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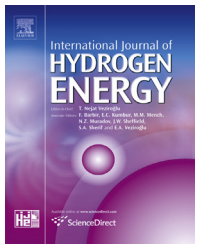

Biohydrogen production from dark fermentation of cheese whey: Influence of pH

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ABSTRACT

Hydrogen production from cheese whey through dark fermentation was investigated in this study in order to systematically analyse the effects of the operating pH. The effluents from pecorino cheese and mozzarella cheese production were the substrates used for the fermentation tests. Either CW only or a mixture of CW and heat-shocked activated sludge were used in mesophilic pH-controlled batch fermentation experiments. The results indicated that hydrogen production was strongly affected by multiple factors including the substrate characteristics, the addition of an inoculum as well as the pH. The process variables were found to affect to a varying extent numerous interrelated aspects of the fermentation process, including the hydrogen production potential, the type of fermentation pathways, as well as the process kinetics. The fermentation products varied largely with the operating conditions and mirrored the H_2 yield. Significant fermentative biohydrogen production was attained at pHs of 6.5–7.5, with the best performance in terms of H_2 generation potential (171.3 NL H_2/kg TOC) being observed for CW from mozzarella cheese production, at a pH value of 6.0 with the heat-shocked inoculum.

Introduction

The dairy industry, like other activities in food manufacturing, is a large contributor to the production of liquid effluents, which are often highly problematic due to both the specific quantities generated (i.e., per unit of manufactured product) and the high associated organic load [1,2]. According to European Commission statistics [3], the overall production of dairy products in 2012 in the EU-28 area accounts for 90.7 million tonnes. The main dairy products include drinking milk (35.0% of the overall production in 2012), whey (47.6%), cheese (10.2%), cream (2.8%), milk powder (2.3%), as well as butter and other yellow fat products (2.2%) [3]. In Europe, Italy is the third cheese producer after Germany and France, with an annual production of 1.2 million tonnes in 2012 [3].

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Cheese manufacturing results in the following two main types of wastewater streams [4]: cheese whey (obtained either from hard cheese production – which is partially reused to produce cottage cheese – or from soft cheese manufacturing) and a lower-strength wastewater stream (resulting from refrigeration and cleaning operations for pipes, tanks and other equipment). Cheese whey (CW) is regarded as the most problematic effluent, due to its high biological (BOD) and chemical oxygen demand (COD) as well as generation rate. According to Carvalho et al. [4], the specific production of CW is estimated to be 0.8–0.9 L per L of processed milk, or 9 kg per kg of cheese produced [5]. The chemical composition and characteristics of CW depend upon the type of milk as well as cheese production techniques used [4]. On average, cheese whey accounts for ~80% of the original fermentation medium [6] and retains ~55% of the milk nutrients [5,7]; 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) [5]; these include NaCl and KCl (>50%), calcium salts and others [8–10]. Other constituents of CW include lactic and citric acids, urea and uric acid as well as B-group vitamins [8].

Appropriate management of CW is mandatory to fulfil the current regulations which ban direct land or water disposal. To this regard, anaerobic digestion may represent a valid option to reduce the organic load of CW while allowing exploitation of its energy content. However, although the high carbohydrate content (mainly due to lactose) makes CW suited for biological processing, the anaerobic treatment of raw CW is quite problematic due to its high organic load, low alkalinity as well as attitude to rapid acidification [8,10] and accumulation of metabolites from lactose degradation (propionic acid, ethanol and acetate) [9,11], that may impair the fermentation reactions, or require appropriate dilution and external alkali addition [9,12]. To this regard, separation of acidogenesis from methanogenesis in the anaerobic process is a promising strategy with a view to separately optimize the different stages of the degradation process thereby attaining improved stabilization yields and energy conversion efficiency (see e.g. Refs. [12–15]). In particular, the first phase of the two-stage process may be optimized in view of biological production of hydrogen gas (H_2).

Among the operating parameters of fermentative H_2 production, pH is considered a key variable that affects substrate hydrolysis, hydrogenase activity and the metabolic pathways [16], in turn influencing substrate and energy utilization, synthesis of proteins and various storage products, and the fermentation products [17]. According to previous studies focussing on different substrates (see e.g. Refs. [18–23]), hydrogenogenic pathways (with associated acetate and butyrate production) were shown to establish at pH levels of ~4.5–6.5, while neutral or higher pHs were found to promote ethanol and propionic fermentation (with associated null or negative H_2 generation). Ethanol fermentation (solventogenesis) is assumed as a detoxification method of the biomass to prevent process inhibition caused by high VFA concentrations and associated low pHs in the liquid solution [24]. However, the type and entity of the effects of pH on the metabolic pathways have also been shown to vary with the inoculum and substrate characteristics [23,25].

Relatively few studies addressed the influence of different pH values on fermentative H_2 production from CW [26–28], however in all cases only the effect of the initial pH was investigated. More commonly, other researchers [6,8,15,26,28–32] focused on the hydrogenogenic process at fixed pH levels or within predefined pH ranges maintained by either initial/periodic addition of a buffer solution or with automated pH control during the process. The pH conditions tested were generally *a priori* adopted on the basis of results from previous studies on similar (pure) substrates; to this regard, the literature reports values of 6.0–6.5 for lactose [33] and 5.0–6.0 for glucose [34], sucrose [35] and starch [36]. Davila-Vazquez et al. [26] studied the effect of the initial pH using glucose, lactose and CW as substrates, with the highest H_2 yield being attained at different initial pHs for glucose and lactose (7.5) compared to CW (6.0). Rosales-Colunga et al. [27], using genetically modified *Escherichia coli* as the inoculum, obtained for CW a yield of 2.74 mol H_2/mol lactose consumed at an initial pH of 7.5. Azbar et al. [28], using a continuous reactor under pH-controlled (5.5) and thermophilic conditions, observed H_2 production yields of 0.3–7.9 L H_2/(L·d) depending on OLR and HRT.

From the literature overview presented, it is clear that a systematic investigation on the effects of pH (specifically, the operating pH) on fermentative H_2 production from CW is currently missing and the different conditions adopted in previous studies often led to inconsistent conclusions as to the optimal pH value, as already emphasized by other authors [8]. In the present study, we attempted at filling the mentioned gaps existing by means of a number of hydrogenogenic batch fermentation tests on undiluted CW performed at various operating pHs.

Materials and methods

Feedstock and seed microorganisms

Samples of fresh raw cheese whey (CW_1 and CW_2) were collected at two different Italian cheese making factories. CW_1 was the effluent from cheese production using a mixture of sheep and cow milk, while CW_2 was the by-product of a mozzarella cheese making factory which made use of cow milk. The wastewater was stored at 4 °C until use. The characterization parameters for CW_1 and CW_2 are reported in Table 1.

CW_1 and CW_2 were directly used as the feed material in H_2 production experiments using the indigenous hydrogenogenic biomass on a first instance. A number of tests were also carried out on mixtures of CW_2 and activated sludge (AS) from the aerobic unit of a municipal wastewater treatment plant, which was used as the biomass source. 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. The activated sludge was kept under anaerobic conditions in 20-L tanks and settled for 24 h before use. The AS was heat-shocked (105 °C, 30 min) before mixing with CW_2 in order to harvest the hydrogenogenic biomass; the

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Table 1 – Main characterization parameters of the two CW samples.

Parameter	Unit of measure	Value	
		CW_1	CW_2
pH	–	4.2 ± 0.3	6.5 ± 0.5
Total solids	g/L	75 ± 5.8	63.7 ± 4.5
Volatile solids	g/L	70 ± 4.9	58.8 ± 4.4
Total organic carbon, TOC_{tot}	g/L	22.8 ± 2.2	27.6 ± 6.5
Soluble organic carbon, TOC_{sol}	g/L	17.6 ± 0.7	20.6 ± 7.3
Soluble carbohydrates (as hexose)	g/L	16.7 ± 4.1	38 ± 6.3
Nitrogen	g/L	1.8 ± 0.2[a]	0.94 ± 0.2[b]
Chloride	mg/L	953 ± 9	1847 ± 89

[a] Total nitrogen.
[b] Total Kjeldahl nitrogen.

heat-shock treatment (HST) conditions were selected on the basis of previous results [23].

Experimental set-up and analytical methods

Batch fermentation tests were carried out at 39 ± 1 °C using, depending on the set of experiments performed, 1-L (working volume = 0.8 L) or 2-L (working volume = 1.8 L) glass fermentation reactors equipped with mechanical stirring and eudiometers for gas measurement using the volume displacement principle. The eudiometers were filled with a NaCl-saturated solution, acidified with H_2SO_4 to pH = 2 to prevent gas dissolution and coloured with methyl orange for easier visual estimation of the liquid level. In the experiments on the CW_2 sample an automatic recording system of total biogas volume was also used, which consisted of an electronic balance that periodically weighed the volume of solution displaced from the eudiometers. The measured gas volume was corrected for ambient temperature and pressure, and converted to standard temperature and pressure (T = 0 °C, p = 1 atm) conditions. The reactors were connected to an automatic system for data acquisition and continuous pH control through NaOH addition. Different pH set-point values in the range 5.5–8.5 were adopted in the experiments. Before the start of the experiments, the reactors were flushed with N_2 gas to drive off air from the reactor headspace, then operated in batch mode. The tests were stopped once biogas production could no longer be detected.

Table 2 provides a summary of the experimental conditions tested in each set of experiments. It is noted that the investigated pH ranges for the CW_1 and CW_2 samples were not the same, since in the experimental study on CW_2 it was deliberately planned to explore in more detail the optimal pH range only. The tests were conducted in triplicate and the results that will be reported are the average value of replicate analyses.

Table 2 – Experimental conditions adopted during the fermentation experiments.

Set of runs	Inoculum	Mixture composition % by weight	Set-point pH
$100CW_1$	–	100% CW_1	5.5, 6.5, 7.5, 8.5
$100CW_2$	–	100% CW_2	5.5, 6.0, 6.5, 7.5
$90CW_2\_10AS$	AS	90% CW_2 + 10% AS	5.5, 6.0, 6.5, 7.5
$45CW_2\_55AS$	AS	45% CW_2 + 55% AS	5.5, 6.0, 6.5, 7.5

Process performance was evaluated by monitoring the amount and composition of the produced biogas and the concentrations of total solids (TS), volatile solids (VS), total organic carbon (TOC_{tot}), soluble organic carbon (TOC_{sol}), soluble carbohydrates, VFAs and alcohols. These parameters were measured according to the Standard Methods for the Examination of Water and Wastewater [37]. Soluble carbohydrates were analysed using the colorimetric phenol–sulphuric acid method using glucose as the standard [38]. The TOC concentration was measured using a Shimadzu TOC analyser equipped with modules for the analysis of both liquid and solid samples.

The biogas was periodically sampled from the eudiometers with a 1-mL gastight syringe. The biogas composition was determined by a gas chromatograph (Model 5890 series II, Hewlett Packard), equipped with a thermal conductivity detector and a 2-m stainless column packed with Porapak Q (50/80 mesh). The operational temperatures of injector, oven and detector were 100, 70 and 100 °C, respectively, with N_2 as carrier gas.

The concentrations of VFAs (acetic [HAc], propionic [HPr], butyric + *iso*-butyric [HBu], valeric + *iso*-valeric [HVal], caproic + *iso*-caproic, heptanoic, lactic) and ethanol (EtOH) were determined using a gas chromatograph (Model 6890N, Agilent Technology) coupled with a mass spectrometry with a triple-axis detector (Model 5975CVL, Agilent Technology) equipped with an HP-FFAP capillary column (30 m, inner diameter 0.53 mm, Agilent Technology), in 0.45-μm filtered and H_3PO_4-acidified (pH < 3) liquid effluent (1 μL). The temperature of detector and injector was 230 and 250 °C, respectively. The temperature of the quadrupole was 150 °C. The oven temperature was initially set at 60 °C, followed by a ramp of 10 °C/min up to 220 °C held for 2 min. Helium (1.6 mL/min, split ratio 20:1) was used as the carrier gas. All the analytical determinations were performed in triplicate.

Results and discussion

Hydrogen production

Fig. 1a) shows the specific cumulative H_2 production from CW_1 for each tested pH, expressed per unit of initial TOC in the mixture. Both the time evolution of biogas production and the final yield were found to be largely affected by the operating pH, clearly showing that adequate pH control is one of the key factors for significant H_2 production to be achieved.

The best performance for CW_1 was observed at an operating pH of 7.5, with an associated final cumulative production of 90.9 NL H_2/kg TOC (45.1 NL H_2/kg VS). By decreasing pH to 6.5, the H_2 production yield also decreased down to 45.2 NL H_2/kg TOC (20.6 NL H_2/kg VS). The H_2 production over time at pHs of 6.5 and 7.5 was found to evolve similarly up to 18 h from the beginning of the test; thereafter, while at pH = 6.5 a plateau in biogas generation was rapidly attained, a more prolonged production phase (~35 h overall) was observed at pH = 7.5. The

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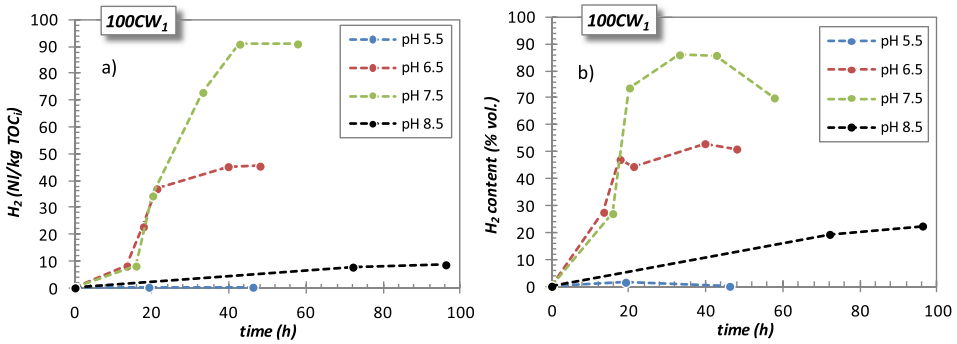

Fig. 1 – a) Specific cumulative H_2 production and b) H_2 content in biogas at different operating pHs ($100CW_1$ runs).

experiments at pHs = 5.5 and 8.5 gave drastically different results in terms of both process evolution over time and H_2 production yield: the final yield was only 0.2 NL H_2/kg TOC (0.1 NL H_2/kg VS) at pH = 5.5 and was limited to 10.2 NL H_2/kg TOC (5.1 NL H_2/kg VS) at pH = 8.5. It is emphasized that previous studies in which pH was controlled during the fermentation process [26–28] adopted set-point values that are close to the range of low performance for the present investigation. Although a direct comparison between the results of different studies is complicated by the different conditions adopted for the other operating variables, the present findings clearly indicate that specific optimization of the operating pH is mandatory to maximize the conversion yield of the process.

The operating pH of the fermentation system was also found to significantly affect biogas composition (see Fig. 1b)). For the runs at pHs = 6.5 and 7.5 the H_2 content in the biogas increased sharply with time reaching maximum values of 53 and 86% vol. after 30 h; the higher H_2 concentration observed at pH = 7.5 is likely a result of the increased CO_2 solubility in water at increasing pHs. At pHs = 5.5 and 8.5 the H_2 content in the biogas was limited to only 1.5 and 22% vol., confirming that the fermentation process was significantly hindered. Gas-chromatographic analyses indicated that, except for CO_2, no additional gaseous constituents were present at detectable concentrations in the biogas. In particular, in all the experiments on CW_1 no methane was ever detected, although some of the adopted pH conditions may have been suitable for methanogenesis and no specific pre-treatment was applied to CW_1 to inhibit the activity of methanogenic bacteria. The absence of methane in biogas can be ascribed to the short duration of the experiments (2–3 days) compared to the typically longer generation time of methanogenic microorganisms.

The results obtained for sample CW_2 are reported in Fig. 2a) in terms of specific cumulative H_2 production as a function of time observed for runs without inoculum addition. Both the overall production yield and pH dependence differed from those observed for sample CW_1. The maximum specific H_2 production yield attained ranged from 87.2 NL H_2/kg TOC (50.7 NL H_2/kg VS) at pH = 7.5 to 166.4 NL H_2/kg TOC (85.8 NL H_2/kg VS) at pH = 6.5, again demonstrating the key role of the operating pH in dictating the process evolution and, in turn, gas production. The optimal condition observed for CW_1 (pH = 7.5) was not confirmed in the experiments on CW_2, conversely yielding the lowest H_2 production. Comparing the yields of the two investigated CW samples, the maximum values attained were found to differ by a factor of ~2. Although more than one factor likely plays its specific role in the process, examining the analysed composition of the two CW samples (Table 1) and considering that H_2 production is acknowledged to preferentially occur due to carbohydrate fermentation [23], it is tempting to hypothesize that the carbohydrate content was the main reason explaining the lower H_2 yield of CW_1 compared to CW_2; the measured carbohydrate concentrations in the two samples differed by a factor of 2.3, a value that closely agrees with the differences observed in the

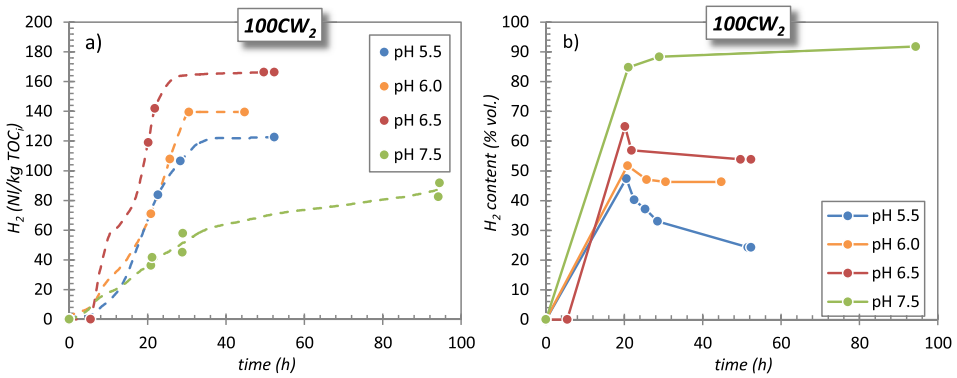

Fig. 2 – a) Specific cumulative H_2 production (dots: direct measurements; dashed lines: data derived from continuous biogas volume recording) and b) H_2 content in biogas at different operating pHs ($100CW_2$ runs).

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biogas yields. Furthermore, other observed differences (i.e. the optimal pH) may also be explained by the use of different starters of the cheese production process. These findings clearly demonstrate that the optimum for fermentative H_2 generation, in terms of both operating conditions to be adopted and process yield attained, is strongly dependent on the specific characteristics of the substrate and should thus be individually determined for each single type of biodegradable material concerned.

No methane was detected during the $100CW_2$ runs, with biogas being again composed by H_2 and CO_2 only. The maximum H_2 concentration in biogas was observed to increase with pH from 47.4% vol. at pH = 5.5 to 91.8% vol. at pH = 7.5 (see Fig. 2b)). As already pointed out above, the volumetric content of H_2 in biogas is not only related to the conversion efficiency of the fermentation process, but is mainly caused by the enhanced solubility of CO_2 in water at increasing pHs. For CW_2, this caused the highest H_2 concentration in the biogas (which would be desirable in view of biogas utilization) to correspond to the lowest specific production yield.

Since CW_2 displayed higher H_2 production yields, further tests were aimed at optimizing the process. CW_2 inoculation with HST sludge produced effects of various nature on the process, which were also found to be dependent on the substrate/inoculum ratio adopted (see Fig. 3). At the lower inoculum addition level ($90CW_2$_10AS runs; Fig. 3a) and b)), the final H_2 production was comparable to the $100CW_2$ runs at all pHs, considering that for mixed microbial cultures variations of 10–20% between the experiments are regarded as falling within the process variability range [31]. This indicates that the external biomass added at this level did not exert any appreciable effect on the process yield. However, the process kinetics was positively affected by the use of the HST sludge, with some reduction in both the initial lag time and the overall duration of the H_2 production process (see below for details). The benefits on the fermentation kinetics were fairly more evident when the sludge addition level was increased to 55% ($45CW_2$_55AS runs), with the H_2 generation process being considerably reduced if compared to the $100CW_2$ experiments (10–23 h as opposed to 30–92 h). The highest yield for the $45CW_2$_55AS mixture was attained at pH = 6.0 (instead of 6.5), and was 171.3 NL H_2/kg TOC; assuming that degradation of whey only contributed to biogas production while AS acted primarily as a biomass source, a yield of 233.4 NL H_2/kg TOC_{CW} was calculated, as high as 1.4 times the optimum yield attained for the $100CW_2$ runs. This result highlights the importance of the food-to-microorganisms ratio on the evolution and performance of the fermentation process.

For both the $90CW_2$_10AS and the $45CW_2$_55AS runs the lowest performance in terms of final H_2 production (90.2 and 138.5 NL H_2/kg TOC_{CW}, respectively) was attained in the experiments at pH = 5.5, followed by those at pH = 7.5.

The H_2 content of the biogas (data not shown here) was maintained at levels comparable to those measured in the $100CW_2$ experiments, confirming the increasing trend with pH but displaying a more constant evolution over time.

In order to derive a number of representative lumped parameters of H_2 production to be used in our experiments for the sake of comparison of the H_2 production performance, the

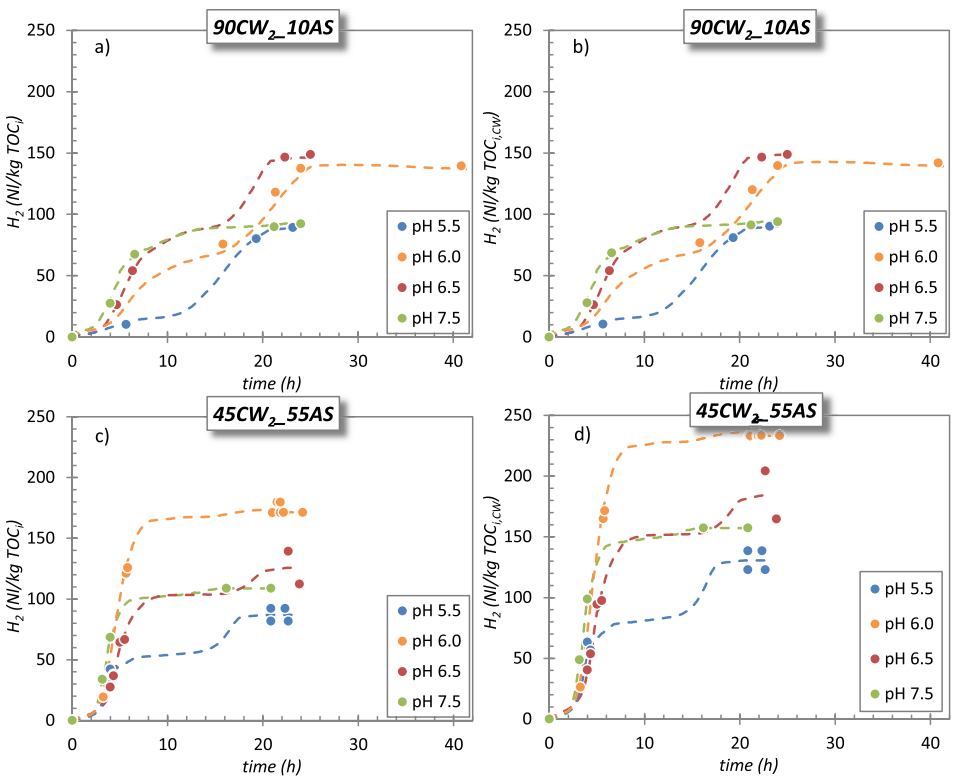

Fig. 3 – Specific cumulative H_2 production (dots: direct measurements; dashed lines: data derived from continuous biogas volume recording) at different operating pHs for the $90CW_2$_10AS runs (a), b)) and the $45CW_2$_55AS runs (c), d)).

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biogas production data were fitted through an empirical growth model. The Gompertz model (Eq. (1)) has been widely used to describe the evolution of fermentative H_2 production over time (see e.g. Ref. [39]):

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

where H is the cumulative H_2 production, P is the maximum H_2 production, R_m is the maximum H_2 production rate, λ is the lag phase duration and t is the time. However, the volumetric biogas production data obtained with the automatic recording system in the runs on the CW_2 sample (see Figs. 2 and 3), which displayed a two-branched evolution over time, revealed that a two-stage model (with different kinetic parameters for each stage) was more appropriate for fitting the experimental data. The presence of kinetically different stages during the fermentation process is 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 (Eq. (2)):

$$H = P_1\exp\left\{-\exp\left[\frac{R_{m1} \cdot e}{P_1}(\lambda_1 - t) + 1\right]\right\} + P_2\exp\left\{-\exp\left[\frac{R_{m2} \cdot e}{P_2}(\lambda_2 - t) + 1\right]\right\} \quad (2)$$

where the model parameters have the same meaning as in Eq. (1) and the subscripts 1 and 2 denote the first and second stage of the fermentation process. The cumulative H_2 production data were fitted with Eq. (2) using TableCurve 2D v. 5.01. Where the density of experimental measurements did not allow for using Eq. (2), as it was the case for the CW_1 sample, the traditional modified Gompertz model (Eq. (1)) was used instead. The resulting kinetic parameters are reported in Table 3. The additional parameter, t_{95}, defined as the time required for H_2 production to attain 95% of the total cumulative yield, was also calculated and reported in the table. Since t_{95} provides a measure of how fast the maximum production is achieved, it is believed to be useful to compare, from a kinetic viewpoint, experimental conditions with different associated H_2 generation yields.

The kinetic parameters of the theoretical models were all found to be pH-dependent and also strongly variable with the specific substrate characteristics, with the maximum H_2 yield being found at pH = 7.5 for CW_1 and pH = 6.0–6.5 for CW_2. As commented above, the H_2 production potential was also notably higher for the CW_2 sample compared to CW_1, while the effect of HST sludge addition on the biogas yield was only evident at the highest level tested (55% by weight). The kinetics of the fermentation process was on the other hand dramatically affected by the inoculum addition and to a lower

Table 3 – Kinetic parameters of the fermentation process.

Parameter	$100CW_1$ runs			
	pH 5.5	pH 6.5	pH 7.5	pH 8.5
P (NL/kg TOC_{CW})	–	45.5	91.7	10.3
R_m (NL/kg TOC_{CW} h)	–	4.3	4.8	0.5
λ (h)	–	11.9	13.8	48.8
T_{95} (h)	–	27.4	41.7	78.9

	$100CW_2$ runs							
	pH 5.5		pH 6.0		pH 6.5		pH 7.5	
	1st stage	2nd stage	1st stage	2nd stage	1st stage	2nd stage	1st stage	2nd stage
P (NL/kg TOC_{CW})	30.3	93.4	74.2	68.0	69.2	96.8	102.4	6.6
P_{tot} (NL/kg TOC_{CW})	123.7		142.1		165.9		109.0	
R_m (NL/kg TOC_{CW}.h)	2.0	6.4	4.0	8.5	14.2	13.8	41.6	1.6
λ (h)	4.1	13.6	3.7	20.2	5.6	16.3	2.4	11.0
t_{95} (h)	33.8		31.5		25.1		11.7	

	90CW2_10AS runs							
	pH 5.5		pH 6.0		pH 6.5		pH 7.5	
	1st stage	2nd stage	1st stage	2nd stage	1st stage	2nd stage	1st stage	2nd stage
P (NL/kg TOC_{CW})	19.9	74.0	74.1	65.8	91.7	59.0	79.7	11.8
P_{tot} (NL/kg TOC_{CW})	93.9		139.9		150.7		91.5	
R_m (NL/kg TOC_{CW}.h)	2.3	10.2	7.8	11.5	15.0	14.1	17.0	1.8
λ (h)	0.6	12.4	2.3	17.9	2.8	16.5	2.0	8.9
t_{95} (h)	22.4		24.7		21.1		13.2	

	45CW2_55AS runs							
	pH 5.5		pH 6.0		pH 6.5		pH 7.5	
	1st stage	2nd stage	1st stage	2nd stage	1st stage	2nd stage	1st stage	2nd stage
P (NL/kg TOC_{CW})	82.1	49.6	229.2	6.3	152.9	32.9	148.0	9.5
P_{tot} (NL/kg TOC_{CW})	87.6		173.0		126.7		109.1	
R_m (NL/kg TOC_{CW} h)	28.5	15.7	68.6	2.6	43.7	9.5	60.0	2.3
λ (h)	2.2	14.4	2.9	15.5	3.0	17.0	2.4	11.0
t_{95} (h)	17.8		8.7		19.7		11.7	

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though still appreciable extent by pH. The maximum H_2 production rate, R_m, was found to be up to one order of magnitude higher for the 45CW$_2$_55AS series than for the other experiments, varying from values below 4.3 NL H_2/kg TOC_{CW}·h for CW_1 to 19.0–50.0 NL H_2/kg TOC_{CW}·h depending on pH. The overall process duration was also found to benefit in all cases from biomass addition, with the parameter t_{95} displaying comparable values for both the 90CW$_2$_10AS (13.2–24.7 h) and the 45CW$_2$_55AS runs (11.7–19.7 h). Furthermore, for the CW_1 sample the time required to attain the steady-state condition was found to increase with pH, while the opposite trend was observed in all the experiments on the CW_2 sample. Although apparent conflicting behaviours may be inferred from such results, they are likely consistent to each other, in fact mirroring the implicit inverse correlation existing between t_{95} and the H_2 production potential; this appears to point out that improved process performances in terms of maximum cumulative biogas production were accompanied by lower overall durations of the fermentation process.

Substrate degradation and metabolites production

Substrate degradation during the fermentation process was monitored through measurements of the concentrations of soluble carbohydrates, TOC and soluble TOC. The concentration of soluble carbohydrates was found to decrease rapidly with time following approximately a first-order decay trend. The final removal yields attained values of 88–99% depending on the test conditions, somehow higher than those reported by Venetsaneas et al. (69%; [8]), but in line with the results of other authors (78% [15] and 92–99% [32]). No clear trend of soluble carbohydrate degradation with the operating pH could be identified, while the mixture characteristics appeared to affect the substrate degradation kinetics to a larger extent. The highest consumption rates were displayed by the 45CW$_2$_55AS series, confirming the faster biogas production rates attained.

The TOC evolution during the fermentation experiments is reported in Fig. 4, which further states the lower degree of substrate degradation as well as the slower consumption kinetics observed for the runs on the CW_1 sample. It is noted that the TOC reduction at the end of the experiments was, as expected, considerably less pronounced than soluble carbohydrates removal, with 70–83% and 50–56% of the original organic matter remaining in the digestate at the end of the runs on the CW_1 and CW_2 sample, respectively. The presence of residual TOC at the end of the hydrogenogenic process is obviously related to the contribution of both non-degraded organic matter and metabolic products (VFAs and alcohols).

It is also noted that TOC removal as a function of the operating pH appeared not to reflect the correlation observed with H_2 production, so that the highest degree of TOC degradation did not correspond to the optimal pH condition for the hydrogenogenic process. This clearly indicates that, with a view to maximizing H_2 production, the type of substrate degradation pathways that establish in the fermentation system is more relevant than the extent of substrate consumption.

The analysis of the metabolic products also provides useful information on the evolution of the process and can be used to explain the observed H_2 generation yields. CW is well known to contain microorganisms deriving from the cheese production process (i.e. starters, mainly *Lactobacillus* and *Streptococcus*). When no specific inoculum is used in the fermentation experiments, the nature of the metabolic products and the

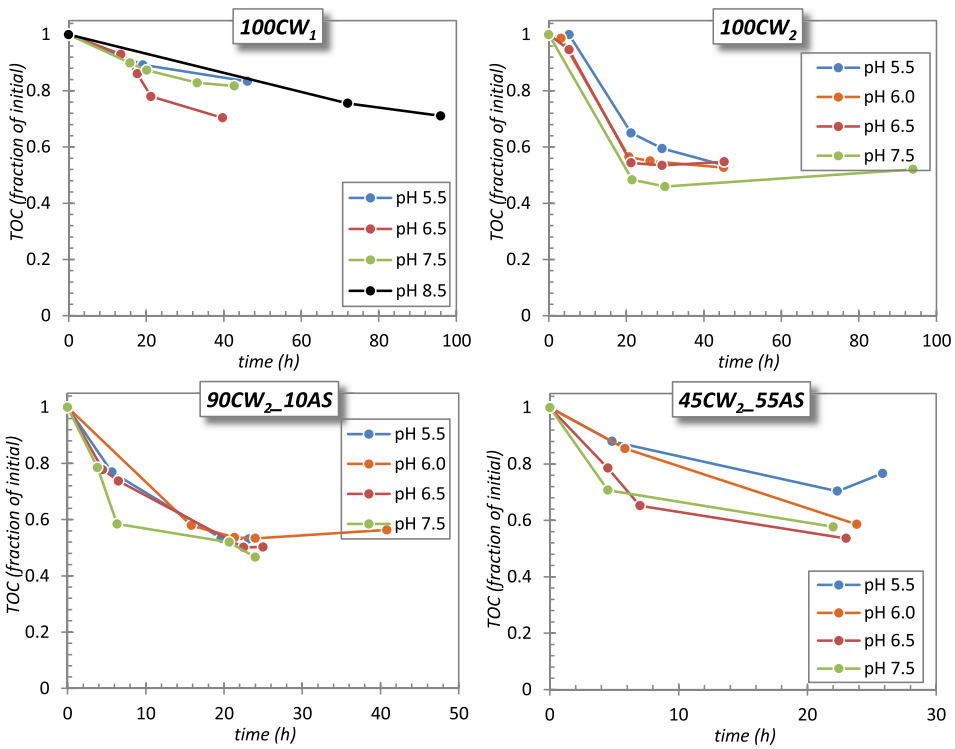

Fig. 4 – TOC evolution over time as a function of pH and mixture composition.

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associated H_2 production depend upon the prevailing endogenous microbial species in CW under the pH conditions adopted. The curves showing the evolution of the main metabolic products during the fermentation experiments are reported in Fig. 5; caproic and heptanoic acids are not reported in the graphs since they were present at much lower concentrations than the other metabolites; the total concentrations of VFAs (from acetic to heptanoic acid) + ethanol are also plotted in the same figure.

In general, the main metabolic products were found to include acetate, butyrate, propionate and ethanol (this particularly for the CW_1 sample); valerate was detected at lower but still appreciable concentrations. The interpretation of the analytical results for VFAs and ethanol is complicated by the fact that multiple metabolic pathways were likely taking place in the fermentation systems, where a mixed microbial culture was responsible for substrate degradation. Theoretically, spore-forming bacteria of the *Clostridium* and *Bacillus* genera produce H_2 via the so-called clostridial fermentation [40,41], with acetate or butyrate as the end products of the fermentation process, accompanied by small amounts of ethanol and other reduced end products to ensure the electron balance. The reactions for lactose fermentation [19,28,42] are written as Eqs. (3) and (4):

$$C_{12}H_{22}O_{11} + 5H_2O \rightarrow 8H_2 + 4CO_2 + 4CH_3COOH \text{ (acetate production)} \quad (3)$$

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 4H_2 + 4CO_2 + 2CH_3CH_2CH_2COOH \text{ (butyrate production)} \quad (4)$$

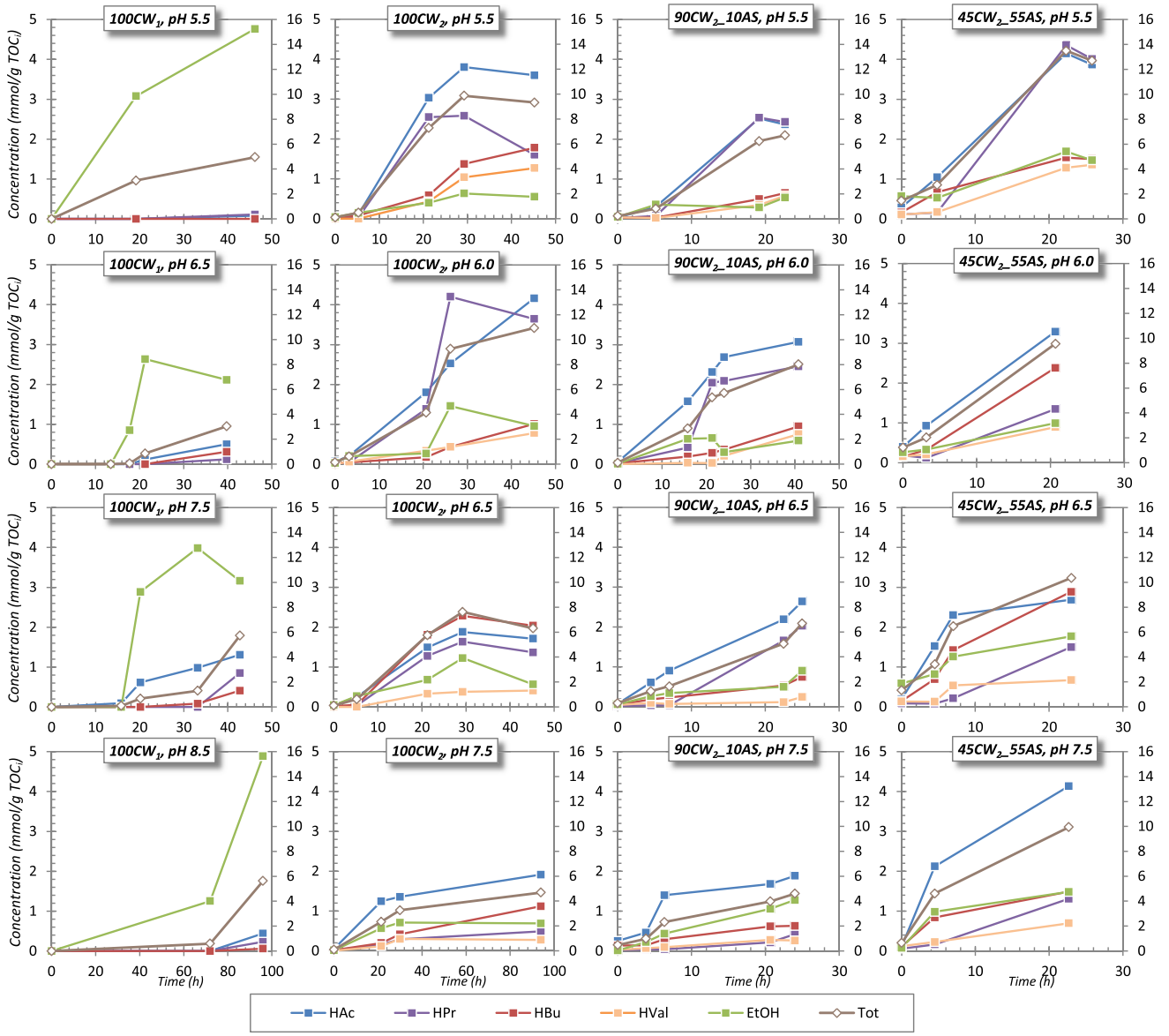

Fig. 5 – VFAs and alcohol evolution over time as a function of pH and mixture composition. Note: The data labelled with "Tot" (which report the sum of VFAs [from acetic to heptanoic acid] and ethanol concentrations) are read on the right-hand y-axis scale.

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In the so-called mixed acid fermentation operated by enteric bacteria, H_2 production is accompanied by a complex mixture of acetate, formate, lactate, succinate and ethanol as the end products [40,41,43]. In propionic-type fermentation, propionate, acetate and carbon dioxide are the major fermentation products [41], while consumption of H_2 occurs at a rate of 1 mol/mol of propionate produced [44].

It may thus be inferred that the metabolic products detected in the experiments derive from multiple fermentation pathways that were likely overlapping and competing during the process. The presence of a variety of fermentation products confirms the results of previous literature studies on CW [6,8,28,29,31,32,45,46], although the relative ratios between the individual species involved have been found to be largely variable with the initial substrate characteristics, the type of microbial consortium present and the fermentation conditions. The results of the present work show that the relative distribution of VFAs and ethanol varied depending on the operating pH and mixture composition.

As expected on the basis of the observed biogas production yields, CW_1 displayed a notably lower VFA production compared to CW_2. Such results reveal that when using CW_1 the degree of substrate conversion was considerably lower than for CW_2; at the same time, the relative ratios between the analysed metabolites in the experiments on CW_1 and CW_2 were also different, indicating that the substrate characteristics (in this case, likely the carbohydrate content and the indigenous biomass) are crucial in dictating the process evolution. In the $100CW_1$ runs high ethanol and lactate concentrations were detected, particularly where the lowest H_2 production yields were attained (pH = 5.5 and 8.5); this appears to be consistent with the fact that both the ethanol and the lactate production are characterized by null H_2 production. However, significant ethanol production was still observed in the highest-yield $100CW_1$ run (pH = 7.5); during the first 35 h, increasing contents of ethanol and acetate were observed along with some appreciable H_2 production, probably due to the mixed acid fermentation which involves simultaneous production of H_2 and ethanol; in the same run, after the first 35 h, the concentration of ethanol started to decrease and the prevailing metabolism was likely of the clostridial type, with associated H_2, acetate and butyrate production. The fact that during this run H_2 production increased sharply when ethanol generation stopped and acetate/butyrate generation started, indicates a competition between ethanol and butyrate producers from the above mentioned fermentation pathways [47] and reflects the higher H_2 yield of the latter metabolism. The influence of pH on simultaneous H_2 and ethanol production has not, to the authors' best knowledge, been specifically investigated in the literature. However, a number of studies [46,48,49] evidenced high ethanol contents during hydrogenogenic fermentation of CW, either alone or co-digested with glucose; under some conditions, ethanol was found to be the main metabolite and its production was favoured by increased CW/glucose proportions [46] and HRT [48].

Alcohol production also occurred in CW_2 runs, but ethanol concentrations were always lower than the other main metabolic products. This finding indicates that for this substrate neither mixed acid fermentation nor solventogenesis occurred during the process; as compared to CW_1, the presence of Enteric bacteria may be negligible in CW_2 due to the high temperatures (~90 °C) adopted during the production of mozzarella cheese. Moreover, CW_2 was inoculated with heat-shocked AS in order to harvest the spore-forming hydrogenogenic biomass, which was therefore probably the prevailing biomass.

High H_2 production yields are generally reported to occur when butyrate is present in excess of acetate [6,8,15,28,29,31]; however, in other cases good process performances were attained with butyrate and acetate at comparable levels [6,26] or when acetate prevailed [45]. In our experiments the best results ($100CW_2$, pH = 6.5; $45CW_2\_55AS$, pH = 6.5) were attained when butyrate concentrations were constantly higher than or comparable to acetate production; in all the other runs on CW_2 acetate was always found to largely prevail over butyrate along the whole fermentation process.

Propionate production was also observed during the experiments, as noted by other investigators [15,28,29,31], although significant concentrations were only observed at later process stages. This clearly suggests that the intense H_2 production phase was mainly associated to the production of acetate and butyrate, with later fermentation stages being accompanied by a progressive change in the prevailing metabolic pathways. The hypothesis made in some literature studies [28,50] that propionic acid is more favourably formed as pH increased was not confirmed in the present study (while the opposite was in fact observed), as also noted by Perna and co-workers [32].

Lactate is another important fermentation product that has frequently been found in fermentation studies on CW [8,15,29,32,42]. Although in the present study lactate could not be analysed in all samples due to analytical constraints, according to the data collected, the concentrations in the fermentation effluent were significantly lower than the corresponding initial values; this indicates a net lactate removal at the end of the fermentation process. Lactate was detected at initial concentrations of ~2–3 g/L, likely due to spontaneous degradation of lactose by the indigenous lactic acid bacteria present in the CW samples [30]. In principle, since lactate is recognized to be an important intermediate product of anaerobic degradation of lactose, it cannot be excluded that it was further produced during the fermentation tests and consumed afterwards until the end of the experiments. Without more detailed data on lactate evolution in the reactors, no conclusive statement on this issue can be derived. However, it should be mentioned that the absence of lactate in the fermentation effluent was also noted by Yang and co-workers [51] in their study on CW permeate. Perna and co-workers [32] have also observed that while acidic pHs (<5) appear to favour lactic fermentation, more alkaline conditions likely promote lactate conversion into butyrate and H_2. The fact that in our experiments the operating pH was constantly controlled at values $\geq$5.5 may thus well explain the low lactate concentrations detected in the fermentation effluent. The scientific literature reports several possible pathways for VFAs and H_2 production from lactate fermentation; while some authors propose the formation of either acetate and H_2 [52–54] or butyrate and H_2 [54,55] from lactate, additional potential metabolic pathways producing butyrate and H_2

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production from lactate and acetate have also been proposed [40] (and references therein) [55].

From the discussion above, it is clear that the net H_2 production observed during the experiments likely resulted from multiple concomitant metabolic pathways occurring in the fermentation system. Theoretical evaluations about the contribution of potential metabolic pathways were derived considering that among the hydrogenogenic fermentation reactions the clostridial metabolism displays the highest H_2 yields, while propionic fermentation involves H_2 consumption. The total predicted H_2 generation ($H_{2,pred}$) was calculated from the measured VFAs productions for each run on the basis of the stoichiometry of such two pathways (2 mol of H_2 generated per mol of acetate and butyrate produced; 1 mol of H_2 consumed per mol of propionate produced). The comparison between $H_{2,pred}$ and $H_{2,obs}$, shown in Fig. 6a), shows that in several cases the theoretical production deviated from the observed values. In such cases, it may be inferred that metabolic pathways other than the clostridial fermentation also occurred during the process. Multiple possible alternatives may explain the observed behaviour. When $H_{2,obs}$ was higher than $H_{2,pred}$, propionic fermentation of lactate [41], which involves propionate production with no H_2 consumption, may have played a role. On the other hand, metabolic pathways including hydrogenotrophic methanogenesis, propionic fermentation (accompanied by acetate production; see e.g. Ref. [41]), mixed acid fermentation (characterized by lower H_2 yields than the clostridial pathway; see e.g. Refs. [43,56]) and homoacetogenesis (producing acetate from H_2 and CO_2 [57,58]) may be claimed for those runs with $H_{2,obs} < H_{2,pred}$. The first two types of reactions may be excluded, since no methane production was observed during the experiments and no apparent link was recognised between the $H_{2,pred}/H_{2,obs}$ ratio and propionate formation. At this stage of the investigation, however, it is not possible to argue which of the remaining fermentation pathways was responsible of the observed differences between $H_{2,obs}$ and $H_{2,pred}$.

In overall terms, the total final amount of metabolic products analysed was calculated to account for 37–63% of soluble TOC for the $100CW_2$ runs, while the corresponding range increased to 65–88% (without any clear influence of either pH or mix composition) when the HST inoculum was added to CW. The residual undegraded carbohydrates in solution contributed on average by 0.5–12.0% to soluble TOC. As a result, it is evident that a portion of TOC in solution (35–61% for the $100CW_2$ runs and 5–30% for the other experiments) was present as species other than carbohydrates and the analysed metabolites (VFAs and ethanol). On the basis of the discussion above, additional end products of mixed acid fermentation, including formate and succinate, were probably also present in the final effluent. However, the large differences in soluble TOC balance between the $100CW_2$ and the rest of the experiments may also be interpreted in the sense that the nature of non-detected soluble constituents varied depending on whether pre-treated AS was added to CW. To this regard it is inferred that, when the indigenous biomass in CW was the only source of microorganisms in the fermentation system, the contribution of non-hydrogenogenic pathways to the substrate degradation process was more pronounced.

The presence of additional metabolic products other than those associated to hydrogenogenic pathways, as suggested by the previous discussion, indicates that the H_2 production potential of the investigated substrate was not fully exploited. In order to quantify the process performance from the viewpoint of conversion of the original substrate into H_2, a conversion yield was estimated for each run. To this aim, the simplifying assumption was made that the soluble carbohydrates initially present in CW were only composed by lactose; such a hypothesis is justified by the fact that lactose is recognized as the major constituent of CW [4,5,8,9]. The values derived under such a hypothesis (see Fig. 6b)) ranged from a minimum of 0.04 to a maximum of 2.6 mol H_2/mol lactose, and were close to the yields reported in the literature for CW fermentation (maximum yields: 3.5 mol H_2/mol lactose consumed [6]; 3.1 mol H_2/mol lactose [26]; 2.5 mol H_2/mol lactose [28]; 2.8 mol H_2/mol lactose [31]; 1.1 mol H_2/mol lactose [32]). This confirms that the existence of reduced end-products of the fermentation process, competing metabolic pathways with null or negative associated H_2 production as well as non-degraded substrate fractions significantly reduce the real conversion yield from the theoretical yield of 4–8 mol H_2/mol lactose predicted from reactions (3) and (4). It is however noted that the conversion yield was appreciably increased when the HST inoculum was used in the fermentation tests. This may suggest that, with specific optimization

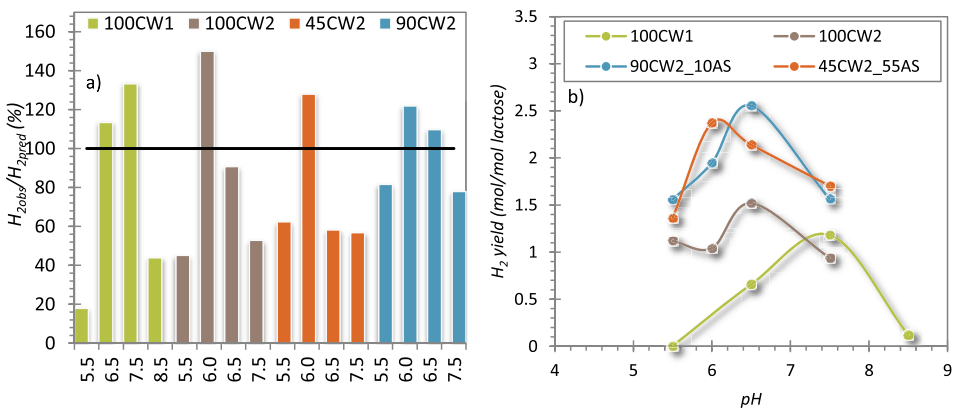

Fig. 6 – a) Comparison between $H_{2,pred}$ and $H_{2,obs}$ and b) process yield as a function of pH.

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of the biomass growth conditions, further improvement in the process performance may be attained.

Conclusions

An experimental investigation on fermentative H_2 production from two types of cheese whey, namely from pecorino and mozzarella cheese production, was conducted by means of batch tests with continuous pH control, either with or without addition of heat-shocked activated sludge as a biomass source. Significant fermentative biohydrogen production was attained at pHs of 6.5–7.5, with the best performance in terms of H_2 generation potential (171.3 NL H_2/kg TOC) being observed for the CW sample from mozzarella cheese production, at a pH of 6.0 with the heat-shocked inoculum. The experimental data revealed that hydrogen production is a very sensitive process, as it strongly depends on multiple factors including the origin and composition of the investigated substrate, the addition of a selected biomass source, the operating pH of the digestion system, in addition to other parameters that were not specifically focused in the present study. The mentioned factors and process variables were found to dramatically affect to a varying extent numerous interrelated aspects of the fermentation process, including the H_2 production potential, the type of the prevailing fermentation pathways, as well as the process kinetics. The fermentation products were found to vary largely with the operating conditions and to mirror the H_2 yield observed. Although specific investigation on this issue was beyond the objectives of the study, the experimental results appear to indicate that the effect of the investigated process parameters are interrelated. Further tests to identify the metabolic pathways of the fermentation process under different combinations of the operating parameters are currently underway in order to identify the mechanisms of biological hydrogen production during dark fermentation and derive information to improve the overall performance of the process.

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