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Hydrogen Production from Enzymatic Hydrolysates of Alkali Pre-Treated Giant Reed (*Arundo donax* L.)

Ciro Vasmara [1], Stefano Cianchetta [2], Rosa Marchetti [1], Enrico Ceotto [1] and Stefania Galletti [2,*]

[1] CREA—Council for Agricultural Research and Economics, Research Centre for Animal Production and Aquaculture, Via Beccastecca 345, 41018 San Cesario sul Panaro, Italy; ciro.vasmara@crea.gov.it (C.V.); rosa.marchetti@crea.gov.it (R.M.); enrico.ceotto@crea.gov.it (E.C.)

[2] CREA—Council for Agricultural Research and Economics, Research Centre for Agriculture and Environment, Via di Corticella 133, 40128 Bologna, Italy; stefano.cianchetta@crea.gov.it

* Correspondence: stefania.galletti@crea.gov.it; Tel.: +39-051-6316836

Abstract: The perennial rhizomatous grass giant reed (*Arundo donax* L.) can be exploited to produce hydrogen by dark fermentation. This implies a high availability of simple sugars, like glucose and xylose, and, thus, a pre-treatment is necessary to remove lignin and expose the holocellulose to enzymatic attack. This study aimed at evaluating the hydrogen production from giant reed hydrolysates. Giant reed dry meal was pre-treated with diluted NaOH (1.2% weight/weight), then the solid fraction was separated from the alkaline black liquor by filtration, enzymatically hydrolyzed with a cellulase blend (Cellic CTec2), and fermented in mesophilic batch conditions with a microbial consortium derived from pig slurry. The impact on hydrogen yield of initial pH was evaluated by comparing the hydrogen production from hydrolysates with not adjusted (5.3) or adjusted initial pH (8.7) using NaOH or alkaline black liquor. The highest hydrogen yield, 2.0 mol/mol of hexoses, was obtained with alkaline initial pH 8.7, regardless of how the pH adjustment was managed. The yield was 39% higher than that obtained in reactors with initial pH 5.3. In conclusion, thermo-alkaline pre-treatment followed by enzymatic saccharification and initial pH adjustment at 8.7 with the black liquor remaining after pre-treatment is a promising strategy to produce hydrogen from giant reeds in dark fermentation.

Keywords: giant reed; alkaline pretreatment; enzymatic hydrolysis; dark fermentation; pH; black liquor; bio-hydrogen

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1. Introduction

In recent years, the perspective of H_2 utilization as an energy carrier is spreading, in transportation or stationary applications, after eventual conversion to electricity utilizing fuel cells. H_2 advantages are its lower heating value (120 MJ kg^{-1}) which is the highest among the fuels, and the absence of polluting emissions [1]. Currently, hydrogen is mostly obtained from fossil sources like natural gas, oil and naphtha, or coal by steam reforming or gasification [2]. Hydrogen can also be obtained from water by electrolytic processes or from renewable sources like biomass, by chemical or physical methods, like gasification or pyrolysis [3]. However, all these processes need a primary source of energy. Recently, there has been a growing interest in the biological production of hydrogen [4] and, in particular, by dark fermentation (DF). This technology does not require high energy or chemical inputs and it utilizes smaller reactor volumes, in comparison with the volumes and surface extensions required in photo-biological H_2 production. The DF greatest limit is the low H_2 yield, which is reported in the range 0.9–3.3 mol/mol hexose from pure carbohydrates [5], although a yield of 5.77 mol/mol hexose using an over-producing engineered strain of hyperthermophilic *T. maritima*, was recently achieved [6]. The H_2 production by DF can be an order of magnitude larger than by other biological processes [4], such as direct and indirect

bio-photolysis, and photo-fermentation; furthermore, the energy conversion efficiency can be improved by integrating DF with anaerobic digestion, producing methane [7,8].

Substrates rich in monosaccharides are ideal for H_2 production in DF [9]. Lignocellulosic materials represent interesting feedstocks to obtain fermentable monosaccharides like pentoses and hexoses [10], after opportune processing, since they are available worldwide at low cost, both as agricultural and agro-industrial waste, or as dedicated energy crops [11]. Among these, the perennial rhizomatous grass giant reed (*Arundo donax* L.), is considered one of the most promising energy crops in warm temperate zones [12]. In these zones, indeed, giant reeds can reach a dry biomass yield of up to 49 t ha^{-1}, much higher than those obtainable from other energy crops, such as Miscanthus [13]. Giant reed clumps are widespread in the wild in Mediterranean regions. Giant reed stands can be easily established providing high biomass yields when properly managed [14,15]. Due to its high adaptability to different soils, the giant reed can also be cultivated on marginal lands, poorly suited to annual row crops.

Lignocellulosic feedstocks are composed of cellulose linked in microfibrils by strong hydrogen bonds and embedded in a matrix of hemicellulose, pectin, and lignin, closely associated in a complex crystalline structure [16]. To release fermentable sugars from this kind of material, a pre-treatment step is required to expose the holocellulose followed by enzymatic hydrolysis, which is necessary to enhance the sugar concentration after the pre-treatment [3]. This process is already applied to industrial bioethanol production from 2G biomass [17]. However, by replacing the yeasts with H_2-producing bacteria, the process can be converted from bioethanol to bio-H_2 production, with all the advantages of using H_2 already described above. Furthermore, H_2 can be used either to generate energy via fuel cells or as a fuel for hydrogen vehicles [6]. A variety of pretreatment methods are described in the literature: from biological to physical, physicochemical, and chemical [18–20], with different effects on the lignocellulosic substrate and different impacts on the subsequent enzymatic hydrolysis step. In previous works, giant reed biomass pretreated by alkaline pretreatments produced fermentable sugars at high yields [21,22]. In addition, it is recognized that alkaline pretreatments produce few inhibitors for the subsequent steps [22,23]. Moreover, alkaline pre-treatments can be carried out at a relatively low temperature and pressure, with obvious advantages [24]. More recently, enzymatic hydrolysis after alkaline pre-treatment, gave superior sugar yield (up to 1.75 folds) using NaOH compared to $Ca(OH)_2$ [25]. However, a detoxification step could be required before the enzymatic hydrolysis to remove inhibitors possibly generated in the black liquor during the pre-treatment [3]. After alkaline pre-treatment, the black liquor is still alkaline and could be used to adjust the initial pH of DF. The pH plays a key role in the DF process [26] and recently it was reported that initial alkaline pH enhanced H_2 yield in DF from a lactose-rich substrate [27]. In that study, initial pH 8.7 was identified as optimum to reach the highest H_2 production. At the initial alkaline pH, the best environmental conditions for the activity of H_2-producing bacteria were promoted [9]. Since *Clostridia* and *Enterobacter* are the major producers of H_2 in DF [28] and since they can ferment several sugars, initial alkaline pH could also increase H_2 production in DF from glucose-like rich substrates.

However, the introduction of black liquor into the DF reactor could be detrimental, due to its inhibitory compounds (i.e., polyphenols) [29].

Previously, a high inhibitory effect on DF by compounds released after pre-treatment of giant reed by steam explosion was reported [30,31]. Careful successive adaptation of the inoculum improved H_2 yield from steam-exploded giant reed [32].

As far as we are aware, little is known about H_2 production by DF of alkali-pretreated giant reeds. In this study, the effects on H_2 production by DF of a thermo-alkaline pre-treatment of giant reed biomass with NaOH, followed by enzymatic saccharification, were evaluated. The impact of initial pH and possible inhibitors released by the pre-treatment was assessed. In addition, the possibility of re-cycling the alkaline residual liquor discarded after the pre-treatment in DF was also evaluated.

2. Materials and Methods

2.1. Feedstock

Giant reed meal was obtained from winter-harvested aboveground biomass collected on a field crop established at the CREA experimental farm, located at Anzola dell' Emilia (Bologna, Northern Italy, Lat. 44°32′ N, Long. 11°11′ E, 38 m a.s.l.). The meal was obtained after milling and sieving (<1.5 mm) the aboveground oven-dried plant organs, i.e., stems and leaves [33]. Samples were stored at room temperature in plastic bags until use.

2.2. Experimental Design

The experiments were performed according to a completely randomised experimental design [34] comparing three DF conditions of giant reed hydrolysates: (i) initial pH 5.3 (not adjusted, hereinafter Not-adj); (ii) initial pH 8.7 (adjusted with NaOH, hereinafter NaOH-adj); (iii) initial pH 8.7 (adjusted with alkaline black liquor addition, hereinafter Liq-adj). As a control, not treated and not hydrolysed giant reed meal, pH 8.7 adjusted with NaOH, was also included, (hereinafter C). All treatments were performed in triplicate.

A total of 12 experimental units were set up and utilised in DF experiments.

2.3. Pre-Treatment, Fibre Recovery, Hydrolysis and Sugar Content Determination

Giant reed meal was pre-treated as previously described [22]. Briefly, the meal was added to dilute alkali (final NaOH 1.2% weight/weight, w/w) up to a concentration of 10% w/w, then the slurry was pre-treated at 121 °C, 20 min, in glass bottles (600 g slurry per bottle, in triplicates).

The pre-treated solid fraction was partially separated from the black liquor by filtration and both the two fractions were recovered. The solid fraction was gently washed with distilled water under vacuum until a clear filtrate was obtained. Washing was aimed at removing possible inhibitors for the following fermentation step. Then, this solid fraction was saccharified (pH 5.0, at 50 °C for 144 h) as a slurry at 7.0% w/w, with a mix of commercial enzymes (Cellic CTec2, SAE0020, Sigma-Aldrich, St. Louis, MO, USA) at a cellulase load of 25 filter paper units per gram of dry weight (FPU/g DW), as detailed in a previous paper [22]. After the saccharification, the hydrolysed biomass was centrifuged, and the supernatant was recovered for the following DF, while the pellet was discarded. Sugar-rich hydrolysates (the recovered supernatants) were opportunely diluted by adding a few millilitres of sterile distilled water to obtain a concentration equal to 50 g/L of equivalent sugars (according to the DNS assay described below).

The supernatant was analysed for reducing sugar content by the 3,5-dinitrosalicylic acid (DNS) method [35] adapted for 96-well microplates, in duplicate [36]. The assay was performed in citrate buffer 50 mM, pH 4.8, 5 min, 95 °C. Pure glucose, as well as a mix of glucose and xylose (1:1), and dilutions of a control enzyme mix, were included, as standards.

All the recovered fractions of interest (pre-treated solids, black liquor and hydrolysate supernatant) were weighted. Samples of pre-treated solids and black liquor were oven-dried at 60 °C for quantification on a dry weight basis.

2.4. Dark Fermentation

Dark fermentation for H_2 production was carried out in laboratory static mesophilic batch conditions [27] in 118 mL reactors.

Each reactor (treatment) contained 30 mL of hydrolysate (1.5 g of sugars). In the case of Not-adjusted reactors, the hydrolysate was used as it was (pH 5.3), and the pH in the reactors was not adjusted. In the other reactors, the initial pH was adjusted to 8.7 with 0.5 mL 32% NaOH solution (NaOH-adj reactors) or 5 mL of alkaline black liquor (Liq-adj reactors). Control reactors (C) were fed with 1.5 g VS untreated giant reed hydrated with 30 mL potassium phosphate-buffered medium [33], as hydration medium (HM).

The hexose equivalent content in each reactor (8.33×10^{-3} mol/reactor) was calculated by dividing the amount of sugar per reactor (g/reactor) by 180 (g/mol hexose). Note that

the theoretical hydrogen and acetate yields per mol of carbon are equal for glucose and xylose [37].

The headspace of the reactors was gassed with 100% N_2, to ensure the initial anaerobiosis conditions, and then each reactor was inoculated with 5 mL of inoculum. The inoculum used (40.2 ± 0.02 g VS L^{-1}) was a non-selected or pre-treated mixed inoculum, prepared as described in a previous paper [38], using pig slurry as raw material.

The control (C) was prepared in the same way. Reactors containing only inoculum and HM were also included as blanks to subtract the endogenous H_2 production.

Reactors were plugged using butyl rubber stoppers and aluminium seals and then they were incubated for 14 days at 35 °C. The DF was carried out in two cycles of 7 days; at the end of the first cycle, the pH of each reactor was brought back to its initial value utilizing an appropriate volume of NaOH solution (3 mL) or black liquor (7 mL) depending on the treatment. This change in the reactor's volume was considered in the calculations of biogas production. The pH resetting at the end of the first cycle was intended to favour further fermentation of residual substrates. This procedure had been previously adopted [39]. After the end of the second cycle, a reiterated pH correction was performed to check if it was possible to stimulate the DF once again.

The biogas production (volume and composition) was measured according to [40], as previously described [41]. Briefly, biogas was collected daily using 100-mL glass syringes. Hydrogen, CO_2, and CH_4 concentrations were determined as described below (par. 2.5).

The cumulative volume of H_2 was calculated by adding the volumes of gas collected in the syringe to that accumulated in the reactor headspace. Gas volume was reported at standard conditions (STP) of temperature (273 K) and pressure (101 kPa). The cumulative hydrogen production was finally determined after subtraction of the hydrogen produced by the inoculum.

Max Rate is the highest measured daily rate of H_2 production per gram of volatile solids added.

The maximum content of H_2 in the biogas during the first or second cycle of DF ($\%H_2$ MAX_1, $\%H_2$ MAX_2) was the highest percentage of H_2 detected in the biogas.

2.5. Analytical Methods

Biogas composition (H_2, CH_4, and CO_2) in the reactor headspace was analysed using a MicroGC Agilent 3000 gas chromatograph, equipped with 2 columns: Molsieve and Plot U; detector: TCD. Carrier gas: argon.

Total solids (TS), VS, ash, and pH of the oven-dried, milled and sieved biomass of giant reed and the washed pre-treated materials were determined according to standard procedures [42]. Total solids were determined gravimetrically after thermal treatment at 105 °C at a constant weight. Volatile solids were determined as the difference between TS and ash which was determined after incineration in a muffle furnace at 550 °C for 10 h. The pH was determined using a Crimson Titromatic 1S pH-meter; in the case of giant reed meal, it was determined after suspension, 2-h stirring and sedimentation of 1.1 g dry matter in 50 mL distilled water.

Total C and total N were determined in duplicate using CHN Truspec elemental analyzer (Leco).

Fibre fractions (neutral detergent fibre, NDF; acid detergent fibre, ADF; and lignin, ADL) of samples dried at 60 °C at constant weight were determined according to [43]. The hemicellulose content was estimated as the difference between NDF and ADF; cellulose as the difference between ADF and ADL.

Total polyphenols were determined as described previously [44]. Acetic acid concentration was determined using a GC-2010 PRO (Shimadzu) gas-chromatograph, equipped with a Nukol™ capillary column (Supelco, cat. No. 24107), 30 m × 0.25 mm ID, 0.25 µm film thickness; detector: FID; carrier gas: Helium; total flow rate: 68.6 mL min^{-1}; split 100; oven programmed temperature: 100 °C (1 min) to 194 °C at 8 °C min^{-1}, 194 °C for 4 min. Samples were prepared as previously described [27]. To quantify the acetic acid concentra-

tion, 2,2-dimethylbutyric acid (Sigma-Aldrich) was used as an internal standard [45]. Peak identification was based on the comparison of the unknown peak retention times with the retention times of a commercial volatile free acid standard mix (46975-U Supelco).

2.6. Statistical Analysis

All the statistical analyses were performed using PAST 4.10 software, Hammer Ø., Oslo, Norway [46]. Factors and factor interaction effects were considered significant at $p < 0.05$. The Tukey Honestly Significant Difference (HSD) at $p = 0.05$ was used to compare the treatment mean values.

3. Results

3.1. Biomass Fractionation Yields Following the Pre-Treatment

The different steps of the dry giant reed meal treatment from the alkaline pre-treatment up to the enzymatic hydrolysis are illustrated in Figure 1.

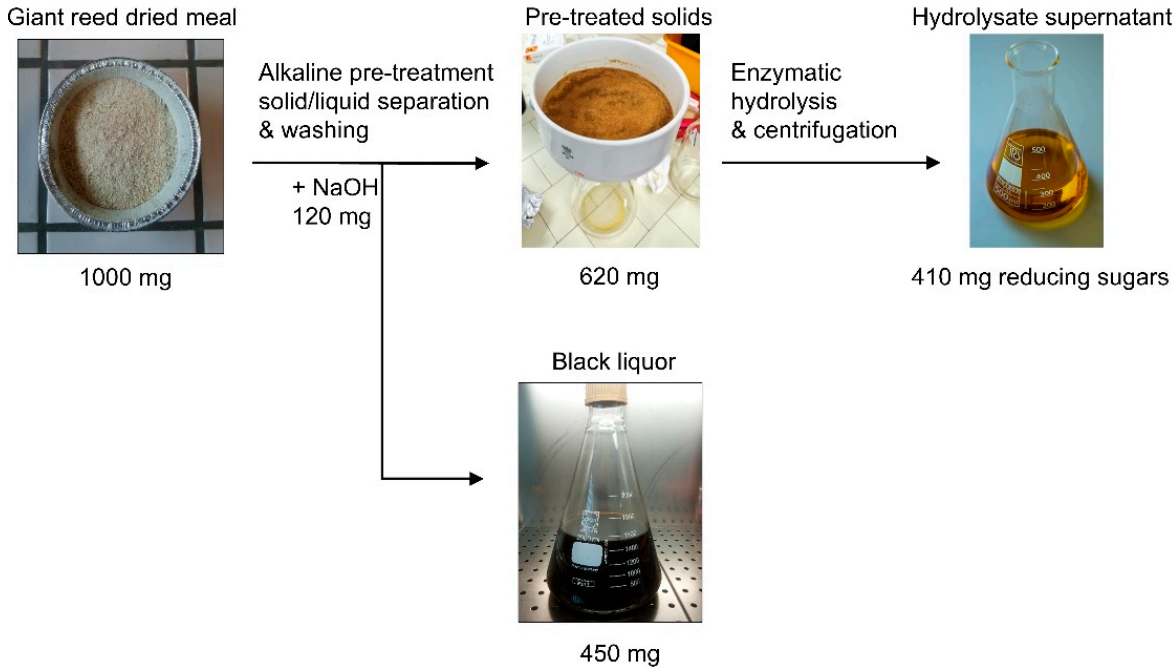


Figure 1. Workflow of the giant reed meal treatment steps, from alkaline pre-treatment to enzymatic hydrolysis. The amounts of the recovered fractions of interest are reported on a dry weight (DW) basis.

After the thermo-alkaline pre-treatment, 620 mg DW of solids per 1000 mg DW of the meal were recovered, which yielded on average 410 mg DW of reducing sugars after enzymatic hydrolysis. A conspicuous amount of liquefied biomass, together with residual NaOH, for a total of 450 mg DW, ended up in the liquid fraction (black liquor).

3.2. Composition of the Materials Utilised in Dark Fermentation

Table 1 shows the composition of all the materials utilized in DF. Hydrolysate supernatant and black liquor displayed similar TS content of around 6%, the former had a higher VS content and mostly contained reducing sugars, the latter had a higher ash content and a lower VS content (50%), in particular polyphenols derived from the lignin de-structuring and acetic acid (Table 1). Both giant reed meal and the hydrolysates were slightly acidic, while the black liquor was strongly alkaline (pH > 11). due to residual NaOH content from the giant reed alkaline pre-treatment.

Table 1. Composition of the materials utilised in dark fermentation.

Trait	Giant Reed Meal	Hydrolysate Supernatant	Black Liquor	Inoculum
Total solids (TS), %	98.21 (0.4)	6.03 (0.02)	6.43 (0.02)	6.33 (0.30)
Volatile solids (VS), %	92.90 (0.2)	5.04 (0.02)	3.22 (0.03)	4.02 (0.02)
Volatile solids, % TS	95	84	50	64
Ash, %	5.31 (0.23)	0.99 (0.01)	3.21 (0.02)	2.31 (0.29)
pH	5.80 (0.02)	5.32 (0.01)	11.12 (0.01)	7.60 (0.01)
Cellulose, % TS	39.11 (0.3)	n.d.	2.38 (0.11)	3.62 (0.85)
Hemicellulose, % TS	23.14 (0.3)	n.d.	n.d.	0.66 (0.07)
Lignin, % TS	11.67 (0.1)	n.d.	4.16 (0.08)	3.78 (0.89)
Acetic acid, g L^{-1}	n.d.	n.d.	10.48 (0.3)	0.43 (0.02)
Total polyphenols, % TS	0.05 (0.02)	n.d.	21.77 (0.2)	n.d.
Reducing sugars, g L^{-1}	n.d.	50.35 (0.2)	trace	n.d.

n.d.: not detected.

3.3. Dark Fermentation Kinetics and Parameters

Figure 2 shows the cumulative H_2 production obtained from the differently prepared giant reed hydrolysates, in comparison with not treated and not hydrolyzed control (C). The dark fermentation proceeded in two cycles of 7 days; at the end of the first cycle, the pH of each reactor was brought back to its initial value utilizing an appropriate volume of NaOH solution or black liquor depending on the treatment (pH resetting) restarting DF.

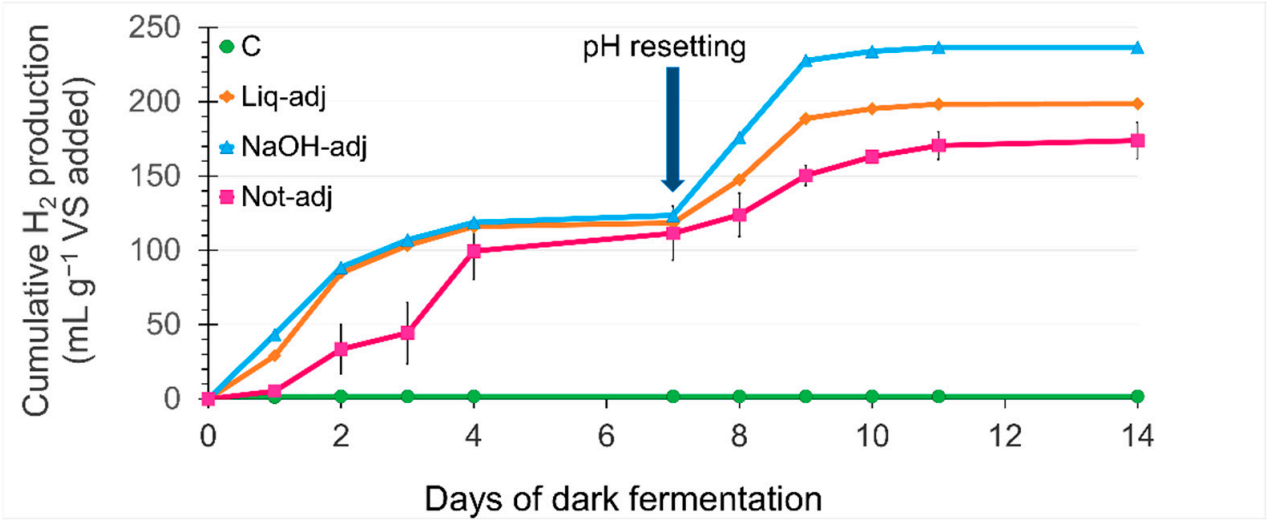


Figure 2. Measurements of the cumulative H_2 production per gram of volatile solids during dark fermentation of differently prepared giant reed hydrolysates: Not-adj (not adjusted initial pH 5.3, pink squares); NaOH-adj (NaOH-adjusted initial pH 8.7, blue triangles); Liq-adj (black liquor-adjusted initial pH 8.7, orange diamonds); C (not treated and not hydrolyzed giant reed control, NaOH-adjusted initial pH 8.7, green circles). The downward vertical arrow indicates the time of pH resetting of all reactors to their initial value with NaOH or black liquor depending on the treatment. Vertical bars represent the standard deviation (n = 3).

It should be noted that Liq-adj reactors contained the same amount of sugar as the other treatments but a higher amount of VS (Table 2), due to the VS content of the black liquor derived from pre-treated biomass solubilization (12 mL in total added for the pH adjustments).

Table 2. Two-cycle dark fermentation parameters of the hydrogen production from giant reed hydrolysates in comparison with not treated and not hydrolyzed control.

	Dark Fermentation Parameter [2]									
Treatment [1]	**Initial pH**	**Total VS Added**	**Reducing Sugars**	**Cumulated H_2**	**Max Rate$_1$**	**Max Rate$_2$**	**Day 7 pH**	**H_2 MAX$_1$ conc.**	**H_2 MAX$_2$ conc.**	**Final pH**
		g/Reactor	**g/Reactor**	**mL H_2 STP g^{-1} VS**	**mL H_2 STP d^{-1} g^{-1} VS**	**mL H_2 STP d^{-1} g^{-1} VS**		**%**	**%**	
Liq-Adj	8.7	1.9	1.5	199 b [3]	56 a	43 b	4.6 a	58 a	48 a	4.5 a
NaOH-Adj	8.7	1.5	1.5	237 a	45 b	52 a	4.4 a	55 a	50 a	4.6 a
Not-Adj	5.3	1.5	1.5	174 c	55 a	27 c	4.5 a	53 a	49 a	4.7 a
C	8.7	1.5	trace	2 d	1 c	0 d	7.2 b	10 b	0 b	7.2 b

[1] *Liq-adj*: hydrolysate with black liquor-adjusted initial pH (8.7); *NaOH-adj*: hydrolysate with NaOH-adjusted initial pH (8.7); *Not-adj*: hydrolysate with not adjusted initial pH (5.3); *C*: not treated and not hydrolyzed control, NaOH adjusted initial pH (8.7). [2] *Total VS added:* volatile solids in first + second cycle; *Cumulated H_2*: cumulated H_2 production per gram of volatile solids at the end of the dark fermentation; *max Rate$_1$*, *max Rate$_2$*: maximum measured daily rate of H_2 production per gram of volatile solids during the first or second cycle of dark fermentation; *pH before resetting*: at the end of the first cycle of dark fermentation; *%H_2 MAX$_1$*, *%H_2 MAX$_2$*: maximum content of H_2 in the biogas during the first or second cycle of dark fermentation. [3] Means sharing common letters are not significantly different at $p = 0.05$ according to Tukey's Honestly Significant Difference test.

In general, H_2 production increased steadily without any lag for all the hydrolysates, while it was almost negligible for the control, where, instead, methanogenic fermentation occurred (data not shown).

At the end of the first cycle, the highest cumulative H_2 production levels were obtained in NaOH-adj or Liq-adj reactors. The H_2 production curves almost overlapped and reached a plateau at 4 days of incubation.

After pH resetting, the second cycle of DF started with a similar profile in all treatments, although in this cycle higher values for NaOH-adj reactors were recorded compared to Liq-adj reactors. The production curve of Not-adj reactors always remained below the other two treatments with pH adjustment. The production from Not-adj reactors was also more variable compared to the other treatments.

Noteworthy is the fact that a substantial fraction of the total cumulative H_2 production was obtained during the second cycle: 48%, 40%, and 36% for NaOH-adj, Liq-adj and Not-adj reactors, respectively.

After the end of the second DF cycle, a further pH resetting did not produce any recovery of the hydrogen production (not shown).

The alkaline pre-treatment followed by enzymatic hydrolysis significantly increased the cumulated H_2 production per gram of VS in all conditions, compared to C (not pre-treated nor hydrolyzed) (Figure 2, Table 2). The pH adjustment at 8.7 of the hydrolysates determined significant increases of 14–36% in the cumulated H_2 production per gram of VS, compared to not-adjusted reactors (pH 5.3). The highest cumulated H_2 value per gram of VS was obtained in NaOH-adj reactors (pH 8.7) with an absolute value of 237 mL H_2 g^{-1} VS while in Liq-adj reactors a statistically significant lower value (−16%) was observed (199 mL H_2 g^{-1} VS).

It should be noted that, while the absolute amount of reducing sugars was the same in all hydrolysate-containing reactors (1.5 g per reactor), Liq-adj reactors also contained 0.4 g VS from the liquor itself (that was added in non-negligible volumes to adjust the reactor pH) up to a total of 1.9 g VS (Table 2). These liquor-derived VS (Table 1) represent 21% of the total VS added in these reactors. Thus, Liq-adj reactors contained more VS, compared to NaOH-adj or Not-adj reactors, despite having the same amount of sugar as the other hydrolysate-containing reactors.

The amount of reducing sugars was negligible in not-treated and not-hydrolyzed giant reed control and these reactors displayed very low cumulated H_2 production per gram VS (Table 2).

The maximum daily rate of H_2 production (max Rate) was 56, 52 and 55 mL H_2 STP d^{-1} g^{-1} VS, respectively, for Liq-adj, NaOH-adj, and Not-adj reactors, with a significant increase, compared to C: notably, the highest max Rate was observed for Liq-adj reactors, and it was significantly higher compared to that of NaOH-adj reactors.

The pH at the end of the first cycle of dark fermentation (day 7, before pH resetting) converged to acidic values (4.4–4.6) in hydrolysate-containing reactors irrespective of their initial pH or kind of adjustment. On the contrary, it was neutral in C reactors, where methanogenesis occurred (data not shown). The maximum content of H_2 in the biogas ranged between 55–58% during the first cycle, and between 48–50% during the second cycle of DF in hydrolysate-containing reactors without significant difference, due to their initial pH or kind of pH adjustment. A significantly lower H_2 content (10%) was observed for the C reactors during the first cycle.

At the end of the second cycle of the DF, the pH converged again to acidic values (4.5–4.7) in hydrolysate-containing reactors without significant differences among treatments.

3.4. Time-Course of the Daily Rate of H_2 Accumulation in Dark Fermentation

Figure 3 shows the daily rate of H_2 accumulation (*R*) in DF per gram of VS (panel a) and per mole of reducing sugars in hexose equivalents (panel b) present in the reactor.

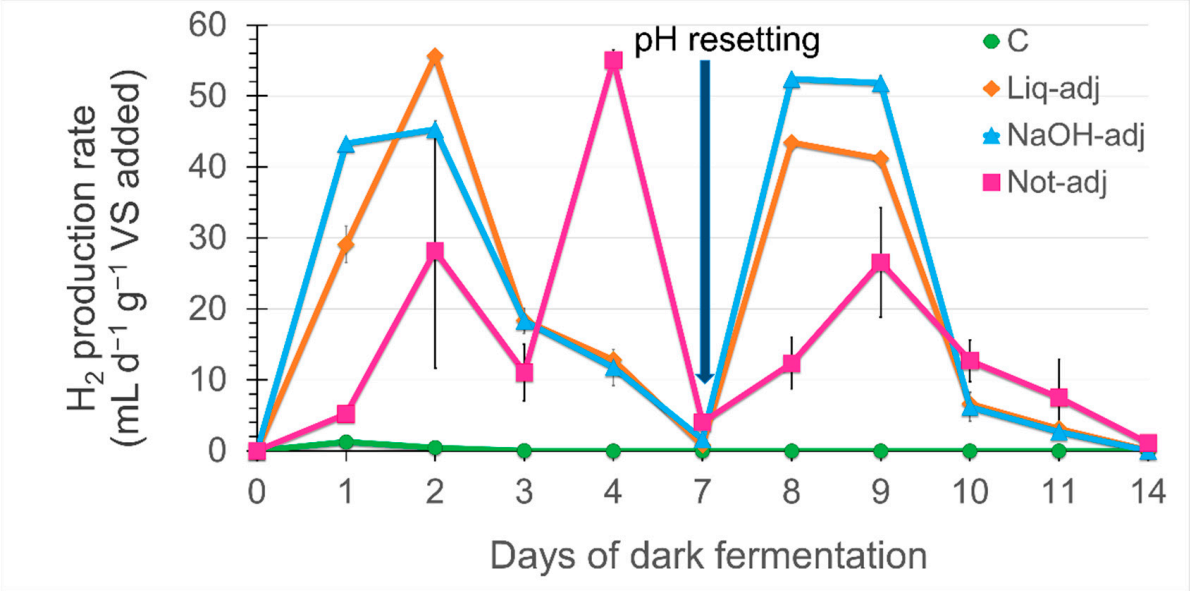
In panel (a), the time-course of the values of *R* for the control was steady and close to zero, *R* being very little from day two onward. On the contrary, two major DF cycles were visible for the hydrolysate-containing reactors with a major peak in each cycle. Not-adj reactors displayed a significantly lower *R* profile in the second cycle compared to the other treatments.

In both DF cycles, the H_2 accumulation was very fast since *R* was significantly different from zero on both days 1 and 8 of DF i.e., H_2 production started within one day in both DF cycles.

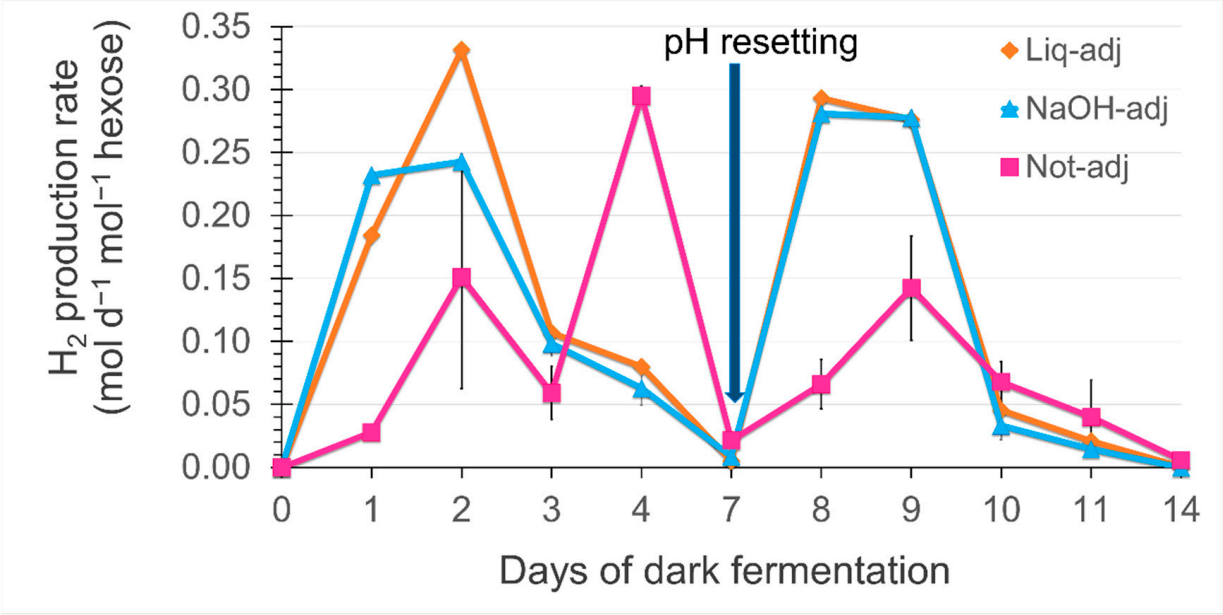
Notably, the maximum *R*-value for each treatment/cycle was reached at different times depending on the treatment and the DF cycle: (i) Not-adj reactors required four and two days to reach the maximum *R*-value after the start of the first and second cycles, respectively; (ii) NaOH-adj and Liq-adj reactors reached the maximum *R*-value faster than Not-adj reactors i.e., at two days and one day from the start of the first and second cycles, respectively. In general, the maxima were reached faster in the second cycle than in the first one. The maximum *R*-values per gram VS were 45, 56, 55 and 52, 43, 27 for NaOH-adj, Liq-adj, and Not-adj reactors, in the first and second cycles, respectively. In particular, during the first DF cycle, NaOH-adj reactors showed a significantly lowest value (45) if compared with the other treatments; on the contrary, in the second cycle, Not-adj reactors showed the lowest value (27).

Notably, during the first cycle, the Liq-adj reactors reached a higher *R*-value than NaOH-adj reactors even though there was a one-day shift (*R*-value 33% lower on day 1 and 23% higher on day 2). During the second cycle, Liq-adj and NaOH-adj profiles were similar in shape but the maximum *R*-value per gram VS was significantly lower in Liq-adj than in NaOH-adj reactors (−21%). However, it should be noted that, although having the same sugar load as in NaOH-adj, Liq-adj reactors contained a significantly higher amount of VS (liquor carry over) depressing the *R* rate per gram VS, and particularly doing so after resetting the pH.

It would seem that the initial pH, and the type of pH correction, significantly affect the *max Rate* and *cumulated H_2* production per g of VS. To isolate the effects of the black liquor addition on the H_2 rate production, the *R* rate per mole of hexose was also reported (Figure 3b). In this case, during the second cycle, NaOH-adj and Liq-adj reactors showed an almost overlapping profile.



(**a**)



(**b**)

Figure 3. Daily rate of H_2 accumulation (*R*) in dark fermentation of differently prepared giant reed hydrolysates, i.e., Not-adj (not adjusted initial pH 5.3, pink squares); NaOH-adj (NaOH-adjusted initial pH 8.7, blue triangles); Liq-adj (black liquor-adjusted initial pH 8.7, orange diamonds); C represents the not treated and not hydrolyzed giant reed (control, NaOH adjusted pH 8.7, green circles): (**a**) Daily rate of H_2 accumulation per g VS; (**b**) Daily rate of H_2 accumulation per mole of hexose. The downward vertical arrow indicates the time of pH resetting of all reactors to their initial value with NaOH or black liquor depending on the treatment. Vertical bars represent the standard deviation (n = 3).

The maximum *R*-values per mole of hexose were 0.24, 0.33, 0.29 and 0.28, 0.30, 0.14 for NaOH-adj, Liq-adj, and Not-adj reactors, in the first and second cycles, respectively.

In the first DF cycle, Liq-adj reactors showed the statistically highest *R*-value (0.33), whereas in the second cycle no significant difference was observed with respect to NaOH-

adj reactors. Not-adj reactors showed the lowest value in the second cycle and were halved with respect to the other reactors.

3.5. Hydrogen Yield Per Mole of Hexose in Dark Fermentation

To evaluate the influence of black liquor on H_2 production, the cumulated H_2 production per mole of hexose from giant reed hydrolysates for the different treatments was reported (Figure 4).

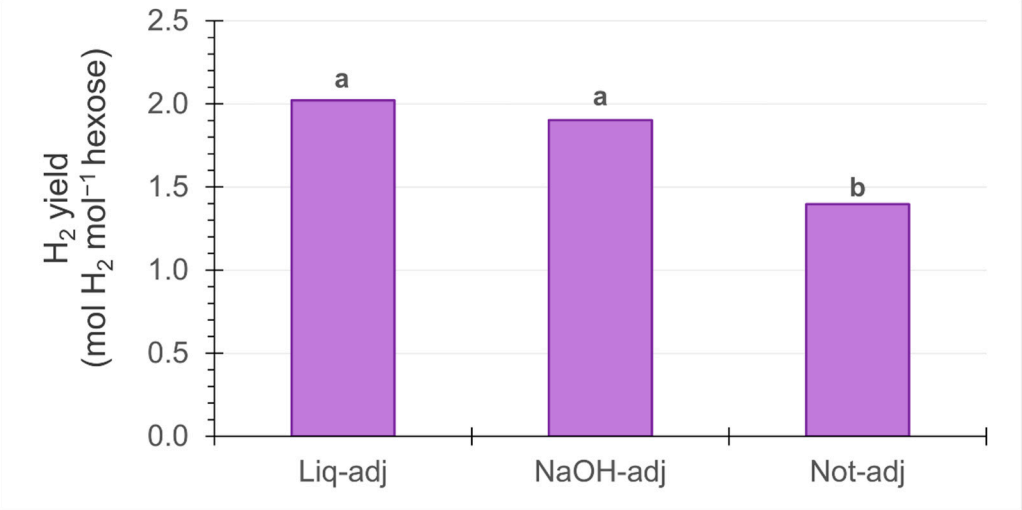


Figure 4. Cumulated H_2 production per mole of hexose from giant reed hydrolysates with different initial pH.; Liq-adj: black liquor-adjusted initial pH 8.7; NaOH-adj: NaOH-adjusted initial pH 8.7; Not-adj: not adjusted initial pH 5.3; Means with different letters differ significantly at $p < 0.05$ according to Tukey's Honestly Significant Difference test.

The highest absolute yield value was observed for Liq-adj, reactors (2.0 mol H_2/mol hexose). However, the value observed for NaOH-adj reactors (1.9 mol H_2/mol hexose) was not statistically different. The statistically significant lowest yield (1.4 mol H_2/mol hexose) was obtained in Not-adj reactors. The initial alkaline pH (8.7) caused an increase of 39% in the hydrogen yield per mole of hexose compared to pH 5.3, without any detrimental effect due to the use of residual black liquor as an alkali source.

4. Discussion

4.1. Biomass Fractionation Yields Following the Pre-Treatment

As previously observed giant reed is a rather recalcitrant substrate and requires a pre-treatment to partially remove lignin and enhance enzymatic hydrolysis to obtain fermentable sugars [47] suitable for DF. However, after the alkali pre-treatment a substantial amount of biomass was liquefied (black liquor) as expected [22,48]. Black liquor re-utilization in the process to adjust pH could improve the overall yield and the sustainability of the process, reducing waste.

The sugar yield obtained in this study from the hydrolysis of the solid pre-treated biomass (410 mg/g) was consistent with previous reports, ranging between 380–489 mg/g [21,22,49].

4.2. Composition of the Materials Utilized in Dark Fermentation

Dark fermentation is more sensitive than anaerobic digestion to furanic and phenolic compounds [29]. Thus, the high content of polyphenols found in the black liquor (Table 1) could hamper H_2 production. Phenolic compounds can have a considerable inhibitory effect on fermentation because they are generally toxic to microorganisms, even at low concentrations. However, at very low concentrations, some phenolic compounds may

improve fermentation kinetics [50,51] since *Clostridium* spp. (typically H_2 producers) provide for their degradation [29].

Black liquor also contains significant amounts of acetic acid (Table 1), which originated from the hemicellulose degradation due to the pre-treatment [29]. Giant reed has, in fact, a relatively high content of acetylated hemicellulose (3.7–4.8% acetate eq. on a DW basis) [52–54] that can be easily de-acetylated in alkaline conditions releasing acetic acid. Moreover, this acid can also be generated from the cleavage of xylose [55]. Several authors found acetic acid after the chemical pre-treatment of giant reed with concentrations ranging between 3.2–11 g/L, depending on solid content and treatment severity [54,56,57]. Notably, acetic acid can have an inhibitory or stimulatory effect on DF, depending on pH, concentration, and microorganisms [58,59]

4.3. Dark Fermentation Kinetics and Parameters

The kinetics of H_2 production from hydrolysates reached a plateau within 96 h (first DF cycle) (Figure 2). Relatively faster kinetics was observed by other authors for giant reed hydrolysates, reaching a plateau within 72 h [30–32]. However, those authors worked with a significantly lower initial sugar concentration (5–20 g/L).

One of the most relevant parameters that influence H_2 production in DF is pH [26] and the optimal pH for H_2 production from carbohydrates is in the range of 5.2–7.0, depending on raw materials, microbial populations, and operational conditions [51]. When biomass hydrolysates were used, the optimal initial pH for H_2 production was found to be dependent on inoculum source: 6.5–7.0 with enrichment cultures from cow dung compost, 5.5 with *Clostridium butyricum*, and 8.0 with dairy manure bacteria [51].

The dark fermentation of hydrolysates of steam-exploded giant reed carried out at initial pH 5.9 with a mixed culture from an anaerobic digestion plant, allowed cumulative H_2 production to double, thanks to a progressive adaptation of the consortium [32]. Notably, the present study's comparison between acidic versus alkaline initial pH highlighted the superior performance of the latter, in the presence of higher sugar concentration than that used in ref. [32] (50 g/L versus 5 g/L of sugars, respectively). The usefulness of alkaline initial pH was already highlighted in a previous study on a substrate with a similar sugar concentration (51 g/L lactose), comparing initial pH in the range 4–10 [27]. Those authors found that H_2 production started only when pH fell below 6. However, it was much higher in the reactors with initial alkaline pH. Alkaline conditions allowed an optimal selection and activity of the microbial consortium, favouring H2-producing *Clostridia* and *Enterobacteria* [9] which are among the most efficient H_2 producers [60].

Notably, the pH resetting at the end of the first DF cycle facilitated a restart of H_2 production in the second DF cycle, significantly increasing the overall yield (Figure 2) as previously reported for scotta permeate [39]. In the case of alkaline pH, the H_2 production rate was also restored. The re-use of the discarded alkaline black liquor as pH corrector did not exert any inhibition phenomena on DF, notwithstanding its considerable acetate and polyphenol content, which are generally considered to be potential inhibitors. Interestingly, no methanogenesis was recorded, notwithstanding acetic acid's ability to induce acetoclastic methanogenesis. Methanogens are considered the main H_2-consuming microorganisms [29]. In particular, *Methanosarcina* spp. can produce CH_4 via acetoclastic and hydrogenotrophic pathways [61].

Some polyphenolic compounds may improve fermentation kinetics at very low concentrations [51]. In the present study, a delay in the H_2 accumulation rate was observed on day 1 of DF. However, this initial inhibition was resolved within 48 h. In a previous study, the start of anaerobic digestion of the giant reed was also affected by black liquor addition [33].

With phenol concentrations up to 1 g/L, no negative effects on DF of wastewater by *C. butyricum* were reported, whereas at 1.5 g/L a complete DF inhibition was observed [29]. Based on Table 1, it can be calculated that the concentration of polyphenols in the Liq-adj reactors at the start of the first cycle was 1.75 g/L (70 mg in 40 mL reaction volume),

whereas polyphenolic load at the start of the second cycle was 2 g/L (98 mg in 47 mL reaction volume). However, no inhibitory effects were detected in the DF process, except for a slight delay at the start of the first DF cycle. In the second DF cycle, on the other hand, no delay was observed. It is known that an adaptation of the microbial community to phenolic compounds can alleviate inhibitory effects [29], which could explain the lack of inhibition in the second DF cycle, whereas the initial pH 8.7 could play a key role in alleviating the initial inhibition since it was previously reported that it created favourable environmental conditions for *Clostridia* [9], which are the main polyphenolic compound degraders [29].

Finally, liquor could also contain other fermentable compounds, like, for instance, cellulose and oligomers from hemicellulose degradation, which could have contributed to stimulating the growth of *Clostridia* [62].

The initial alkaline pH obtained with liquor or NaOH was clearly beneficial in terms of final cumulated H_2 calculated per gram of VS, if compared to DF performed with acidic initial pH (Table 2). The positive effect of alkaline pH on H_2 production has been previously reported [27,63]. However, the significant VS carry-over of low (or no) hydrogen-yielding compounds in the liquor in Liq-adj reactors may explain the relatively lower performance per VS gram of these latter compared to NaOH-adj reactors. The lack of detrimental effect on the maximum rate of H_2 production in Liq-adj reactors could be explained by the fast recovery from initial inhibition clearly visible on day 1 (Figure 3). The observed cumulated H_2 values per gram of VS (up to 237 mL H_2 STP g^{-1} VS) were higher than the values previously reported by other authors performing DF experiments with lignocellulosic hydrolysates. Some authors reviewed H_2 yields from different lignocellulosic hydrolysates corresponding to 20–140 mL/g at high VS concentration (VS range 20–40 g/L) [64]. More recently H_2 yield corresponding to over 200 mL/g VS was achieved with hydrolysed sugar beet pulp [65]. The high H_2 yield observed in the present study can be explained by the very high sugar content per gram VS of the hydrolysate.

The H_2 concentrations in the biogas observed in this work (48–58%) were lower than those reported by other authors utilizing giant reed hydrolysates (69–75%) [30–32]. However, these authors worked with significantly lower initial sugar concentrations (5–20 g/L) and with thermally pre-treated inocula and/or were enriched in H_2-producing bacteria. Both these latter conditions favoured a high percentage of H_2 in the biogas. Pre-treatment and enrichment in H_2-producing bacteria eliminated most of the H_2-consuming microorganisms, but also those that consume sugar by producing CO_2 but not H_2 [26]. On the other hand, a low sugar concentration may lead to slow acidification. The acidification of the medium promotes a metabolic pathway shift [66] from acetic acid (Equation (1)) to butyric acid (Equation (2)) pathway [67]:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

The butyric acid metabolic pathway is activated to counteract pH drop and, therefore, it is favoured at pH below 6 [68]; whereas the metabolic pathway of Equation (1) allows for biogas richer in H_2. If the metabolic shift is delayed or avoided, it is obvious that the % H_2 in the biogas remains high. Indeed, under similar conditions of initial sugar concentration to the present study, H_2 production via acetic and butyric acid pathways coexisted from the beginning of the exponential H_2 production phase [27]. This co-production at initial alkaline pH was also reported more recently on DF of brewery spent grains at a similar organic load (47 g/L) [67] with 58% of maximum H_2 concentration in the biogas. At 28 g/L sugar concentration, with Miscanthus hydrolysate fermented by *Thermotoga neapolitana*, H_2 and CO_2 production of 82 and 62 mmol/L, respectively, were reported [69], which corresponded to a 57% H_2 concentration in the biogas, thus, a value consistent with our experiment performed at high sugar concentration.

4.4. Daily Rate of H_2 Accumulation in Dark Fermentation

Concerning the daily rate of H_2 accumulation (*R*), the comparison of panel (a) with panel (b) of Figure 3, shows that the initial pH adjustment (8.7) was always beneficial compared to not adjusting the initial pH (5.3), giving rise to a faster rise to maximum in both cycles and higher maxima in the second. Notably, the pH correction with liquor did not negatively affect the *R*-values per mole of hexose, if compared to pH correction with NaOH. In fact, in both cycles, the highest *R*-values were obtained in Liq-adj reactors (0.34 and 0.30 moles of H_2 per mole of hexose per day in the first and second cycle, respectively).

Based on data reported by other authors [32] concerning DF of steam-exploded giant reed hydrolysate, a rate *R* of 1.6 moles of H_2 per mole of hexose per day could be calculated, which was higher than what we found in this study. It must be taken into account that in the previous work, that value was recorded after an inoculum enrichment process. Furthermore, the hydrolysate was supplemented with nutrient solution and minimal medium. Besides, it must be considered that the inoculum-to-substrate ratio (ISR) was higher than that used in the current study. These conditions enhanced the maximum R value. In the present study, conversely, the hydrolysate was used without additives and was inoculated with an ISR = 0.13 because, using non-selected mixed inoculum, the aim was to keep the methanogens content low, considering the origin of the inoculum (pig slurry).

A maximum volumetric H_2 productivity (Q) of 5.4–6.2 mmol/L/h in reactors containing 28 g/L of sugars from alkaline-pretreated Miscanthus hydrolysates during DF with *Caldicellulosiruptor saccharolyticus* and *T. neapolitana*, was reported [69] corresponding to 0.83–0.96 moles of H_2 per mole of hexose per day, respectively. Furthermore, Q values were almost halved and H_2 yields strongly decreased by utilizing 28 g/L sugars instead of 14 g/L [69]. However, it must be considered that in that study the DF was carried out in thermophilic conditions, which favour the kinetics of H_2 accumulation [70]. Furthermore, these values were obtained with pure cultures and in very particular conditions, such as controlled pH and the headspace of the reactors constantly flushed with N_2 to remove H_2 by lowering the partial pressure of H_2.

Thus, the comparatively lower rates observed in our study may be explained by the very high initial sugar concentration used [64], as well as the non-adequate ISR possibly slowing down the process. However, all the conditions reported in the various studies were very peculiar. In more similar conditions, as in [27], the maximum daily rate was consistent with the *R max* reported in this study.

4.5. Hydrogen Yield Per Mole of Hexose in Dark Fermentation

The addition of alkaline black liquor to adjust pH did not interfere with the cumulated H_2 production per mole of hexose, indicating a lack of inhibitory effect on DF. Thus, the black liquor can be advantageously re-used instead of NaOH to adjust pH and obtain a significant H_2 yield increase.

The H_2 yields per mole of hexose from different lignocellulosic hydrolysates were generally below 1.8 and tended to be lower for high sugar concentrations [64]. In the present study, H_2 yield reached 2 mol/mol hexose, which was higher than those reported by Toscano et al. [30] (0.17–0.3 mol H_2/mol hexose) or by Ausiello et al. [31] (1.14 mol H_2/mol hexose), using hydrolysates from steam-exploded giant reed (10 and 20 g/L initial sugars, respectively) obtained with thermally pre-treated or thermally pre-treated and enriched inoculum.

The yields reported in this study are consistent with those previously obtained using giant reed hydrolysate (2.59 mol H_2/mol hexose), after three consecutive steps of inoculum adaptation and low sugar concentration (5 g/L) [32] on supplemented hydrolysate. In the current study, such high yields were obtained notwithstanding the high initial sugar concentration, only adjusting the initial pH to an alkaline value (8.7), without any inoculum adaptation. These findings are of the same order of magnitude as those obtained previously under similar conditions on lactose (51 g/L) at initial pH 8 and per moles of hexose-equivalent consumed [27].

5. Conclusions

The opportunity to combine thermo-alkaline pre-treatment with saccharification and to adjust initial pH to alkaline values to improve H_2 production from giant reed, was highlighted. In particular, when the initial pH was adjusted with alkaline black liquor residues after biomass pre-treatment, no inhibitory effect was observed. Thus, the alkaline black liquor can be recycled in the DF process, instead of being discarded. The integration of DF with other technologies, such as anaerobic digestion, could allow valorizing DF effluent, rich in organic acids, closing the loop to produce more energy. In this case, the high acetate content in the black liquor would be added to the volatile fatty acids produced in DF and could be conveniently exploited in anaerobic digestion.

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Data Availability Statement: Data is contained within the article. The raw data utilized in this study are available on request from the corresponding author.

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