

Manuscript Number: BITE-D-17-04051R1

Title: A PARAMETRIC RESPONSE SURFACE STUDY OF FERMENTATIVE HYDROGEN PRODUCTION FROM CHEESE WHEY

Article Type: Original research paper

Keywords: biological hydrogen production; cheese whey; pH; inoculum-to-substrate ratio; response surface analysis

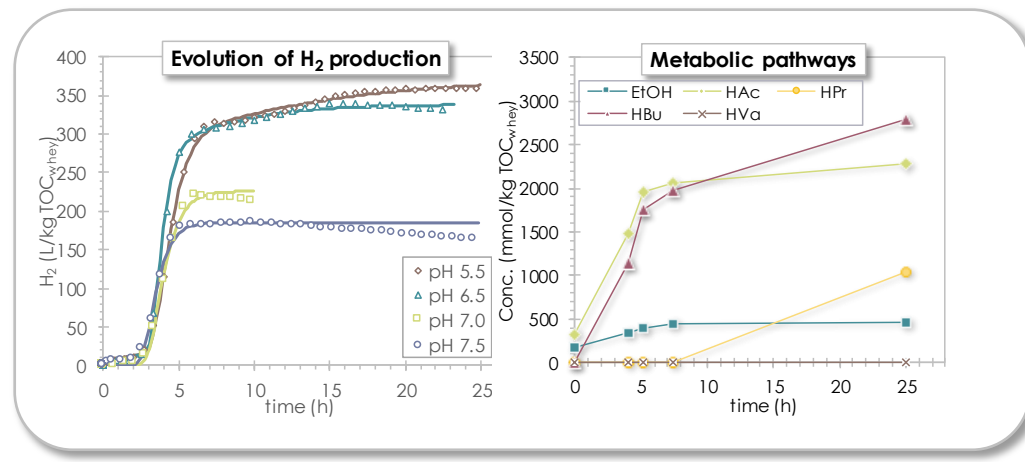
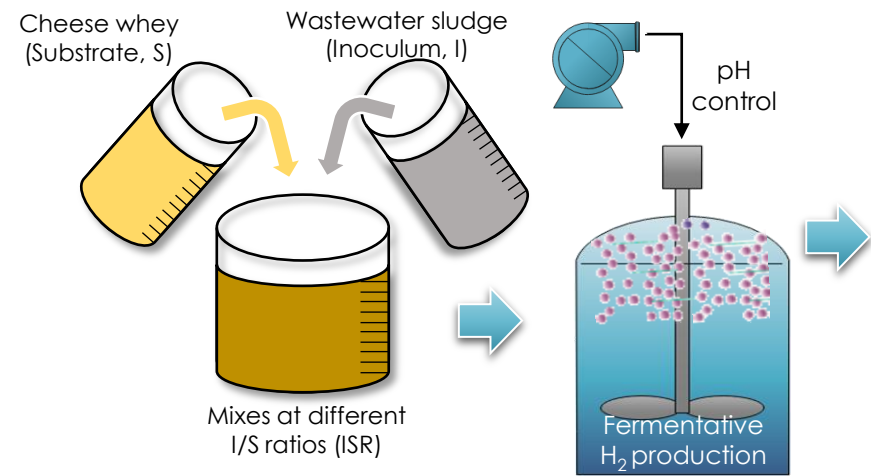
Corresponding Author: Professor Alessandra Polettini, Prof.

Corresponding Author's Institution: University of Rome "La Sapienza"

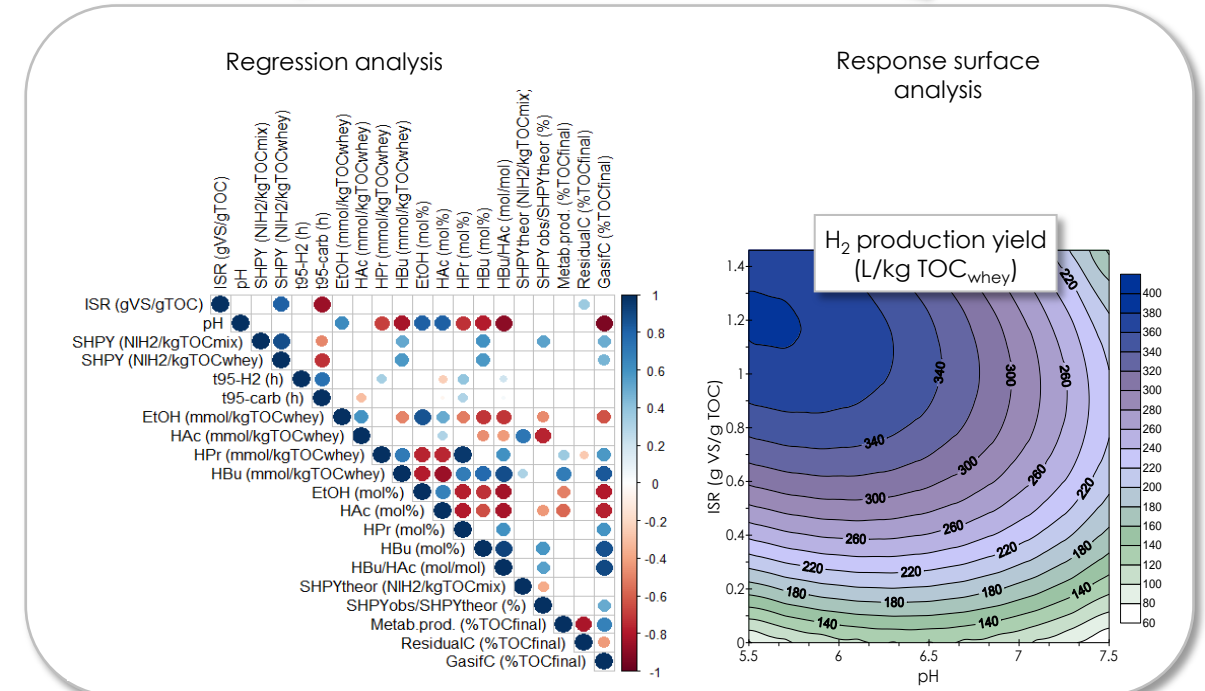
First Author: Masoumeh Akhlagi

Order of Authors: Masoumeh Akhlagi; Maria Rosaria Boni, Prof.; Giorgia De Gioannis, Prof.; Aldo Muntoni, Prof.; Alessandra Polettini, Prof.; Raffaella Pomi, Prof.; Andreina Rossi, Dr.; Daniela Spiga, Dr.

Abstract: Batch factorial experiments were performed on cheese whey + wastewater sludge mixtures to evaluate the influence of pH and the inoculum-to-substrate ratio (ISR) on fermentative H<sub>2</sub> production and build a related predictive model. ISR and pH affected H<sub>2</sub> potential and rate, and the fermentation pathways. The specific H<sub>2</sub> yield varied from 61 (ISR = 0, pH = 7.0) to 371 L H<sub>2</sub>/kg TOCwhey (ISR = 1.44 g VS/g TOC, pH = 5.5). The process duration range was 5.3 (ISR = 1.44 g VS/g TOC, pH = 7.5) □ 183 h (ISR = 0, pH = 5.5). The metabolic products included mainly acetate and butyrate followed by ethanol, while propionate was only observed once H<sub>2</sub> production had significantly decreased. The multiple metabolic products suggested that the process was governed by several fermentation pathways, presumably overlapping and mutually competing, reducing the conversion yield into H<sub>2</sub> compared to that expected with clostridial fermentation.



Evaluation of the effect of pH and ISR on H<sub>2</sub> production and metabolic pathways



- Biohydrogen production from cheese whey was studied through factorial experiments
- The role of pH and ISR on H<sub>2</sub> production was specifically assessed
- Regression and response surface analyses were used to interpret the results
- Multiple fermentation pathways were likely to overlap during fermentation
- The maximum yield was 371 L H<sub>2</sub>/kg TOC<sub>whey</sub> for ISR = 1.44 g VS/g TOC and pH = 5.5

## A PARAMETRIC RESPONSE SURFACE STUDY OF FERMENTATIVE HYDROGEN PRODUCTION FROM CHEESE WHEY

M. Akhlagi<sup>1</sup>, M.R. Boni<sup>1</sup>, G. De Gioannis<sup>2,3</sup>, A. Muntoni<sup>2,3</sup>, A. Poletti<sup>1\*</sup>, R. Pomi<sup>1</sup>, A. Rossi<sup>1</sup>,  
D. Spiga<sup>2</sup>

<sup>1</sup> Department of Civil and Environmental Engineering, University of Rome “La Sapienza”, Italy

<sup>2</sup> Department of Civil and Environmental Engineering and Architecture, University of Cagliari, Italy

<sup>3</sup> IGAG – CNR (Environmental Geology and Geoengineering Institute of the National Research Council), Italy

\* Corresponding author: Prof. Alessandra Poletti, Department of Civil and Environmental Engineering, University of Rome “La Sapienza”, Via Eudossiana 18 – 00184 Rome (Italy); tel/fax: +39/06 44585037; e-mail: [alessandra.poletti@uniroma1.it](mailto:alessandra.poletti@uniroma1.it)

### ABSTRACT

Batch factorial experiments were performed on cheese whey + wastewater sludge mixtures to evaluate the influence of pH and the inoculum-to-substrate ratio (ISR) on fermentative H<sub>2</sub> production and build a related predictive model. ISR and pH affected H<sub>2</sub> potential and rate, and the fermentation pathways. The specific H<sub>2</sub> yield varied from 61 (ISR = 0, pH = 7.0) to 371 L H<sub>2</sub>/kg TOC<sub>whey</sub> (ISR = 1.44 g VS/g TOC, pH = 5.5). The process duration range was 5.3 (ISR = 1.44 g VS/g TOC, pH = 7.5) – 183 h (ISR = 0, pH = 5.5). The metabolic products included mainly acetate and butyrate followed by ethanol, while propionate was only observed once H<sub>2</sub> production had significantly decreased. The multiple metabolic products suggested that the process was governed by several fermentation pathways, presumably overlapping and mutually

competing, reducing the conversion yield into H<sub>2</sub> compared to that expected with clostridial fermentation.

**Keywords:** biological hydrogen production; cheese whey; pH; inoculum-to-substrate ratio; response surface analysis.

## 1. INTRODUCTION

Like other food manufacturing activities, the dairy industry is known to generate large specific amounts of liquid effluents, which are also typically characterized by a high organic load.

According to European Commission statistics (European Commission and Eurostat, 2015), the overall amount of dairy products generated in the EU-28 area accounted for 114 million tonnes in 2015 (latest official statistics available). The main dairy products include drinking milk (27.5% of the overall production in 2015) and other miscellaneous fresh products (13.7%), as well as whey (43.7%), cheese (8.4%), milk powder (2.6%) and butter and other yellow fat products (2.0%) among the manufactured products (European Commission and Eurostat, 2015).

In Europe, Italy is the third cheese producer after France and Germany, with an annual production of 1.2 million tonnes in 2015 (European Commission and Eurostat, 2015). Cheese manufacturing results in two main types of wastewater streams (Carvalho et al., 2013), including cheese whey (CW) and a lower-strength wastewater stream resulting from refrigeration and cleaning operations. The specific production of CW is estimated to be 0.8–0.9 L per L of processed milk (Carvalho et al., 2013), or 9 kg per kg of cheese produced (Siso, 1996). The chemical composition and characteristics of CW depend upon the type of milk as well as cheese production techniques used (Carvalho et al., 2013). On average, CW accounts for ~80% of the original fermentation medium (Azbar et al., 2009b) and retains ~55% of the milk nutrients (Siso, 1996). The main components include lactose (45-50 g/L), proteins (6-8 g/L), lipids (4-5 g/L) and

mineral salts (8-10% dried extract) (Siso, 1996); these include NaCl and KCl (>50%), calcium salts and others (Prazeres et al., 2012; Venetsaneas et al., 2009). Other constituents of CW include lactic and citric acids, urea and uric acid as well as B-group vitamins (Venetsaneas et al., 2009).

The dairy industry has explored over the last decades different alternatives to exploit the valuable components of CW. Simple options practiced in the past such as land application or direct use as farm animal feed have been considerably restricted by dedicated regulations due to obvious concerns about the associated environmental impacts and the potential negative effects on the nutritional and health conditions of the animals. In some geographical areas, CW is used to produce ricotta/cottage cheese, generating a secondary CW effluent of different characteristics. More ambitious and process-intensive alternatives aim at exploiting the nutritional content of CW by recovering proteins and lactose. The main outputs include lactose, minerals, whey powder, whey protein concentrate and whey protein isolate which can be variously reused as food or beverage ingredients. Further options involve CW treatment, typically through biological anaerobic processes, to exploit its main constituents producing either biofuels or chemical products (sugars, organic acids, biopolymers, etc.) for industrial applications. More specifically, due to the typically high carbohydrate content of CW, bio-hydrogen production through dark fermentation has recently deserved some significant attention by the scientific community as a strategy to optimize the degradation process and allow for improved substrate stabilization and conversion into biofuels (Azbar et al., 2009a, 2009b, Davila-Vazquez et al., 2009, 2008; De Gioannis et al., 2014; Ferchichi et al., 2005; Fernández et al., 2015; Ferreira Rosa et al., 2014b; Ghimire et al., 2017; Perna et al., 2013; Rosales-Colunga et al., 2010; Stamatelatou et al., 2011; Venetsaneas et al., 2009; Yang et al., 2007).

Fermentative H<sub>2</sub> production has proved to be a very sensitive process as it strongly depends on multiple factors (substrate characteristics, organic loading rate, inoculum type and addition ratio,

reactor type and operation regime, type of pre-treatment applied, temperature, pH) that are also strictly interrelated and mutually interactive (Alibardi and Cossu, 2015a; De Gioannis et al., 2013; Ghimire et al., 2016; Van Ginkel et al., 2001). Consequently, attaining significant and stable generation rates in fermentative H<sub>2</sub> production requires careful optimization of the operating parameters of the process.

In particular, for biological H<sub>2</sub> production to proceed properly, a balanced amount of active inoculum, consisting of a microbial community catalyzing a series of interdependent biochemical reactions (hydrolysis and acidogenesis), is required. The relative amount of biomass available in the system and substrate to be treated is commonly expressed through either the so-called inoculum-to-substrate ratio (ISR) or its reciprocal, the food-to-microorganisms (F/M) ratio. A microbial culture can shift from substrate-limited to substrate-sufficient growth depending on the relative availability of substrate and biomass, with higher growth yields being commonly reported at lower F/M ratios (Liu, 1996). Excessive substrate availability may cause an unbalance between anabolic and catabolic reactions leading to energy spilling (Liu, 1996) and thus affecting the yield of substrate conversion into the metabolic products (Argun and Dao, 2017; Cappai et al., 2015; Ghimire et al., 2017).

The operating pH is also recognized as an extremely important parameter that can affect the evolution of the fermentation process, as it determines the degree of substrate hydrolysis, the activity of hydrogenase, the efficiency of energy utilization by the microbial cells as well as the metabolic pathways (Kim et al., 2011). Changes in pH thereby result in different rates of substrate and energy utilization, synthesis of proteins and various storage products, as well as metabolites production (Rodríguez et al., 2006). In mixed cultures, the relative abundance of microbial species, and in turn the amount of hydrogenogenic biomass, has also proved to be pH-dependent (Fang and Liu, 2002; Khanal et al., 2003; Nazlina et al., 2011; Song et al., 2011; Yuan et al., 2015). Extreme pH values can negatively affect the activity of hydrogen-producing

bacteria, since ATP is used to ensure cell neutrality rather than to produce  $H_2$  (Nazlina et al., 2011); low pHs can also result in inhibition of the hydrogenase activity (Khanal et al., 2003; Nazlina et al., 2011). Several authors reported that acetate and butyrate pathways, which are widely recognized as being associated to high  $H_2$  production yields, are prevalent at operating pH values from 4.5–5.0 up to 6.0 (Cappai et al., 2014; De Gioannis et al., 2014; Moon et al., 2015). Operating pHs outside this range are on the other hand known to promote unfavourable conditions for bio-hydrogen production.  $H_2$ -neutral pathways including solventogenesis and lactate production are commonly reported to occur under acidic conditions, while  $H_2$  consumption as caused by propionic fermentation has been documented at alkaline pHs (De Gioannis et al., 2014; Perna et al., 2013; Rodríguez et al., 2006; Van Ginkel and Logan, 2005). It should however be mentioned that deviations from such behaviours have also been reported depending on the specific process conditions adopted, as well as the characteristics of the inoculum and substrate (Cappai et al., 2014; Nazlina et al., 2011), in particular for complex substrate compositions.

From the discussion provided above, the effects of the operating pH of the system and the ISR on the overall performance of bio-hydrogen production appear to require further elucidation in order to reconcile the inconsistencies derived from different literature studies. On account of the fact that the parameters governing the fermentation process are recognized to be mutually interrelated, investigations based on a “one variable at a time” approach are considered to fail to detect and quantify interactions between the variables of concern, at the same time requiring a considerably high number of experiments. Considering that only a limited number of studies on the influence of pH and ISR on fermentative  $H_2$  production from CW are currently available in the literature (Davila-Vazquez et al., 2008), **the present work attempts** to fill in the existing gaps by means of a number of hydrogenogenic batch fermentation tests on undiluted CW. **The main innovation from the study lies on the systematic investigation of the relationships and mutual**



interactions among the operating conditions and response variables of fermentative H<sub>2</sub> production, using an approach based on factorial design of the experiments, statistical analysis of results and predictive modelling of parameters effects.

## **2. MATERIALS AND METHODS**

### **2.1 Feedstock and inoculum**

Samples of fresh raw CW were collected at an Italian dairy industry producing mozzarella cheese from a mixture of cow and buffalo milk. The sample was stored at 4 °C until use.

Activated sludge (AS) from the aerobic unit of a municipal wastewater treatment plant was used as the inoculum. AS was considered a suitable biomass source due to the presence of facultative bacteria, which are recognized to be capable of enhancing the fermentative stage of the process due to their high growth rate and ability to rapidly recover from accidental oxygen intrusion.

Before use, the activated sludge was stored in 20-L closed tanks and settled for 24 h before use.

The AS was heat-shocked (105 °C, 30 min) before mixing with CW in order to harvest the hydrogenogenic biomass; the heat-shock treatment (HST) conditions were selected on the basis of previous investigations (Cappai et al., 2014; De Gioannis et al., 2014).

The characterization parameters for the CW and AS samples are reported in Table 1.

### **2.2 Experimental set-up**

Batch fermentation tests were carried out in glass reactors connected to an automatic system for data acquisition and pH control through continuous NaOH addition. The reactors (total volume = 1 liter, working volume = 0.5 liters) were equipped with a mechanical stirring device and maintained under mesophilic conditions ( $T = 39 \pm 1$  °C). A eudiometer was connected to each reactor to allow for gas measurement using the volume displacement principle; to this aim, each eudiometer was filled with a NaCl-saturated solution acidified with H<sub>2</sub>SO<sub>4</sub> to pH = 2 to prevent

gas dissolution. An automatic recording system of biogas volume was used, which consisted of an electronic balance that weighed the volume of solution displaced from the eudiometers into a storage tank. Corrections for liquid and gas densities were adopted to convert the measured liquid weight to the corresponding gas volume. The latter was then further converted to standard temperature and pressure conditions ( $T = 273.15 \text{ K}$ ,  $P = 10^5 \text{ Pa}$ ).

Before the onset of the experiments, the reactors were flushed with  $\text{N}_2$  gas for a few minutes to drive off air from the reactor headspace.

Sixteen batch fermentation runs were arranged according to a full factorial design in two factors (pH and ISR). In particular, four pH set-point values (5.5, 6.5, 7.0 and 7.5) and four CW/AS ratios (25:75, 50:50, 75:25 and 100:0 on a wet weight basis, corresponding to ISR values of 1.44, 0.48, 0.16 and 0 g VS/g TOC) were adopted during the tests. Table 2 provides a summary of the experimental conditions tested in each experiment. Each test was performed in duplicate and the results will be reported in the following as the average of replicate data. The tests were stopped once any appreciable biogas production could be no longer detected.

## **2.3 Analytical methods**

A 10-mL volume of digestate was periodically withdrawn from the reactors during the experimental runs, with a sampling frequency that was adjusted on the basis of the observed evolution of biogas production. An aliquot of the samples to be analyzed for soluble parameters was filtered onto a  $1.2 \mu\text{m}$  membrane.

The process performance was evaluated by monitoring the volumetric amount and composition of the biogas produced, as well as the concentration of total solids (TS), volatile solids (VS), total organic carbon (TOC), soluble carbohydrates, volatile fatty acids (VFAs) and ethanol. For details on the analytical methods adopted in the study, the reader is referred to previous papers (Cappai et al., 2014; De Gioannis et al., 2014).

The biogas was periodically sampled from the eudiometers with a 25-mL gastight syringe and analyzed through a gas chromatograph (Model 3600 CX, VARIAN) equipped with a thermal conductivity detector and 2-m stainless-steel packed column (ShinCarbon ST) with an inner diameter of 1 mm. The operation temperatures of 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 VFAs (acetic [HAc], propionic [HPr], butyric + iso-butyric [HBu], valeric + isovaleric [HVal], hexanoic + isohexanoic [HHex], heptanoic [HHep]) concentration in the digestate was determined in 0.2-µm filtered (to prevent fouling) and HCl acidified (pH = 2) liquid effluent (1 µl) with a gas chromatograph equipped with a flame ionization detector (FID) and a 30 m capillary column (TRB-WAX) 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. All the analytical determinations were performed in duplicate.

The modified Gompertz bacterial growth model was used to calculate the kinetic parameters of the H<sub>2</sub> production process (Lay et al., 1999):

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

where  $H$  is the cumulative H<sub>2</sub> production,  $P_s$  is the maximum H<sub>2</sub> production,  $R_m$  is the maximum H<sub>2</sub> production rate,  $\lambda$  is the lag phase duration and  $t$  is the time. However, the volumetric biogas production data, which displayed in some cases a two-branched evolution over time, revealed that for a number of tests a two-stage model was more appropriate for fitting the experimental data. The presence of kinetically different stages during the fermentation process was likely related to substrate constituents of various nature displaying specific degradation rates. Thus, stemming from the modified Gompertz equation, the following theoretical model was built for

the purpose of data fitting:

$$H = P_{s1} \exp \left\{ - \exp \left[ \frac{R_{m1} \cdot e}{P_{s1}} (\lambda_1 - t) + 1 \right] \right\} + P_{s2} \exp \left\{ - \exp \left[ \frac{R_{m2} \cdot e}{P_{s2}} (\lambda_2 - t) + 1 \right] \right\} \quad (2)$$

The kinetic parameters in model (2) have for either stage the same meaning as explained for Eq. (1).

The experimental data were fitted with Equations (1) or (2) by means of least-square linear regression using Table Curve2D<sup>®</sup>. In order to evaluate the overall duration of the process, the time ( $t_{95}$ ) required for H<sub>2</sub> production to attain 95% of the maximum yield was also calculated.

## 2.4 Statistical analyses

Dedicated statistical analyses were performed to identify the correlations between the variables of concern. A correlation matrix showing the existence of monotonic relationships between the operating parameters and the main response variables of the process was derived using the Spearman rank-order correlation criterion. The graphical visualization of the correlation matrix was made using the *corrplot* package (Wei and Simko, 2016) developed for application with the R software (R Development Core Team, 2009).

The results of the fermentation tests were further processed to identify the significant effects and interactions of pH and ISR on the process performance. To this aim, the statistical t-test was adopted assuming a confidence level of 95%. The experimental results were used to derive a second-order polynomial relationship (Eq. (3)), describing the response surface for the variable of concern,  $y$ , as a function of the factor effects and interactions:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \varepsilon \quad (3)$$

where  $y$  is expressed as a linear combination of zero-, first- and second-order terms, plus a random error component ( $\varepsilon$ ); second-order terms are in turn further subdivided into a two-way interaction and pure quadratic terms. In the model,  $x_1$  and  $x_2$  are the levels of the two factors,  $\beta_0$

is the zero-order coefficient,  $\beta_1$  and  $\beta_2$  those of the linear component, while  $\beta_{11}$ ,  $\beta_{22}$  and  $\beta_{12}$  those of the quadratic component. Such coefficients were estimated through least-square linear regression of the experimental data using the *rsm* package (Lenth, 2009) implemented in R.

### 3. RESULTS AND DISCUSSION

#### 3.1 H<sub>2</sub> production

The investigated operating conditions proved in all cases suitable to produce a significant amount of H<sub>2</sub>, although specific features were identified that will be illustrated in detail below. It should be emphasized that methane production was never detected during the experimental runs, the generated biogas being only composed of H<sub>2</sub> and CO<sub>2</sub>. The volumetric H<sub>2</sub> content of biogas was always found to be higher than 40% and to increase with the operating pH of the system up to values of 90–96%, clearly as a consequence of the increased CO<sub>2</sub> solubility in water under increasingly alkaline conditions rather than due to biochemical reasons.

Figure 1 shows the specific cumulative H<sub>2</sub> production (i.e., per unit of initial TOC of CW in the mixture) for the full set of experimental runs performed. In the figure, the measured data are depicted as individual data points, while the continuous lines represent the single-stage or two-stage Gompertz curves (Eqs. (1) and (2)) derived from the fitting procedure described in section 2.3. It was evident that the hydrogenogenic process was largely dependent on both pH and ISR, clearly showing the importance of a careful control and optimization of such parameters with a view to maximize the process performance. In particular, large variations were observed depending on the experimental conditions adopted, as for both time evolution and specific H<sub>2</sub> production yield (SHPY). More specifically, SHPY (as derived from the Gompertz curves) was observed to vary by a factor of up to 6, ranging from a maximum of 371 L H<sub>2</sub>/kg TOC<sub>whcy</sub> (194 L/kg TOC<sub>mix</sub>) at CW = 25% (ISR = 1.44 g VS/g TOC) and pH = 5.5 to a minimum of 61 L H<sub>2</sub>/kg TOC<sub>whcy</sub> at CW = 100% (ISR = 0) and pH = 7.0. The combination CW = 25% (ISR = 1.44

g VS/g TOC) and pH = 6.5 also yielded some remarkable final H<sub>2</sub> production, with a value of 338 L H<sub>2</sub>/kg TOC<sub>whey</sub> (192 L/kg TOC<sub>mix</sub>).

It is worth mentioning that the maximum SHPY achieved was quite notable when compared to the results reported in previous studies. The maximum observed SHPY was calculated to correspond to 186 L H<sub>2</sub>/kg VS<sub>whey</sub>, 236 L H<sub>2</sub>/kg hexose<sub>whey</sub>, 1.9 mol H<sub>2</sub>/mol hexose<sub>whey</sub>, 3.8 mol H<sub>2</sub>/mol lactose<sub>whey</sub> (assuming a 2:1 molar carbon equivalence between lactose and hexose), 6.2 mmol H<sub>2</sub>/g COD<sub>whey</sub> (adopting the simplifying hypothesis of TOC being present in the form of lactose only, which yields a COD of 2.67 g O<sub>2</sub>/g TOC). Table 3 provides a summary of the performance reported in previous studies on fermentative H<sub>2</sub> production from CW. As evident, the process optimization with respect to pH and ISR proved to result in a **fairly** significant gain in the H<sub>2</sub> production performance of the system, SHPY being up to 3.4 times the highest values documented in previous literature studies. Only in two cases (for which yields of 10.285 mmol/g COD (Yang et al., 2007) and 9.2 mmol/g COD (Azbar et al., 2009a) were reported) were **SPHY values from the present study** lower than those documented in the literature.

A first attempt at identifying the existence of simple monotonic correlations between pH or ISR and the process performance variables as well as among the response variables themselves was made by analyzing the Spearman's correlation matrix. The results are depicted in Figure 2 a) in terms of Spearman's rank correlation coefficients for those pairs of variables for which the calculated correlation was found to be statistically significant (significance level = 95%). Given the complexity of the resulting relationships, these will be discussed at different points in the subsequent manuscript sections. It should be emphasized here that the results of the correlation analysis are to be interpreted with care, and in particular the fact that no significant correlation between two given variables is detected would not *a priori* exclude the existence of a non-monotonic relationship between the two.

What is relevant from Figure 2 a) at this point of the discussion is that SHPY was found to be

positively correlated with ISR but not with pH. Nevertheless, the fact that lower pHs and higher ISRs qualitatively appeared to promote H<sub>2</sub> production suggested that the dependence of SHPY on pH may have been non-monotonic and could have also possibly been explained by interaction effects. In order to assess the validity of such a hypothesis, the response surfaces for SHPY as a function of the factor levels were derived by accounting for first-order and quadratic effects of pH and ISR according to the quadratic model in Eq. (3). All zero-, first- and second-order terms were indeed found to be statistically significant. Figure 2 b) reports the results of model fitting in terms of contour plots of SHPY as a function of pH and ISR as well as the relationship between the corresponding observed and predicted values. The high correlation ( $R^2 = 0.86$  - Figure 2 c)) between these proves the good degree of data fitting by the adopted model and the validity of the above mentioned hypothesis of non-monotonic dependence of SHPY on pH. The curvature of the contour lines suggests that adequate prediction of substrate conversion into biogas during CW fermentation implies that higher than first-order effects of the factors are accounted for. Furthermore, the response surfaces show that the effect of pH was more relevant for higher ISR values ( $\geq \sim 0.6$  g VS/g TOC), while below a certain inoculum addition ratio H<sub>2</sub> production was relatively low and poorly dependent on pH, which also justifies the Spearman's correlation results shown in Figure 2 a). The statistical analysis showed that the stationary point, which in this case represents the theoretical maximum of SHPY in the investigated region and corresponds to a value of 382 L H<sub>2</sub>/kg TOC<sub>whey</sub>, was attained at pH = 5.6 and ISR = 1.2 g VS/g TOC. The predictive model in Eq. (3) also indicated that SHPY in excess of 250 L H<sub>2</sub>/kg TOC<sub>whey</sub>, which represents the upper value of the commonly documented yield in literature studies, can be attained for a relatively wide range of operating conditions provided that suitable combinations of pH ( $\leq 7.3$ ) and ISR ( $\geq 0.4$  g VS/g TOC) are selected.

The fermentation kinetics was analyzed through the parameter  $t_{95}$  defined in section 2.3, referred to here as  $t_{95}\text{-H}_2$ , which provides a measure of the overall duration of the hydrogenogenic

reactions. The other kinetic parameters  $R_m$  and  $\lambda$  were not used for comparison purposes among the different runs since their numeric values are affected both by the SHPY attained and by the existence of multiple stages during the degradation process. The estimated values of  $t_{95-H_2}$ , depicted in Figure 3, were found to range from 5.3 h (at pH = 7.5 and ISR = 1.44 g VS/g TOC) to 183 h (at pH = 5.5 and null inoculum addition). The overall process duration also appeared not to be significantly affected by pH and to depend on ISR only in the lower range of values tested ( $< 0.3$  g VS/g TOC), which explains the lack of correlation indicated by the Spearman's analysis. Lower inoculum additions to the feed mixture appeared to require longer process durations for an adequate microbial community to develop and degrade a given amount of substrate. The second-order model (Eq. (3)) derived to describe the response surfaces for  $t_{95-H_2}$  was found to produce inconsistent results especially for those experimental conditions that corresponded to faster fermentation kinetics, predicting negative  $t_{95-H_2}$  in the lower range of values. This shows that for such a parameter the model failed to yield accurate predictions of the overall process durations within the whole range of operating conditions investigated. It is tempting to hypothesize that the actual relationship between  $t_{95-H_2}$  and the process conditions would only be explained if third- or higher-order effects were included in the predicting model, although **it was not possible** to assess this hypothesis given the resolution of the factorial design used in the experimental campaign. Dropping out the notably higher  $t_{95-H_2}$  values (corresponding to zero inoculum addition and pHs of 5.5 and 6.5) removed the above mentioned inconsistencies of negative  $t_{95-H_2}$  values and somehow improved the predictions by the model, although the degree of fitting remained relatively low ( $R^2 = 0.64$  – results not reported here).

### 3.2 Organic matter degradation

Substrate degradation during the process was evaluated by following the time evolution of carbohydrates, TOC, DOC as well as the concomitant production of metabolites. Figure 4 reports



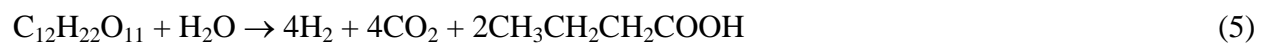
the profiles of soluble carbohydrates and TOC as a function of time for the different operating conditions tested. TOC removal at the end of the experiments was relatively low (< 20% in most cases), which well mirrors the fact that the hydrogenogenic process is an intermediate stage of anaerobic degradation and most of the organic matter in the system is maintained in the form of both non-degraded substances and metabolic products. TOC removal showed no correlation with either ISR or pH, which is likely a result of the fact that similar levels of organic matter degradation may be the result of different metabolic pathways, with different associated H<sub>2</sub> production yields. Therefore, as already pointed out in previous studies (see e.g. (Alibardi and Cossu, 2015a; De Gioannis et al., 2014)), the degree of TOC degradation attained cannot be taken as an indicator of the evolution of the hydrogenogenic process.

Different features were observed for carbohydrates. The concentration of soluble carbohydrates showed a notably fast decrease over time, confirming the well established **characteristic** of their being the preferred substrate for H<sub>2</sub> production (Alibardi and Cossu, 2015a, 2015b; Argun et al., 2008; De Gioannis et al., 2014, 2013; Kim et al., 2004; Lay et al., 1999; Nazlina et al., 2011). Carbohydrate utilization appeared to be described by a first-order type kinetics, in agreement with previous results **of the current research** (De Gioannis et al., 2014). Carbohydrate consumption at the end of the runs was fairly more pronounced than that of TOC, with values above 95%, indicating that an almost complete degradation of such species occurred in all tests. High carbohydrate utilization yields (> 75%) in fermentation of organic waste materials are commonly reported in literature studies (De Gioannis et al., 2014; Ferreira Rosa et al., 2014a; Kargi et al., 2012a; Ottaviano et al., 2017; Perna et al., 2013; Stamatelatou et al., 2011; Venetsaneas et al., 2009), although it has also been shown that non-optimized process conditions may significantly reduce carbohydrate degradation leading to extremely low removal yields (Ottaviano et al., 2017). A 95% yield of carbohydrate degradation was calculated to be associated to  $t_{95}$  values, referred to here as  $t_{95\text{-carb}}$ , varying from 5.5 h at ISR = 0.48

g VS/g TOC and pH = 7.5 to 53 h at null inoculum addition and pH = 5.5. While a monotonic correlation was only observed with ISR and not with pH (see Figure 2 a)), the second-order polynomial model in Eq. (3) showed that the influence of pH and ISR on the values of  $t_{95}\text{-carb}$  differed from that observed for SHPY. This suggests that, despite the similarities in carbohydrate degradation in terms of both removal yields and rates, the prevailing metabolic pathways and the related substrate conversion into  $\text{H}_2$  varied largely depending of the operating parameters adopted. A close relationship was on the other hand observed to exist between  $t_{95}\text{-H}_2$  and  $t_{95}\text{-carb}$  (see Figure 2 a)), which was interpreted as a clear indication of  $\text{H}_2$  generation ceasing as a result of carbohydrate depletion, in spite of organic matter being still largely available in the fermentation system in other chemical forms. The fact that SHPY was found to be negatively associated with  $t_{95}\text{-carb}$  may support this assumption and further suggest that higher substrate conversions into  $\text{H}_2$  are accompanied by faster carbohydrate utilization.

Figure 5 shows the time evolution of the measured soluble metabolites of the fermentation process (VFAs from acetate to valerate, as well as ethanol) compared to the observed  $\text{H}_2$  production. It is noted that both caproic and heptanoic acids were always below the analytical detection limit (10 ppm) and their contribution to the total amount of metabolites produced was thus negligible. The main metabolic products were found to include acetate and butyrate, followed by ethanol; propionate production was evident at the later stages of the fermentation process, while valerate was detected at appreciable concentrations in a very limited number of cases only. The concomitant presence of multiple metabolic products, which has also been observed in other studies on CW (Davila-Vazquez et al., 2009; De Gioannis et al., 2014; Ferreira Rosa et al., 2014b; Perna et al., 2013; Venetsaneas et al., 2009), suggests that the hydrogenogenic process was likely governed by several fermentation pathways, which presumably overlapped and competed with each other. The results reported in the literature also show that the relative amount of the metabolic products formed is largely variable depending on

the specific composition of the substrate, the characteristics of the inoculum and the ISR, as well as the fermentation conditions (pH, temperature, OLR, HRT and others). All such variables evidently affect the nature and relative contribution of the metabolic pathways governing the process, determining the type and concentration of the associated metabolic products and in turn the net H<sub>2</sub> production yield. Given the very complex nature of the microbial reactions involved, particularly in a mixed culture like the one being established in a system fed with real substrates and inocula, providing a univocal interpretation of all the mechanisms involved becomes a challenging task. Nevertheless, a number of distinguishing features can be identified based on the analytical results. Specifically, acetate turned out to be the most abundant metabolic product among the analyzed species, apart from the ISR = 0 and pH = 5.5 combination, where butyrate prevailed. As already mentioned above, butyrate was also present in the digestate at significant concentrations in all fermentation tests, with the exception of the runs at ISR = 0 and pH = 7.0 and 7.5. The observed concentration ranges for acetate and butyrate at the end of the runs were, respectively, 1.9–3.2 and 1.9–3.0 mol/kg TOC<sub>whely</sub> at pH = 5.5, 2.7–5.8 and 2.0–3.5 mol/kg TOC<sub>whely</sub> at pH = 6.5, 2.6–4.1 and 0.2–1.7 mol/kg TOC<sub>whely</sub> at pH = 7.0, 4.3–4.9 and 0–1.5 mol/kg TOC<sub>whely</sub> at pH = 7.5. It was also interesting to note that the H<sub>Bu</sub>/H<sub>Ac</sub> ratio tended to systematically increase as time elapsed, closely mirroring the shape of the measured H<sub>2</sub> production curves. One explanation to such a feature may be found in the fermentation reactions associated to the metabolic activity of spore-forming bacteria of the *Clostridium* and *Bacillus* genera, which produce H<sub>2</sub> via the so-called clostridial fermentation (Moat et al., 2003). In the case of lactose as the substrate, the fermentation reactions can be represented as (Azbar et al., 2009a; Collet et al., 2004; Khanal et al., 2003):



Such reactions are also accompanied by the formation of smaller amounts of ethanol and other

reduced end products, the presence of which is necessary to ensure the electron balance. It is thus tempting to hypothesize that reactions (4) and (5) proceeded concomitantly during the process, but the rate of butyrate production was higher than that of acetate generation, so that the H<sub>Bu</sub>/H<sub>Ac</sub> ratio increased over time. The increasing fraction of butyrate over acetate may well result from the fact that the former may serve to consume the excess reducing equivalents generated during the process, thus meeting the electron balance condition (Ljungdahl et al., 1989). To this regard, the fact that the H<sub>Bu</sub>/H<sub>Ac</sub> ratio remained below 1 in all cases (except for the final value of 1.2 observed at ISR = 1.44 g VS/g TOC and pH = 5.5) may possibly further indicate that acetate production derived not only from clostridial fermentation, but also from other pathways including either heterotrophic/autotrophic homoacetogenesis or lactate plus acetate production by homofermentative lactic acid bacteria (Ljungdahl et al., 1989). Other potential pathways such as propionic fermentation with associated acetate production (Moat et al., 2003), which in principle may explain acetate-generating mechanisms other than hydrogenogenic reactions, should in this case be excluded since propionate was not detected at appreciable concentrations during the H<sub>2</sub> production period. This fact, which further confirms the findings of previous studies (Cappai et al., 2014; De Gioannis et al., 2014), clearly indicates that propionate generation only occurred once more competitive metabolic pathways got exhausted, which was in turn likely due to the reduced availability of readily degradable substrate constituents such as carbohydrates. Furthermore, while some literature studies (Azbar et al., 2009a; De Gioannis et al., 2014; Perna et al., 2013; Rodríguez et al., 2006; Van Ginkel and Logan, 2005) appeared to suggest that propionate production may predominantly occur at alkaline pHs, this was not the case of the current study, where its formation was in fact more appreciable at pHs 5.5 and 6.5.

Similarly, the commonly acknowledged feature that solventogenesis is favoured under acidic conditions was not confirmed by the present experimental findings, whereas the data in Figure 2

a) in fact indicated a positive correlation between pH and ethanol production in terms of both absolute and relative contents in the digestate at maximum cumulative  $H_2$  generation.

Interestingly, it was also observed that the percent molar distribution of ethanol in the digestate as a function of time was positively correlated with that of acetate and negatively with that of butyrate and propionate. This may tentatively be interpreted as an indication of the fact that alcohol production, rather than from dedicated pathways, derived from fermentation reactions in which reduced products are generated to act as electron scavengers consuming residual reducing equivalents.

The relative contribution of the overlapping and possibly competing metabolic pathways that were likely to take place during the fermentation process is not easy to evaluate. An attempt at identifying the role of the prevailing metabolic pathways was made by deriving the theoretical  $H_2$  production yield that one would expect if the clostridial and propionic fermentation were the only ongoing reactions. In particular, the theoretical  $H_2$  generation yield ( $SHPY_{theor}$ ) was calculated from the measured VFAs productions for each experiment assuming a generation of 2 mol of  $H_2$  per mol of acetate or butyrate produced (see reactions (4) and (5)) and a consumption of 1 mol of  $H_2$  per mol of propionate produced (Cappai et al., 2014). The ratio between the observed and the theoretical SHPY for the different experiments is reported in Figure 6 a). The  $SHPY_{obs}$  was found to range between 24 and 137% of  $SHPY_{theor}$ , and in most cases the observed production deviated from the corresponding theoretical yield. As discussed above, numerous potential metabolic pathways may be claimed to explain the observed differences between the two. It is worth mentioning here that in most cases  $SHPY_{obs}$  was lower than  $SHPY_{theor}$ , indicating that part of the degraded substrate was in fact utilized by non-hydrogenogenic pathways having a number of metabolites in common with clostridial fermentation. To this regard, the fact that the  $SHPY_{obs}/SHPY_{theor}$  ratio turned out to be negatively correlated with acetate production (see Figure 2 a)) appears to support the hypothesis mentioned above that acetate production derived

not only from clostridial fermentation, but mainly from other non-hydrogenogenic pathways. Furthermore, the presence of metabolic products that are not directly generated by hydrogenogenic pathways indicates that the original substrate is only partly exploited in terms of its H<sub>2</sub> generation potential, a portion of it being converted into “undesired” species. To this regard, a measure of the process efficiency may be derived considering the existence of an upper threshold for hydrogenogenic fermentation, referred to as the Thauer limit (Thauer et al., 1977), of 4 moles of H<sub>2</sub> that can be generated per mole of glucose. In the present study, the observed substrate conversion into H<sub>2</sub> was calculated to range from 0.3 to 1.9 mol H<sub>2</sub>/mol hexose, thus corresponding to a conversion efficiency of 8–47% of the theoretical threshold.

Additional considerations about the fate of the original substrate during the process and the conversion yield into the final product were drawn through a mass balance of carbon, taking into account the following contributions (see Figure 6 b)): 1) C in the form of the analyzed metabolic products (C2-C7 VFAs and ethanol); 2) residual C (including C present as non-degraded organic compounds and/or additional metabolic products); 3) C removed through periodic digestate sampling; 4) gasified C (in the form of CO<sub>2</sub>, given the fact that CH<sub>4</sub> was never detected in biogas). The term “balance” in Figure 6 b) represents the C mass that was apparently lost due either to inaccuracies in the analytical measurements or sample inhomogeneity and was thus required to close the materials balance. It was evident that the largely major portion (76–98%) of the initial C mass was retained in the digestate as residual C, out of which 13–44% was associated to the measured metabolic products and 36–79% to residual unaccounted C.

#### 4. CONCLUSIONS

- H<sub>2</sub> yield, kinetics and fermentation pathways were variously affected by pH and ISR.
- Distinctive monotonic relationships between the variables were identified. The response surfaces provided a useful estimation of the optimal operating range for hydrogenogenesis.

- A major portion of carbon remained as metabolites or non-degraded species. Soluble carbohydrates were largely removed. H<sub>2</sub> generation ceased when carbohydrate depletion occurred.
- Metabolites included acetate, butyrate and ethanol. Propionate was observed as H<sub>2</sub> production decreased. The process was likely governed by several overlapping and competing fermentation pathways, which reduced the expected SHPY.

## SUPPLEMENTARY INFORMATION

E-supplementary data for this work reporting additional more specific experimental results of the study (specifically: 1) the influence of pH and ISR on the  $t_{95}$ -carb; and 2) the time evolution of the HBU/HAc ratio) can be found in the on-line version of the present paper.

## REFERENCES

- Alibardi, L., Cossu, R., 2015a. Effects of carbohydrate, protein and lipid content of organic waste on hydrogen production and fermentation products. Waste Manag. doi:10.1016/j.wasman.2015.07.049
- Alibardi, L., Cossu, R., 2015b. Composition variability of the organic fraction of municipal solid waste and effects on hydrogen and methane production potentials. Waste Manag. 36, 147–55. doi:10.1016/j.wasman.2014.11.019
- Argun, H., Dao, S., 2017. Bio-hydrogen production from waste peach pulp by dark fermentation: Effect of inoculum addition. Int. J. Hydrogen Energy 42, 2569–2574. doi:10.1016/j.ijhydene.2016.06.225
- Argun, H., Kargi, F., Kapdan, I., Oztekin, R., 2008. Batch dark fermentation of powdered wheat starch to hydrogen gas: Effects of the initial substrate and biomass concentrations. Int. J. Hydrogen Energy 33, 6109–6115. doi:10.1016/j.ijhydene.2008.08.004

- Azbar, N., Dokgöz, F.T., Keskin, T., Eltem, R., Korkmaz, K.S., Gezgin, Y., Akbal, Z., Öncel, S., Dalay, M.C., Gönen, Ç., Tutuk, F., 2009a. Comparative evaluation of bio-hydrogen production from cheese whey wastewater under thermophilic and mesophilic anaerobic conditions. *Int. J. Green Energy* 6, 192–200. doi:10.1080/15435070902785027
- Azbar, N., Dokgöz Çetinkaya, F.T., Peker, Z., 2009b. Optimization of basal medium for fermentative hydrogen production from cheese whey wastewater. *Int. J. Green Energy* 6, 371–380. doi:10.1080/15435070903107049
- Cappai, G., De Gioannis, G., Friargiu, M., Massi, E., Muntoni, A., Polettini, A., Pomi, R., Spiga, D., 2014. An experimental study on fermentative H<sub>2</sub> production from food waste as affected by pH. *Waste Manage.* 34, 1510–1519. doi:10.1016/j.wasman.2014.04.014
- Cappai, G., De Gioannis, G., Muntoni, A., Polettini, A., Pomi, R., Spiga, D., 2015. Effect of inoculum to substrate ratio (ISR) on hydrogen production through dark fermentation of food waste, in: Cossu, R., He, P., Kjeldsen, P., Matsufuji, Y., Reinhart, D., Stegmann, R. (Eds.), *Sardinia 2015, Fifteenth International Waste Management and Landfill Symposium*, S. Margherita Di Pula, Cagliari, Italy, 5-9 October 2015.
- Carvalho, F., Prazeres, A.R., Rivas, J., 2013. Cheese whey wastewater: characterization and treatment. *Sci. Total Environ.* 445–446, 385–96.
- Collet, C., Adler, N., Schwitzguébel, J.-P., Péringer, P., 2004. Hydrogen production by *Clostridium thermolacticum* during continuous fermentation of lactose. *Int. J. Hydrogen Energy* 29, 1479–1485. doi:10.1016/j.ijhydene.2004.02.009
- Davila-Vazquez, G., Alatraste-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
- De Gioannis, G., Friargiu, M., Massi, E., Muntoni, A., Polettini, A., Pomi, R., Spiga, D., 2014. Biohydrogen production from dark fermentation of cheese whey: Influence of pH. *Int. J. Hydrogen Energy* 39, 20930–20941. doi:10.1016/j.ijhydene.2014.10.046
- De Gioannis, G., Muntoni, A., Polettini, A., Pomi, R., 2013. A review of dark fermentative hydrogen production from biodegradable municipal waste fractions. *Waste Manage.* 33, 1345–1361.
- European Commission, Eurostat, 2015. Milk and milk product statistics - Statistics Explained [WWW Document]. [http://ec.europa.eu/eurostat/statistics-explained/index.php/Milk\\_and\\_milk\\_product\\_statistics](http://ec.europa.eu/eurostat/statistics-explained/index.php/Milk_and_milk_product_statistics). doi:;
- Fang, H.H.P., Liu, H., 2002. Effect of pH on hydrogen production from glucose by a mixed culture. *Bioresour. Technol.* 82, 87–93. doi:10.1016/S0960-8524(01)00110-9
- Ferchichi, M., Crabbe, E., Gil, G.-H., Hintz, W., Almadidy, A., 2005. Influence of initial pH on hydrogen production from cheese whey. *J. Biotechnol.* 120, 402–409. doi:10.1016/j.jbiotec.2005.05.017
- Fernández, C., Cuetos, M.J., Martínez, E.J., Gómez, X., 2015. Thermophilic anaerobic digestion of cheese whey: Coupling H<sub>2</sub> and CH<sub>4</sub> production. *Biomass and Bioenergy* 81, 55–62. doi:10.1016/j.biombioe.2015.05.024
- Ferreira Rosa, P.R., Santos, S.C., Sakamoto, I.K., Amâncio Varesche, M.B., Silva, E.L., 2014a. Hydrogen production from cheese whey with ethanol-type fermentation: Effect of hydraulic retention time on the microbial community composition. *Bioresour. Technol.* 161, 10–19. doi:10.1016/j.biortech.2014.03.020
- Ferreira Rosa, P.R., Santos, S.C., Silva, E.L., 2014b. Different ratios of carbon sources in the

- fermentation of cheese whey and glucose as substrates for hydrogen and ethanol production in continuous reactors. *Int. J. Hydrogen Energy* 39, 1288–1296.
- 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
- Ghimire, A., Sposito, F., Frunzo, L., Trably, E., Escudié, R., Pirozzi, F., Lens, P.N.L., Esposito, G., 2016. Effects of operational parameters on dark fermentative hydrogen production from biodegradable complex waste biomass. *Waste Manage.* 50, 55–64. doi:10.1016/j.wasman.2016.01.044
- Kargi, F., Eren, N.S., Ozmihci, S., 2012a. Hydrogen gas production from cheese whey powder (CWP) solution by thermophilic dark fermentation. *Int. J. Hydrogen Energy* 37, 2260–2266. doi:10.1016/j.ijhydene.2011.11.018
- Kargi, F., Eren, N.S., Ozmihci, S., 2012b. 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
- Khanal, S., Chen, W., Li, L., Sung, S., 2003. Biological hydrogen production: effects of pH and intermediate products. *Int. J. Hydrogen Energy* 29, 1123–1131. doi:10.1016/j.ijhydene.2003.11.002
- Kim, D.-H., Kim, S.-H., Jung, K.-W., Kim, M.-S., Shin, H.-S., 2011. Effect of initial pH independent of operational pH on hydrogen fermentation of food waste. *Bioresour. Technol.* 102, 8646–52. doi:10.1016/j.biortech.2011.03.030
- Kim, S.H., Han, S.K., Shin, H.S., 2004. Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. *Int. J. Hydrogen Energy* 29, 1607–1616.
- Lay, J.-J., Lee, Y.-J., Noike, T., 1999. Feasibility of biological hydrogen production from

- organic fraction of municipal solid waste. *Water Res.* 33, 2579–2586. doi:10.1016/S0043-1354(98)00483-7
- Lenth, R.V., 2009. Response-surface methods in R, using rsm. *J. Stat. Softw.* 32, 1–17.
- Liu, Y., 1996. Bioenergetic interpretation on the S<sub>0</sub>/X<sub>0</sub> ratio in substrate-sufficient batch culture. *Water Res.* 30, 2766–2770. doi:10.1016/S0043-1354(96)00157-1
- 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
- Moat, A.G., J.W., F., Spector, M.P., 2003. Fermentation pathways, in: *Microbial Physiology*. Wiley-Liss, New York, pp. 412–433.
- Moon, C., Jang, S., Yun, Y.-M., Lee, M.-K., Kim, D.-H., Kang, W.-S., Kwak, S.-S., Kim, M.-S., 2015. Effect of the accuracy of pH control on hydrogen fermentation. *Bioresour. Technol.* 179, 595–601. doi:10.1016/j.biortech.2014.10.128
- Nazlina, H.M.Y., Rahman, N.A., Hasfalina, C.M., Yusoff, M.Z.M., Hassan, M.A., 2011. Microbial characterization of hydrogen-producing bacteria in fermented food waste at different pH values. *Int. J. Hydrogen Energy* 36, 9571–9580. doi:10.1016/j.ijhydene.2011.05.048
- Ottaviano, L.M., Ramos, L.R., Botta, L.S., Amâncio Varesche, M.B., Silva, E.L., 2017. Continuous thermophilic hydrogen production from cheese whey powder solution in an anaerobic fluidized bed reactor: Effect of hydraulic retention time and initial substrate concentration. *Int. J. Hydrogen Energy* 42, 4848–4860. doi:10.1016/j.ijhydene.2016.11.168
- Perna, V., Castelló, E., Wenzel, J., Zampol, C., Fontes Lima, D.M., Borzacconi, L., Varesche, M.B., Zaiat, M., Etchebehere, C., 2013. Hydrogen production in an upflow anaerobic packed bed reactor used to treat cheese whey. *Int. J. Hydrogen Energy* 38, 54–62. doi:10.1016/j.ijhydene.2012.10.022
- Prazeres, A.R., Carvalho, F., Rivas, J., 2012. Cheese whey management: a review. *J. Environ.*

Manage. 110, 48–68.

R Development Core Team, 2009. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rodríguez, J., Kleerebezem, R., Lema, J.M., van Loosdrecht, M.C.M., 2006. Modeling product formation in anaerobic mixed culture fermentations. *Biotechnol. Bioeng.* 93, 592–606. doi:10.1002/bit.20765

Rosales-Colunga, L.M., Razo-Flores, E., Ordoñez, L.G., Alatraste-Mondragón, F., De León-Rodríguez, A., 2010. Hydrogen production by *Escherichia coli*  $\Delta$ hycA  $\Delta$ lacI using cheese whey as substrate. *Int. J. Hydrogen Energy* 35, 491–499. doi:10.1016/j.ijhydene.2009.10.097

Siso, M.I.G., 1996. The biotechnological utilization of cheese whey: A review. *Bioresour. Technol.* 57, 1–11. doi:10.1016/0960-8524(96)00036-3

Song, J., An, D., Ren, N., Zhang, Y., Chen, Y., 2011. Effects of pH and ORP on microbial ecology and kinetics for hydrogen production in continuously dark fermentation. *Bioresour. Technol.* 102, 10875–10880. doi:10.1016/j.biortech.2011.09.024

Stamatelatou, K., Antonopoulou, G., Tremouli, A., Lyberatos, G., 2011. Production of gaseous biofuels and electricity from cheese whey. *Ind. Eng. Chem. Res.* 50, 639–644. doi:10.1021/ie1002262

Thauer, R.K., Jungermann, K., Decker, K., 1977. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* 41, 100–80.

Van Ginkel, S., Logan, B.E., 2005. Inhibition of Biohydrogen Production by Undissociated Acetic and Butyric Acids. *Environ. Sci. Technol.* 39, 9351–9356. doi:10.1021/ES0510515

Van Ginkel, S., Sung, S., Lay, J.-J., 2001. Biohydrogen Production as a Function of pH and Substrate Concentration. *Environ. Sci. Technol.* 35, 4276–4730. doi:10.1021/ES001979R

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

Wei, T., Simko, V., 2016. Corrplot: Visualization of a Correlation Matrix.

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

Yuan, Y., Wang, S., Liu, Y., Li, B., Wang, B., Peng, Y., 2015. Long-term effect of pH on short-chain fatty acids accumulation and microbial community in sludge fermentation systems. *Bioresour. Technol.* 197, 56–63. doi:10.1016/j.biortech.2015.08.025

## Figure captions

Figure 1. Specific cumulative H<sub>2</sub> production yield as a function of pH and mixture composition

Figure 2. a) Correlation matrix showing the Spearman's rank correlation coefficients for each pair of variables. Blank cells indicate non-significant correlations ( $p > 0.05$ ); b) Contour plot for SHPY (L H<sub>2</sub>/kg TOC<sub>whey</sub>) according to Eq. (3); c) Relationship between predicted and observed SHPY

Figure 3. Plot of t<sub>95</sub>-H<sub>2</sub> as a function of pH and ISR

Figure 4. Time evolution of soluble carbohydrates and TOC as a function of pH and mixture composition

Figure 5. Time evolution of VFAs and ethanol (left-hand y-axis) as a function of pH and mixture composition, and comparison with H<sub>2</sub> production (right-hand y-axis)

Figure 6. a) Comparison between observed and theoretical SHPY; b) Carbon mass balance for the experimental runs

Table 1. Average composition of CW and AS

Parameter	Unit of measure	CW	AS
pH	-	5.5±0.3	n.a.
Total Solids (TS)	g/L	63±3.8	18±1.6
Volatile Solids (VS)	g/L	58±3.7	14±1.3
Total Organic Carbon (TOC)	g/L	29.2±1.5	6.9±0.24
Soluble organic carbon (DOC)	g/L	25.9±3.0	0.59±0.05
Total ammonia	mg N-NH <sub>4</sub> /L	262.6±30.7	379±5.5
Soluble ammonia	mg N-NH <sub>4</sub> /L	215.9±18.3	234±10.1
Total Kjeldahl nitrogen	g N-NH <sub>4</sub> /L	1.3±0.02	2.3±0.1
Soluble Kjeldahl nitrogen	g N-NH <sub>4</sub> /L	1.2±0.10	0.25±0.1
Total carbohydrates	g hexose/L	47.4±0.2	6.8±0.16
Soluble carbohydrates	g hexose/L	45.9±1.2	0.08±0.001

Table 2. Experimental conditions adopted during the fermentation experiments

Run no.	Run code	Mixture composition (% wet wt.)	ISR (g VS/g TOC)	pH
1	25CW pH 5.5	25% CW, 75% AS	1.44	5.5
2	25CW pH 6.5	25% CW, 75% AS	1.44	6.5
3	25CW pH 7.0	25% CW, 75% AS	1.44	7.0
4	25CW pH 7.5	25% CW, 75% AS	1.44	7.5
5	50CW pH 5.5	50% CW, 50% AS	0.48	5.5
6	50CW pH 6.5	50% CW, 50% AS	0.48	6.5
7	50CW pH 7.0	50% CW, 50% AS	0.48	7.0
8	50CW pH 7.5	50% CW, 50% AS	0.48	7.5
9	75CW pH 5.5	75% CW, 25% AS	0.16	5.5
10	75CW pH 6.5	75% CW, 25% AS	0.16	6.5
11	75CW pH 7.0	75% CW, 25% AS	0.16	7.0
12	75CW pH 7.5	75% CW, 25% AS	0.16	7.5
13	100CW pH 5.5	100% CW	0	5.5
14	100CW pH 6.5	100% CW	0	6.5
15	100CW pH 7.0	100% CW	0	7.0
16	100CW pH 7.5	100% CW	0	7.5



Table 3. Summary of previous studies on H<sub>2</sub> production from CW

Substrate	Inoculum	Operating conditions	SHPY	Ref.
CW	<i>C. saccharoperbutyl-acetonicum</i> ATCC 27021	Batch reactor T = 30 °C	7.89 mol/kg lactose	(Ferchichi et al., 2005)
CW permeate powder	Anaerobic sludge	Batch reactor T = 35–38 °C	10.285 mmol/g COD	(Yang et al., 2007)
CW permeate powder	Anaerobic sludge	CSTR T = 35–38 °C HRT = 24 h OLR = 14 kg COD/m <sup>3</sup> /d	2.3 mmol/g COD	(Yang et al., 2007)
CW powder	Anaerobic granular sludge	Batch reactor T = 37 °C	3.1 mol/mol lactose	(Davila-Vazquez et al., 2008)
CW	HST anaerobic granular sludge	Batch reactor T = 36 °C	9.2 mmol/g COD	(Azbar et al., 2009a)
CW	Anaerobic granular sludge	Batch reactor T = 36 °C	3.5 mol/mol lactose consumed	(Azbar et al., 2009b)
CW	HST anaerobic granular sludge	CSTR T = 37 °C HRT = 6 h OLR = 138.6 kg lactose/m <sup>3</sup> /d	2.8 mol/mol lactose	(Davila-Vazquez et al., 2009)
CW	None	CSTR T = 35 °C HRT = 24 h OLR = 30 g COD/d	0.78 mol/mol glucose consumed	(Venetsaneas et al., 2009)
CW	<i>E. coli</i> W3110 $\Delta$ hycA strain	Batch reactor T = 37 °C	2.74 mol/mol lactose consumed	(Rosales-Colunga et al., 2010)
CW powder	Anaerobic sludge	Batch reactor T = 55 °C	111 l/kg glucose	(Kargi et al., 2012b)
CW powder	Anaerobic sludge	Batch reactor T = 55 °C	1.03 mol/mol glucose	(Kargi et al., 2012a)
CW powder	Acclimated CW biomass	Continuous UAPB reactor T = 30 °C pH = 5.6 HRT = 24 h OLR = 37 kg COD/m <sup>3</sup> /d	0.668 mol/mol lactose	(Perna et al., 2013)
CW	HST aerobic activated sludge	Batch reactor T = 39 °C pH = 6.0	171.3 L/kg TOC	(De Giannnis et al., 2014)
CW powder	HST granular sludge	Continuous AFB reactor T = 30 °C HRT = 4 h OLR = 30 kg COD/m <sup>3</sup> /d	1.33 mol/mol lactose	(Ferreira Rosa et al., 2014a)
CW	HST anaerobic granular sludge	Continuous AFB reactor T = 30 °C HRT = 6 h OLR = 20 kg COD/m <sup>3</sup> /d	1.27 mol/ mol lactose	(Ferreira Rosa et al., 2014b)
CW	None	CSTR T = 35 °C pH = 4.5–5.5 HRT = 3 d OLR = 12.7 kg COD/m <sup>3</sup> /d	12 L/kg COD	(Fernández et al., 2015)
CW + buffalo manure	HST anaerobic sludge	CSTR HRT = 12 h	152.2 L/kg VS	(Ghimire et al., 2017)

		OLR = 2.6 kg VS/m <sup>3</sup> /d		
CW powder	Thermophilic anaerobic granular sludge	Continuous AFB reactor T= 55 °C HRT = 4 h OLR = 30 kg COD/m <sup>3</sup> /d	3.67 mol/mol lactose	(Ottaviano et al., 2017)

Abbreviations:

CSTR = continuous-flow stirred tank reactor

HRT = hydraulic retention time

OLR = organic loading rate

UAPB = upflow anaerobic packed bed reactor

AFB = anaerobic fluidized bed reactor

HST = heat-shock treated

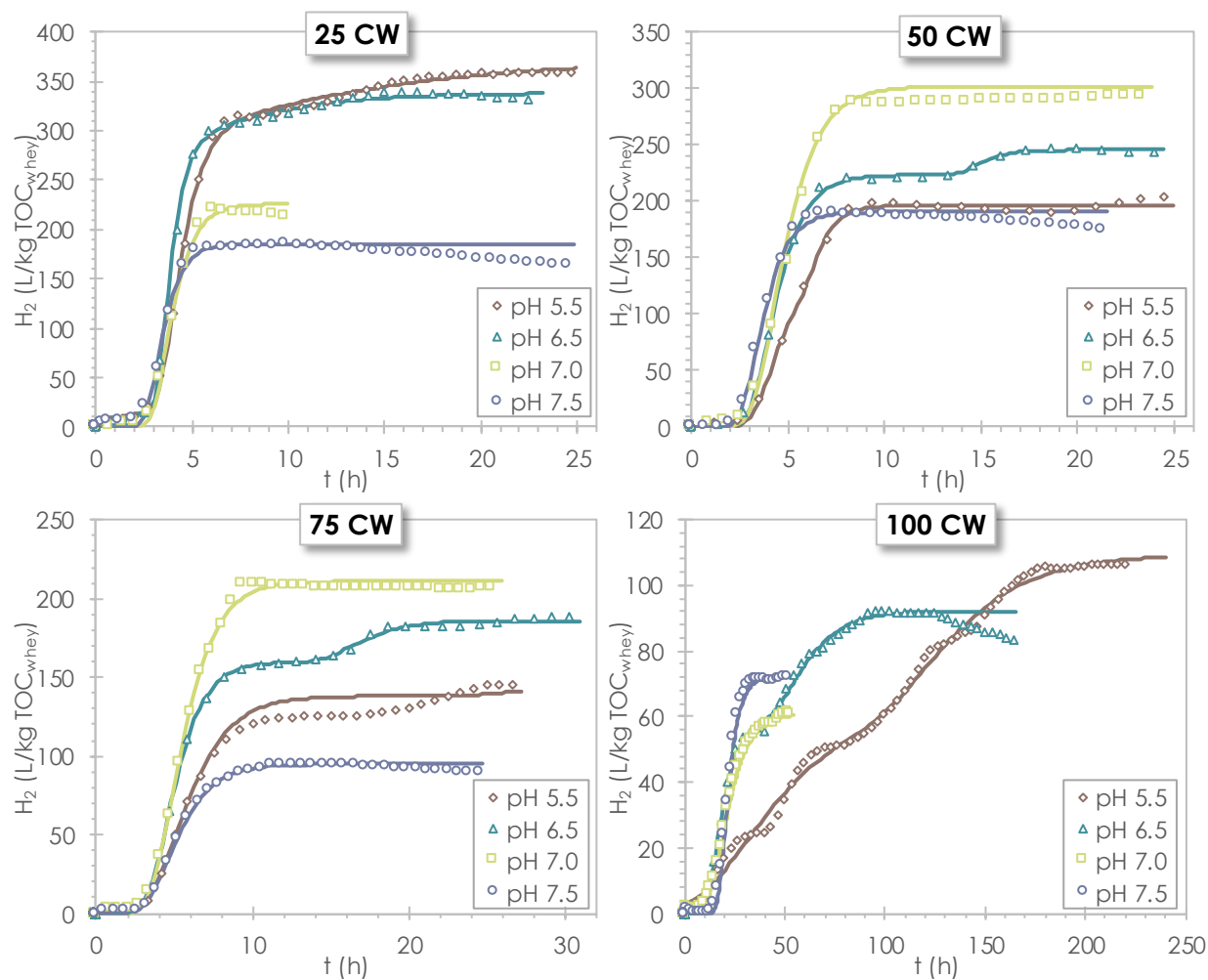


Figure 1

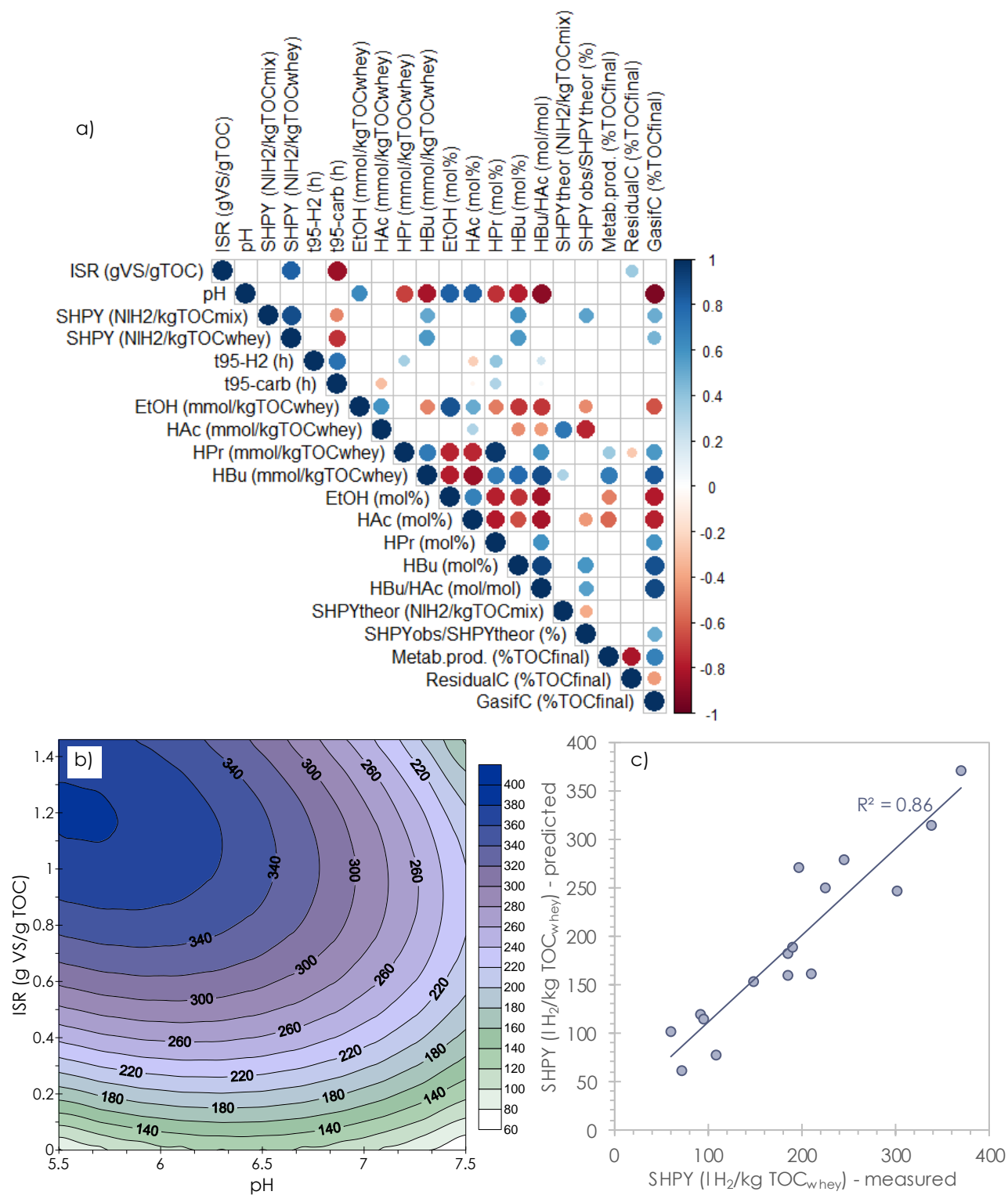


Figure 2

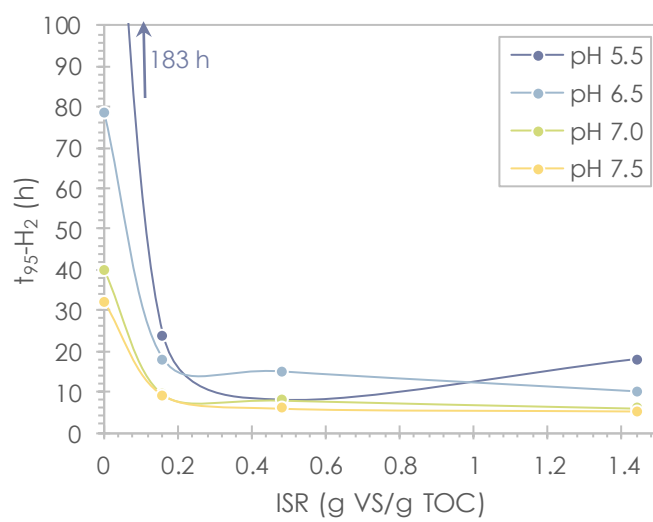


Figure 3

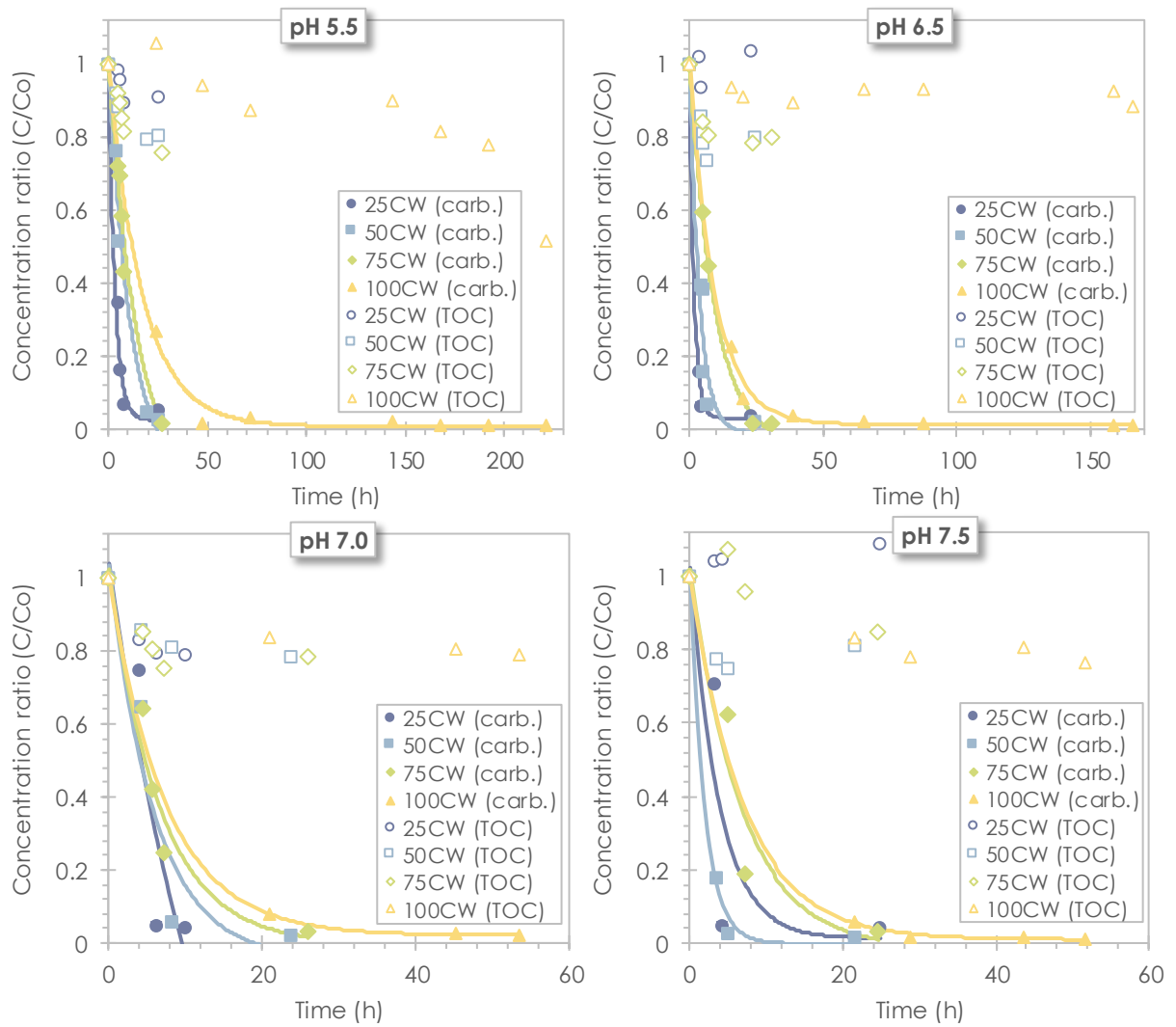


Figure 4

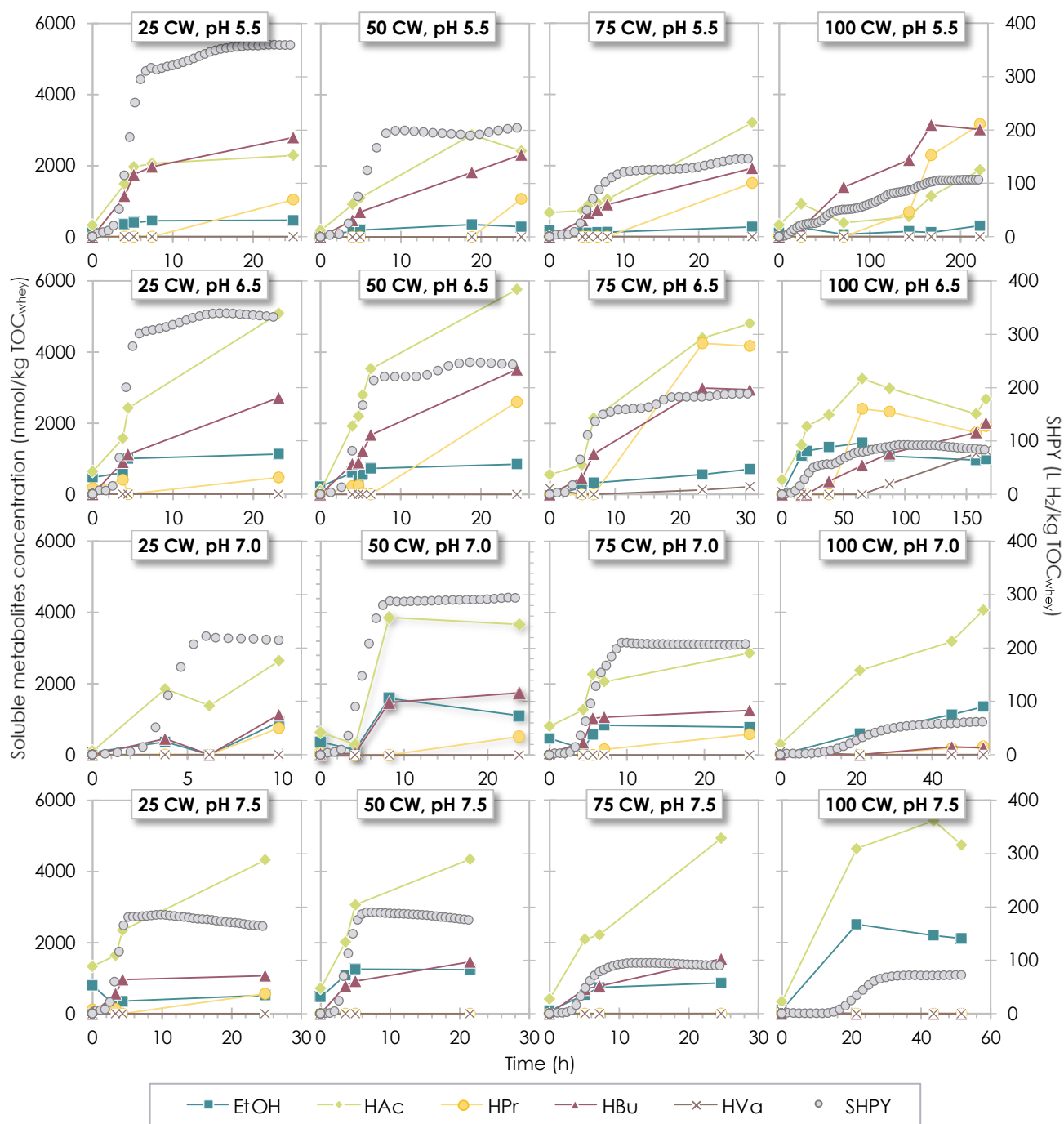


Figure 5

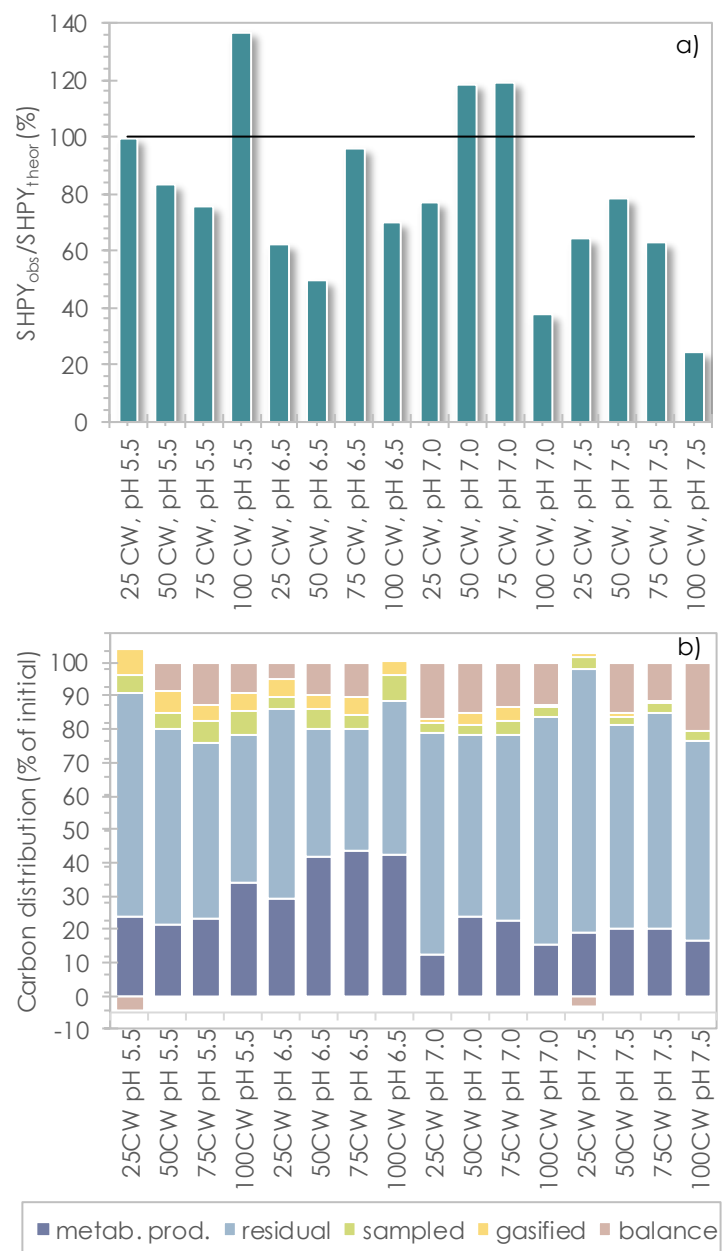


Figure 6



## Supplementary material

[Click here to download Electronic Annex: paper CW batch analisi stat \(Suppl Info\).docx](#)