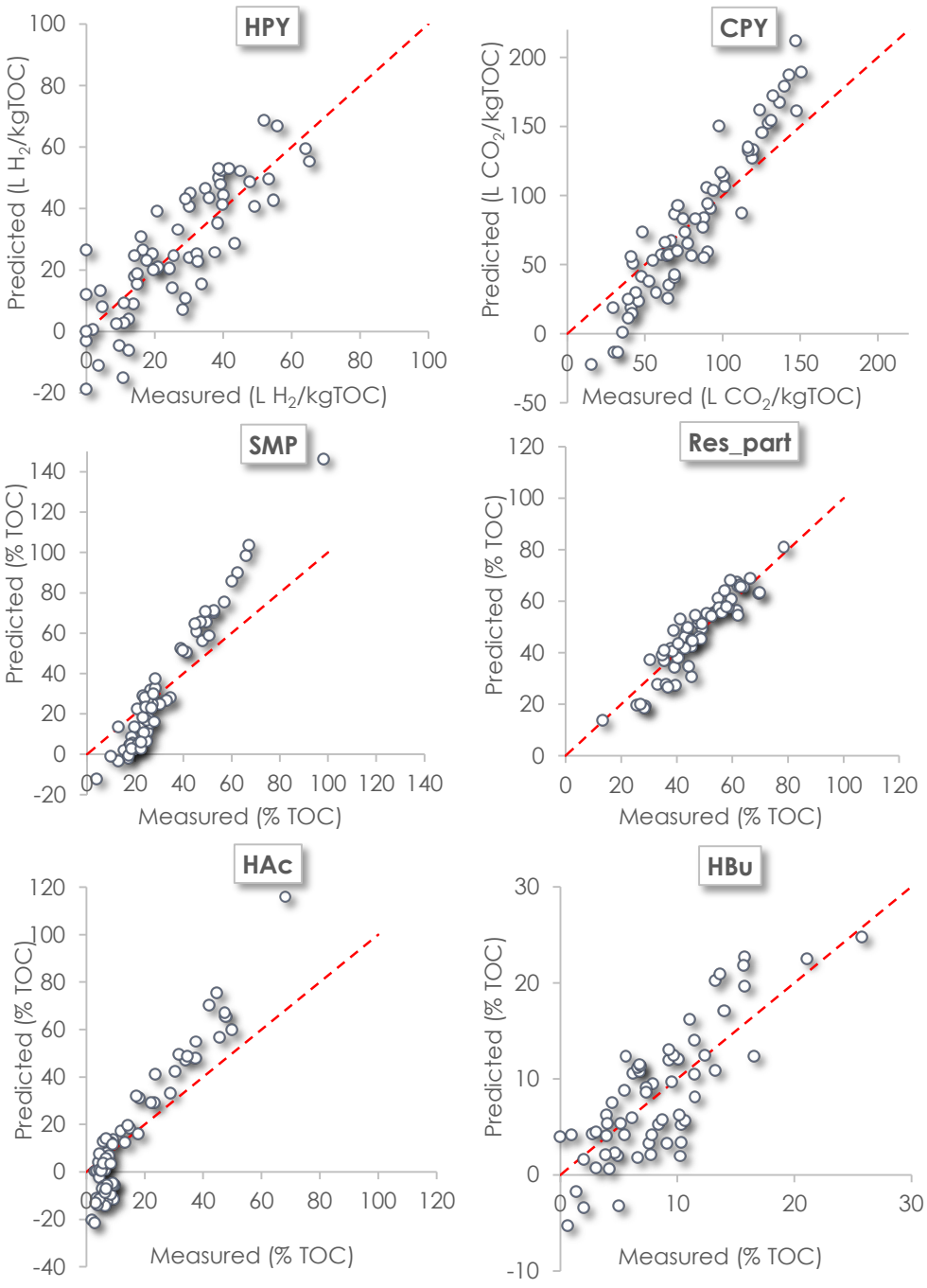

Figure 6. Predicted-vs.-fitted values of selected parameters (model: 5-PCs PCA)

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 experiments, being related to both the amount and the relative distribution of the individual metabolites generated. In most cases, the pool of metabolic products was dominated by acetate and butyrate; however, they were found to derive not only from hydrogenogenic pathways but also from competing reactions and the contribution of the different metabolic routes was in turn a function of the specific operating conditions. For some experimental runs, lactate and – to a lesser extent – succinate were also detected in the fermentate, indicating the occurrence of a more complex set of metabolic reactions that had negative implications in terms of substrate conversion into H_2.

The statistical analysis of the experimental results also showed that ~80% of the information associated to the set of process parameters analysed could be described by five factors that individually explained specific features of the process itself. Among these, the first three components were able to explain cumulatively the largest share (65%) of the sample variance. In particular, the first principal component grasped the effect of retention time and total metabolic products formed, with shorter retention times being accompanied by higher total metabolites (and acetate) production and in turn decreased fractions of residual soluble carbon. The second factor mainly accounted for biogas (H_2, CO_2) generation and butyrate production, showing a correlation between the two despite the fact that H_2-neutral butyrate production from chain elongation reactions was also likely to play a role during the experiments. The third principal component mainly explained the effect of the organic loading rate and that of propionate production, which showed a mutual negative correlation.

The statistical analysis was also capable of grasping the stability features of the process, which were mainly explained by the second factor.

In summary, processing of the experimental data proved useful to group the performance and monitoring indicators of fermentation into a reduced set of variables, each representing a specific feature of the process.

As pointed out in the introduction, the production of biohydrogen through dark fermentation is a process that is very sensitive to a plurality of aspects, which also mutually influence each other. This complexity, although predictable for transitional biochemical processes, represents an obstacle to full-scale implementation, which is still a long way off despite the remarkable number of studies conducted so far on various substrates. It is hoped that studies such as the present, which aims at analysing, organizing and systematically understanding the results of the experimental activity, will contribute to confirm the main research outcomes and consolidate the knowledge, in turn providing the scientific community with reliable indicators that may represent a solid basis for the transition to full-scale application.

References

Akhlaghi, M., Boni, M.R., De Gioannis, G., Muntoni, A., Polettini, A., Pomi, R., Rossi, A., Spiga, D., 2017. A parametric response surface study of fermentative hydrogen production from cheese whey. Bioresour. Technol. 244, 473–483. doi:10.1016/j.biortech.2017.07.158

Akhlaghi, M., Boni, M.R., Polettini, A., Pomi, R., Rossi, A., De Gioannis, G., Muntoni, A., Spiga, D., 2019. Fermentative H2 production from food waste: Parametric analysis of factor effects. Bioresour. Technol. 276, 349–360. doi:10.1016/J.BIORTECH.2019.01.012

Antonopoulou, G., Gavala, H.N., Skiadas, I. V., Lyberatos, G., 2012. Modeling of fermentative hydrogen production from sweet sorghum extract based on modified ADM1. Int. J. Hydrogen Energy 37, 191–208. doi:10.1016/J.IJHYDENE.2011.09.081

Antonopoulou, G., Gavala, H.N., Skiadas, I. V, Angelopoulos, K., Lyberatos, G., 2008a. Biofuels generation from sweet sorghum: fermentative hydrogen production and anaerobic digestion of the remaining biomass. Bioresour. Technol. 99, 110–9. doi:10.1016/j.biortech.2006.11.048

Antonopoulou, G., Stamatelatou, K., Venetsaneas, N., Kornaros, M., Lyberatos, G., 2008b. Biohydrogen and methane production from cheese whey in a two-stage anaerobic process. Ind. Eng. Chem. Res. 47, 5227–5233. doi:10.1021/ie071622x

APHA, AWWA, WEF, 2017. Standard methods for the examination of water and wastewater, 23rd ed. American Public Health Association, Washington DC.

Azbar, N., Dokgöz Çetinkaya, F.T., Keskin, T., Korkmaz, K.S., Syed, H.M., 2009. Continuous fermentative hydrogen production from cheese whey wastewater under thermophilic anaerobic conditions. Int. J. Hydrogen Energy 34, 7441–7447. doi:10.1016/j.ijhydene.2009.04.032

Boni, M.R., De Gioannis, G., Muntoni, A., Polettini, A., Pomi, R., Rossi, A., Spiga, D., Zonfa, T., 2021. Bio-H2 production from cheese whey and wastewater sludge in semi-continuous

systems, in: SIDISA 2021 – XI INTERNATIONAL SYMPOSIUM ON ENVIRONMENTAL ENGINEERING. Politecnical University of Turin, Turin (Italy).
Cai, G., Jin, B., Saint, C., Monis, P., 2010. Metabolic flux analysis of hydrogen production network by Clostridium butyricum W5: Effect of pH and glucose concentrations. Int. J. Hydrogen Energy 35, 6681–6690. doi:10.1016/J.IJHYDENE.2010.04.097
Carvalho, F., Prazeres, A.R., Rivas, J., 2013. Cheese whey wastewater: characterization and treatment. Sci. Total Environ. 445–446, 385–96.
Castelló, E., Braga, L., Fuentes, L., Etchebehere, C., 2018. Possible causes for the instability in the H2 production from cheese whey in a CSTR. Int. J. Hydrogen Energy 43, 2654–2665. doi:10.1016/J.IJHYDENE.2017.12.104
Castelló, E., García y Santos, C., Iglesias, T., Paolino, G., Wenzel, J., Borzacconi, L., Etchebehere, C., 2009. Feasibility of biohydrogen production from cheese whey using a UASB reactor: Links between microbial community and reactor performance. Int. J. Hydrogen Energy 34, 5674–5682. doi:10.1016/j.ijhydene.2009.05.060
Castelló, E., Nunes Ferraz-Junior, A.D., Andreani, C., Anzola-Rojas, M. del P., Borzacconi, L., Buitrón, G., Carrillo-Reyes, J., Gomes, S.D., Maintinguer, S.I., Moreno-Andrade, I., Palomo-Briones, R., Razo-Flores, E., Schiappacasse-Dasati, M., Tapia-Venegas, E., Valdez-Vázquez, I., Vesga-Baron, A., Zaiat, M., Etchebehere, C., 2020. Stability problems in the hydrogen production by dark fermentation: Possible causes and solutions. Renew. Sustain. Energy Rev. 119, 109602. doi:10.1016/J.RSER.2019.109602
Castillo Martinez, F.A., Balciunas, E.M., Salgado, J.M., Domínguez González, J.M., Converti, A., Oliveira, R.P. de S., 2013. Lactic acid properties, applications and production: A review. Trends Food Sci. Technol. 30, 70–83. doi:10.1016/J.TIFS.2012.11.007
Cooney, M., Maynard, N., Cannizzaro, C., Benemann, J., 2007. Two-phase anaerobic digestion for production of hydrogen–methane mixtures. Bioresour. Technol. 98, 2641–2651. doi:10.1016/J.BIORTECH.2006.09.054
Cota-Navarro, C.B., Carrillo-Reyes, J., Davila-Vazquez, G., Alatriste-Mondragón, F., Razo-Flores, E., 2011. Continuous hydrogen and methane production in a two-stage cheese whey fermentation system. Water Sci. Technol. 64, 367–374. doi:10.2166/WST.2011.631
Dareioti, M.A., Vavouraki, A.I., Kornaros, M., 2014. Effect of pH on the anaerobic acidogenesis of agroindustrial wastewaters for maximization of bio-hydrogen production: A lab-scale evaluation using batch tests. Bioresour. Technol. 162, 218–227. doi:10.1016/j.biortech.2014.03.149
Davila-Vazquez, G., Alatriste-Mondragón, F., de León-Rodríguez, A., Razo-Flores, E., 2008. Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: Influence of initial substrate concentration and pH. Int. J. Hydrogen Energy 33, 4989–4997. doi:10.1016/j.ijhydene.2008.06.065
Davila-Vazquez, G., Cota-Navarro, C.B., Rosales-Colunga, L.M., de León-Rodríguez, A., Razo-Flores, E., 2009. Continuous biohydrogen production using cheese whey: Improving the hydrogen production rate. Int. J. Hydrogen Energy 34, 4296–4304. doi:10.1016/j.ijhydene.2009.02.063
Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28, 350–356. doi:10.1021/ac60111a017
Eurostat, 2020. Agriculture, forestry and fishery statistics: 2020 edition. Agric. For. Fish. Stat. 2020 Ed.
García-Depraect, O., Diaz-Cruces, V.F., Rene, E.R., León-Becerril, E., 2020. Changes in performance and bacterial communities in a continuous biohydrogen-producing reactor subjected to substrate- and pH-induced perturbations. Bioresour. Technol. 295, 122182. doi:10.1016/J.BIORTECH.2019.122182
Ghimire, A., Luongo, V., Frunzo, L., Pirozzi, F., Lens, P.N.L., Esposito, G., 2017. Continuous

biohydrogen production by thermophilic dark fermentation of cheese whey: Use of buffalo manure as buffering agent. Int. J. Hydrogen Energy 42, 4861–4869. doi:10.1016/j.ijhydene.2016.11.185

Hamilton, C., Hiligsmann, S., Beckers, L., Masset, J., Wilmotte, A., Thonart, P., 2010. Optimization of culture conditions for biological hydrogen production by Citrobacter freundii CWBI952 in batch, sequenced-batch and semicontinuous operating mode. Int. J. Hydrogen Energy 35, 1089–1098. doi:10.1016/J.IJHYDENE.2009.10.073

Jung, K.W., Kim, D.H., Shin, H.S., 2010. Continuous fermentative hydrogen production from coffee drink manufacturing wastewater by applying UASB reactor. Int. J. Hydrogen Energy 35, 13370–13378. doi:10.1016/J.IJHYDENE.2009.11.120

Kargi, F., Eren, N.S., Ozmihci, S., 2012. Bio-hydrogen production from cheese whey powder (CWP) solution: Comparison of thermophilic and mesophilic dark fermentations, International Journal of Hydrogen Energy. doi:10.1016/j.ijhydene.2012.02.162

Kim, D.H., Han, S.K., Kim, S.H., Shin, H.S., 2006. Effect of gas sparging on continuous fermentative hydrogen production. Int. J. Hydrogen Energy 31, 2158–2169. doi:10.1016/J.IJHYDENE.2006.02.012

Lay, C.H., Wu, J.H., Hsiao, C.L., Chang, J.J., Chen, C.C., Lin, C.Y., 2010. Biohydrogen production from soluble condensed molasses fermentation using anaerobic fermentation. Int. J. Hydrogen Energy 35, 13445–13451. doi:10.1016/J.IJHYDENE.2009.11.128

Lê, S., Josse, J., Husson, F., 2008. FactoMineR: An R Package for Multivariate Analysis. J. Stat. Softw. 25, 1–18. doi:10.18637/jss.v025.i01

Lee, H.-S., Salerno, M.B., Rittmann, B.E., 2008. Thermodynamic evaluation on H2 production in glucose fermentation. Environ. Sci. Technol. 42, 2401–2407. doi:10.1021/es702610v

Lee, H.S., Rittmann, B.E., 2009. Evaluation of metabolism using stoichiometry in fermentative biohydrogen. Biotechnol. Bioeng. 102, 749–758. doi:10.1002/BIT.22107

Ljungdahl, L.G., Hugenholtz, J., Wiegel, J., 1989. Acetogenic and Acid-Producing Clostridia, in: Clostridia. Springer US, Boston, MA, pp. 145–191. doi:10.1007/978-1-4757-9718-3_5

Lopez-Hidalgo, A.M., Alvarado-Cuevas, Z.D., De Leon-Rodriguez, A., 2018. Biohydrogen production from mixtures of agro-industrial wastes: Chemometric analysis, optimization and scaling up. Energy 159, 32–41. doi:10.1016/J.ENERGY.2018.06.124

Moat, A.G., J.W., F., Spector, M.P., 2002. Fermentation pathways, in: Microbial Physiology. Wiley-Liss, New York, pp. 412–433.

Muñoz-Páez, K.M., Poggi-Varaldo, H.M., García-Mena, J., Ponce-Noyola, M.T., Ramos-Valdivia, A.C., Barrera-Cortés, J., Robles-González, I. V., Ruiz-Ordáz, N., Villa-Tanaca, L., Rinderknecht-Seijas, N., 2014. Cheese whey as substrate of batch hydrogen production: Effect of temperature and addition of buffer. Waste Manag. Res. 32, 434–440. doi:10.1177/0734242X14527333

OECD-FAO, 2021. Agricultural Outlook 2021-2030, OECD-FAO Agricultural Outlook. OECD. doi:10.1787/19428846-EN

Palomo-Briones, R., Razo-Flores, E., Bernet, N., Trably, E., 2017. Dark-fermentative biohydrogen pathways and microbial networks in continuous stirred tank reactors: Novel insights on their control. Appl. Energy 198, 77–87. doi:10.1016/j.apenergy.2017.04.051

Palomo-Briones, R., Trably, E., López-Lozano, N.E., Celis, L.B., Méndez-Acosta, H.O., Bernet, N., Razo-Flores, E., 2018. Hydrogen metabolic patterns driven by Clostridium-Streptococcus community shifts in a continuous stirred tank reactor. Appl. Microbiol. Biotechnol. 102, 2465–2475. doi:10.1007/S00253-018-8737-7/TABLES/3

Park, Jeong Hoon, Kumar, G., Park, Jong Hun, Park, H.D., Kim, S.H., 2015. Changes in performance and bacterial communities in response to various process disturbances in a high-rate biohydrogen reactor fed with galactose. Bioresour. Technol. 188, 109–116. doi:10.1016/J.BIORTECH.2015.01.107

Patel, A.K., Vaisnav, N., Mathur, A., Gupta, R., Tuli, D.K., 2016. Whey waste as potential

feedstock for biohydrogen production. Renew. Energy 98, 221–225. doi:10.1016/J.RENENE.2016.02.039

Polettini, A., Pomi, R., Rossi, A., Zonfa, T., De Gioannis, G., Muntoni, A., 2022. Continuous biohydrogen production from cheese whey - Part 1: New insights into process stability. J. Clean. Prod. (submitted.

Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. Int. J. Hydrogen Energy 38, 13172–13191. doi:10.1016/j.ijhydene.2013.07.122

Sarma, S., Anand, A., Dubey, V.K., Moholkar, V.S., 2017. Metabolic flux network analysis of hydrogen production from crude glycerol by Clostridium pasteurianum. Bioresour. Technol. 242, 169–177. doi:10.1016/J.BIORTECH.2017.03.168

Sikora, A., Błaszczyk, M., Jurkowski, M., Zielenkiewicz, U., 2013. Lactic Acid Bacteria in Hydrogen-Producing Consortia: On Purpose or by Coincidence?, in: Kongo, J.M. (Ed.), Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes. InTech. doi:10.5772/2825

Vasmara, C., Pindo, M., Micheletti, D., Marchetti, R., 2018. Initial pH influences microbial communities composition in dark fermentation of scotta permeate. Int. J. Hydrogen Energy 43, 8707–8717. doi:10.1016/j.ijhydene.2018.03.122

Venetsaneas, N., Antonopoulou, G., Stamatelatou, K., Kornaros, M., Lyberatos, G., 2009. Using cheese whey for hydrogen and methane generation in a two-stage continuous process with alternative pH controlling approaches. Bioresour. Technol. 100, 3713–7. doi:10.1016/j.biortech.2009.01.025

Yang, P., Zhang, R., McGavey, J., Benemann, J., 2007. Biohydrogen production from cheese processing wastewater by anaerobic fermentation using mixed microbial communities. Int. J. Hydrogen Energy 32, 4761–4771. doi:10.1016/j.ijhydene.2007.07.038

Ziara, R.M.M., Miller, D.N., Subbiah, J., Dvorak, B.I., 2019. Lactate wastewater dark fermentation: The effect of temperature and initial pH on biohydrogen production and microbial community. Int. J. Hydrogen Energy 44, 661–673. doi:10.1016/j.ijhydene.2018.11.045