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Enhancing Dark Fermentative Hydrogen Production from Problematic Substrates via the Co-Fermentation Strategy

Grazia Policastro [1,2,*], Rosetta Lamboglia [1], Massimiliano Fabbricino [1] and Francesco Pirozzi [1]

[1] Department of Civil, Architectural and Environmental Engineering, University of Naples Federico II, via Claudio 21, 80125 Naples, Italy
[2] Department of Civil Engineering, Pegaso Telematic University, 80100 Naples, Italy
* Correspondence: grazia.policastro@unina.it

Abstract: The aim of the present paper is the improvement of dark fermentative hydrogen production from problematic substrates. In detail, the study is aimed at (i) investigating the inhibiting effect of two problematic biomasses (i.e., of olive mill wastewater, containing recalcitrant/toxic compounds and cheese whey, lacking pH buffering capacity) on the dark fermentation process, (ii) as well as verifying the possibility to apply a co-fermentation strategy to enhance the process. To investigate the inhibiting effect of the substrates, two experimental sets were conducted using olive mill wastewater and cheese whey alone, under different food-to-microorganism ratios (i.e., 1, 2.5, and 5). Further experiments were conducted to verify the possibility of improving hydrogen production via the co-fermentation strategy. Such experiments included two tests conducted using different volumetric percentages of olive mill wastewater and cheese whey (90% olive mill wastewater + 10% cheese whey and 80% olive mill wastewater + 20% cheese whey). Results show that using olive mill wastewater alone, the inhibiting effect increased at a higher food-to-microorganism ratio. Moreover, because of the occurrence of a metabolic shift, hydrogen was not produced using 100% cheese whey. Interestingly, compared to the 100% olive mill wastewater condition, the use of 20% cheese whey allowed to double the hydrogen yield, reaching the high cumulative hydrogen production of 2.08 LL^{-1}. Obtained results confirm that the two investigated substrates exert inhibiting effects on microorganisms. Nevertheless, co-fermentation is an effective strategy to improve the dark fermentation process of problematic biomass.

Keywords: biohydrogen; cheese whey; co-fermentation; dark fermentation; olive mill wastewater

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1. Introduction

The most recent European strategies promoting sustainable development stress the importance of developing biorefining systems to replace fossil-based materials and energy sources. The concept of "environmental biorefineries" relies on biological processes aimed at producing materials and energy from waste biomass, contributing to sustainable management of waste disposal, in agreement with the Waste-to-Energy approach [1].

Dark Fermentation (DF) is a key biotechnology in many microbial-based biorefineries due to its capability to transform organic waste into valuable organic compounds (i.e., organic acids and alcohols) and energy (i.e., hydrogen) [2]. Compared to anaerobic digestion, DF can be considered more sustainable and environmental-friendly because of its capacity to produce hydrogen instead of methane. Indeed, energy production from hydrogen combustion is not associated with any carbon dioxide emission. Moreover, hydrogen has higher heat value than any other fuel [3].

DF efficiency depends on several factors, including the substrate and inoculum characteristics, the required pre-treatment, the food-to-microorganisms (F/M) ratio and substrate-to-inoculum ratio, the process temperature, and the pH conditions [3].

Among these factors, the characteristics and the composition of the substrate play a crucial role in the process development. Indeed, to be suitable for DF processes (and,

of course, sustainable), substrates must be abundant and rich in highly biodegradable compounds [4]. Unfortunately, many waste substrates with the mentioned characteristics lack pH buffering capacity or contain recalcitrant/toxic compounds. As such substrates may lead to bacteria inhibition, they can be considered "problematic biomass".

A typical example of it is represented by Olive Mill Wastewater (OMW). OMW is one of the most abundant waste substrates being produced in large amounts in olive oil factories (0.5–1.5 m^3 per ton of olives) [5]. Because of its high content of biodegradable compounds (35–110 g L^{-1} $BOD_{5,}$), it represents a promising substrate for DF processes. However, OMW is also rich in recalcitrant compounds, such as lignin, tannins, and other phenolic compounds (0.5–24 g L^{-1}), which are toxic to microorganisms [5]. Therefore, its use as DF substrate has several limitations. Ghimire et al., for example, detected reduced hydrogen productivity from OMW DF, compared to food waste and rice straw DF conducted in the same process conditions. Such evidence was attributed to the presence of polyphenols [3].

Another typical example is represented by Cheese Whey (CW). CW is also very abundant, as its production is estimated at 0.8–0.9 L per liter of treated milk [6]. It is very rich in biodegradable organic substances, is characterized by a high content of lactose (0.18–60 kg/m^3), protein (1.4–33.5 kg/m^3), and fats (0.08–10.58 kg/m^3) [7], and is, therefore, potentially, an ideal substrate for DF [8]. However, its characteristic low pH makes it difficult to conduct the fermentative process without the use of an external buffer solution. Indeed, previous studies have reported that DF of CW at uncontrolled pH may lead to lactic acid bacteria enrichment at the detriment of hydrogen-producing strains [9,10].

The present paper is based on the hypothesis that the co-fermentation of OMW and CW can enhance the process performances compared to the fermentation of the two substrates alone. Indeed, OMW can increase the buffer capacity of the substrates mix, avoiding the detriment of hydrogen-producing strains, and, at the same time, CW can prevent the inhibition of the process due to the presence of polyphenols because of the dilution of the toxic compounds contained in OMW.

While previous studies have shown that CW dilution with other waste, such as sugarcane vinasse [9], brewery spent grain [11], fruit vegetable wastes [12], or buffalo manure [13], can improve hydrogen production from CW, no attempt has been made so far to improve hydrogen production from OMWW using a co-fermentative process, although the co-digestion of OMW with secondary substrates (e.g., cattle excreta, piggery effluent) has been successful in increasing biomethane production in traditional anaerobic digestion processes [14,15].

To confirm the overmentioned hypothesis, the present study investigates the inhibiting effect of OMW and CW on the DF process under different F/M ratios, and verifies the performances of fermentative tests conducted using CW and OMW as co-substrates. The work represents the first attempt to apply the mentioned strategy to OMW in DF and to overcome the specific problems of both substrates contextually. The obtained promising findings represent a step forward to improve the DF process using problematic waste biomass.

2. Materials and Methods

2.1. Substrates and Inoculum

Substrates used in the experimental study were CW and OMW. OMW was collected from a real-scale plant producing extra virgin olive oil, located in the Campania Region (South of Italy), immediately after the filtration phase. CW was obtained from a dairy company producing mozzarella cheese, located in the same region. After sampling, both substrates were stored at −20 °C to keep their characteristics as unaltered as possible. Anaerobic digestate was used as a start-up inoculum for all experimental tests. The digestate was collected from a full-scale plant, treating buffalo manure, close to the above-mentioned dairy company. Before use, the digestate was subjected to a thermal pre-treatment to inhibit methanogens, as detailed elsewhere [16]. The main characteristics of the used inoculum and substrates, evaluated in triplicates, are reported in Table 1.

Table 1. Characteristics of the used inoculum and substrates.

Sample	TS [3] (gL^{-1})	VS [4] (gL^{-1})	COD ($g_{COD}L^{-1}$)
OMW [1]	39 ± 2	32 ± 1	71 ± 3
CW [2]	52 ± 2	44 ± 3	72 ± 8
Digestate	67 ± 1	47 ± 1	69 ± 5

[1] OMW = olive mill wastewater; [2] CW = cheese whey; [3] TS = total solids; [4] VS = volatile solids.

2.2. Experimental Setup and Operating Conditions

Three different sets of experiments were conducted during the study. The first two sets (SET_{OMW} and SET_{CW}) were aimed at individuating the possible inhibition effects during the fermentative conversion of OMW and CW alone. Each set consisted of three tests conducted using OMW or CW as single substrates, under three different F/M ratios (i.e., 1, 2.5, and 5 g_{VS}/g_{VS}). The third set (SET_{CO}) was conducted to verify the possibility of improving hydrogen production via the co-fermentation of OMW and CW. SET_{CO} included two tests conducted using different volumetric percentages of OMW and CW (90% OMW + 10%CW and 80%OMW + 20%CW) under the same F/M ratio (2.5 g_{VS}/g_{VS}). The experimental plan is summarized in Figure 1, whilst the detailed characteristics of reactors are reported in Table 2.

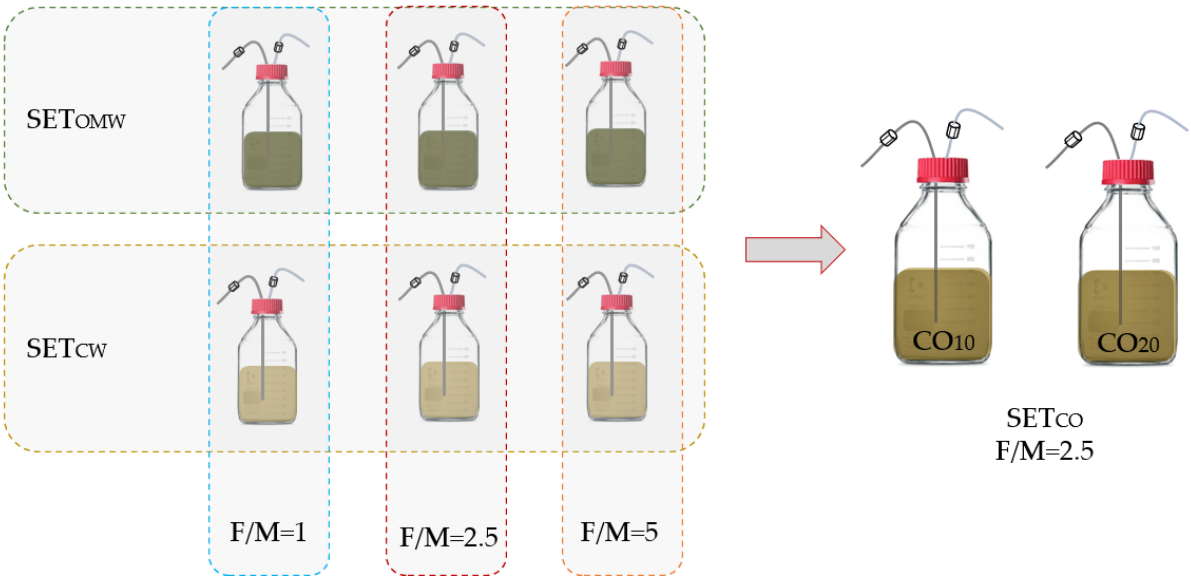

Figure 1. Experimental setup.

Table 2. Characteristics of reactors.

SET	Test	F/M	Digestate [2] [L]	OMW [2] [L]	CW [2] [L]	Substrate	COD_{IN} [1] [g L^{-1}]
SET_{OMW}	O_1	1	0.16	0.24	-	100% OMW	16.9
	$O_{2.5}$	2.5	0.09	0.31	-	100% OMW	22.3
	O_5	5	0.05	0.35	-	100% OMW	25
SET_{CW}	C_1	1	0.19	-	0.21	100% CW	14.9
	$C_{2.5}$	2.5	0.11	-	0.29	100% CW	20.9
	C_5	5	0.06	-	0.34	100% CW	24.2
SET_{CO}	CO_{10}	2.5	0.09	0.28	0.03	90% OMW–10% CW	22.17
	CO_{20}	2.5	0.09	0.25	0.06	80% OMW–20% CW	22

[1] Initial COD concentration inside the reactor; [2] Volume added in the reactor (total working volume: 400 mL).

All tests were performed in triplicate, using 0.5 L glass reactors with a working volume of 0.4 L. Each reactor consisted of the not-diluted inoculum and the selected substrate/substrates. Reactors operated under uncontrolled pH and batch feeding mode. Mesophilic conditions (35 ± 2 °C) were ensured by placing the reactors in a thermostatic bath. Reactors were equipped with two ports on the top, which were used for gaseous and liquid sampling operations. Before use, the reactor-top systems were tested with pressurized air to be sure that no communication with atmospheric air was possible. The volume of produced gas was measured once a day, together with gas composition, organic acids (OAs) concentration, and pH. Tests were stopped when hydrogen was no longer produced.

2.3. Analytical Methods

Gas volume was measured using the water displacement method [11]. Gas composition, instead, was analyzed by gas chromatography, using a Varian Star 3400 gas chromatograph equipped with Shin-Carbon ST 80/100 column and a thermal conductivity detector. OAs concentration was determined by high-pressure liquid chromatography (HPLC), using a LC 25 Chromatography Oven (Dionex, Sunnyvale, CA, USA), equipped with an Organic Acids column (Metrohom, Herisau, Switzerland) and a UVD 340U detector (Dionex, USA). The eluent was a 1 mM H_2SO_4 solution, pumped at 0.7 mL/min by a GD 500 Gradient Pump (Dionex, USA). The oven temperature was 50 °C. pH was measured using an inoLab pH meter (WTW, Munich, Germany). COD was determined by colorimetry, according to Standard Methods [17]. TS and VS content were evaluated by oven drying at 105 °C and 550 °C [17].

3. Results

3.1. Effect of Olive Mill Wastewater and Cheese Whey on Dark Fermentation

As said, the first two sets of experiments were conducted using CW and OMW alone, under different F/M ratios, to study the occurrence of inhibiting effects. Obtained results are reported in Figure 2 and Table 3.

Table 3. Results of SET_{OMW} and SET_{CW}.

Test	V_{biogas} [$L \cdot L^{-1}$]	V_{H2} [$L \cdot L^{-1}$]	r_{H2} [$mL \cdot L^{-1} \cdot h^{-1}$]	Y_{H2} [$mL \cdot g_{COD}{}^{-1}$]	OAs [$g \cdot L^{-1}$]	COD Conversion [%] [1]	pH Range [-]
O_1	2.42 ± 0.3	1.04 ± 0.31	10.80	61.53	5 ± 1.2	35	5.6–5.3
$O_{2.5}$	2.34 ± 0.01	1.17 ± 0.01	11.21	52.46	13 ± 1.7	65	5.9–5.6
O_5	0.76 ± 0.26	0.36 ± 0.24	2.17	14.40	15 ± 2.4	64	6.0–5.4
C_1	1.61 ± 0.01	<0.01	-	-	14 ± 1.3	86	4.5–3.9
$C_{2.5}$	3.05 ± 0.30	<0.01	-	-	9 ± 1.3	45	4.5–3.8
C_5	1.17 ± 0.02	<0.01	-	-	12 ±2.3	61	4.4–3.5

[1] Calculated as the sum of the COD contribution of all detected products over the initial COD. The COD of products has been obtained using the theoretical COD value of each product.

In more detail, Figure 2 reports the biogas trend and composition, whilst Table 2 reports: (i) The specific production of biogas referred to the volume of treated substrate (V_{biogas}), (ii) the specific production of hydrogen referred, once more, to the volume of treated substrate (V_{H2}), (iii) the hydrogen production rate (r_{H2}), (iv) the specific production of H_2, referred this time to the initial amount of organic substrate, i.e., the hydrogen yield (Y_{H2}), (v) the total concentration of organic acids (OAs) at the end of the process, and (vi) the pH range. Cumulative biogas and hydrogen volume ($L \cdot L^{-1}$) refer to liters of produced biogas or hydrogen over the reactor volume in liters (working volume). Rates were calculated as cumulative production over productive hours (from t = 0 to the last day on which production was detected). A blank test was conducted using the pre-treated digestate alone. The reactor produced 30 $mL \cdot L^{-1}$ of cumulative total biogas, composed of CO_2 only. As this value was lower than the 4% of the cumulative production

of all the other reactors, it was considered negligible, and the following results were not adjusted accordingly.

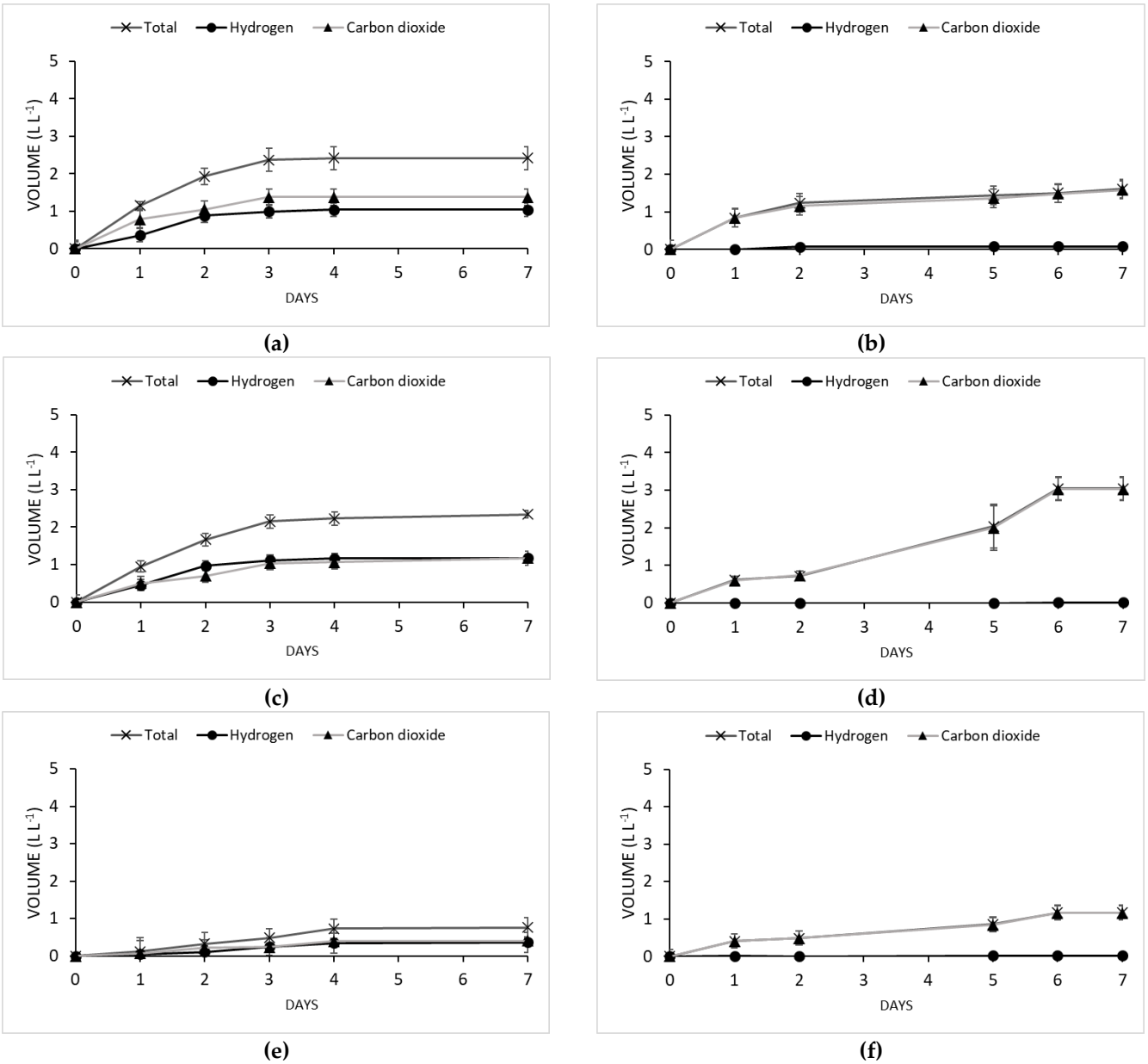

Figure 2. Total biogas, hydrogen and carbon dioxide cumulative production for SET_{OMW} (**a**,**c**,**e**) and SET_{CW} (**b**,**d**,**f**). (**a**,**b**): F/M = 1; (**c**) and (**d**): F/M = 2.5; (**e**,**f**): F/M = 5.

As it can be easily seen from Table 3, during the fermentation of OMW alone, the biogas production, the hydrogen production, and the hydrogen production rates were similar for the two lower tested F/M ratios (O_1 and $O_{2.5}$) and drastically dropped to the highest F/M ratio (O_5). Concerning the hydrogen yield, it was already reduced at F/M 2.5 (52.46 $mL \cdot g_{COD}{}^{-1}$) compared to F/M 1 (61.53 $mL \cdot g_{COD}{}^{-1}$). As observed for the rate and cumulative production, the minimum value of 2.17 $mL \cdot L^{-1} \cdot h^{-1}$ was reached at F/M 5. Overall, test O_5 led to very low biogas and hydrogen production. On the other hand, the concentration of organic acids was higher at the end of tests $O_{2.5}$ and O_5, reaching, in the two cases, similar values (between 13 and 15 $g \cdot L^{-1}$)

During the fermentation of CW, on the other hand, for all tested F/M ratios, cumulative hydrogen production was negligible (<0.01 $L \cdot L^{-1}$), and the biogas was composed only of

CO_2. Indeed, the use of CW led to the acidification of reactors, as indicated by the low pH values (below 4.5). In more detail, from further analyses conducted on liquid samples, a very high lactic acid accumulation equal to 14 g/L emerged during test C_1, 9 g/L during test $C_{2.5}$, and 12 g/L during test C_5. From Figure 2, it can be noticed that the duration of the biogas production phase was different for the two different substrates. In particular, after 3–4 days, no biogas production was detected in OMW reactors. Conversely, for CW reactors a 6–7-day process occurred. The theoretical COD conversion results indicate that in all reactors, the fermentation process was not complete. However, the detection of unknown peaks during HPLC analyses did not allow for a proper comparison between such values.

3.2. Co-Fermentation Strategy

After having tested the substrates alone, two co-fermentation tests were performed to assess the possible improvement of the process using the two substrates together. This time, F/M ratio was set to 2.5 g_{VS}/g_{VS}, and two different percentages of CW were added to OMW. The F/M ratio of 2.5 was chosen for the CO set to study the possible reduction of the inhibition, which, according to previous sets, was exerted by both substrates under the chosen F/M ratio. Conversely, the F/M = 1 ratio was too low to detect the inhibition of OMW. The F/M = 5 was found to be extremely high for hydrogen production purposes. Obtained results are summarized in Figure 3.

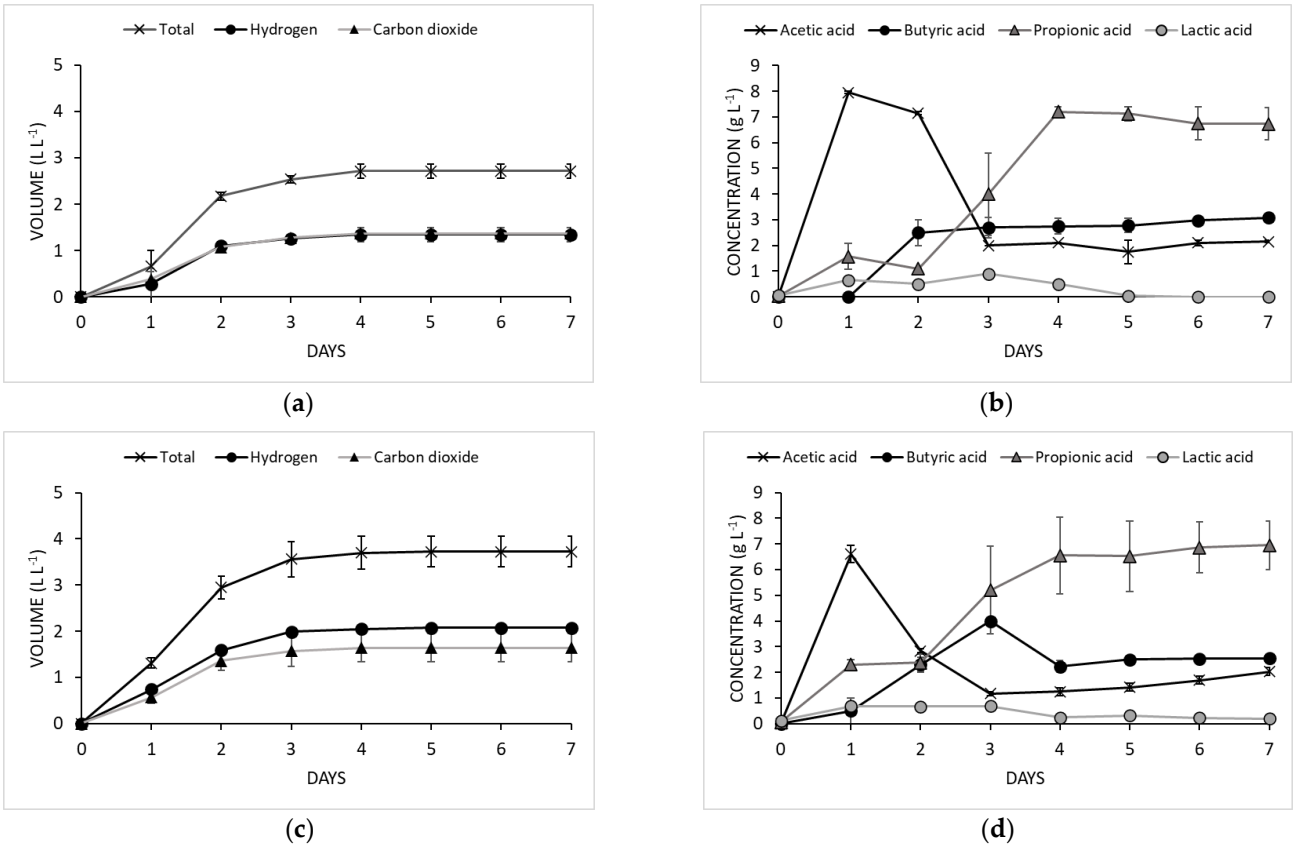

Figure 3. Experimental results of test $CO_{10\%}$ (**a**,**b**) and test $CO_{20\%}$ (**a**,**b**). (**a**,**c**) Cumulative total biogas, hydrogen, and carbon dioxide trends; (**b**,**d**) organic acids concentration (OAs) over time.

For both $CO_{10\%}$ and $CO_{20\%}$ reactors, hydrogen production was observed during the first 4 days of fermentation. However, the use of 20% CW led to the highest hydrogen production, which reached 2.08 $L \cdot L^{-1}$. The hydrogen production rate was around 14 $mL \cdot L^{-1} \cdot h^{-1}$ when the lowest amount of CW was added to OMW and increased to almost 22 $mL \cdot L^{-1} \cdot h^{-1}$ when the highest amount was tested. Similarly, hydrogen yield reached 60.44 mL $gCOD^{-1}$ during test $CO_{10\%}$ and 135.45 during test $CO_{20\%}$. CO_2 produc-

tion was almost not affected by the increase of the CW percentage in the substrate. Similarly, no significative variations were observed concerning OAs trends. In both experiments, the first day of fermentation was characterized by a high rate of acetic acid production. Successively, acetic acid was consumed, whilst an increase in butyric and propionic acid concentration was observed. Propionic acid was the main final by-product, reaching the final concentration of 7.17 in $CO_{10\%}$ and 7.41 gL^{-1} in $CO_{20\%}$.

4. Discussion

The initial substrate and inoculum amounts represent a key factor for the DF process, as they strongly influence the process performance in terms of hydrogen production. Indeed, the relative availability of the substrate to biomass determines whether the culture is under substrate-limited or substrate-sufficient conditions [18]. This aspect has been investigated in previous works via the substrate-to-microorganisms (S_o/X_o) or food-to-microorganisms (F/M) ratio [19].

Pan et al. tested different F/M ratios within the range of 1–10, to maximize hydrogen production from waste peach pulp. The most convenient F/M ratio was found to be 7 [10]. Compared to the optimal value, higher F/M ratios resulted in the acidification of the reactor, promoting alternative metabolic pathways rather than hydrogen production. On the other hand, Ghimire et al. reported an optimal F/M ratio value of 1. The authors investigated the fermentative hydrogen production from food waste and concluded that higher F/M ratios inhibited the process due to shock substrate loadings [3].

Such different optimal values confirm that the F/M ratio strongly depends on the adopted substrate characteristics.

In this work, the highest hydrogen production from OMW (SET_{OMW}) was observed using the F/M ratio of 2.5. On the other hand, the hydrogen yield was higher under the low F/M ratio of 1. Such results highlight that the DF process was already inhibited by the substrate excess. The inhibiting effect of OMW was attributed to the presence of phenolic compounds, as expected [3,20].

Concerning the SET_{CW}, all the adopted experimental conditions promoted the lactic acid production pathway rather than the hydrogen one. Such results were due to the low fermentation pH ranges, determined by the acidic characteristics of the CW substrate. As previously reported, due to their ability to regulate the intracellular pH, lactic acid bacteria are more acid-tolerant than other fermentative bacteria [21]. Moreover, they can grow at extremely low pH values, similar to those observed in the present study [22]. Indeed, in previous studies, acidic pre-treatments on fermentation inoculum were adopted to favor lactic acid bacteria growth rather than hydrogen-producing bacteria [23].

Overall, SET_{OMW} and SET_{CW} confirmed that OMW and CW are problematic substrates for DF processes and can hardly be used alone in biorefining systems without pre-treatment, dilution, or pH control.

Co-fermentation processes tested in SET_{CO} experiments allowed to keep pH ranges favorable for the mixed culture DF process aimed at hydrogen production (i.e., 5.5–6.3) [24]. Contextually, the replacement of part of the OMW with CW exerted a dilution effect. As a consequence, the performance of the process improved compared to experiments conducted using OMW and CW as single substrates. Aside from the dilution effect, the microbial community contained in the CW, most likely, played an important role in enhancing the DF process of phenolic compounds. Indeed, previous studies conducted on the biological treatment of OMW reported that *Lactobacilli*, which are the main strains contained in dairy effluents, are able to reduce the phenolic content by 46% [25,26].

Regarding the production of intermediate metabolites during the process, Figure 3 indicates that the main product at the end of the process was propionic acid. Moreover, as usually observed in DF process, acetic acid and butyric acid were detected too [13]. Usually, in DF processes, there are two main possible propionic acid production pathways. The first one is related to hydrogen, and it is a hydrogen-consuming pathway [24]. The other one relies on propionic acid generation from acetic acid, whilst hydrogen is not

consumed [27]. OAs trends over time suggested that propionic acid was generated from acetic acid consumption. Such a hypothesis is consistent with previous observations reporting propionic acid production from acetate in the presence of *Clostridia* species, which are the main fermentative bacteria contained in digestates [27].

Figure 4 reports hydrogen-cumulative productions obtained in the present study under the same F/M ratio.

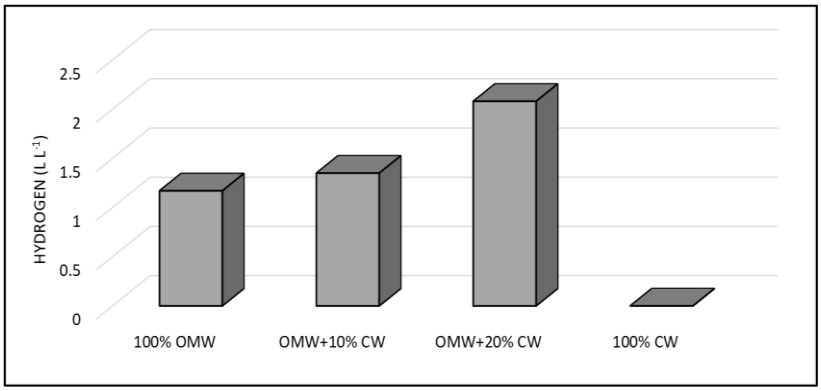

Figure 4. Hydrogen yield of experiments conducted using the F/M ratio of 2.5 g_{SV} g_{SV}^{-1}.

From Figure 4, it is possible to observe that hydrogen production from OMW was enhanced by the presence of CW. In more detail, using 20% CW in the substrate, cumulative hydrogen production almost doubled compared to the 100% OMW condition. Moreover, the hydrogen production rate in this latter condition was 102 $mL \cdot L^{-1} d^{-1}$per gram of VS, i.e., much higher than the maximum value obtained by Mugnai et al. [28] during DF of non-pre-treated OMW, which was only 30 $mL \cdot L^{-1} d^{-1}$per gram of VS.

Similar process improvements were observed by other authors, as a consequence of the substrate pre-treatment, during OMW fermentation. For instance, Eroglu et al. pre-treated the OMW to remove polyphenols using clay. Pre-treated OMW was compared with the raw one in the photo fermentative hydrogen production process. Results showed that the hydrogen productivity of the reactor fed with the pre-treated OMW was doubled compared to the reactor fed with the raw OMW [29].

Of course, in view of the process scale-up, recurring to pre-treatments is more complicated to manage and more expensive than the recourse to the co-fermentation strategy substrates. Moreover, as demonstrated in this study, co-fermentation allows the valorization for hydrogen production also of substrates, such as CW, which, otherwise, could not be used in traditional DF processes [16,30]. Such a result is extremely important, considering the high organic carbon content and the prevalent rapidly biodegradable COD fraction of CW, which makes it a promising substrate for dark fermentative processes [8]. Of course, further investigations are required before the process scale-up. Future directions should include the optimization of hydrogen production by finding the optimal OMW and CW percentages as well as the other operating condition. Specific studies on phenol degradation and analyses of the involved microbial communities are required as well. Moreover, investigations of further processes aimed at producing hydrogen and bioplastics from the liquid dark fermentation effluent are worth to be performed [1].

5. Conclusions

A wide variety of abundant agri-food waste streams own a very high organic carbon content and, therefore, could be valorized via the DF process. Unfortunately, if not properly pre-treated, many of them present problems leading to process inhibition. As pre-treatments should be avoided for scale-up purposes, DF of problematic substrates represents a major challenge. The present study shows that complementing two waste streams, which present two different problems (i.e., lacking pH buffering capacity or containing recalcitrant/toxic compounds), is an effective strategy to improve the DF process. In particular, the strategy

was applied to olive mill wastewater and cheese whey. The inhibiting effect of such biomass was confirmed by testing the substrates individually. Nonetheless, the co-fermentation strategy allowed to double the cumulative hydrogen production compared to the best result obtained using the substrates alone. Therefore, the present study represents a promising starting point for future research aimed at the full-scale fermentative valorization of problematic substrates.

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