Journal of Environmental Engineering and Landscape Management
ISSN 1648–6897 / eISSN 1822-4199
2019 Volume 27 Issue 2: 101–113
https://doi.org/10.3846/jeelm.2019.9806

BIOHYDROGEN PRODUCTION FROM WASTES OF PLANT AND ANIMAL ORIGIN VIA DARK FERMENTATION

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Received 31 January 2018; accepted 31 January 2019

Abstract. This study investigated the batch experiments on biohydrogen production from wastes of plant and animal origin. Several substrates including sugar beet pulp (SBP), sugar beet leaves (SBL), sugar beet stillage (SBS), rye stillage (RS), maize silage (MS), fruit and vegetable waste (FVW), kitchen waste (KW) and slaughterhouse waste (SHW) including intestinal wastes, meat tissue, post flotation sludge were tested for their suitability for hydrogen production. Generally, the substrates of plant origin were found to be appropriate for dark fermentation, and the highest hydrogen yield of 280 dm^3 H_2/kg VS was obtained from fruit and vegetable waste. Contrary to these findings, slaughterhouse waste as well as kitchen waste turned out to be unsuitable for hydrogen production although their methane potential was high. It was also concluded that the combined thermal pretreatment with substrate acidification was needed to achieve high hydrogen yields from wastes.

Keywords: biohydrogen, plant biomass, food waste, dark fermentation, anaerobic digestion.

Introduction

Consumption of fossil fuels such as brown coal or crude oil is considered to be the main reason for air pollution and unfavorable climate changes as a result of increasing concentrations of carbon dioxide, nitrogen oxide and ashes in the atmosphere (Ozkan, Erguder, & Demirer, 2011). Moreover, coal and petroleum are finite resources and this forces the search for new, alternative energy sources. Among various alternatives, hydrogen with its high-energy yield (122 kJ/g) plays an important role. Hydrogen is known as an environmentally friendly energy source since its combustion does not emit any carbon-based gases, which would induce the climate changes by greenhouse effect intensification (Argun & Dao, 2017; Chu et al., 2008). Hydrogen can be generated in various strategies; in thermochemical (thermo-chemical gasification), electrochemical (water electrolysis) or biological processes. Conventional methods of hydrogen production are energy consuming, require high temperatures and need using fossil fuels (natural gas, coal) to generate power (Argun & Dao, 2017). Contrary to them, biological hydrogen production attracts more and more attention, is less energy intensive (Ozkan et al., 2011) and can utilize wastes. Bioydrogen can be generated via several different metabolic pathways, including direct water biophotolysis by green algae, indirect water bio-photolysis by cyanobacteria, photo-fermentation by photosynthetic purple non-sulfur bacteria or dark fermentation (DF) by heterotrophic anaerobic bacteria (Escamilla-Alvarado, Rios-Leal, Ponce-Noyola, & Poggi-Varaldo, 2012; Urbaniec & Bakker, 2015). The latter process is of particular interest due to several advantages including production of hydrogen with no light needed, the use of various kinds of substrates and that it is one of the options for recycle the waste biomass. In dark fermentation, hydrogen is produced by a variety of anaerobic and facultative microorganism among which *Clostridiaceae* and *Enterobacteriaceae* families play a crucial role (Fang, Zhang, & Liu, 2002). In particular, acidogenic bacteria of *Clostridium* sp., *Enterobacter* sp., *Citrobacter* sp., *Bacilluss* p. and *Alcaligenes* sp. could ferment mono- and disaccharides to hydrogen, carbon dioxide and organic acids (acetic acid, lactic acid, butyric acid, propionic acid) (Urbaniec & Bakker, 2015; Ghimire et al., 2015). *Clostridium* and *Bacillus* genera are characterized by the formation of spores in response to unfavorable environmental conditions, which is of importance regarding practical applications of these bacteria for hydrogen generation (Argun & Dao, 2017). Considering feedstock for hydrogen production, they have to meet specific criteria.

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A substrate should be rich mainly in carbohydrates, be received from sustainable feedstocks and be easily accessible. Moreover, it should require only a minimum pretreatment, which must be performed at a low cost. Hydrogen has been produced from various raw materials including, barley straw, corn stalk, corn stover, corn cob, apple pomace (Urbaniec & Bakker, 2015), fruit peel waste, wheat straw (Pawar, Nkemka, Zeidan, Murto, & van Niel, 2013), cassava stillage (Luo, Xie, Zou, Zhou, & Wang, 2010), sugar beet molasses (Urbaniec & Grabarczyk, 2014), peach pulp (Argun & Dao, 2017), sugarcane bagasse (Pattra, Sangyoka, Boonmee, & Reungsang, 2008) and sugar cane stillage (Santos, Rosa, Sakamoto, Varesche, & Silva, 2014). Furthermore, kitchen waste (Li & Jin, 2015) and industrial waste like distillery effluents (Wicher, Seifert, Zagrodnik, Pietrzyk, & Łaniecki, 2013), or starch-containing wastewater (Urbaniec & Bakker, 2015) have also been applied.

The objective of this study was to investigate the dark fermentative production of hydrogen from various substrates of agri-food origin using different pretreatment strategies. The following raw materials were used in the research: sugar beet pulp, sugar beet leaves, sugar beet and rye stillage, maize silage, kitchen waste, and slaughterhouse waste. These materials, especially the ones of plant origin, are generated in large amounts whereas their utilization and disposal still creates great technological and environmental problems. In particular, the problem of sugar beet pulp, sugar beet leaves and stillage should be considered. Poland produces as much as 3.4 million tons of beet pulp in sugar factories the utilization of which in a traditional way can consume up to 40% of the total energy used in the plant for heating operations. Moreover, plant materials like beet pulp, leaves or maize silage tend to uncontrolled decomposition, especially when they are improperly prepared and stored. They are often not suitable for feeding animals due to the presence of harmful molds easily growing under low pH conditions. In light of this, production of hydrogen via dark fermentation could be an alternative to traditional utilization methods of agri-food materials also considering hydrogen as a clean form of energy with high calorific value. To the best of the authors' knowledge, this study is the first to investigate sugar beet pulp after steam and hydrolysis pretreatment, maize silage, rye stillage and sugar beet stillage as the feedstocks for hydrogen production.

1. Material and methods

1.1. Inoculum characteristics

Anaerobic sludge collected from the anaerobic mesophilic digester at the Municipal Wastewater Treatment Plant in Lodz, Poland was served as inoculum for the experiments. The inoculum had total and volatile solids concentrations of 25.34 g TS/kg and 16.00 g VS/kg, respectively. The full characteristic of inoculum used in this study is shown in Table 1.

Table 1. Characteristics of substrates

Substrate	Indicator								
	Total solids (TS)	Volatile solids (VS)	Chemical oxygen demand (COD)	Carbon	Nitrogen	Phosphorus	Hydrogen	Sulfur	C/N
Unit	g/kg	g/kg	gO_2/kg	% TS	% TS	% TS	% TS	% TS	–
Inoculum	25.34±0.81	16.00±0.20	2.90±0.06	59.7±1.21	3.20±0.23	1.94±0.07	5.90±0.12	0.91±0.25	18.66±1.24
Rye stillage	145.18±0.10	122.83±0.26	133.55±0.21	65.1±0.89	1.86±0.08	0.11±0.02	5.1±0.47	0.29±0.01	35.00±3.08
Fruit and vegetable wastes	148.63±5.66	127.06±4.78	198.48±0.06	59.7±1.87	6.7±0.15	0.29±0.1	6.3±0.25	0.04±0.00	8.91±2.14
Sugar beet leaves	160.62±5.49	138.74±4.54	128.16±0.42	61.3±1.54	0.29±0.01	0.02±0.00	7.9±0.21	0.02±0.00	211.38±9.53
Maize silage	409.84±1.48	396.73±4.78	56.33±0.01	63.1±0.24	1.86±0.31	0.11±0.01	5.1±0.22	0.02±0.00	33.92±2.37
Sugar beet stillage	144.96±0.27	122.60±0.45	6.44±0.01	62.3±0.57	5.8±0.09	4.92±0.14	6.1±0.14	0.36±0.01	10.74±2.54
Sugar beet pulp	202.82±3.51	193.36±3.90	252.24±0.06	69.8±1.02	0.19±0.02	0.02±0.00	7.9±0.87	0.02±0.00	364.37±10.81
Intestinal wastes	258.61±1.32	229.82±1.36	346.53±0.01	61.4±1.38	9.69±1.65	0.12±0.01	4.9±0.35	0.02±0.00	6.34±1.72
Meat tissue	306.67±1.30	288.56±1.74	204.78±0.06	62.1±0.56	10.63±1.87	0.09±0.00	4.8± 0.76	0.01±0.00	5.84±1.13
Post flotation sludge	261.90±1.78	210.17±1.94	70.40±0.01	59.2±1.14	3.45±1.08	0.06±0.00	5.8±0.39	0.07±0.00	17.16±2.41
Kitchen wastes	247.34±5.50	237.39±8.66	147.85±0.1	58.4±1.78	5.2±0.58	0.29±0.01	6.1±0.24	0.32±0.02	14.60±1.37
± Standard deviation									

1.2. Substrate characteristics

The experiments were performed using the following substrates: sugar beet (*Beta vulgaris)* pulp (fresh, hydrolyzed, autoclaved), sugar beet leaves, maize (*Zea mays*) silage, fruit and vegetable wastes, kitchen wastes, sugar beet stillage, rye stillage, slaughterhouse waste (meat tissue, intestinal wastes and post flotation sludge). Due to the seasonality of most substrates, the raw materials were stored at −18 °C before use and the pretreatment was performed after thawing. Prior the fermentation process, the substrate were ground in a grinder (FIMAR TS-32D400V) to obtain the particles of 0.3–1.3 cm in diameter. Sugar beet pulp (SBP) was delivered from Dobrzelin Sugar Factory, Poland. Sugar beet pulp hydrolysates (SBPH) were obtained by enzymatic pretreatment of SBP using a mixture of two commercial multienzyme preparations: Viscozyme and Ultraflo Max (Novozymes, Denmark). The saccharification of sugar beet pulp was conducted at the temperature of 50 °C and pH of 5.0 with mixing for 24 h. Then, the hydrolysate was filtered to obtain the liquid and solid fractions. Fresh sugar beet leaves (SBL) from a farm in Dobrzelin were ground and then steamed in autoclave at 121 °C for 40 minutes. Maize silage was collected from a local farm in Bełchatów, Poland. Fruit and vegetable waste (FVW) as well as kitchen waste (KW) were daily collected in individual households. FVW were composed of fruit and vegetable residues exclusively, whereas kitchen waste also contained other wastes, including meat residues and non-biodegradable contaminants (large bones, egg shells). Kitchen waste as well as fruit and vegetable waste were collected for a few weeks, then ground and thoroughly mixed to prepare a relatively homogenous mass for all the experimental processes. Slaughterhouse waste: meat tissue, intestinal wastes and post flotation sludge, were collected at PINI Polonia Company in Kutno, Poland. *Sugar beet stillage as well as rye stillage were* byproducts of bioethanol production from an installation operated in Institute of Fermentation Technology and Microbiology, Lodz University of Technology. The characteristics of substrates used for the investigation are shown in Table 1.

1.3. Experimental setup and operational conditions

The experiments were performed using batch fermentation systems (Figure 1) consisting of 1 dm^3 glass bottles with a working volume of 0.7 dm3. Each bottle was connected to a 1 dm^3 gas collecting tank to measure daily biogas production by a water displacement method as described elsewhere. The reactors were filled in with 0.5 kg of inoculum and then the substrates were added to achieve the inoculum to substrate (X_o/S_o) ratio of 2:1 (g VSinoculum/g VSsubstrate) based on volatile solids concentration without any nutrient supplementation (Angelidaki et al., 2009; Tsapekos, Kougiasp, Treu, Campanaro, & Angelidaki, 2017). Before shutting down, the headspace of each bottle was rinsed with nitrogen gas for 3 minutes to ensure anaerobic conditions. The bottles were then incubated at 35 °C in a thermostat, which maintained constant mesophilic temperature, and they were manually shaken once a day. Each experiment was continued to the point at which only residual or no biogas production was measured. For each substrate, four experimental runs were performed as shown in Figure 1 – first variant: feedstock and inoculum without any pretreatment, second variant: with pH adjustment to 5.5, third variant: with thermal pretreatment (80 °C for 1.5 h) and fourth variant: with pH adjustment and thermal pretreatment. These operations have been aimed to inactivate hydrogen consuming microorganisms (primarily methanogens) (Akobi, Hafez, & Nakhla, 2016). The individual runs were performed in triplicates, the results of which are expressed as averages.

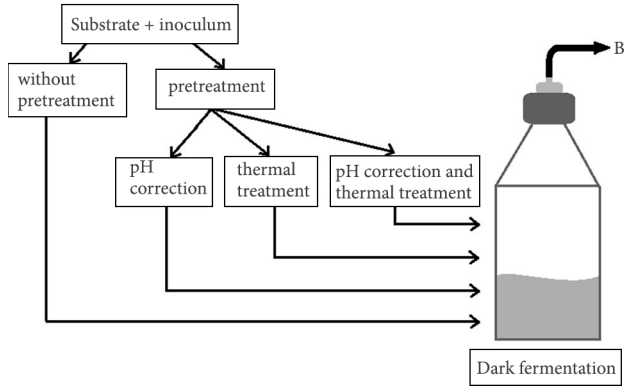

Figure 1. Schematic diagram of the experiments

1.4. Analytical methods

Total and volatile solids (TS, VS) as well as pH were analyzed based on Standard Methods for the Examination of Water and Wastewater (Rice, Baird, Eaton, & Clesceri, 2012). Chemical oxygen demand (COD) was determined using a DR 6000 spectrophotometer and HACH-Lange test LCK914. Elemental analysis (C, N, H, P, S) was performed with a 2500 elemental analyzer (CE Instruments, UK) following the manufacturer's procedure. The total carbon was divided by the total nitrogen to obtain the C/N ratio. Biogas yield was monitored on a daily basis by the water displacement method (Zhong et al., 2011). Biogas composition was analyzed using a portable gas analyzer (Madur, GA-21 plus).

The analyses of individual samples were performed in at least triplicates. The calculation of the average values, standard deviations, and the analysis of variance (single factor ANOVA) were performed in Microsoft Excel 2010. The significance of differences between experimental groups was calculated by Tukey's test (R version 3.5.0) with an alpha level of $p < 0.05$.

2. Results and discussion

2.1. Characteristics of substrates

The characteristics of substrates and inoculum used in this study are depicted in Table 1 and 2. The inoculum had an average VS concentration of 16 g/kg, and the average COD

value was 24.90 g O_2/kg. The initial pH of the inoculum was in the range of 7.34–7.76. The substrates significantly differed in terms of both organics and nutrient contents. Sugar beet pulp had the highest carbon content of 69.8% TS, but also contained as low as 0.19% TS of nitrogen and 0.02% TS of phosphorus. This gives a very high C/N ratio value of 364 for this substrate and suggests nutrient supplementation prior digestion. Likewise, sugar beet leaves were also poor in both nitrogen and phosphorus with the C/N ratio of around 211. The optimum range of C/N ratio for classical anaerobic digestion was suggested to be 20–30 (Xia, Cheng, & Murphy, 2016). However, for dark fermentation, this ratio differs, for example O-Thong, Prasertsana, Intrasungkhab, Dhamwichukornc, and Birkelandd (2008) reported an optimum hydrogen production at a C/N ratio of 74 and a C/P ratio of 559. Contrary to sugar beet pulp and leaves, slaughterhouse wastes (especially meat tissue and intestinal wastes) were abundant in nitrogen but also poor in phosphorus.

2.2. Batch experiments

The results of the batch digestion tests are summarized in Tables 2–6, whereas the plots with cumulative biogas yields are shown in Figures 2 and 3. A statistical comparison of hydrogen yields from the individual substrates is depicted in Tables 1S-4S in supplementary materials.

Generally, the amount of produced hydrogen depends on several factors of which the substrate type, pH and pretreatment temperature play a crucial role. Heat pretreatment and acidic pH inactivate hydrogen consumers (mainly methanogens) whereas spore-forming bacteria (*Clostridium sp., Bacillus sp.*) responsible for hydrogen production easily survive (Lee, Ebie, Xu, Li, & Inamori,

Table 2. Parameters of the batch digestion tests

Substrate	Mass of substrate [g]	Substrate VS [g/kg]	Mass of inoculum [g]	Inoculum VS [g/kg]	Duration time [d]
Rye stillage	33	122.83±0.26	500	16.00±1.20	14
Fruit and vegetable wastes	28	127.06±4.78	500	14.02±1.11	14
Sugar beet stillage	50	122.60±0.45	500	18.77±1.02	14
Intestinal wastes	15	229.82±1.36	500	14.02±1.11	14
Meat tissue	12	288.56±1.74	500	14.02±1.11	14
Post flotation sludge	17	210.17±1.94	500	14.02±1.11	14
Kitchen wastes	17	237.39±8.66	500	18.77±1.02	14
Maize silage	12	127.06±4.78	500	18.56±1.57	14
Sugar beet leaves	25	138.74±4.54	500	18.56±1.57	14
Fresh sugar beet pulp	18	194.28±6.13	500	14.02±1.11	14
Hydrolyzed sugar beet pulp	32	146.34±3.34	500	18.77±1.02	14
Steamed sugar beet pulp	24	193.36±1.90	500	18.56±1.57	14
± Standard deviation					

Table 3. Parameters of the batch digestion tests without pretreatment

Substrate	Specific gas production (SGP) dm^3/kg VS	Specific methane production (SMP) dm^3 CH_4/kg VS	Specific hydrogen production (SHP) dm^3 H_2/kg VS
Rye stillage	696.74±12.26	33.04±3.65	39.34±9.32
Fruit and vegetable wastes	649.93±42.9	234.91±50.97	102.83±23.24
Sugar beet stillage	508.43±12.58	226.22±22.47	109.26±9.45
Intestinal wastes	680.94±54.35	421.20±21.71	74.23±6.11
Meat tissue	1096.68±31.65	782.33±0.55	30.36±1.57
Post flotation sludge	637.44±10.89	280.18±39.43	47.74±0.69
Kitchen wastes	613.56±45.83	270.30±21.36	88.13±12.49
Maize silage	545.08±48.26	242.40±56.15	116.73±21.47
Sugar beet leaves	378.49±36.21	180.57±21.45	31.53±1.13
Fresh sugar beet pulp	230.91±3.03	125.34±6.34	11.20±6.79
Hydrolyzed sugar beet pulp	1032.31±89.27	389.91±24.15	143.43±24.11
Steamed sugar beet pulp	923.08±68.85	416.12±23.59	121.12±25.39

Table 4. Parameters of the batch digestion tests with pH adjustment

Substrate	Specific gas production (SGP) dm^3/kg VS	Specific methane production (SMP) dm^3 CH_4/kg VS	Specific hydrogen production (SHP) dm^3 H_2/kg VS
Rye stillage	193.28±5.57	2.15±0.57	31.87±5.17
Fruit and vegetable wastes	302.82±57.17	1.71±0.28	94.9±17.56
Sugar beet stillage	386.27±45.73	3.26±0.89	51.16±2.49
Intestinal wastes	151.56±32.29	74.84±8.94	8.02±1.33
Meat tissue	398.53±59.22	127.59±19.39	26.39±8.67
Post flotation sludge	197.32±15.21	57.40±3.11	3.62±2.54
Kitchen wastes	338.18±21.47	4.15±0,56	62.72±4.35
Maize silage	181.17±24.83	2.39±0.49	74.74±3.24
Sugar beet leaves	121.52±22.39	1.17±0.09	25.46 ±1.36
Fresh sugar beet pulp	130.82±38.51	34.68±4.50	13.58±6.65
Hydrolyzed sugar beet pulp	407.16±35.61	13.56±1.25	77.85±8.37
Steamed sugar beet pulp	177.95±30.19	2.78±0.01	61.75±12.74

Table 5. Parameters of the batch digestion tests with thermal pretreatment

Substrate	Specific gas production (SGP) dm^3/kg VS	Specific methane production (SMP) dm^3 CH_4/kg VS	Specific hydrogen production (SHP) dm^3 H_2/kg VS
Rye stillage	377.78±77.44	14.90±1.14	8.98±3.72
Fruit and vegetable wastes	339.41±33.94	0.71±0.99	97.69±10.82
Sugar beet stillage	287.27±24.19	94.26±13.64	45.14±18.39
Intestinal wastes	575.06±50.25	389.53±30.58	56.38±7.18
Meat tissue	30.32±2.04	0.00±0.00	0.00±0.00
Post flotation sludge	447.82±1.97	244.89±17.19	49.33±14.72
Kitchen wastes	236.73±28.14	6.42±1.37	51.46±12.57
Maize silage	218.45 ±32.24	0.61±0.05	268.78±24.16
Sugar beet leaves	25.32±2.14	0.00±0.00	0.00±0.00
Fresh sugar beet pulp	278.09±3.03	71.94±5.72	30.85±7.69
Hydrolyzed sugar beet pulp	317.47±24.16	5.34±0.78	150.98±5.24
Steamed sugar beet pulp	170.84±60.39	1.56±0.54	102.02±19.61

Table 6. Parameters of the batch digestion tests with pH adjustment and thermal pretreatment

Substrate	Specific gas production (SGP) dm^3 /kg VS	Specific methane production (SMP) dm^3 CH_4/kg VS	Specific hydrogen production (SHP) dm^3 H_2/kg VS
Rye stillage	203.53±9.29	8.23±1.67	104.75±2.28
Fruit and vegetable wastes	523.63±8.92	0.00±0.00	280.33±14.5
Sugar beet stillage	152.56±12.14	5.03±0.54	76.94±16.32
Intestinal wastes	0.00±0.00	0.00±0.00	0.00±0.00
Meat tissue	7.22±0.21	0.00±0.00	0.00±0.00
Post flotation sludge	129.45±32.66	0.00±0.00	0.00±0.00
Kitchen wastes	232.00±22.15	0.13±0.08	53.45±2.47
Maize silage	157.54±8.37	0.00±0.00	115.28±20.57
Sugar beet leaves	91.77±2.15	2.25±0.29	5.69±1.28
Fresh sugar beet pulp	14.30±0.12	1.03±0.23	6.17±1.38
Hydrolyzed sugar beet pulp	364.00±12.53	4.88±1.24	212.17±17.59
Steamed sugar beet pulp	76.16±17.25	0.00±0.00	34.90±1.55

2010; Sikora, Błaszczyk, Jurkowski, & Zielenkiewicz, 2013). In our experiments, pH of the samples before digestion was adjusted to 5.5, which was a value within an optimal range for dark fermentative hydrogen production reported in the literature (Ghimire et al., 2015; Guo, Trably, Latrinne, Carrere, & Steyer, 2010). As shown in Tables 3–6, the experiments with neither pH correction nor thermal pretreatment gave the highest biogas yields, but the main biogas component was methane. The highest biogas productions of 1097 dm^3/kg VS and 681 dm^3/kg VS were obtained for meat tissue and intestinal wastes, respectively, which corresponded to the methane yields of 782 dm^3 CH_4/kg VS and 421 dm^3 CH_4/kg VS, respectively. Regarding the substrates of plant origin, the greatest biogas yields of 1032 dm^3/kg VS and 923 dm^3/kg were achieved for hydrolyzed and steamed sugar beet pulp, and the corresponding methane yields reached 390 dm^3 CH_4/kg VS and 416 dm^3 CH_4/kg VS. Moreover, the cumulative hydrogen yields from hydrolyzed and steamed SBP reached 143 dm^3 H_2/kg VS and 121 dm^3 H_2/kg VS, respectively, and these values were the highest among all the experiments performed without any pretreatment (Table 3). The cumulative hydrogen yield of over 100 dm^3 CH_4/kg VS was also reported for sugar beet stillage, maize silage and fruit and vegetable waste, and the statistical analysis showed no significant differences in hydrogen production between these substrates (Table 1S).

The pH adjustment alone did not significantly improve hydrogen production as the specific yields of this gas did not exceed 100 dm^3/kg VS (Table 4). The heat pretreatment followed by the pH correction to 5.5 was needed to achieve considerable hydrogen yields and to inhibit methane production. As reported in the literature (Lee et al., 2010) heat treatment inactivates most hydrogen consumers. The highest specific hydrogen production after combined pretreatment was achieved for fruit and vegetable yields (280 dm^3 CH_4/kg VS) and for hydrolyzed sugar beet pulp (212 dm^3 CH_4/kg VS). The hydrogen yield greater than 100 dm^3 CH_4/kg VS was also achieved for maize silage, rye stillage and steamed SBP (Table 5). Only trace amounts of methane not exceeding 5 dm^3 CH_4/kg VS were recorded for these substrates. Interestingly, the hydrogen production from maize silage was 4-fold higher that the yields obtained from similar substrate in the study of Benito Martin, Schlienz, and Greger (2017). The statistical analysis showed that the production of hydrogen from fruit and vegetable waste as well as hydrolyzed SBP (both exceeding 200 dm^3 H_2/kg VS) was significantly higher ($p < 0.05$) compared to the other substrates after combined pH and thermal pretreatment (Table 4S). It is interesting to note, that maize silage produced as high as 269 dm^3/kg VS of hydrogen in the experiment with only thermal pretreatment (Table 5), whereas the hydrogen yields in the samples after pH adjustment did not exceed 115 dm^3/kg VS (Table 4). This finding might be linked to the high lactic acid production from maize silage reported in the literature and its negative impact on dark fermentation. In the literature, there are only few reports on the use of maize silage for hydrogen production. Generally, most reports have focused on the anaerobic co-digestion of maize silage with other organic substrates. Likewise sugar beet pulp, maize silage is abundant in polysaccharides, mainly hemicelluloses (17.9% TS) and cellulose (17.5% TS), whereas the content of lignin is relatively low (1.07% TS) (Benito Martin et al., 2017). The anaerobic digestion process of ensilaged substrates is characterized by an occurrence of lactic acid bacteria (LAB), particularly in its first phase. Sikora el al. (2013) observed the presence of LAB (mainly *Leuconostocaeae*) in hydrogen-producing consortia with *Clostridiaceae* as predominant ones. A potential impact of lactic acid bacteria on dark fermentation still remains unknown. Noike, Takabatake, Mizuno, and Ohba (2002) as well as Ren et al. (2007) observed competition for substrate between hydrogen producers and LAB, which led to lower hydrogen yield. Moreover, an inhibitory effect of LAB on hydrogen yield is strongly dependent on pH and the digestion temperature. Noike et al. (2002) have also suggested the use of thermal pretreatment as an effective method of LAB inactivation. In contrast to these findings, some scientists suggest a positive effect of LAB activity on hydrogen production (Yang, Zhang, McGarvey, & Benemann, 2007). During the dark fermentation process, lactates and acetates are converted to butyrates with the release of hydrogen (Chojnacka et al., 2011). Furthermore, Yang et al. (2007) have found that some lactic acid bacteria are able to produce hydrogen. However, these speculations are not fully recognized and need to be confirmed.

Regarding methane production during dark fermentation, it might be concluded that significantly higher and more stable yield of this gas was observed in the experiments with no thermal pretreatment. Moreover, lower hydrogen yield could be linked to the simultaneous H_2 and CH_4 production, despite pH adjustment. As mentioned earlier, inoculum used for the experiments derived from the anaerobic digester treating municipal sewage sludge. Such inoculum has a variety of anaerobic microorganisms including methanogens including species capable of growing at low pH. Hence, pH adjustment with no thermal pretreatment does not guarantee a complete inactivation of methanogens and lack of methane in biogas. Furthermore, some *Archae* species like *Methanosaeta* or *Methanospharea* metabolize H_2 and CO_2 to methane, and use formic acid or alcohols (mainly methanol) as electron donors (Akobi et al., 2016). Another group of microorganisms involved in hydrogen consumption are homoacetogenic bacteria. As reported in the literature, homoacetogenesis can consume 11–43% of H2 yielded in batch tests (Saady, 2013). Moreover, homoacetogenic bacteria can compete for the substrate with hydrogenothropic methanogens and utilize H_2 to reduce CO_2 to acetic acid. The presence of H_2 in anaerobic reactor can had a positive effect on methanogenesis and no identified negative effect on acetogenesis (Saady, 2013).

Regarding the other carbohydrate-rich substrates – fresh sugar beet leaves and fresh sugar beet pulp – they produced almost no hydrogen, even in the experiments with the pH correction and thermal pretreatment (Table 6). This can be linked to the structure of these materials, mainly composed of lignocellulostic substances. As reported in the literature, sugar beet pulp is mainly composed of polysaccharides (41–61% hemicelluloses, 20–24% cellulose, 1–2% lignin), which form a dense, complex structure. Other components of SBP are proteins, which constitute around 7–8% of its dry mass (Dredge, van Dyk, Radloff, & Pletschke, 2011). In view of the above, fresh sugar beet pulp is hardly decomposed by microorganisms (Akobi et al., 2016). However, approximately 60–70% of its dry mass are potentially available polysaccharides (Panagiotopoulos et al., 2010), which makes sugar beet pulp

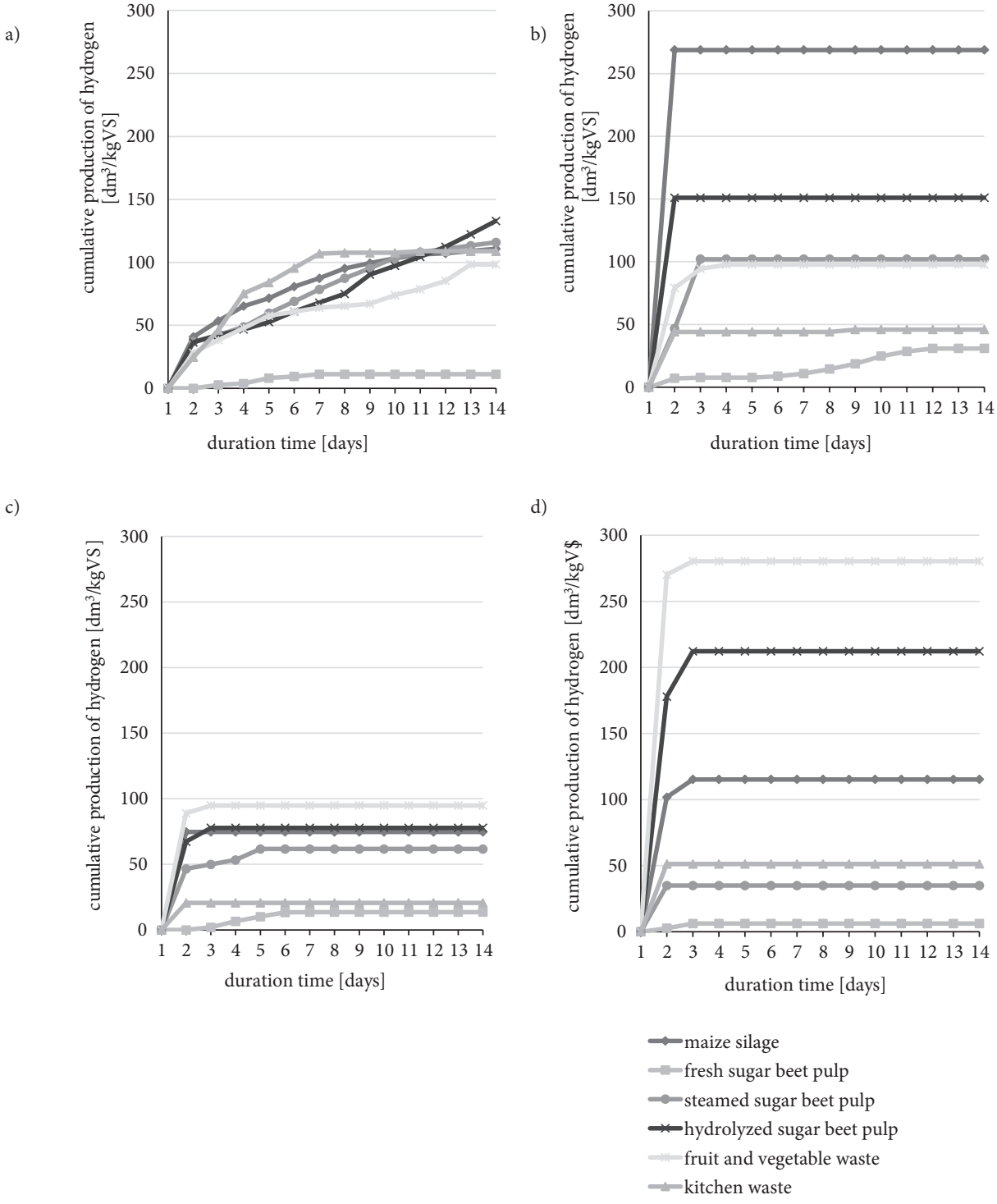

Figure 2. Cumulative hydrogen production a) – without correction, b) – with pH adjustment, c) – with thermal correction, d) – with pH adjustment and thermal correction in batch experiments from MS, steamed SBP, hydrolyzed SBP, fresh SBP, FVW and KW

attractive for hydrogen production. In our research, sugar beet pulp was preliminary treated by steaming or by means of two commercial multienzyme preparations: Viscozyme and Ultraflo Max. These operations significantly improved both hydrogen and methane production, especially enzymatic pretreatment enabled as much as 212 dm^3/kg VS of hydrogen (as discussed above) to be achieved, in contrast to 6 dm^3/kg VS obtained for fresh SBP (Table 6). The experiments have also confirmed that sugar beet pulp is relatively susceptible to any pretreatment due to a relatively low content of lignin, compared to other materials of plant origin (Panagiotopoulos et al., 2010). The effect of different pretreatment methods (alkaline, thermal, microwave, thermal-alkaline and microwave-alkaline) on hydrogen production was also studied by Ozkan et al. (2011). These authors achieved cumulative hydrogen yields in the range of 112–149 dm^3 whereas the samples with no pretreatment produced only 0.096 dm^3 of hydrogen.

Considering slaughterhouse wastes, they were confirmed to be well-suitable substrates for methane

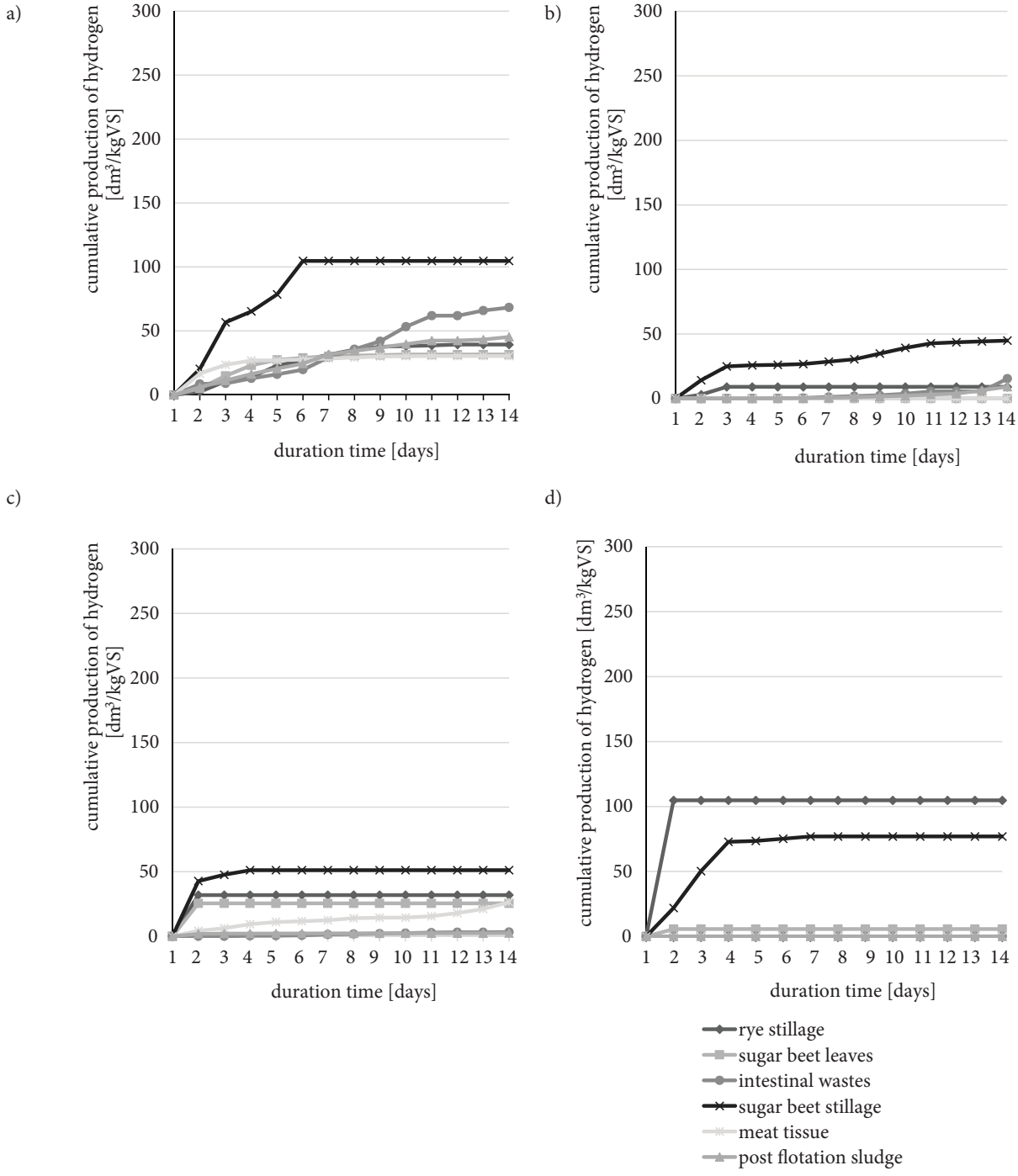

Figure 3. Cumulative hydrogen production a) – without correction, b) – with pH adjustment, c) – with thermal correction, d) – with pH adjustment and thermal correction in batch experiments from RS, SBL, SBS and SHW

production. In this study, the highest methane yield of 782.33 dm^3 CH_4/kg VS was achieved for meat tissue in the experiment without any pretreatment (Table 3). However, these substrates were found to be not suitable for hydrogen production, which can be linked to the high contents of lipids and proteins and low concentrations of carbohydrates. Proteins are firstly decomposed to amino acids, which, in turn, are fermented in pairs via Strickland reactions with no hydrogen yield (Hallenbeck, 2009). Lipids, in turn, are hydrolyzed to glycerol and long chain fatty acids. Furthermore, decomposition of slaughterhouse waste leads to accumulation of volatile fatty acids, sulfides and ammonia (Ghimire et al., 2015; Cuetos, Gómez, Otero, & Morán, 2010). Fatty acids are then decomposed to acetates and hydrogen by syntrophic bacteria, but this process is only possible at a very low hydrogen partial pressure, which can only be provided by an activity of hydrogen consuming methanogens (Hallenbeck, 2009). Furthermore, LCFAa may also cause problems in dark fermentation because these compounds are toxic to various anaerobic microorganisms including acetogens, which are involved in hydrogen production (Angelidaki & Ahring, 1992). These findings may also explain the differences between hydrogen production from fruit and vegetable wastes and kitchen wastes in our study. Fruit and vegetable waste exhibited the highest hydrogen yield of 280 dm^3/kg VS among all tested substrates, whereas the corresponding value for kitchen waste was only 53 dm^3 H_2/kg VS (Table 6). For comparison, Li, Zhao, Guo, Qijan, and Niu (2008) obtained 196 dm^3 H_2/kg VS from kitchen waste whereas Lay et al. (2005) yielded 125 dm^3 H_2/kg VS, and both cited authors used similar conditions in their research. Significantly different hydrogen yields obtained from fruit and vegetable waste and kitchen waste can be attributed to the compositions of both waste types. Fruit and vegetable wastes are exclusively composed of plant biomass abundant in carbohydrates, while kitchen wastes, apart from FVW, contain other edible components, including meat and fish residues, mayonnaise and sauces, rise, noodles etc. Many of these ingredients are not suitable for hydrogen production due to the high content of lipids and proteins. Interestingly, both substrates (FVW and KW) gave similar, moderate methane yields of 235–270 dm^3/kg VS in the experiments with no pretreatment (Table 3). This might have been due to a C/N balance of fresh substrate far from optimal for anaerobic digestion.

Conclusions

Batch tests have been found to be effective in showing the potential of hydrogen production from various substrates of plant and animal origin.

It was confirmed that the materials of plant origin were generally much more susceptible to dark fermentation, even without any pretreatment, compared to the substrates rich in proteins and fats.

A cumulative hydrogen yield greater than 100 dm^3 H_2/kg VS was obtained from fruit and vegetable waste, sugar beet pulp stillage, maize silage as well as steamed and hydrolyzed sugar beet pulp.

Hydrogen production from plant waste and biomass was considerably improved by applying thermal pretreatment and pH adjustment to 5.5 in order to inhibit methanogens and stimulate hydrogen producers. In particular, fruit and vegetable waste yielded around 280 dm^3 H_2/kg VS whereas maize stillage nearly 270 dm^3 H_2/kg VS.

However, some carbohydrate-rich substrates needed additional preliminary treatment to be suitable for hydrogen production. Especially, sugar beet pulp subjected to hydrolysis followed by thermal pretreatment and pH adjustment gave a hydrogen yield of 212 dm^3/kg VS in contrast to almost no hydrogen production from fresh SBP.

It was also reported that slaughterhouse waste and kitchen waste are not suitable for hydrogen production but they have high potential of methanation, whereas hydrolyzed sugar beet pulp can successfully be used to produce both gases.

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APPENDIX
Supplementary material

Table 1S. Statistical comparison of hydrogen production from batch test with no pretreatment (ANOVA, p value, Tukey's test)

Without pretreatment	Fresh sugar beet pulp	Steamed sugar beet pulp	Hydrolyzed sugar beet pulp	Sugar beet leaves	Kitchen wastes	Fruit and vegetable wastes	Maize silage	Rye stillage	Sugar beet stillage	Meat tissue	Intestinal wastes	Post flotation sludge
Fresh sugar beet pulp	–	–	–	–	–	–	–	–	–	–	–	–
Steamed sugar beet pulp	<0.01	–	–	–	–	–	–	–	–	–	–	–
Hydrolyzed sugar beet pulp	<0.01	0.259	–	–	–	–	–	–	–	–	–	–
Sugar beet leaves	<0,01	<0.01	<0.01	–	–	–	–	–	–	–	–	–
Kitchen wastes	<0.01	0.830	0.939	<0.01	–	–	–	–	–	–	–	–
Fruit and vegetable wastes	<0.01	0.198	0.175	<0.01	0.145	–	–	–	–	–	–	–
Maize silage	<0.01	0.297	0.407	<0.01	0.506	0.457	–	–	–	–	–	–
Rye stillage	<0.01	<0.01	<0.01	0.612	<0.01	<0.01	<0.01	–	–	–	–	–
Sugar beet stillage	<0.01	0.951	0.927	<0.01	0.884	0.215	0.351	<0.01	–	–	–	–
Meat tissue	<0.01	<0.01	<0.01	0.817	<0.01	<0.01	<0.01	0.663	<0.01	–	–	–
Intestinal wastes	<0.01	<0.01	<0.01	0.023	<0.01	0.027	<0.01	0.047	<0.01	0.019	–	–
Post flotation sludge	<0.01	<0.01	<0.01	0.151	<0.01	<0.01	<0.01	0.535	<0.01	0.391	0.118	–

– p value < 0.01 (highly significant)

– p value < 0.05 (significant)

– p value > 0.05 (not significant)

Table 2S. Statistical comparison of hydrogen production from batch test with pH adjustment (ANOVA, p value, Tukey's test)

pH adjustment	Fresh sugar beet pulp	Steamed sugar beet pulp	Hydrolyzed sugar beet pulp	Sugar beet leaves	Kitchen wastes	Fruit and vegetable wastes	Maize silage	Rye stillage	Sugar beet stillage	Meat tissue	Intestinal wastes	Post flotation sludge
Fresh sugar beet pulp	–	–	–	–	–	–	–	–	–	–	–	–
Steamed sugar beet pulp	<0.01	–	–	–	–	–	–	–	–	–	–	–
Hydrolyzed sugar beet pulp	<0.01	<0.01	–	–	–	–	–	–	–	–	–	–
Sugar beet leaves	<0.01	<0.01	<0.01	–	–	–	–	–	–	–	–	–
Kitchen wastes	<0.01	<0.01	<0.01	<0.01	–	–	–	–	–	–	–	–
Fruit and vegetable wastes	<0.01	<0.01	<0.01	<0.01	<0.01	–	–	–	–	–	–	–
Maize silage	<0.01	<0.01	0.865	<0.01	<0.01	0.023	–	–	–	–	–	–
Rye stillage	<0.01	<0.01	<0.01	0.051	0.169	<0.01	<0.01	–	–	–	–	–
Sugar beet stillage	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	–	–	–	–
Meat tissue	0.146	<0.01	<0.01	0.035	0.025	<0.01	<0.01	<0.01	<0.01	–	–	–
Intestinal wastes	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	–	–
Post flotation sludge	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.206	–

– p value < 0.01 (highly significant)

– p value < 0.05 (significant)

– p value > 0.05 (not significant)

Table 3S. Statistical comparison of hydrogen production from batch test with thermal treatment (ANOVA, p value, Tukey's test)

Thermal treatment	Fresh sugar beet pulp	Steamed sugar beet pulp	Hydrolyzed sugar beet pulp	Sugar beet leaves	Kitchen wastes	Fruit and vegetable wastes	Maize silage	Rye stillage	Sugar beet stillage	Meat tissue	Intestinal wastes	Post flotation sludge
Fresh sugar beet pulp	–	–	–	–	–	–	–	–	–	–	–	–
Steamed sugar beet pulp	<0.01	–	–	–	–	–	–	–	–	–	–	–
Hydrolyzed sugar beet pulp	<0.01	<0.01	–	–	–	–	–	–	–	–	–	–
Sugar beet leaves	<0.01	<0.01	0	–	–	–	–	–	–	–	–	–
Kitchen wastes	<0.01	<0.01	<0.01	<0.01	–	–	–	–	–	–	–	–
Fruit and vegetable wastes	<0.01	0.645	<0.01	<0.01	<0.01	–	–	–	–	–	–	–
Maize silage	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	–	–	–	–	–	–
Rye stillage	0.016	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	–	–	–	–	–
Sugar beet stillage	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	–	–	–	–
Meat tissue	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	–	–	–
Intestinal wastes	0.595	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.143	<0.01	–	–
Post flotation sludge	0.983	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.025	<0.01	0.435	–

– p value < 0.01 (highly significant)

– p value < 0.05 (significant)

– p value > 0.05 (not significant)

Table 4S. Statistical comparison of hydrogen production from batch test with thermal treatment and pH adjustment (ANOVA, p value, Tukey's test)

pH adjustment and thermal treatment	Fresh sugar beet pulp	Steamed sugar beet pulp	Hydrolyzed sugar beet pulp	Sugar beet leaves	Kitchen wastes	Fruit and vegetable wastes	Maize silage	Rye stillage	Sugar beet stillage	Meat tissue	Intestinal wastes	Post flotation sludge
Fresh sugar beet pulp	–	–	–	–	–	–	–	–	–	–	–	–
Steamed sugar beet pulp	<0.01	–	–	–	–	–	–	–	–	–	–	–
Hydrolyzed sugar beet pulp	<0.01	<0.01	–	–	–	–	–	–	–	–	–	–
Sugar beet leaves	0.053	<0.01	<0.01	–	–	–	–	–	–	–	–	–
Kitchen wastes	<0.01	<0.01	<0.01	<0.01	–	–	–	–	–	–	–	–
Fruit and vegetable wastes	<0.01	<0.01	<0.01	<0.01	<0.01	–	–	–	–	–	–	–
Maize silage	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	–	–	–	–	–	–
Rye stillage	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.435	–	–	–	–	–
Sugar beet stillage	<0.01	<0.01	<0.01	<0.01	0.016	<0.01	<0.01	<0.01	–	–	–	–
Meat tissue	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	–	–	–
Intestinal wastes	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	–	–	–
Post flotation sludge	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	–	–	–

– p value < 0.01 (highly significant)

– p value < 0.05 (significant)

– p value > 0.05 (not significant)