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Boosting dark fermentation with co-cultures of extreme thermophiles for biohythane production from garden waste

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HIGHLIGHTS

- Co-cultures can synergistically intensify hydrogen production from sugars and garden waste (GW).
- Biohythane with 15% of H_2 was produced from GW.
- Potential energy generation of 22.2 MJ kg^{-1} (VS) was achieved from GW in two-step process.

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ABSTRACT

Proof of principle of biohythane and potential energy production from garden waste (GW) is demonstrated in this study in a two-step process coupling dark fermentation and anaerobic digestion. The synergistic effect of using co-cultures of extreme thermophiles to intensify biohydrogen dark fermentation is demonstrated using xylose, cellobiose and GW. Co-culture of *Caldicellulosiruptor saccharolyticus* and *Thermotoga maritima* showed higher hydrogen production yields from xylose (2.7 ± 0.1 mol mol^{-1} total sugar) and cellobiose (4.8 ± 0.3 mol mol^{-1} total sugar) compared to individual cultures. Co-culture of extreme thermophiles *C. saccharolyticus* and *Caldicellulosiruptor bescii* increased synergistically the hydrogen production yield from GW (98.3 ± 6.9 L kg^{-1} (VS)) compared to individual cultures and co-culture of *T. maritima* and *C. saccharolyticus*. The biochemical methane potential of the fermentation end-products was 322 ± 10 L kg^{-1} (COD_t). Biohythane, a biogas enriched with 15% hydrogen could be obtained from GW, yielding a potential energy generation of 22.2 MJ kg^{-1} (VS).

1. Introduction

The production of fuels from renewable biomass has been a long-standing research goal and is a priority for the sustainable development of our society. Biodegradable biomass can be directly converted by dark-fermentation, to hydrogen, CO_2 and other chemicals such as butyric acid, acetic acid or ethanol (Ren et al., 2011). Yet, due to the low production rates and yields, dark-fermentation for hydrogen production is still not economically competitive per se, compared to non-biological hydrogen production methods.

However, biohydrogen production can be integrated in a hybrid two-step process that overcomes the drawback of a low substrate conversion, where the hydrolysates resulting from dark fermentation, rich in volatile fatty acids (VFA), can be converted to CH_4 and CO_2 through an anaerobic digestion process. Alternatively, a photofermentation process can follow the dark fermentation step, but this alternative has shown limited cost efficiency especially concerning the nutrients requirements and the costs of photobioreactors (Ochsa et al., 2010). By applying a second step of methanogenesis it is possible to produce biohythane, a mixture of hydrogen and methane, usually with 10–25% hydrogen in volume. Biohythane is considered an important future fuel since it combines the advantages of hydrogen and methane, namely: (i) reduction of greenhouse gas emissions, (ii) improvement of fuel efficiency since the narrow range of flammability of methane is extended, (iii) the flame speed of methane is greatly increased and the quenching distance of methane can be reduced, making the engine easy to ignite with less input energy (Liu et al., 2013).

Dark fermentation can achieve a maximum H_2-production efficiency of 33%, i.e., four molecules of H_2 can be achieved per molecule of glucose with acetate and CO_2 as the other fermentation end products. The maximum yield is only possible when the H_2 partial pressure (pH_2) is kept sufficiently low (Stams, 1994), e.g. by continuous stripping of the produced H_2 with nitrogen. Still, there is a

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challenge in this field concerning the possibility of obtaining significantly high H_2 yields at relatively elevated pH_2 (van Groenestijn et al., 2002). In general, based on thermodynamics, extreme thermophilic bacteria and Archaea may produce up to the theoretical maximum of 4 mol H_2 per mol of hexose (Verhaart et al., 2010; Willquist et al., 2010). Extreme thermophilic bacteria such as, *Thermotoga maritima*, *Caldicellulosiruptor saccharolyticus* and *Caldicellulosiruptor bescii* have high polysaccharide-hydrolyzing capacity and are able to use most of the reducing equivalents formed during glycolysis for the production of hydrogen (Verhaart et al., 2010). Moreover, *C. saccharolyticus* is referred as relatively insensitive to high pH_2 (Willquist et al., 2010). This organism has recently gained increased interest due to its ability to produce thermostable cellulolytic and xylanolytic enzymes, to grow on complex lignocellulosic carbon sources, and to co-metabolize a wide spectrum of monosaccharides including both pentose and hexose sugars (de Vrije et al., 2009). Additionally, *C. bescii* has been recognized as degrading switchgrass, a model plant for bioenergy production without thermochemical pretreatment (Basen et al., 2014). *T. maritima* can grow either on various C5 and C6 sugars, starch, glycogen or on complex organic substrates (e.g. on peptone) and can achieve hydrogen yields close to the maximum theoretical (Frock et al., 2010). The importance of extremely thermophilic bacteria for biohydrogen production has been recognized but is not yet fully exploited. On the other hand, several co-culture approaches have been used for biohydrogen production, but co-cultures of hyperthermophilic bacteria, though promising, are not common (Pachapur et al., 2015). Liu et al. (2008) found that *C. thermocellum* JN4 degraded microcrystalline cellulose to produce hydrogen, ethanol, acetic acid and lactic acid, but could not completely utilize the cellobiose and glucose produced by the cellulose degradation. When co-cultured with *T. thermosaccharolyticum* GD17, hydrogen production increased about 2-fold.

Because few data are available on the potential synergistic effects of co-culturing hyperthermophilic bacteria for biohydrogen production from complex substrates, in this work, we study the effect of using co-cultures of *C. saccharolyticus* DSM 8903 and *T. maritima* DSM 3109 for biohydrogen production from xylose and cellobiose and co-cultures of *C. saccharolyticus* DSM 8903 and *C. bescii* DSM 6725, for biohydrogen production from garden waste. Moreover, the potential biohythane production from garden waste in a two-step process coupling dark fermentation and anaerobic digestion is evaluated.

2. Material and methods

2.1. Microorganisms and medium

C. saccharolyticus DSM 8903, *C. bescii* DSM 6725, *T. maritima* DSM 3109 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). *C. saccharolyticus* and *T. maritima* were individually cultured in modified DSM 640 medium containing 5 mmol L^{-1} glucose. The culture medium consisted of (per L) KH_2PO_4 0.75 g, K_2HPO_4 1.5 g, $MgCl_2{\cdot}6H_2O$ 0.4 g, NH_4Cl 0.9 g, yeast extract 1.0 g, $FeCl_3{\cdot}6H_2O$ 2.5 mg, NaCl 2.7 g, trypticase 2 g, SL-10 (medium 320 DSMZ) trace elements 1 ml, and resazurin 0.5 mg. The pH was adjusted to 7.2 at room temperature. Medium was reduced with 0.75 g L^{-1} Cysteine-HCl monohydrated. The medium was made anoxic by flushing with 100% N_2. *C. bescii* was cultured in DSM 516 medium containing 5 mmol L^{-1} glucose. The pH was adjusted to 7.2 at room temperature. The medium was reduced with 0.5 g L^{-1} $Na_2S{\cdot}9H_2O$. The medium was made anoxic by flushing with 80%/20% N_2/CO_2. The experiments were carried out under sterile conditions. *C. saccharolyticus*, *C. bescii* and *T. maritima* were grown at 70 °C. After reaching an optical density (OD) at 620 nm of 0.2–0.3, cell dry mass concentration was determined and the cultures were used as inocula for the subsequent batch assays.

2.2. Hydrogen production from sugars

Hydrogen production assays with simple sugars were previously performed to assess the possible synergetic effect of using co-cultures. Co-culture ($1{:}1_{(w/w)}$) of *T. maritima* and *C. saccharolyticus*, as well as individual cultures, were tested for biohydrogen production from xylose and cellobiose the main sugars resulting from hydrolyse of lignocellulosic materials. Initial sugar concentrations of 26 mmol L^{-1} of xylose and 12.5 mmol L^{-1} of cellobiose were used corresponding to theoretical hydrogen production yield 87 and 100 mmol L^{-1}, respectively. For each sugar tested, a set of batch assays were performed with mono-cultures and co-culture in triplicate including blanks without culture. Assays were performed in 120 mL serum bottles containing 50 mL of modified DSM 640 medium supplemented with 50 mmol L^{-1} 4-morpholine propanesulfonic acid (MOPS) and reduced with 0.75 g L^{-1} cysteine-HCl monohydrated. The medium was made anoxic by flushing with 100% N_2 and pH was adjusted to 7.2 at room temperature. The mono-culture assays were inoculated with 5 mL of culture containing a dry cell mass concentration of 1.6×10^{-3} g mL^{-1}. The co-culture assays were inoculated with 2.5 mL of the same cultures used in mono-culture assays. Afterwards, the bottles were incubated at 70 °C with shaking (90 rpm).

2.3. Hydrogen production from garden waste

2.3.1. Substrate characterization

Garden waste (GW) is composed by two fractions, one is garden grass and the other fraction is composed by small bushes. GW was dried at room temperature and then milled into pieces with less than 0.5 cm. The chemical characterization indicated that total and soluble COD were 934 ± 15 and 174 ± 0.7 mg g^{-1}, respectively, and the total and volatile solids (TS and VS) content were 914 ± 1 and 847 ± 2 mg g^{-1}, respectively. Glucan (glucose, cellobiose, cellulose) and xylan (xylose and hemicellulose) content represented, in percentage of VS, 23.5 ± 5.9% and 8.8 ± 0.9%, respectively. The Klason lignin content represented 32.1 ± 0.3% of the VS. Soluble COD was low (174 ± 0.7 mg g^{-1}) and an autoclaving pre-treatment (121 °C and 0.1 MPa for 20 min) was applied to increase the soluble COD. After autoclaving the soluble COD increased to 214.0 ± 5.9 mg g^{-1}, corresponding to 23% of the total COD.

2.3.2. Experiment set-up

Hydrogen production assays were performed in 160 mL serum bottles. Preliminary assays were done to evaluate the growth ability of *T. maritima* and *C. Saccharolyticus* in the culture media recommended for both strains. The aim was to select the medium to use in the co-culture experiments. It was observed, by visual inspection, that *C. Saccharolyticus* did not grow well in the *T. maritima* medium, but *T maritima* could grow well in the *C. Saccharolyticus* medium. Therefore, all the assays with *T. maritima*, *C. saccharolyticus* individual cultures and co-culture ($1{:}1_{(w/w)}$) were performed with 50 mL of phosphate-buffered medium (20 mmol L^{-1}) containing 50 mmol L^{-1} 4-morpholine propanesulfonic acid (MOPS) flushed with N_2 (100%). The medium was supplemented with trace elements solution SL-10 according to DSMZ 320 medium, yeast extract and resazurin were added to a final concentration of 0.5 g L^{-1} and 0.5 mg L^{-1}, respectively. Medium was reduced with 0.75 g L^{-1} Cysteine-HCl monohydrated.

In the assays with *C. bescii*, *C. saccharolyticus* and co-culture, it was observed that *C. bescii* did not grow in the medium of *C. saccharolyticus* but this bacterium could grow well in the *C. bescii*

medium. Therefore, the assay with *C. bescii*, *C. saccharolyticus* (in *C. bescii* medium) individual cultures and co-culture ($1{:}1_{(w/w)}$) were performed with anaerobic medium containing 0.33 g L^{-1} KH_2PO_4 and $NaHCO_3$ 1.5 g L^{-1} flushed with N_2/CO_2 (80/20 (v/v)). The medium was supplemented with trace elements solution SL-10 according to DSMZ 320 medium, yeast extract and resazurin were added to a final concentration of 0.5 g L^{-1} and 0.5 mg L^{-1}, respectively. Medium was reduced with 0.5 g L^{-1} $Na_2S{\cdot}9H_2O$. Before inoculation the bottles containing the GW were autoclaved at 121 °C and 0.1 MPa for 20 min. The autoclave functioned as thermal and pressure pre-treatment for the GW.

For the different inocula tested it was used a ratio of garden waste (g VS) to initial inoculum cell dry weight (g CDW) of 85. Afterwards, the bottles were incubated at 70 °C with shaking (90 rpm). All the experiments were performed in quadruplicate and included controls without GW and without inoculum. Production of hydrogen gas and soluble fermentation products were monitored.

2.4. Methane production from dark fermentation end products of garden waste

2.4.1. Inoculum

Anaerobic granular sludge from a brewery industry was used as inoculum in the methanogenic assays. The sludge contained 0.08 ± 0.01 g volatile solids (VS) g^{-1}. The specific methanogenic activity (SMA) in the presence of acetate (30 mmol L^{-1}) was 156 ± 5 mL g^{-1} (VS) d^{-1}, and in the presence of H_2/CO_2 (80/20 (v/v), 1 bar) was 375 ± 8 mL g^{-1} (VS) d^{-1}. SMA was determined according to the guidelines of Angelidaki et al. (2009).

2.4.2. Experiment set-up

Methanogenic assays were also performed according to the guidelines defined by Angelidaki et al. (2009), with a working volume of 120 mL, at 37 °C. The hydrolysates obtained after H_2 production were added to 600 mL serum bottles containing 20 g of inoculum and basal medium containing $NaHCO_3$ (5 g L^{-1}). pH of the medium was corrected to 7.0–7.2 with NaOH or HCl 2 mol L^{-1}. 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 also performed. The methane accumulated in the headspace of the closed bottles was measured by gas chromatography (GC), with a flame ionization detector (FID), using a gas tight syringe to sample 500 μL. Methane production was corrected for standard temperature and pressure (STP) conditions (0 °C and 0.1 MPa). Biochemical methane potential (BMP) was defined by the volume of methane produced per unit of COD of substrate added to the assay.

2.5. Analytical methods

Determination of lignin, xylan and glucan was performed according to Sluiter et al. (2008). Total and soluble COD were determined using standard kits (Hach Lange, Düsseldorf, Germany). Sample filtration was performed prior to soluble COD determination. Hydrogen concentration in the gas phase was determined by gas chromatography using a column molsieve (MS-13x 80/100 mesh) and thermal conductivity detector Bruker Scion 456 Chromatograph, (Bruker, Massachusetts, USA) with argon (30 mL min^{-1}) as the carrier gas. The injector, detector and column temperatures were 100, 130, and 35 °C respectively. Methane content in the biogas was analysed in a gas chromatograph (Chrompack 9000) equipped with a FID detector 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.

Volatile fatty acids (VFA), lactic acid, sugars were determined by high performance liquid chromatography using an HPLC (Jasco, Japan) with a *Chrompack column* (6.5 × 30 mm^2); sulfuric acid (0.005 mol L^{-1}) at a flow rate of 0.6 mL min^{-1} was used as mobile phase. Column temperature was set at 60 °C. Detection of VFA, lactic acid, sugars was made sequentially using a UV detector at 210 nm and a RI detector. Cells dry weight was determined using a 0.2 μm filter.

2.6. Data analysis

The modified Gompertz equation was used to describe the progress of cumulative hydrogen production obtained from the batch experiments. Using the cumulative hydrogen production data, corrected to STP conditions (0 °C and 0.1 MPa), the maximum hydrogen production rates were estimated from the fit of the modified Gompertz equation (Eq. (1)).

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

where $H(t)$ is cumulative hydrogen production (mL), P hydrogen production potential (mL), R_m maximum hydrogen production rate (mL h^{-1}), e = 2.71828…, λ lag-phase time (h), and, t time (h). Dissolved hydrogen concentration was calculated using the Henry's law at 70 °C: $K_H{*}Pi$, where K_H is the Henry's law constant for hydrogen (8.7 × 10^{-9} MPa^{-1} at 70 °C).

3. Results and discussion

3.1. Co-culture experiments for biohydrogen production from sugars

Hydrogen production from xylose and cellobiose by extreme thermophiles *T. maritima* and *C. saccharolyticus* was evaluated either as individual cultures or as co-cultures. The results showed a significant improvement of the hydrogen production by the utilization of co-culture of *T. maritima* and *C. saccharolyticus,* suggesting a synergistic effect between these two extreme thermophiles.

Significantly higher hydrogen production from xylose and cellobiose, 71 and 60 mmol L^{-1}, respectively were obtained for the co-culture (Figs. 1 and 2) than with individual cultures of *T. maritima* ($p < 0.001$: *t*-test) and *C. saccharolyticus* ($p < 0.001$: *t*-test). These results correspond to 83 and 60% of the maximum theoretical hydrogen production from each sugar tested. Moreover, the highest hydrogen production yields for both sugars were obtained in co-culture (4.8 ± 0.3 and 2.7 ± 0.1 mol mol^{-1} total sugar for cellobiose and xylose, respectively) (Table 1). Co-culture and *T. maritima* assays showed no lag phases for both sugars tested. Longer lag phases (24 h) observed in *C. saccharolyticus* assays were shortened using *T. maritima* in co-culture (Figs. 1 and 2). Probably the hydrogen production in the first hours of co-culture assays was a result of sugars conversion by *T. maritima*.

In *T. maritima* and co-culture experiments, cellobiose was not all consumed (Fig. 2). In these assays glucose started to accumulate after 83 h from the beginning of the experiment. Total sugar consumption balance, taking into account the cellobiose consumed and the glucose formed, shows that the percentage of sugar consumption in the cellobiose experiments is approximately the same (49–52%) (Table 1 and Fig. 2). The highest hydrogen yield (4.8 mol mol^{-1} total sugar) was, however, achieved in the co-culture. Also, the percentage of xylose utilization in the co-culture, 86%, was higher than in the individual culture assays (Table 1 and Fig. 1). Moreover, the ratio acetate produced/sugar

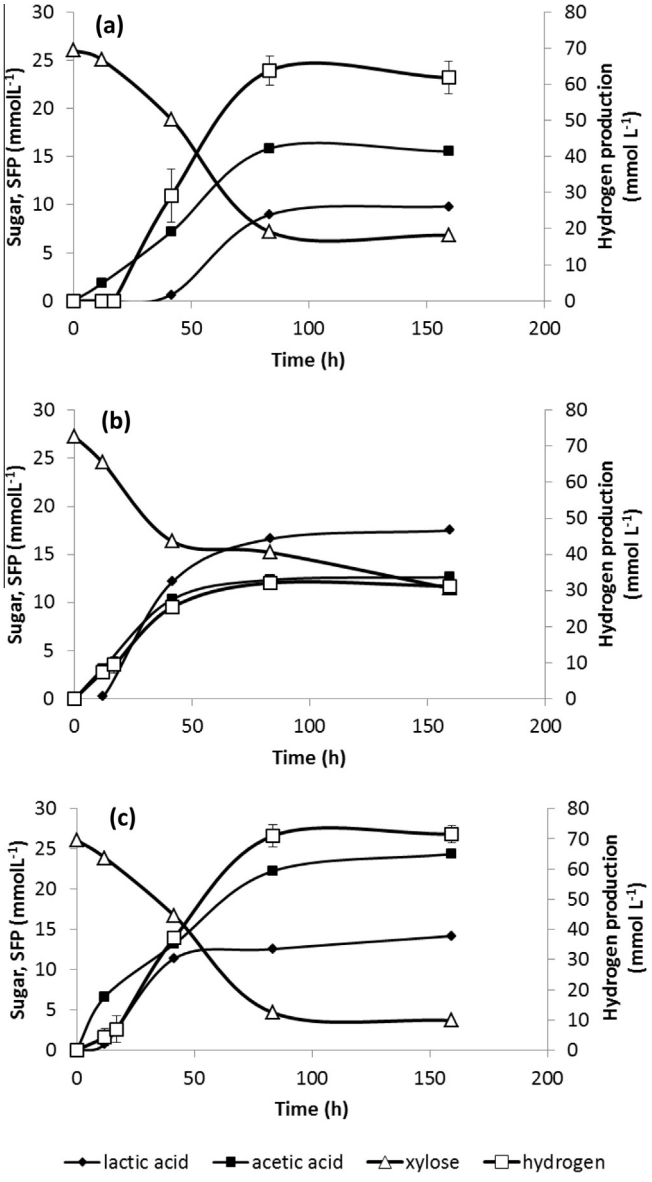

Fig. 1. Hydrogen production from xylose, soluble fermentation products, xylose consumption, using (a) *C. saccharolyticus* and (b) *T. maritima* individual cultures and (c) co-culture.

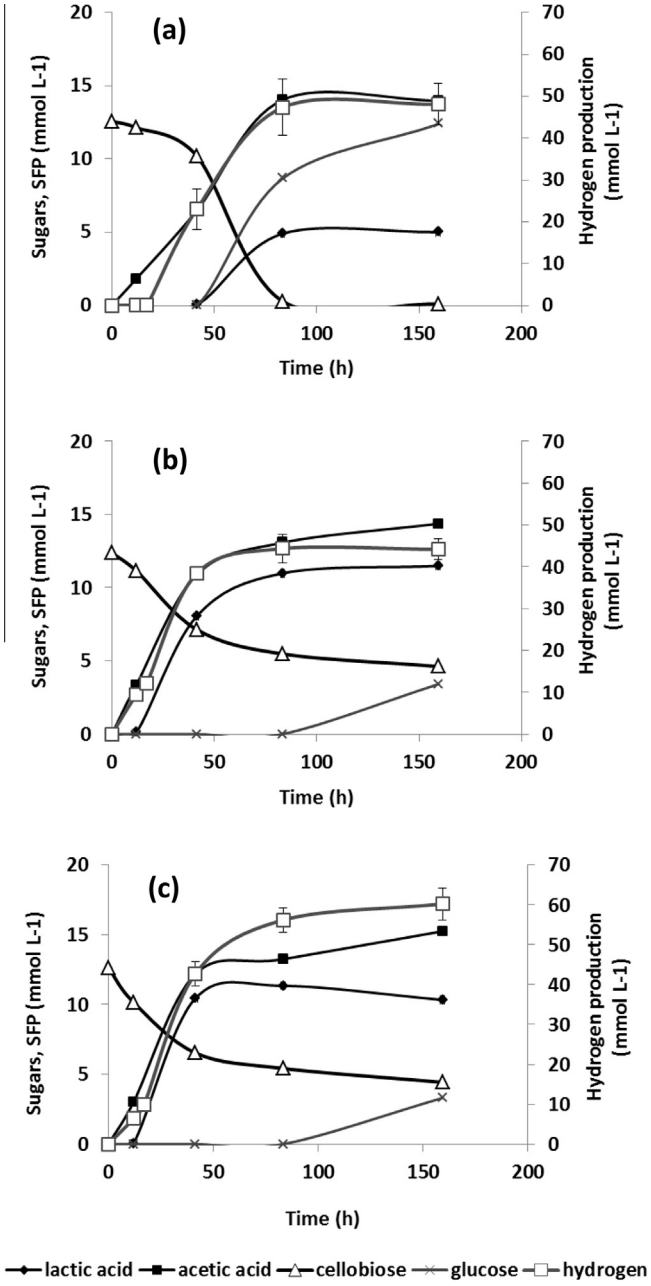

Fig. 2. Hydrogen production from cellobiose, soluble fermentation products, cellobiose consumption, using (a) *C. saccharolyticus* and (b) *T. maritima* individual cultures and (c) co-culture.

consumed was higher in the co-culture than in individual culture assays (Fig. 1).

The hydrogen partial pressure (pH_2) is normally used as a measure of H_2 tolerance and the critical value is in general referred as the pH_2 at which lactate formation is initiated as an alternative way to reoxidize NADH (Verhaart et al., 2010). In the *C. saccharolyticus* assays, lactate formation was observed when pH_2 achieved 59 ± 14 kPa and 46 ± 9 kPa for xylose and cellobiose as substrates, respectively. Contrary, lactate formation was observed, for both substrates, at pH_2 lower than 24 kPa in *T. maritima* and co-culture assays. Therefore *C. saccharolyticus* was less affected by pH_2 compared to *T. maritima* and co-culture.

Hydrogen partial pressure of 10–20 kPa has been referred as critical for *C. saccharolyticus* growing in sucrose (Willquist et al., 2010). In the present study, lactate formation was initiated in *C. saccharolyticus* assays at higher pH_2 for both substrates tested (59 ± 14 kPa for xylose and 46 ± 9 kPa for cellobiose). Possible explanation for this inconsistency could be the difference in organism's metabolic activity on the different substrates, i.e., the volumetric H_2 productivity is lower on xylose than on sucrose which results in different concentrations of dissolved H_2 (Willquist et al., 2010). Also, substrates more difficult to hydrolyse, such as cellobiose, could originate different distribution pattern of catabolic and anabolic fluxes leading to different metabolite levels that modulate lactate dehydrogenase (LDH) activity (Willquist and van Niel, 2010). Therefore, the critical *p*H2 has no strict value, but will depend on the organism, the substrate, among other environmental factors of the fermentation process.

Despite lactate being formed, growth and hydrogen production was maintained in all the experiments. For both sugars tested, the highest final pH_2 (146 ± 7 and 121 kPa for xylose and cellobiose, respectively) was achieved in the co-culture assays (Table 1). In these assays, the shift to lactate formation was similar to the observed in the *T. maritima assays*. However, the presence of *C.*

Table 1
Hydrogen production yields, sugar consumption, critical and maximum hydrogen partial pressure and dissolved hydrogen for *C. saccharolyticus* and *T. maritima* individual cultures and co-culture.

		H_2 yield (mol mol^{-1} total sugars)	Sugar consumption (%)	Critical P(H_2)* (kPa)	Maximum P(H_2)** (kPa)	Critical dissolved hydrogen (μmol L^{-1})
C. saccharolyticus	Xylose	2.4 ± 0.2	74	59 ± 14	128 ± 8	509.7
	Cellobiose	3.8 ± 0.4	51	46 ± 9	100 ± 0	403.8
T. maritima	Xylose	1.1 ± 0.1	58	19 ± 5	64 ± 3	165.7
	Cellobiose	3.6 ± 0.2	49	24 ± 1	89 ± 7	211.6
Co-culture *C. saccharolyticus* and *T. maritima*	Xylose	2.7 ± 0.1	86	14 ± 9	146 ± 7	121.0
	Cellobiose	4.8 ± 0.3	52	20 ± 1	121 ± 0	173.1

* Hydrogen partial pressure $p(H_2)$ at witch lactate formation is initiated.
** Above this hydrogen partial pressure $p(H_2)$ no hydrogen was produced.

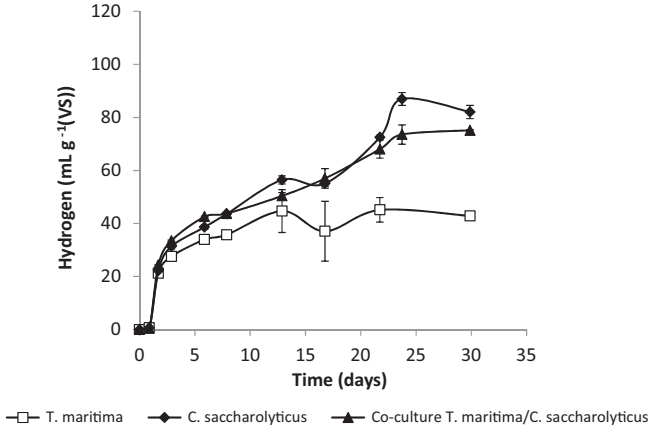

Fig. 3. Hydrogen production yield (mL of hydrogen per volatile solids (VS) of garden waste) using *T. maritima* and *C. saccharolyticus* individual cultures and co-culture of *T. maritima* and *C. saccharolyticus*.

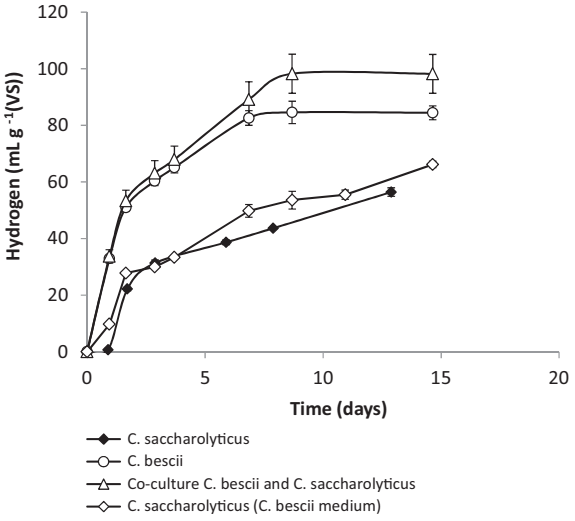

Fig. 4. Hydrogen production yield (mL of hydrogen per volatile solids (VS) of garden waste using *C. bescii* and *C. saccharolyticus* individual cultures and co-culture of *C. bescii* and *C. saccharolyticus*.

saccharolyticus, which is less affected by pH_2, promoted an overall increase of hydrogen production.

3.2. Biohydrogen production from garden waste

Hydrogen production from the lignocellulosic garden waste (GW) was evaluated using individual cultures of the extreme thermophiles *T. maritima*, *C. saccharolyticus* and *C. bescii*. Co-cultures (1:1$_{(w/w)}$) of *T. maritima* and *C. saccharolyticus*, as well as, *C. saccharolyticus* and *C. bescii* were also tested to assess a possible synergistic effect on hydrogen production from GW. Co-culture of *C. saccharolyticus* and *C. bescii* showed a positive synergistic effect on hydrogen production from GW.

The lowest hydrogen production potential (45.1 ± 4.6 L kg^{-1} (VS)) obtained from GW was achieved with individual culture of *T. maritima* (Fig. 3). Hydrogen production potential using co-culture of *T. maritima* and *C. saccharolyticus* was similar to the one obtained with individual culture of *C. saccharolyticus* (Fig. 3 and Table 2). This result suggests that the presence of *T. maritima* was not contributing for an improvement of hydrogen production. A possible explanation could be the fact that GW is a very complex residue and possibly *T. maritima* is not able to use the more recalcitrant fraction.

For cost effective hydrogen fermentation, it is very important to obtain high yields under high pH_2. Co-culture of closely related organisms (*C. bescii and C. saccharolyticus*) exhibited a remarkable stability and tolerance to high pH_2 pressure. Maximum specific hydrogen production of 98.3 ± 6.9 L kg^{-1} (VS) was achieved with co-culture of *C. bescii* and *C. saccharolyticus* (Fig. 4 and Table 2). Moreover, maximum hydrogen production rate was attained in the same assay (46.3 ± 7.3 L kg^{-1} (VS) d^{-1}). The higher hydrogen production observed with the co-culture of *C. bescii* and *C. saccharolyticus* was associated to higher acetate and lower lactate production in the co-culture compared with the *C. bescii* individual

Table 2
Hydrogen production yields and rates. Final concentration of acetate and lactate produced from garden waste dark fermentation process for each culture and co-culture tested.

	Dark fermentation (First step process)				
	Maximum H_2 production rate (L kg^{-1}(VS) d^{-1})*	R^2	H_2 yield (L kg^{-1} (VS))	Acetate (mmol L^{-1})	Lactate (mmol L^{-1})
C. saccharolyticus	33.8 ± 2.4	0.99	82.0 ± 2.5	10.4 ± 0.5	–
T. maritima	34.0 ± 1.3	0.99	45.1 ± 4.6	3.8 ± 0.2	–
C. bescii	44.2 ± 2.7	0.99	84.6 ± 4.0	14.0 ± 0.7	2.7 ± 0.1
Co-culture *C. saccharolyticus*/*T. maritima*	39.2 ± 0.8	0.99	75.1 ± 0.3	10.1 ± 0.5	–
Co-culture *C. saccharolyticus*/*C. bescii*	46.3 ± 3.2	0.99	98.3 ± 6.9	15.6 ± 0.8	1.2 ± 0.1

* Calculated by adjusting modified Gompertz equation.

Table 3
Comparison of biohydrogen yield from different types of feedstocks.

Substrate	Inoculum	Hydrogen yield (L kg^{-1} (VS))	References
Sweet sorghum biomass	Indigenous microflora	10.4	Antonopoulou et al. (2008)
Wheat straw hydrolysate	Anaerobic granular sludge	89.1	Kongjan et al. (2010)
Grass silage	Cow manure	6.46	Pakarinen et al. (2009)
Lipid-extracted microalgae biomass	Anaerobic sludge	46.0	Yang et al. (2011)
Corn stover	*Clostridium butyricum* AS1.209	68.0	Li and Chen (2007)
Beer lees	Mixed microflora	53.0	Cui et al. (2009)
Garden Waste (GW)	Co-culture *C. saccharolyticus*/*C. bescii*	98.3	This study

culture (Table 2). The results put forward that the use of co-culture of *C. saccharolyticus* and *C. bescii* increased synergistically the hydrogen production yield from GW.

Biohydrogen production yield obtained from GW using the co-culture of *C. saccharolyticus* and *C. bescii* was higher than the yields obtained from other lignocellulosic feedstocks, as shown in Table 3. Furthermore, the hydrogen production yield obtained in the present study was achieved without any additional hydrolytic pretreatment, besides autoclaving (121 °C, 0.1 MPa, 20 min).

3.3. Methane production and biohythane

After hydrogen production from GW using the co-culture of *C. bescii* and *C. saccharolyticus*, the biochemical methane potential of the resulting fermentation end products was assessed.

The initial total and soluble COD was 15.3 ± 4.2 and 5.0 ± 0.1 g L^{-1}, respectively. Specific methane production of 322 ± 10 L kg^{-1} (COD_t) was attained with the dark fermentation end products of GW, after 42 days of experiment. The pH at the end of the anaerobic biodegradability assay did not reach inhibitory values and the percentage of soluble COD removal was 86.4%.

The present study demonstrates that is possible to produce biohythane with 15% H_2 from GW (Table 3), which is in the optimal H_2 concentration range (10–25%). According to the results obtained, a maximum potential energy generation of 22 MJ kg^{-1} (VS) can be achieved by using a two-step process (dark fermentation and anaerobic digestion) for the conversion of GW. This value compares well with the energy content of green waste (Hla and Roberts, 2015). Kongjan et al. (2011) found an increased energy conversion from only 7.5% in the hydrogen stage to 87.5% of the potential energy, in a similar two-step process, corresponding to total energy of 13.4 MJ kg^{-1} of wheat straw hydrolysate. Pawar et al. (2008) also presented several scenarios for the energy output of combined hydrogen and methane production from wheat straw, and compared with reference scenario of a single methanogenic process. A value of 19.1 MJ kg^{-1} for wheat straw and 16.3 MJ kg^{-1} for its sugar fraction is mentioned in that study. The potential of energy recovery with this approach is remarkable and deserves future investment in research and development. For example, considering only the amount of household yard clippings of about 20 Mton y^{-1} available in the EU, the theoretical energy potential would reach 373 PJ y^{-1} with this combined process. This interesting energy output estimated is significantly amplified, when crop residues are included, which account for a value of 122 Mton available in 2011 in the EU (Searle and Malins, 2013). Future developments on biohythane production from lignocellulosic residues should consider pilot scale demonstration plants, being mandatory to include accurate life cycle and cost assessment studies, in order to fully consider the integration of technical, economic and environmental implications of this promising process.

4. Conclusions

The present study demonstrates the feasibility of producing biohythane containing 15% of hydrogen, from garden waste (GW), recovering a maximum potential energy of 22 MJ kg^{-1} (VS). It is concluded from this study that a pre-fermentation, with co-cultures of extreme thermophilic bacteria, followed by a methanogenic process, is a promising strategy to recover energy from complex biomass sources, through the production of biohythane. Although biohythane production and utilization processes are still under development, this hydrogen enriched biogas will be certainly a future fuel to consider in the scenarios of renewable energy production.

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