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Garden and food waste co-fermentation for biohydrogen and biomethane production in a two-step hyperthermophilic-mesophilic process

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ABSTRACT

Co-fermentation of garden waste (GW) and food waste (FW) was assessed in a two-stage process coupling hyperthermophilic dark-fermentation and mesophilic anaerobic digestion (AD). In the first stage, biohydrogen production from individual substrates was tested at different volatile solids (VS) concentrations, using a pure culture of *Caldicellulosiruptor saccharolyticus* as inoculum. FW concentrations (in VS) above 2.9 g L^{-1} caused a lag phase of 5 days on biohydrogen production. No lag phase was observed for GW concentrations up to 25.6 g L^{-1}. In the co-fermentation experiments, the highest hydrogen yield (46 ± 1 L kg^{-1}) was achieved for GW:FW 90:10% (w/w). In the second stage, a biomethane yield of 682 ± 14 L kg^{-1} was obtained using the end-products of GW:FW 90:10% co-fermentation. The energy generation predictable from co-fermentation and AD of GW:FW 90:10% is 0.5 MJ kg^{-1} and 24.4 MJ kg^{-1}, respectively, which represents an interesting alternative for valorisation of wastes produced locally in communities.

1. Introduction

Overexploitation of fossil fuels has contributed for a rapid depletion of natural energy sources, causing also environmental pollution and climate changes. The development of alternative energy sources has thus been pursued, with a special focus on renewable and low-carbon fuels. Biomass-derived fuels (biofuels) have an important role in the transition to more sustainable and green energy-based societies, and its production has been increasing worldwide over the last decades.

Comparing with other biofuels, biohydrogen has the highest energy content per unit of weight and it enables clean power generation, since it is a carbon-free fuel which does not emit greenhouse gas after its combustion/oxidation (Sivagurunathan et al., 2017). Major concerns on the sustainability of many first-generation biofuels turned the attention to second-generation biofuels, which have the potential to consume waste residues and reduce CO_2 emissions (IEA Bioenergy, 2009). In this framework, biohydrogen fermentation processes have evolved as clean and cost-effective solutions relying on the activity of hydrogen-producing microorganisms, and on the use of agricultural or forestry residues. (Cheng et al., 2011; Ren et al., 2011; Wang et al., 2018a,b).

Among the possible different feedstock for fermentative biohydrogen production, food waste (FW) is one of the most abundant organic wastes, representing 15–63% of total municipal solid wastes (Yun et al., 2018). FW is being targeted as a promising carbon source due to its characteristics, such as high moisture content (72–85%), high organic concentration (*i.e.* 20–346 g L^{-1} as chemical oxygen demand (COD), 26–143 g L^{-1} as carbohydrates) and high carbon to nitrogen ratio (9–21) (Elbeshbishy et al., 2011; Hwang et al., 2011; Braguglia et al., 2018; Yun et al., 2018). Lignocellulosic agricultural wastes such as rice straw, wheat straw, barney straw and corn stalks have also received special attention concerning biohydrogen production due to their widespread abundance and wide availability (Datar et al., 2007; Cao et al., 2009; Kongjan and Angelidaki, 2010; Lo et al., 2010; Urbaniec and Bakker, 2015; Sivagurunathan et al., 2017). In general, biohydrogen production from these wastes depend on its chemical composition, pre-treatment methods, the microorganisms used as inoculum and the process conditions (Urbaniec and Bakker, 2015). For example, higher hydrogen yields and production rates are generally accomplished by dark fermentation processes performed at thermophilic or hyperthermophilic conditions, relatively to mesophilic processes (Urbaniec and Bakker, 2015). The significance of biohydrogen production was also shown for locally relevant wastes, e.g. oat straw hydrolysate (Arriaga et al., 2011) and mushroom farm waste hydrolysate (Li et al., 2011). Among agricultural wastes, garden waste (GW) appears as a very attractive raw material for biohydrogen production (Boldrin, 2009; Shi et al., 2013; Abreu et al., 2016), and its production has been increasing considerably with rapid urbanization worldwide.

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GW typically includes different fractions such as grass clippings, hedge cuttings, small branches, leaves and wood debris, and consists primarily of renewable polysaccharides and lignin (Shi et al., 2013; Abreu et al., 2016).

In the last years, several studies using FW or GW for biohydrogen production were performed (Cheng et al., 2011; Pan et al., 2013; Yasin et al., 2013; Nissilä et al., 2014; Abreu et al., 2016; Algapani et al., 2018; Ghimire et al., 2018), but the potential utilization of both wastes simultaneously by co-fermentation was not evaluated. In GW the fermentable sugars are present in complex and hardly digestible forms, while FW is an easier biodegradable substrate which is also a source of nitrogen (that is lacking in GW). Nevertheless, the presence of lipids and proteins in FW composition is generally associated with a lower hydrogen production than carbohydrate-rich organic wastes (Bharathiraja et al., 2016). Co-fermentation of FW and GW may contribute to overcome the disadvantages of single fermentation and potentially improve the hydrogen production from these wastes.

Fermentative hydrogen production involves either facultative and strict anaerobic bacteria (De Gioannis et al., 2013). Extreme thermophiles belonging to the genera *Caldicellulosiruptor* and *Thermotoga* are commonly referred as very efficient H_2 producers (Pawar and van Niel, 2013). Among *Caldicellulosiruptor* genera, the interest on *C. saccharolyticus* is increasing due to the capacity to metabolize various carbon sources ranging from simple sugars to complex lignocellulosic materials (Willquist et al., 2010). *C. saccharolyticus* is reported to approach the theoretical maximum H_2 yield from glucose of 4 mol mol^{-1} and is referred as relatively insensitive to high pH_2 (Willquist et al., 2010). Moreover, *C. saccharolyticus* lacks carbon catabolite repression, enabling the simultaneous fermentation of hexoses and pentoses (Bielen et al., 2013).

Biohydrogen dark fermentation can be coupled with a second stage process, where the fermentation end products, rich in volatile fatty acids (VFA), can be converted to methane through an anaerobic digestion process, coupling energy carrier generation and waste treatment. After upgrading, the generated biomethane can be used directly as renewable fuel or fed into the established natural gas grid, thus replacing fossil fuels and contributing for reducing the greenhouse gas emissions. The two-step approach for hydrogen and methane production has been tested with different substrates, from pure sugars to diverse feedstocks such as municipal, agricultural and food industry wastes (Wang and Zhao, 2009; Liu et al., 2013; Costa et al., 2015; Abreu et al., 2016). However, only few studies were reported on hydrogen and methane production from more than one residue in co-fermentation, e.g. FW + pulp and paper sludge (Lin et al., 2013) and FW + waste activated sludge (Liu et al., 2013). Furthermore, there are no studies describing possible solutions for the treatment and valorisation of both GW and FW. This is important because these two types of wastes account for a significant fraction of municipal solid wastes and therefore, considering the amounts produced, may constitute a relevant source for bioenergy production.

The main objective of this study is the optimization of bioenergy production from food waste and garden waste, by co-fermentation using an efficient hydrogen-producing culture of *C. sacharolyticus* coupled with methane production from the end-products of biohydrogen co-fermentation. Hyperthermophilic conditions were selected for the first fermentation step because higher wastes solubilization and biohydrogen production are generally achieved, relatively to mesophilic conditions. The methanogenic step was then performed at mesophilic temperatures, since heat will be transferred from the first to the second step with the digestate and is expected to be sufficient to exempt the heating of the anaerobic digester.

2. Materials and methods

2.1. Food and garden waste characterization

Samples of milled food waste (FW) were collected during 5 days in the University of Minho canteen, at Campus de Gualtar, Braga, Portugal, and stored at −20 °C. A composed substrate was then prepared by mixing and homogenizing all the samples. Garden waste (GW) was composed by grass and small bushes collected by the municipal waste management company AGERE, Braga, Portugal. GW was dried and milled into pieces smaller than 5 mm. FW and GW were characterized in terms of total and soluble COD, total solids (TS), volatile solids (VS), ash content, total Kjeldahl nitrogen (TKN), ammonium (NH_4^+), fat content, proteins, Klason lignin, glucan and xylan content. Soluble COD of FW and GW was also analysed after autoclaving (121 °C and 0.1 MPa for 20 min), that functioned as thermal and pressure pretreatment.

2.2. Biohydrogen production from FW and GW

2.2.1. Inoculum and medium composition

Caldicellulosiruptor saccharolyticus DSM 8903 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). The culture medium consisted of (per L) KH_2PO_4 0.75 g, K_2HPO_4 1.5 g, $MgCl_2{\cdot}6H_2O$ 0.33 g, NH_4Cl 0.4 g, yeast extract 1.0 g, $FeCl_3{\cdot}6H_2O$ 2.5 mg, NaCl 0.9 g, trypticase 2 g, SL-10 (medium 320 DSMZ) trace elements 1 mL, and resazurin 0.5 mg. The culture medium was supplemented with 50 mmol L^{-1} 4-morpholine propanesulfonic acid (MOPS) to increase the buffering capacity and was reduced with 0.75 g L^{-1} cysteine-HCl monohydrated. Cellobiose (2 g L^{-1}) was used as the carbon source and the medium was made anoxic by boiling and flushing with 100% N_2. *C. saccharolyticus* was grown at 70 °C with agitation (90 rpm). After reaching an optical density (OD) at 620 nm of 0.2–0.3 the culture was used as inoculum for the subsequent batch assays.

2.2.2. Biohydrogen production assays

Biohydrogen production from FW and GW was assessed in 160 mL serum bottles containing 50 mL of phosphate-buffered medium (20 mmol L^{-1}) supplemented with MOPS (50 mmol L^{-1}) and flushed with N_2 (100%), as described in Section 2.2.1. Different waste VS concentrations were added to the assays: 0.7, 1.5, 2.9 and 4.4 g L^{-1} of FW; 4.3, 8.5, 17.0 and 25.6 g L^{-1} of GW. The bottles were then autoclaved at 121 °C for 20 min, thus functioning as a wastes pre-treatment. After autoclaving, yeast extract was added to a final concentration of 0.5 g L^{-1} and the medium was reduced with 0.75 g L^{-1} cysteine-HCl monohydrated. Finally, inoculation with 5 mL of pre-cultured *C. saccharolyticus* (OD at 620 nm of 0.2–0.3) was performed and the bottles were incubated at 70 °C under agitation at 90 rpm. All the experiments were performed in quadruplicate and included controls (prepared without *C. saccharolyticus*) and blanks (inoculated with *C. saccharolyticus* but without waste). Production of hydrogen gas, soluble fermentation products and final pH were monitored. Cumulative hydrogen production values were corrected for standard temperature and pressure (STP) conditions (0 °C and 0.1 MPa).

2.3. Hydrogen production by co-fermentation of FW and GW

Co-fermentation assays were prepared as described above for the biohydrogen production assays (Section 2.2.2.), using different GW and FW ratios (90:10%, 50:50%, 100:0% and 0:100% w/w in VS) with a final waste concentration (in VS) of 8.5 g L^{-1}. All the experiments were performed in sextuplicate and included controls without waste and without *C. saccharolyticus*. Production of hydrogen gas, soluble fermentation products and final pH were monitored. Total and soluble COD were also analyzed at the end of the assays.

2.4. Biomethane production assays

Anaerobic granular sludge from a brewery wastewater treatment plant was used as inoculum. The specific methanogenic activity (SMA) of this sludge, determined as described in Eiroa et al. (2012) and expressed in volume of methane produced at STP conditions per mass unit of VS of inoculum and time ($mL\,g^{-1}\,d^{-1}$), was 118.1 ± 1.2 $mL\,g^{-1}\,d^{-1}$ and 540.2 ± 49.9 $mL\,g^{-1}\,d^{-1}$ in the presence of acetate (30 $mmol\,L^{-1}$) and H_2/CO_2 (80/20% v/v, 1 bar overpressure), respectively.

Methane production from the end-products of the co-fermentation tests (GW:FW of 90:10%, 100:0% and 0:100%) was studied in batch assays performed according to the guidelines defined by Angelidaki et al. (2009). A total of 50 mL of the end-products of each co-fermentation assays were added to 600 mL serum bottles, containing 20 g (wet weight) of inoculum and 50 mL of basal medium containing $NaHCO_3$ (5 $g\,L^{-1}$). pH of the medium was corrected to 7.0–7.2. The vials were sealed and the headspace flushed with N_2/CO_2 (80:20% v/v). Before incubation, the medium was amended with $Na_2S{\cdot}9H_2O$, to a final concentration of 1 $mmol\,L^{-1}$. Blank assays to discount for the residual substrate present in the inoculum were performed, as well as controls with hydrolysate and without sludge. Additionally, a control with GW:FW 90:10% (w/w in VS) without being subjected to the first step of dark fermentation was performed. All the assays were made in triplicate and were incubated at 37 °C with agitation (90 rpm). The methane accumulated in the headspace of the closed bottles was measured, the values were corrected for STP conditions and converted to its equivalent COD (considering that 1 g of COD generates 0.35 L of methane at STP conditions). Biochemical methane potential (BMP) was defined by the volume of methane produced (L@STP) per unit of COD (kg) of substrate added to the assay.

2.5. Analytical methods

Determination of lignin, xylan and glucan was performed according to Sluiter et al. (2008). TKN, NH_4^+, TS, VS and ash content were measured according to standard methods (APHA et al., 1998). Total and soluble COD were analysed spectrophotometrically using standard kits (Hach Lange, Düsseldorf, Germany). Sample filtration was performed prior to soluble COD (CODs) determination. Lipids concentration was quantified as described by Bligh and Dyer (1959). Protein content was determined according to the method established by FAO (FAO, 2003). Hydrogen concentration in the gas phase was determined by gas chromatography (GC) using a Bruker Scion 456 Chromatograph (Bruker, Massachusetts, USA) equipped with a molsieve column (MS-13x 80/100 mesh) and a thermal conductivity detector. Argon (30 $mL\,min^{-1}$) was used as the carrier gas. The injector, detector and column temperatures were 100, 130, and 35 °C respectively. Methane content in the biogas was analysed by GC (Chrompack 9000) equipped with a flame ionization detector (FID) and a 2 m × 1/8″ Chromosorb 101 (80–120 mesh) column, using nitrogen as carrier gas (30 $mL\,min^{-1}$); column, injector, and detector temperatures were 35, 110, and 220 °C, respectively. VFA, lactic acid, alcohols and sugars were determined by high performance liquid chromatography (Jasco, Tokyo, Japan) with a Chrompack column (6.5 × 30 mm) and sulfuric acid (5 $mmol\,L^{-1}$) as mobile phase at a flow rate of 0.9 $mL\,min^{-1}$. Column temperature was set at 80 °C. Detection of VFA and lactic acid was made using an ultraviolet (UV) detector at 210 nm; for sugars a refractive index (RI) detector was used.

2.6. Data analysis

The modified Gompertz equation (Eq. (1), Zwietering et al., 1990) was used to describe the progress of cumulative hydrogen production obtained from the batch experiments.

Table 1
Results of food waste and garden waste characterization.

Parameters	Food waste (FW)	Garden waste* (GW)
Total COD (mg g^{-1})	160 ± 11	934 ± 15
Soluble COD (mg g^{-1})	45 ± 0	174 ± 1
TS (mg g^{-1})	168 ± 4	914 ± 1
VS (mg g^{-1})	148 ± 1	847 ± 2
Ash content (mg g^{-1})	20 ± 4	67 ± 2
TKN (mg N g^{-1})	8.1 ± 0.5	n.d.
Ammonium (mg N-NH_4 g^{-1})	1.6 ± 0.9	n.d.
Fat content (mg g^{-1})	48 ± 3	n.a.
Protein (mg g^{-1})	46 ± 3	n.d.
Klason Lignin (% VS)	n.a.	32.1 ± 0.3
Glucan (% VS)	n.a.	23.5 ± 5.9
Xylan (% VS)	n.a.	8.8 ± 0.9

n.d. – not detected; n.a. – not analyzed.
* Abreu et al., 2016.

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

H(t) = cumulative hydrogen production ($mL\,g^{-1}$); P = maximum hydrogen production ($mL\,g^{-1}$); R_m = hydrogen production rate ($mL\,g^{-1}\,day^{-1}$); e = 2.71828…; λ = lag-phase time (d); t = time (d). All parameters were expressed per amount (g) of waste VS added initially. Data analysis was performed using Sigma Plot for Windows 10.0 software (Systat Software Inc, Germany).

3. Results and discussion

3.1. Biohydrogen production from FW and GW

The characteristics of the studied wastes are presented in Table 1. The two wastes presented different moisture content (approx. 9% and 83% humidity for GW and FW, respectively), but similar organic matter concentration (expressed as the ratio between total COD and TS), *i.e.* around 1 $g\,g^{-1}$. Glucan (glucose, cellobiose, cellulose) and xylan (xylose and hemicellulose) content of GW totalize 32% of the volatile solids, and the Klason lignin content represents 32.1 ± 0.3% of the VS. After autoclaving, soluble COD increased up to 23% and 46% of the total COD for GW and FW, respectively.

Biohydrogen was produced from the autoclaved FW and GW, at all the concentrations tested (Fig. 1), while in blanks and controls only vestigial hydrogen amounts were detected (< 0.5 and < 0.08 mL, respectively). In general, for both wastes, volumetric hydrogen production increased with the increase of waste concentration (in VS). However, hydrogen yields (Y_{H2}) decreased with the increase of waste VS concentrations, suggesting a possible inhibition by the substrate. Y_{H2} (expressed relatively to the amount of waste VS added initially) varied from 133.5 ± 2.7 $mL\,g^{-1}$ to 82.9 ± 0.9 $mL\,g^{-1}$ for the range of FW concentrations tested (0.7–4.4 $g\,L^{-1}$ in VS), and the lag phase preceding the onset of hydrogen production increased from 1 day to 5 days in the assays with FW concentrations of 2.9 and 4.4 $g\,L^{-1}$ (Fig. 1a). Elbeshbishy et al. (2011) summarized the results from several studied performed with FW, and referred hydrogen yields between 57 and 250 $mL\,g^{-1}$. Thus, the values obtained in this assay for FW are within the range reported in the literature. For GW, although no lag phase was observed for concentrations up to 25.6 $g\,L^{-1}$ (Fig. 1b), Y_{H2} varied from 53.3 ± 1.4 to 15.3 ± 0.1 $mL\,g^{-1}$ for the range of concentrations tested (4.2–25.6 $g\,L^{-1}$ in VS). Slightly higher hydrogen production yields (around 80 $mL\,g^{-1}$) were obtained from GW by Abreu et al. (2016) when using pure cultures of the extreme thermophiles *C. saccharolyticus* or *Caldicellulosiruptor bescii* as inoculum, and co-culturing these two strains increased synergistically the hydrogen production yield up to 98 $mL\,g^{-1}$. Values between 1 and 150 $mL\,g^{-1}$ have been reported in the literature for different agricultural wastes, as reviewed

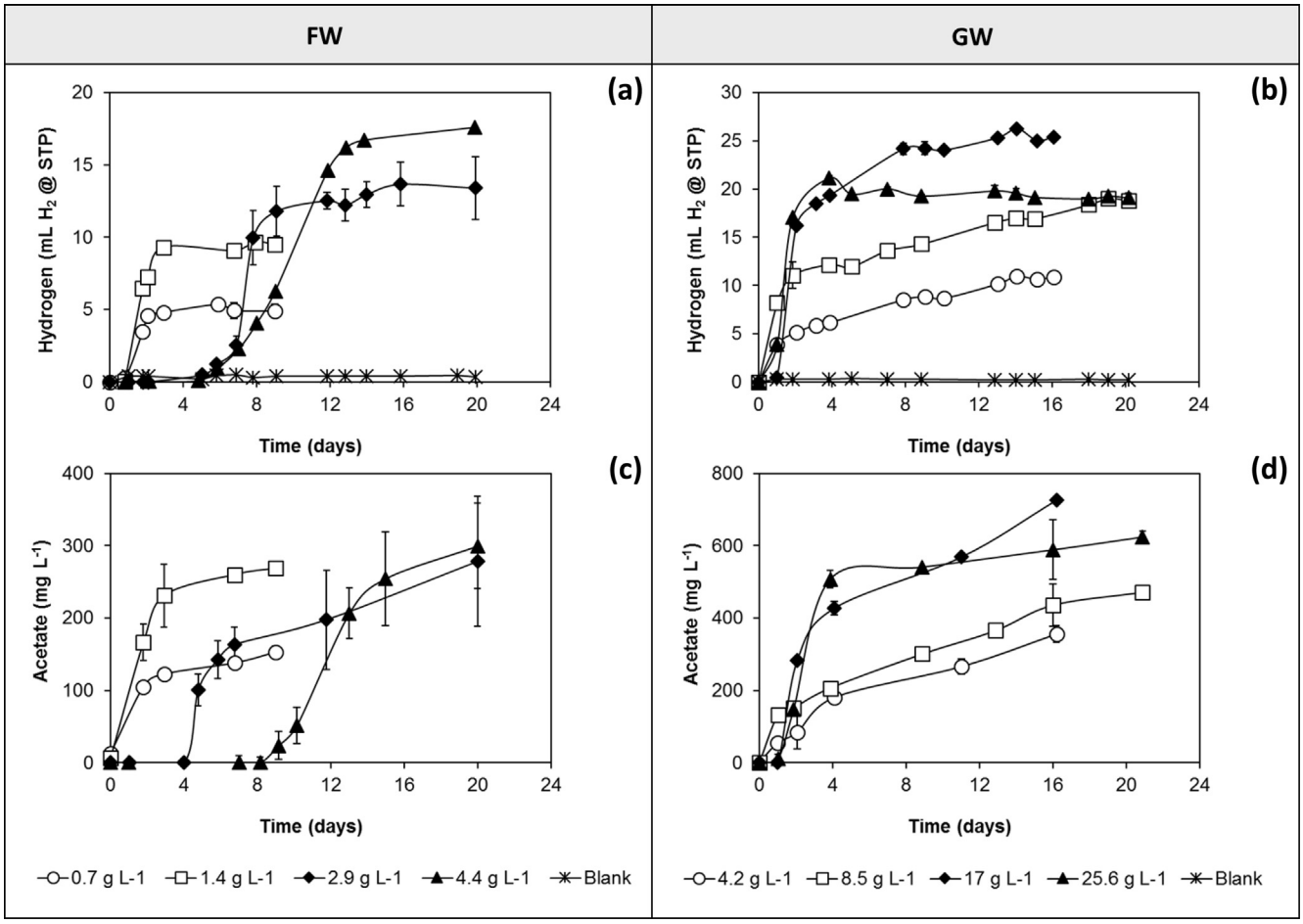

Fig. 1. Dark fermentation of FW and GW: cumulative biohydrogen production from FW (a) and GW (b); acetate production from FW (c) and GW (c).

by Guo et al. (2010), and our results fall within this range. In the case of FW, the potential inhibition by the substrate may be due to the release of ammonium and long chain fatty acids during the autoclaving pretreatment applied. These compounds are known to be potentially inhibitory to different anaerobic microorganisms, although no information is available on this topic for *C. saccharolyticus*. For GW, potentially formed by-products capable of inhibiting hydrogen production may include furan-derivatives (e.g. furfural), carbonic acids (methanoic acid, ethanoic acid and levulinic acid) or phenolic compounds, as previously reported for other lignocellulosic materials (Sivagurunathan et al., 2017). The inhibitory effects and threshold concentrations of these by-products were shown to be highly specific to the microorganisms used as inocula (Sivagurunathan et al., 2017).

Acetate was the only soluble fermentation product detected for both wastes (Fig. 1c, d). Acetate concentration increased with the added FW concentrations, up to a maximum of 300 mg L^{-1} (Fig. 1c), and for GW the highest acetate concentration (725 mg L^{-1}) was obtained with 17 g L^{-1} (Fig. 1d), corresponding to the highest volumetric hydrogen production.

The COD conversion efficiency of the wastes, expressed in percentage, was calculated considering the amount of hydrogen and acetate produced, converted to its equivalent COD, and the amount of soluble or total COD of the waste added to each assay (Table 2). In the range of FW concentrations tested, 65–26% of the soluble COD and 30–12% of the total COD were converted to hydrogen + acetate. For GW this varied between 50 and 15 % and 11–3% for soluble and total COD, respectively (Table 2). These results show that *C. saccharolyticus* was not being able to completely ferment the wastes, probably due to the presence of inhibitory compounds, as discussed before, and to possible difficulties in the hydrolysis of the wastes. Hydrogen production accounted for 20–12% and 15–5% of FW and GW soluble COD, respectively (Table 2), which represent only 9–5% (FW) and 3–1% (GW) of the total COD. Similar percentages of total COD conversion to hydrogen have been previously reported from fermentation of FW (Liu et al., 2013; Algapani et al., 2018; Yun et al., 2018). A significant fraction of the energy content of the substrate is generally kept in the end-products from the H_2 fermentation, which justifies the interest in applying two-

Table 2
COD conversion efficiency in the dark fermentation of FW and GW.

Substrate	Concentration (g L^{-1})[a]	H_2 + Ac (% CODs)[b]	H_2 + Ac (% CODt)[b]	H_2 (% CODs)[c]	H_2 (% CODt)[c]
FW	0.7	65	30	20	9
	1.4	58	27	18	8
	2.9	33	15	13	6
	4.4	26	12	12	5
GW	4.2	50	11	15	3
	8.5	36	8	12	3
	17.0	27	6	9	2
	25.6	15	3	5	1

[a] Expressed in g of waste VS per litre.
[b] Represents the relation between the amount of hydrogen and acetate produced, converted to its equivalent COD, and the amount of soluble COD (CODs) or total COD (CODt) added to each assay.
[c] Represents the relation between the amount of hydrogen produced, converted to its equivalent COD, and the amount of soluble COD (CODs) or total COD (CODt) added to each assay.

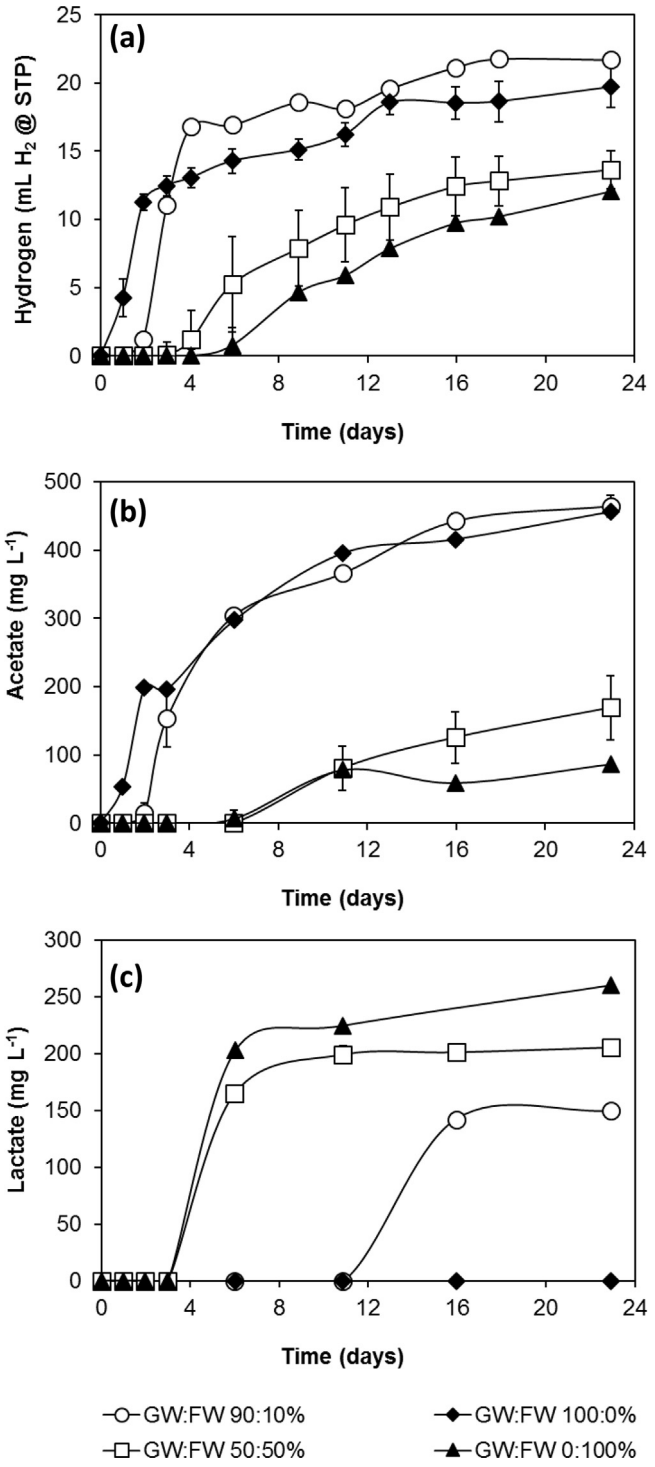

Fig. 2. Biohydrogen production (a), acetate (b) and lactate (c) during the co-fermentation of GW and FW.

phase hydrogen-methane producing systems to improve the overall FW conversion yields (Braguglia et al., 2018).

3.2. Hydrogen production by co-fermentation of FW and GW

Different ratios of GW and FW (90:10 and 50:50%) were tested for biohydrogen production and compared with the individual substrates (GW:FW ratios of 100:0% and 0:100%), using a final waste concentration (in VS) of 8.5 g L^{-1}. Highest cumulative hydrogen production (21.7 mL H_2) was achieved in the assay with GW:FW 90:10% (Fig. 2a), and the lowest value was obtained from GW:FW 0:100%. The onset of hydrogen production was delayed in all the assays with FW, being this effect directly related with the FW concentration (Fig. 2a and Table 3), as already described in the biohydrogen production assays made only with FW (Fig. 1a). These results suggest an inhibitory effect of FW on the hydrogen production, possibly associated with the presence of inhibitory by-products released by autoclaving, even though the co-fermentation of GW with small percentage of FW (90:10%) was beneficial. Higher values of hydrogen production rate and maximum hydrogen production were achieved in this situation (*i.e.* 21.6 ± 3.3 mL g^{-1} d^{-1} and 46.2 ± 0.9 mL g^{-1}, respectively) (Table 3). Analysis of the soluble fermentation products showed acetate and lactate accumulation in most assays (Fig. 2b, c). Acetate concentrations followed the hydrogen production trend (Fig. 2b), while higher lactate concentrations were attained for GW:FW 0:100% and 50:50% (Fig. 2c). No lactate was formed with only GW (GW:FW 100:0%). In general, lactate is formed when hydrogen partial pressure (pH_2) achieves a critical value, the metabolism shifts and NADH is used by lactate dehydrogenase to produce lactate instead of acetate and hydrogen (van de Werken et al., 2008). Different pH_2 critical values for *C. saccharolyticus* have been found, e.g. 10–20 kPa for *C. saccharolyticus* growing in sucrose (van Niel et al., 2003) and up to 60 kPa when using xylose (Willquist et al., 2010). In the present study, the higher pH_2 values attained were 39 kPa and 35 kPa, in the GW:FW 90:10% and 100:0% assays, respectively (Table 3). Unexpectedly, these assays were the ones with lower lactate production, which suggests that lactate formation was not directly related with pH_2.

The COD conversion efficiency of the wastes into hydrogen and organic acids, *i.e.* acetate and lactate (Table 4), was higher in the assays with GW:FW 90:10%, in which the sum of these products accounted for 49% and 12% of the soluble and total COD added (Table 4). In this case, and also in the assay with GW:FW 100:0%, the hydrogen produced represented 13% of the soluble COD and 3% of the total COD, as previously reported (Liu et al., 2013; Algapani et al., 2018; Yun et al., 2018). These results point that co-fermentation of the wastes was not complete, still remaining non-converted soluble COD and non-hydrolyzed waste in the end of the experiment. Moreover, an important amount of the wastes energy remains in the liquid phase as organic acids.

3.3. Biomethane production assays

Biomethane was successfully produced from the resulting end-products of the co-fermentation assays (GW:FW 90:10, 100:0 and 0:100%) (Fig. 3), and no methane was produced in the blanks (with hydrolysate and without sludge). The initial total and soluble COD values were similar in all the conditions tested, *i.e.* around 15 g L^{-1} and 9 g L^{-1}, respectively (Table 3). The highest methane production (1247 ± 70 mg COD-CH_4) and BMP (276.8 ± 15.4 L kg^{-1} relatively to the substrate COD added) was attained with the end-products of dark fermentation carried out with GW:FW 0:100% (Fig. 3, Table 3), possibly due to the presence of lactate that might have been used as an alternative source of hydrogen for the hydrogenotrophic methanogens. With the end-products from GW:FW 90:10% and 100:0%, similar methane production (around 800 mg COD-CH_4) and BMP values were obtained (Fig. 3, Table 3), although the presence of 10% FW in the first step dark-fermentation increased subsequent initial methane production rate comparatively to 100% GW (Fig. 3). When not subjected to the first step of biohydrogen production, the lowest methane production was obtained with a mixture of GW:FW 90:10% (Fig. 3), thus reinforcing the advantage of combining the two sequential steps. At the end of the experiment, soluble COD removal was approximately the same in all the experiments, ranging from 72 to 77% (Table 3). Methane production from the soluble end-products of the co-fermentation assays was almost complete, accounting for more than 88% of the soluble COD, as shown in Table 4. A value higher than 100% was even attained in the assay

Table 3
Overall results from biohydrogen production in co-fermentation assays (1st step) coupled with methane production (2nd step).

Assay	1st step (Hydrogen Co-Fermentation)							2nd step (Methanogenesis)	
	Maximum H_2 production* (mL g^{-1})	H_2 production rate* (mL $g^{-1} d^{-1}$)	Lag phase* (d)	R^{2**}	Maximum pH_2 (kPa)	Final COD_t (g L^{-1})	Final COD_s (g L^{-1})	BMP*** (L kg^{-1})	COD_s removal (%)
GW:FW 90:10%	46.2 ± 0.9	21.6 ± 3.3	1.8 ± 1.2	0.97	39.0 ± 0.2	31.9 ± 4.8	17.7 ± 0.5	181.8 ± 3.8	44.7 ± 1.7
GW:FW 50:50%	31.3 ± 1.8	3.0 ± 0.5	2.6 ± 0.8	0.96	24.5 ± 2.5	–	–	–	–
GW:FW 100:0%	45.8 ± 1.2	10.5 ± 1.9	≈ 0.0	0.95	35.3 ± 2.7	32.2 ± 1.7	18.2 ± 0.6	174.3 ± 23.8	47.9 ± 1.0
GW:FW 0:100%	16.5 ± 0.8	2.3 ± 0.1	4.8 ± 0.3	0.99	12.8 ± 0.7	31.5 ± 1.1	19.3 ± 0.1	276.8 ± 15.4	53.3 ± 2.1
GW:FW 90:10% w/ out 1st step	–	–	–	–	–	26.2 ± 1.6	19.4 ± 0.4	177.9 ± 25.4	50.0 ± 0.8

* Calculated by adjusting modified Gompertz equation. All parameters are expressed per g of waste VS added initially.
** From data adjustment with modified Gompertz equation.
*** BMP – Biochemical methane potential, expressed in volume of methane produced (L@STP) per kg of total COD added.

containing only FW (GW:FW 0:100%), which shows the occurrence of waste solubilization during the experiment, in an amount that corresponds to approximately 16% of the initial total COD. In the co-digestion of GW:FW 90:10% performed without previous fermentation by *C. saccharolyticus*, the methane produced represented only 69% of the soluble COD. Regarding total COD, approximately 50% was converted to methane in all the assays, with the exception of GW:FW 0:100% in which the methane produced accounted for 78% of the total COD (Table 4).

The efficient conversion of organic wastes into biohydrogen and methane, applying two step processes, has been reported by several authors. For example, hydrogen and methane production yields (expressed relatively to the waste VS added) of 105 ± 55 mL g^{-1} and 526 ± 137 mL g^{-1}, respectively, were attained by Algapani et al. (2018) from FW in a continuous thermophilic (55 °C)-mesophilic (37 °C) two-stage system. Hydrogen and methane accounted for 4% and 55% of the total COD in FW, respectively. These authors also showed that the contribution of the hydrogen production step to the overall energy was small, but it played an important role in maintaining the stability of the anaerobic treatment of FW. Concerning lignocellulosic wastes, Y_{H2} and Y_{CH4} of 6 mL g^{-1} and 476 mL g^{-1}, respectively, were reported by Pakarinen et al. (2009) from grass silage using a thermophilic-mesophilic two-step process. Kongjan et al. (2011) reported maximum hydrogen and methane yields of 89 mL g^{-1} and 307 mL g^{-1} from wheat straw hydrolysate during the operation of UASB reactors in series. The hydrogen-producing reactor was operated at 70 °C, and subsequent methane reactor at 55 °C.

In summary, the two step hyperthermophilic-mesophilic process applied in the present work allowed the conjugated treatment of GW and FW, with energy recovery in the form of biohydrogen and biomethane, two renewable and eco-friendly fuels. Combining hydrogen and methane production was beneficial because it increased the energy efficiency of the treatment of both wastes. Indeed, the first fermentation step appears to have facilitated further FW conversion to methane. For GW, the addition of a relatively small amount of FW improved the hydrogen production yield and rate and a significant amount of methane was also produced from this mixture of wastes in the second step. Moreover, more methane was produced in this case than from the same mixture of wastes that was not previously fermented by *C. saccharolyticus* at 70 °C.

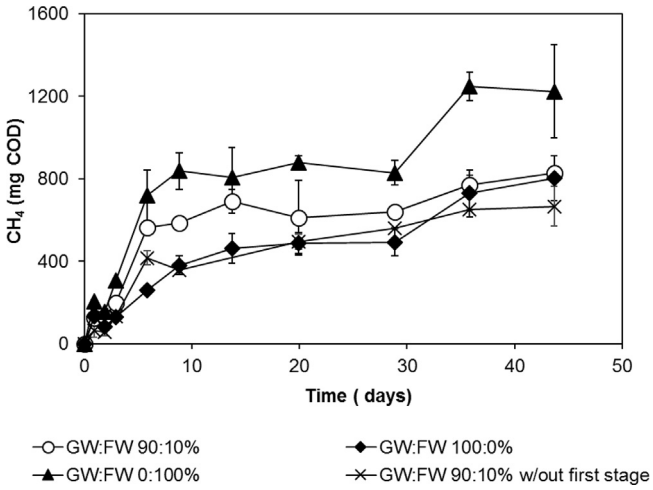

Fig. 3. Methane production from the end-products of the co-fermentation assays.

Table 4
COD conversion efficiency in the hydrogen co-fermentation of GW and FW (1st step) coupled with methane production (2nd step).

Assay	1st step (Hydrogen Co-Fermentation)				2nd step (Methanogenesis)	
	H_2 + Ac + Lac (% CODs)[a]	H_2 + Ac + Lac (% CODt)[a]	H_2 (% CODs) [b]	H_2 (% CODt)[b]	CH_4 (% CODs)[c]	CH_4 (% CODt)[c]
GW:FW 90:10%	49	12	13	3	94	52
GW:FW 50:50%	25	9	6	2	–	–
GW:FW 100:0%	38	9	13	3	88	50
GW:FW 0:100%	18	8	4	2	127	78
GW:FW 90:10% w/out 1st step	–	–	–	–	69	51

[a] Represents the relation between the amount of hydrogen, acetate and lactate produced, converted to its equivalent COD, and the amount of soluble COD (CODs) or total COD (CODt) added to each assay.
[b] Represents the relation between the amount of hydrogen produced, converted to its equivalent COD, and the amount of soluble COD (CODs) or total COD (CODt) added to each assay.
[c] Represents the relation between the amount of methane produced, converted to its equivalent COD (considering that 1 g of COD generates 0.35 L of methane at STP conditions), and the amount of soluble COD (CODs) or total COD (CODt) added to each assay.

4. Conclusions

Hydrogen and methane production from co-fermentation of GW and FW was successfully attained in a two-stage process. A maximum hydrogen yield of 46.2 ± 0.9 L kg^{-1} was achieved in the first stage process of dark fermentation with co-fermentation of GW and FW at a ratio of 90:10% by *C. saccharolyticus*. The co-fermentation end-products of GW and FW 90:10% yielded a maximum methane production of 682 L kg^{-1} in the second stage process of anaerobic digestion. The hydrogen and methane yields obtained in the present study allow a potential total energy generation of 24.9 MJ kg^{-1}.

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