Fuel 258 (2019) 116180

Contents lists available at ScienceDirect

Fuel

journal homepage: www.elsevier.com/locate/fuel

Full Length Article

Effects of several inocula on the biochemical hydrogen potential of sludge-vinasse co-digestion

Miriam Tena, Montserrat Perez, Rosario Solera*

Department of Environmental Technologies, IVAGRO, Faculty of Marine and Environmental Sciences (CASEM), University of Cádiz, Pol. Río San Pedro s/n, 11510 Puerto Real, Cádiz, Spain

ARTICLE INFO

Keywords:
Biochemical hydrogen potential
Inoculum
Sludge
Vinasse
Anaerobic co-digestion
Eubacteria

ABSTRACT

The influence of the inoculum on the Biochemical Hydrogen Potential test (BHP) was investigated. Thermophilic BHP from sludge-vinasses co-digestion (50:50) was studied employing three types of inocula: Acidogenic Inoculum, Sludge Inoculum and Thermal Sludge Inoculum. The maximum hydrogen yield was obtained with a sludge inoculum (177 mL H_2/g VS_{added}). This yield was 21 and 36% higher than for acidogenic inoculum and thermal sludge inoculum, respectively. The results revealed that the choice of inoculum had significant impact on the hydrogen yield and the sludge inoculum is the most beneficial for BHP tests. The percentages between Eubacteria:Archaea increased from 59.2:40.8 to 92.0:9.0 during BHP tests using the sludge inoculum while it remained stablish in the others cases around 50:50. Furthermore, hydrogen production was accompanied by the generation of volatile fatty acids, mainly acetic, butyric and propionic acids. There were no differences in the rate of hydrogen production in any of the BHP.

1. Introduction

In recent years, the energy crisis has imposed the necessity to achieve a sustainable future built on alternative sources of energy and materials. Molecular hydrogen represents a storable form of energy [1]. Moreover, its combustion does not generate polluting products and it has high specific energy [2–4].

Hydrogen production can occur during the anaerobic digestion (AD) process. This process can be divided into two stages: dark fermentation (DF) and methanogenesis. The first stage involves the production of volatile fatty acids (VFAs), H_2 and CO_2, while the second one converts VFAs into CH_4 and CO_2 [5,6]. Simple operation conditions, low operating cost, low energy demand and fast reaction rate are some one-off advantages of dark fermentation [7]. Hydrogen generation using the DF process is possible with a wide range of waste materials such as sludge [8], food waste [9], cheese whey [10], algal biomass [11] and vinasse (V) [12]. Recently, numerous studies have found that co-digestion of two or more substrates can increase the load of biodegradable organic matter, improve the balance of nutrients, improve microbial diversity leading to enhance hydrogen production [13,14]. Although there are numerous studies on hydrogen production by co-digestion of sludge with different substrates such as perennial ryegrass [2], food waste [15] and glycerol [16], no prior studies have been published on the production of hydrogen via sludge-vinasse co-digestion.

In Spain, around 1.2 million tons of sludge are generated every year in the wastewater treatment plants (WWTPs). Waste activated sludge (WAS) is the main by-product of these plants. WAS is an extremely complex and heterogeneous solid waste, composed mainly of biomass from cell growth and decay during activated sludge treatment process in the WWTPs [17]. On the other hand, vinasse is an effluent generated during the production of alcohol in the wine distillation process. This effluent can be highly damaging in the areas in which it is discarded due to its high organic load, low pH and high corrosivity. Instead of harmful, vinasse may be considered as a substrate for hydrogen generation through the dark fermentation process because of the surplus organic load.

Biochemical hydrogen potential (BHP) corresponds to the maximum hydrogen production at dark fermentation infinite time and is a key parameter to evaluate the suitability of substrates to obtain biohydrogen. Batch methods have recently been applied to evaluate the BHP of numerous substrates, although the operating conditions (such as pH, temperature) have yet to be standardized. Moreover, there is no consensus regarding the nature of the inoculum to use in these tests or the type of pre-treatment they should receive (Table 1). One of the most widely used types of inoculum is the anaerobic sludge, though from different sources such as municipal sewage [18,19], wastewater [2], poultry slaughterhouse wastewater [20,21] and citrate-producing wastewater have also been used.

* Corresponding author.
E-mail address: rosario.solera@uca.es (R. Solera).

https://doi.org/10.1016/j.fuel.2019.116180
Received 22 July 2019; Received in revised form 5 September 2019; Accepted 6 September 2019
Available online 12 September 2019
0016-2361/

Table 1
Comparative study on hydrogen production in anaerobic reactors.

Inoculum			Substrates	Experimentation conditions	Maximum hydrogen yield	References
Type	Source	Pretreatment				
Anaerobic sludge	Wastewater	100 °C, 15 min	Sludge and perennial ryegrass	Batch 37 °C	60 mL H_2/g VS_{added}	[2]
Anaerobic sludge	Municipal sewage	100 °C, 30 min	Food waste and crude glycerol	Batch 35 °C	180 mL H_2/g $VS_{consumed}$	[18]
Anaerobic sludge	Municipal sewage	100 °C, 30 min	Food waste, sewage sludge and crude glycerol	Batch 35 °C	179 mL H_2/g $VS_{consumed}$	[19]
Anaerobic sludge	OFMSW		OFMSW and sewage sludge	Batch 55 °C	51 mL H_2/g $VS_{consumed}$	[3]
Anaerobic sludge (Upflow anaerobic sludge blanket UASB)	Wastewater from poultry slaughterhouse	90 °C, 10 min	Cheese whey and glycerin	Anaerobic sequencing batch biofilm reactor (AnSBBR) 30 °C	5.4 mol H_2/kg COD 2.3 mol H_2/kg COD	[20]
Granular (UASB)	Wastewater from poultry slaughterhouse	90 °C, 10 min	Glycerin-based wastewater	AnSBBR 30 °C	20 mol H_2/kg $COD_{consumed}$ 19.8 mol H_2/kg $COD_{consumed}$	[24]
Granular mesophilic sludge (UASB)	Potato wastes	105 °C, 4 h	Glucose Wheat bran from common wheat Wheat bran from durum wheat Wastes from mashed potatoes Wastes from steam-peeling potatoes	Batch 35 °C	185L H_2/kg COD 47L H_2/kg COD 76L H_2/kg COD 177L H_2/kg COD 134L H_2/kg COD	[26]
Anaerobic granular sludge	Municipal sewage	100 °C, 10 min	Citrus vinasse	Batch 37 °C	2.0 mmol H_2/g COD	[22]
Anaerobic sludge	Citrate-producing wastewater	102 °C, 30 min	Brewery wastewater	Batch 36 °C	149.6 mL H_2/g COD	[25]
Anaerobic (Fixed-bed)	Sucrose-based synthetic wastewater		Domestic sewage Glycerin wastewater Sugarcane vinasse	Batch 25 °C	6.01 mmol H_2/g COD 6.03 mmol H_2/g COD 24.97 mmol H_2/g COD	[27]
Anaerobic (Fixed-bed)	Sucrose-based synthetic wastewater		Vinasse	Batch 25 °C	20.8 mL H_2/g COD	[12]
Anaerobic mixed microflora (UASB)	Chemical wastewater	100 °C, 2 h pH 3, 24 h	Synthetic wastewater and domestic sewage wastewater	Batch 29 °C	0.71 mmol H_2/g COD	[23]
Anaerobic sludge (UASB)	Wastewater from poultry slaughterhouse	90 °C, 10 min	Sugarcane vinasse	Batch 55 °C	2.31 mmol H_2/g COD	[21]
Anaerobic granular sludge		 90 °C, 1 h Chloroform 0.2% pH 12 pH 3 Loading-shock	Cassava stillage	Batch 60 °C	65.3 mL H_2/g VS 57.4 mL H_2/g VS 32.9 mL H_2/g VS 59.0 mL H_2/g VS 46.5 mL H_2/g VS 64.4 mL H_2/g VS	[39]
Anaerobic sludge	Wastewater from poultry slaughterhouse	90 °C, 15 min	Vinasse and molasses	AnSBBR	0.8 mol H_2/kg $COD_{consumed}$ 0.5 mol H_2/kg $COD_{consumed}$	[41]
Anaerobic sludge	Waste activated sludge and vinasse Waste activated sludge Waste activated sludge	 100 °C, 15 min	Waste activated sludge and vinasse	Batch 55 °C	146.37 mL H_2/g Vs_{added} 177.23 mL H_2/g VS_{added} 130.17 mL H_2/g VS_{added}	The present study

Most research studies use inocula subjected to thermal pre-treatment in order to enrich the inoculum in terms of the hydrogen-producing bacteria. The pre-treatment is generally carried out at a temperature of 100 °C [2,18,19,22,23], although it has been carried out at 90 °C in other cases [20,21,24] or even at a temperature above 100 °C [25,26]. The exposure times of the inoculum to thermal shock vary greatly, ranging from 15 to 30 min in most cases [2,18,19]. However, in the studies by Giordano et al. [26] and Mohan et al. [23], the exposure time was longer (2–4 h). Other authors use a hydrogen-producing inoculum [3,12,27]. The results of these studies are inconclusive; hence the lack of consensus regarding the type of inoculum or the thermal pre-treatment conditions to be employed in BHP tests.

In this study, BHP tests with different natural inocula and pre-treatment conditions were carried out to study their influence on BHP results. The main purpose of this research is to discern which type of inoculum to use for future BHP tests.

2. Materials and methods

2.1. Substrates

Waste activated sludge and vinasse were used as substrates. The WAS was collected from Guadalete municipal wastewater treatment plant, Jerez de la Frontera, Cadiz, Spain. The V was provided by the González Byass winery located in Jerez de la Frontera, Cadiz, Spain, and kept frozen (−20 °C) until use.

A mixture of both substrates in a 50:50 ratio was used as the feedstock in all the BHP tests.

2.2. Inocula

Three types of inocula were used: Acidogenic Inoculum (AI), Sludge Inoculum (SI) and Thermal Sludge Inoculum (TSI). The AI was collected from a laboratory scale semi-continuous acidogenic thermophilic anaerobic digester treating waste activated sludge-vinasse (50:50) for hydrogen production. The reactor operated at pH 5.5, a temperature of 55 °C and a HRT of 4 days. The AI was thus already conditioned to treat the mixture of WAS-vinasse co-substrates and is, therefore, a hydrogen -producing inoculum. The SI and TSI were collected from a laboratory scale semi-continuous thermophilic anaerobic digester treating waste activated sludge operating at pH 7.0, a temperature of 55 °C and a HRT of 20 days. The TSI was heat-treated in a hot oven at 100 °C for 15 min.

Three BHP tests were carried out, Tests 1, 2 and 3, with the aforementioned inocula, AI, SI and TSI, respectively.

The physic-chemical characteristics of the inocula and substrates are summarized in Table 2.

2.3. Biochemical hydrogen potential

Hydrogen fermentation was performed in 250 mL glass bottles with a 120 mL working volume and a 130 mL headspace volume. For each reactor, a mixing ratio of inoculum to feedstock of 1:1 (v/v) was used [2,3]. The initial pH of each bottle was set at 5.5, a value at which methanogenic *Archaea* are inhibited [18]. Nitrogen was fluxed for 5 min to displace any air present in the bottles and hence ensure an anaerobic environment. All the bottles were maintained at constant temperature under thermophilic conditions (55 °C) in an orbital shaker incubator.

All the experiments were carried out in triplicate and inoculum control bottles were also prepared. Three bottles were used as control for each inoculum without any substrate. The hydrogen production from the control was subtracted from the hydrogen production obtained in the substrate assays prior to data analysis.

Table 2
Physico-chemical characteristics of the inocula and substrates.

Parameters	Units	AI	SI	TSI	WAS + V
pH		5.32	5.49	5.52	5.39
TS	g/L	28.68	40.71	38.01	41.07
VS	g/L	21.50	31.85	29.39	33.51
TCOD	g/L	51.81	49.51	42.27	63.75
SCOD	g/L	37.52	22.58	22.62	28.06
Total VFA	g/L	4.93	2.67	3.41	2.14

2.4. Analytical methods

Both the volume and composition of the biogas were determined daily. The produced biogas was determined indirectly, by measuring the pressure inside the bottles via pressure transducers. The measured pressure is converted to volume of biogas according to the ideal gas law [28]. Gas volumes were converted to standard conditions and corrected by subtracting the production of the blank. The composition of the biogas was determined by gas chromatography separation (Shimadzu GC-2010 system). H_2, CO_2, CH_4 and O_2 were analysed by means of a thermal conductivity detector (TCD) using a Supelco Carboxen 1010 Plot column [29]. Total solids (TS), volatile solids (VS), total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD) were analysed according to the Standard Methods [30] at the beginning and end of each experiment. Volatile fatty acids (VFA) were determined by gas chromatography on a Shimadzu GC-2010 system equipped with a flame ionization detector (FID) and a capillary column filled with Nukol [31]. The pH was measured at the beginning and end of the tests using a Crison 20 Basic pH meter [30].

2.5. Microbial analyses

Fluorescence *in situ* hybridization (FISH) was used to count the microorganisms contained in the reactors. The main steps of FISH of whole cells using 16S rRNA-targeted oligonucleotide probes are cell fixation followed by permeabilization and hybridization with the desired probe(s). Samples from batch reactors were collected in sterile universal bottles at the beginning and end of the BHP test. A 1:1 (v/v) ratio of absolute ethanol was added to the bottles. The samples were stored at −20 °C until they were fixed. Further details of this procedure are given in Montero et al. [32].

The technique used for fixing and permeabilizing cells was based on the method described by Amann et al. [33,34]. The 16S rRNA-targeted oligonucleotide probes used in this study are shown in Table 3: bacterial-universal probe EUB338 [33,34], and *Archaea*-universal probe ARC915 [35]. The cellular concentration and percentages of *Eubacteria* and *Archaea* were obtained by FISH. The total population was estimated as the sum of the populations of *Eubacteria* and *Archaea* for the reason that most anaerobic microorganisms in anaerobic reactors belong to these two groups [36]. Samples were examined visually and the cells were counted under an Axio Imager Upright epifluorescence microscope (Zeiss) equipped with a 100 W mercury lamp and a 100 × oil objective lens. The filter employed depended on the identity of the labelled probe: a B-2A filter (DM 510, Excitation 450–490 and Barrer 520) was used for 6-FAM; while a G-2A filter (DM 580, Excitation 510–560 and Barrer 590) was used for Cy3. In addition, microbial activity was evaluated from biochemical activity according to the methods reported by Montero et al. [32] and Zahedi et al. [37]. The activity was calculated as the ratio of H_2 generated and the number of microorganisms inside the reactor obtained by FISH staining.

3. Results and discussion

3.1. Physico-chemical analysis

The physical-chemical characteristics of three tests at the beginning and end of the tests are summarized in Table 4. The pH remained

Table 3
Oligonucleotide probes used in this study.

	Probe sequences (from 5′ to 3′)	Target	Formamide (%)	Time (h)	T (°C)	Reference
EUB338	GCTGCCTCCCGTAGGAGT	*Eubacteria*	20	1.5	46	[33,34]
ARC915	GTGCTCCCCCGCCAATTCCT	*Archaea*	35	1.5	46	[35]

Table 4
Physico-chemical and microbial characterization of the three tests.

Parameters	Units	Test 1		Test 2		Test 3	
		Initial	Final	Initial	Final	Initial	Final
Physico-chemical characteristics							
pH		5.35	5.07	5.32	5.27	5.46	5.39
TS	g/L	34.99	34.35	41.24	35.80	40.54	34.26
Removal TS	%	1.83		13.20		15.49	
VS	g/L	27.67	27.19	32.25	27.83	31.33	25.90
Removal VS	%	1.73		13.71		17.33	
TCOD	g/L	68.00	33.78	65.63	28.35	86.38	30.64
Removal TCOD	%	50.32		56.80		64.53	
SCOD	g/L	35.38	27.17	26.44	22.46	25.75	22.83
Removal SCOD	%	23.21		15.05		11.34	
Total VFA	g/L	3.40	5.31	2.53	5.27	2.80	5.90
Microbial characterization							
Total population	10^8 cells/mL	13.51	13.29	14.95	85.90	15.29	13.27
Eubacteria	%	41.3	42.6	59.2	92.1	46.3	44.6
Archaea	%	58.7	57.5	40.8	8.0	53.7	55.4

relatively stable during experimentation, varying from 5 to 5.5. There were no abrupt variations in pH, demonstrating that the systems were capable of self-regulating in order to favour microbial activity [36].

VS and TS removal rates ranged between 1.7 and 17.3%. The lowest rate was achieved with the acidogenic inoculum (Test 1).

As for SCOD removal, this was lower than 23% in all tests. Yang and Wang [2] also found that the SCOD concentration decreased, with significant reductions in removal rates of 7.1–31.3%. These authors state that their results indicated that the hydrolysis amount of particular organics by hydrolytic bacteria was lower than the utilization amount of soluble organics by hydrogen producers. In terms of TCOD, the removal rate was greater, with percentages ranging between 50 and 60%. These results are in line with those obtained by Torquato et al. [22], in which the maximum removal rate of 41% was obtained in the digestion of vinasse to produce hydrogen. However, Silva et al. [18,19] reported that COD removal was lower than 20% when testing the co-digestion of food waste, sewage sludge and crude glycerol.

As regards intermediate compounds, a large amount of VFAs was produced during the tests. At the end of the BHP tests, the dominant species were acetic, butyric and propionic acids, the concentrations for each inoculum being shown in Fig. 1. Generally, hydrogen production via dark fermentation produces acetic and butyric acids as by-products [38]. Butyric acid was predominant in Test 2 (using SI), which presents a higher hydrogen yield. Luo et al. [39]and Chen et al. [40] also found that the highest hydrogen production was obtained when butyric acid predominated. Butyric acid-type fermentation is considered one of the most effective pathways for hydrogen production [18]. On the other hand, TSI showed the highest production of propionic acid, which is detrimental for hydrogen production [20]. Tyagi et al. [3] found that hydrogen yield decreases with increasing propionic acid concentration.

3.2. Biogas production

Fig. 2 shows the cumulative hydrogen production for sludge-vinasse co-digestion with different inocula. In all the BHP tests, hydrogen production commenced in the first hours, as the lag phase was short. Furthermore, the biogas generated in all three tests was composed of hydrogen and carbon dioxide, no methanogenic activity being observed

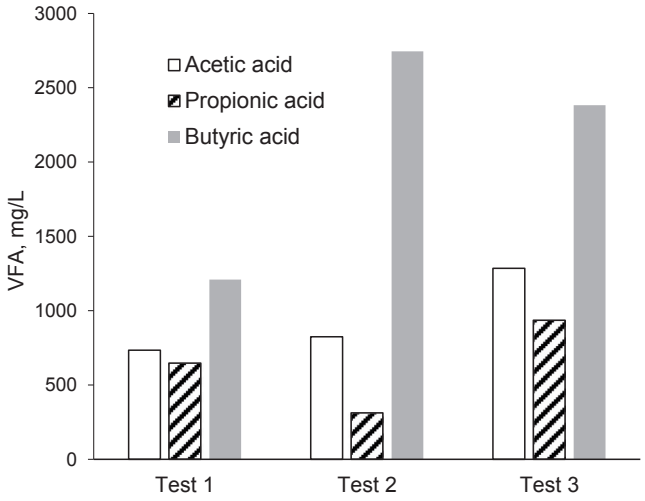

Fig. 1. Volatile fatty acids generated during the tests.

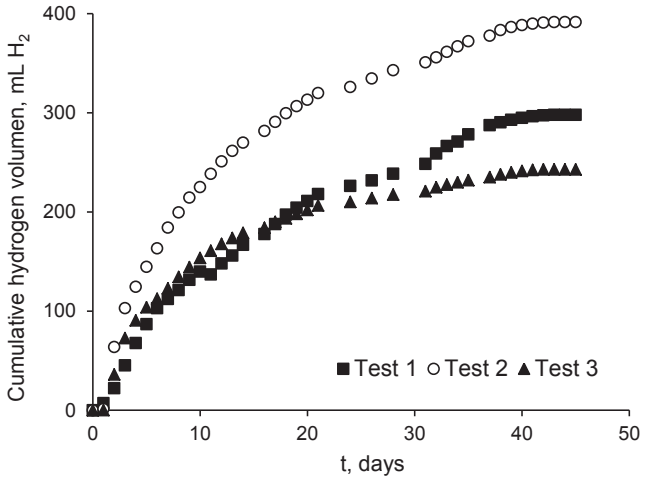

Fig. 2. Cumulative hydrogen production during the operating of batch reactors with different inocula.

(i.e. the biogas was methane free). All this is due to the fact that the pH values fell within the 5–6 range, which is optimal to enhance H_2 generation and avoid methanogenesis [19].

The sludge inoculum led to the highest maximum accumulated H_2 volume (391 mL H_2) compared to the acidogenic inoculum (298 mL H_2) and the thermally pre-treated inoculum (TSI) (243 mL H_2). In terms of H_2 yield (as per millilitres of hydrogen per gram of volatile solids of the substrate initially added to each reactor), the highest value was also achieved at the test using the SI (177 mL H_2/g VS_{added}), corresponding to an increase of 21 and 36% in relation to that obtained in the Test 1 (146 mL H_2/g VS_{added}) and the Test 3 (130 mL H_2/g VS_{added}) (Fig. 3). According to these results, hydrogen production is inhibited rather than enhanced when the inoculum is submitted to a thermal pre-treatment with the purpose of inactivating H_2-consuming *Archaea* and avoiding methane generation, as proposed by several authors [2,18–24,26]. These results are concordant and discrepant at the same time with those collected in the literature. Thus, Luo et al. [39] also observed this

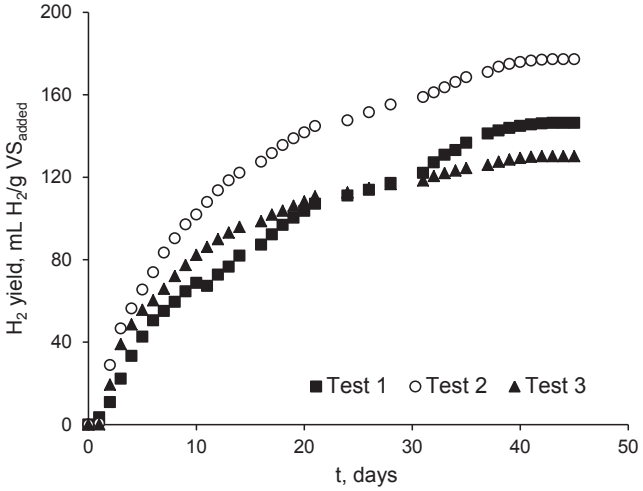

Fig. 3. Hydrogen yield for batch tests using different inocula.

tendency, the best condition was without any inoculum treatment. However, Albanez et al. [41] observed a slight improvement was noticed when performing the inoculum heat shock pretreatment in the co-digestion of vinasse and molasses. In a recent study, Lovato et al. [20] subjected the inoculum used in the co-digestion of cheese whey and glycerin to a heat shock pre-treatment (90 °C for 10 min), obtaining significantly higher values for hydrogen productivities and yields than using untreated inoculum. In other studies using the same inoculum though treating glycerin-based wastewater, the thermally pre-treated inoculum was not found to be significantly different from the untreated sludge in terms of molar productivity and molar hydrogen yield [24]. It is important to emphasize that the last three studies were done in AnSBBR at mesophilic conditions.

3.3. Hydrogen production rate

In order to ease identification of differences between the inocula, the hydrogen production rate of the first ten days is shown in Fig. 4. As for the SI and STI inocula behaved similarly with a significant lead of SI inoculum. This could be expected because both inocula have the same source. As for AI inoculum, a broader and lower peak than in the other inocula was detected. The maximum hydrogen production rate observed in the tests 1 and 2, with the AI and STI inocula, reached the peak after three days, and amounted to 11 mL H_2/(gVS_{added} d) and 20 mL H_2/(gVS_{added} d), respectively. On the other hand, the maximum

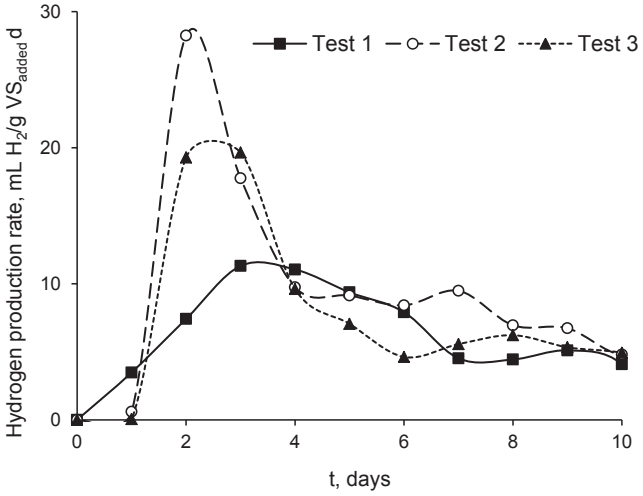

Fig. 4. Hydrogen production rate for batch tests using different inocula.

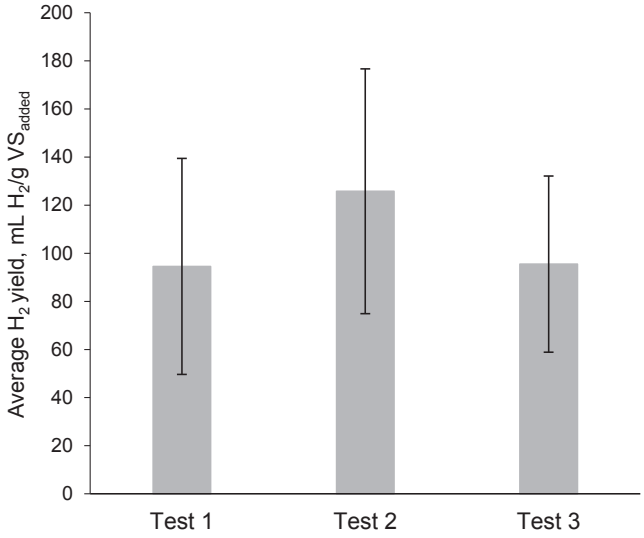

Fig. 5. Average hydrogen yield for batch tests using different inocula with standard deviation.

hydrogen production rate observed in the Test 2 with the sludge inoculum reached the peak at about the second day of experimentation, amounting to 28 mL H_2/(gVS_{added} d). As could be expected, the highest maximum hydrogen production rate was noticed in the experiment with higher hydrogen yield. Lavagnolo et al. [42] claim that generally, faster production rates are associated with higher production yields even though their results disagree with this assertion. Although there are studies that analyse the hydrogen production rate [18,19], no data was found in the literature concerning the effect of inoculum on hydrogen production rate.

3.4. Statistical analysis

Fig. 5 shows the average of hydrogen yield produce to each inoculum with their standard deviation. In order to evaluate differences between results of the three inocula, hydrogen yield and hydrogen production rate results were analysed statistically by single-factor analysis of variance (ANOVA). Table 5 shows the results of this analysis. A confidence level of 95% was selected for all comparisons.

In the matter of hydrogen yield, for the comparison between inocula, the p value is smaller than 0.05 in all cases, therefore there is significant difference between the yields of hydrogen produced in SI inoculum and those of the other two.

Conversely, for the hydrogen production rate there is no significant difference ($p > 0.05$) between all of the three tested inocula.

3.5. Microbial population dynamics

The concentrations of microorganisms in the samples before and after the different tests were studied. The amounts and relative percentages of the main microbial groups are shown in Table 4. In Test 2, in which the highest hydrogen yield (177 mL H_2/g VS_{added}) was obtained, the population size increased during the time of experimentation. Instead, in Test 1 and Test 3, the population size remained stable at the end of the BHP tests in all cases; significantly, the amount of substrate for acidogenic phase was sufficient. *Eubacteria* was the major phylogenetic domain in all cases. It is possible that AI (Test 1), which was already producing hydrogen, during the experiment the microbial population is steady and there is no increase. On the other hand, SI (Test 2), which was previously producing methane, by inhibiting methanogenic *Archaea*, the bacteria that undergo a better adaptation to the new conditions are *Eubacteria* and microbial growth is favoured. In the case of TSI (Test 3), the thermal pretreatment may have removed

Table 5
ANOVA results for the hydrogen yield and hydrogen production rate.

		Degrees of freedom	Sum of squares	Mean square	F value	P value
Hydrogen yield	Inoculas AI and SI	1	18,559	18,559	8.057	0.00585
	Inoculas SI and STI	1	17,435	17,435	8.865	0.00393
	Inoculas AI and STI	1	18	17.6	0.01	0.919
Hydrogen production rate	Inoculas AI and SI	1	12.5	12.53	0.691	0.409
	Inoculas SI and STI	1	29.1	29.13	1.238	0.269
	Inoculas AI and STI	1	3.5	3.452	0.249	0.619

part of the microorganisms present in the SI (Test 2)

No significant variation was found in *Eubacteria: Archaea* ratios at the beginning and end of the experiments in Test 1 or Test 3: 41–46% and 59–54%, respectively. In Test 2, however, the percentages of *Eubacteria* increased from 59% to 92%. Thus, BHP test with sludge inoculum could increase the abundance of the specific bacteria in the reactor, which were beneficial for the hydrogen production.

Although methane is not generated, the analyses showed the largest number of *Archaea* present. In terms of productivity, it may be stated that *Archaea* were inactive [28].

4. Conclusions

H_2 generation from sludge vinasse co-digestion, using different inocula, was studied. The batch tests were successfully in all cases. Significant differences have been found in the production of hydrogen among the three inoculums. The highest hydrogen yield, 177 mL H_2/g VS_{added}, was obtained with a sludge inoculum. This means that, in terms of hydrogen yield, a sludge inoculum is to be preferred in the BHP tests. Even though, Eubacteria was the major phylogenetic domain in all cases, sludge inoculum showed a greater growth of Eubacteria during the test, increasing the percentage of this population from 59.2 to 92.1. The rate of hydrogen production was comparable between the different inocula, that is, the duration of the test is independent of the type of inoculum used. Furthermore, hydrogen production was chiefly accompanied by the production of acetic and butyric acids.

Acknowledgements

This research was supported by the Spanish Ministry of Economy, Industry and Competitiveness, specifically via project CTM2015-64810R entitled “Coproducción de hidrógeno y metano mediante codigestión de biosólidos y vinazas”, financed by the European Regional Development Fund (ERDF).

References

[1] Silva Benavides AM, Rudin MC, Villalobos N, Touloupakis E, Torzillo G. Growth and hydrogen production by three Chlamydomonas strains cultivated in a commercial fertilizer. Int J Hydrogen Energy 2019;4. https://doi.org/10.1016/j.ijhydene.2018.11.209.

[2] Yang G, Wang J. Co-fermentation of sewage sludge with ryegrass for enhancing hydrogen production: performance evaluation and kinetic analysis. Bioresour Technol 2017;243:1027–36. https://doi.org/10.1016/j.biortech.2017.07.087.

[3] Tyagi VK, Angériz Campoy R, Álvarez-Gallego CJ, Romero García LI. Enhancement in hydrogen production by thermophilic anaerobic co-digestion of organic fraction of municipal solid waste and sewage sludge – optimization of treatment conditions. Bioresour Technol 2014;164:408–15. https://doi.org/10.1016/j.biortech.2014.05.013.

[4] Seo YH, Yun YM, Lee H, Han JI. Pretreatment of cheese whey for hydrogen production using a simple hydrodynamic cavitation system under alkaline condition. Fuel 2015;150:202–7. https://doi.org/10.1016/j.fuel.2015.01.100.

[5] Pecorini I, Baldi F, Iannelli R. Biochemical hydrogen potential tests using different inocula. Sustainability 2019;11:1–17. https://doi.org/10.3390/su11030622.

[6] Paudel S, Kang Y, Yoo YS, Seo GT. Effect of volumetric organic loading rate (OLR) on H2and CH4 production by two-stage anaerobic co-digestion of food waste and brown water. Waste Manage 2017;61:484–93. https://doi.org/10.1016/j.wasman.2016.12.013.

[7] Yang G, Wang J. Enhancement of biohydrogen production from grass by ferrous ion and variation of microbial community. Fuel 2018;233:404–11. https://doi.org/10.1016/j.fuel.2018.06.067.

[8] Assawamongkholsiri T, Reungsang A, Pattra S. Effect of acid, heat and combined acid-heat pretreatments of anaerobic sludge on hydrogen production by anaerobic mixed cultures. Int J Hydrogen Energy 2013;38:6146–53. https://doi.org/10.1016/j.ijhydene.2012.12.138.

[9] Cappai G, De Gioannis G, Friargiu M, Massi E, Muntoni A, Polettini A, et al. An experimental study on fermentative H2 production from food waste as affected by pH. Waste Manage 2014;34:1510–9. https://doi.org/10.1016/j.wasman.2014.04.014.

[10] Akhlaghi M, Boni MR, De Gioannis G, Muntoni A, Polettini A, Pomi R, et al. A parametric response surface study of fermentative hydrogen production from cheese whey. Bioresour Technol 2017;244:473–83. https://doi.org/10.1016/j.biortech.2017.07.158.

[11] Sivagurunathan P, Kumar G, Kobayashi T, Xu K, Kim SH. Effects of various dilute acid pretreatments on the biochemical hydrogen production potential of marine macroalgal biomass. Int J Hydrogen Energy 2017;42:27600–6. https://doi.org/10.1016/j.ijhydene.2017.05.106.

[12] Peixoto G, Pantoja-Filho JLR, Agnelli JAB, Barboza M, Zaiat M. Hydrogen and methane production, energy recovery, and organic matter removal from effluents in a two-stage fermentative process. Appl Biochem Biotechnol 2012;168:651–71. https://doi.org/10.1007/s12010-012-9807-4.

[13] Mamimin C, Kongjan P, O-Thong S, Prasertsan P. Enhancement of biohythane production from solid waste by co-digestion with palm oil mill effluent in two-stage thermophilic fermentation. Int J Hydrogen Energy 2019;44:3347–55. https://doi.org/10.1016/j.ijhydene.2018.08.021.

[14] Pan Y, Zhi Z, Zhen G, Lu X, Bakonyi P, Li YY, et al. Synergistic effect and biodegradation kinetics of sewage sludge and food waste mesophilic anaerobic co-digestion and the underlying stimulation mechanisms. Fuel 2019;253:40–9. https://doi.org/10.1016/j.fuel.2019.04.084.

[15] Li Z, Chen Z, Ye H, Wang Y, Luo W, Chang JS, et al. Anaerobic co-digestion of sewage sludge and food waste for hydrogen and VFA production with microbial community analysis. Waste Manage 2018;78:789–99. https://doi.org/10.1016/j.wasman.2018.06.046.

[16] Rivero M, Solera R, Perez M. Anaerobic mesophilic co-digestion of sewage sludge with glycerol: enhanced biohydrogen production. Int J Hydrogen Energy 2014;39:2481–8. https://doi.org/10.1016/j.ijhydene.2013.12.006.

[17] Otieno B, Apollo S, Kabuba J, Naidoo B, Simate G, Ochieng A. Ozonolysis pretreatment of waste activated sludge for solubilization and biodegradability enhancement. J Environ Chem Eng 2019;7:1–8. https://doi.org/10.1016/j.jece.2019.102945.

[18] Silva FMS, Oliveira LB, Mahler CF, Bassin JP. Hydrogen production through anaerobic co-digestion of food waste and crude glycerol at mesophilic conditions. Int J Hydrogen Energy 2017;42:22720–9. https://doi.org/10.1016/j.ijhydene.2017.07.159.

[19] Silva FMS, Mahler CF, Oliveira LB, Bassin JP. Hydrogen and methane production in a two-stage anaerobic digestion system by co-digestion of food waste, sewage sludge and glycerol. Waste Manage 2018;76:339–49. https://doi.org/10.1016/j.wasman.2018.02.039.

[20] Lovato G, Albanez R, Stracieri L, Ruggero LS, Ratusznei SM, Rodrigues JAD. Hydrogen production by co-digesting cheese whey and glycerin in an AnSBBR: temperature effect. Biochem Eng J 2018;138:81–90. https://doi.org/10.1016/j.bej.2018.07.007.

[21] Lazaro CZ, Perna V, Etchebehere C, Varesche MBA. Sugarcane vinasse as substrate for fermentative hydrogen production: the effects of temperature and substrate concentration. Int J Hydrogen Energy 2014;39:6407–18. https://doi.org/10.1016/j.ijhydene.2014.02.058.

[22] Torquato LDM, Pachiega R, Crespi MS, Nespeca MG, de Oliveira JE, Maintinguer SI. Potential of biohydrogen production from effluents of citrus processing industry using anaerobic bacteria from sewage sludge. Waste Manage 2017;59:181–93. https://doi.org/10.1016/j.wasman.2016.10.047.

[23] Venkata Mohan S, Vijaya Bhaskar Y, Murali Krishna P, Chandrasekhara Rao N, Lalit Babu V, Sarma PN. Biohydrogen production from chemical wastewater as substrate by selectively enriched anaerobic mixed consortia: Influence of fermentation pH and substrate composition. Int J Hydrogen Energy 2007;32:2286–95. https://doi.org/10.1016/j.ijhydene.2007.03.015.

[24] Lovato G, Moncayo Bravo IS, Ratusznei SM, Rodrigues JAD, Zaiat M. The effect of organic load and feed strategy on biohydrogen production in an AnSBBR treating glycerin-based wastewater. J Environ Manage 2015;154:128–37. https://doi.org/10.1016/j.jenvman.2015.02.014.

[25] Shi XY, Jin DW, Sun QY, Li WW. Optimization of conditions for hydrogen production from brewery wastewater by anaerobic sludge using desirability function

approach. Renew Energy 2010;35:1493–8. https://doi.org/10.1016/j.renene.2010.01.003.
[26] Giordano A, Cantù C, Spagni A. Monitoring the biochemical hydrogen and methane potential of the two-stage dark-fermentative process. Bioresour Technol 2011;102:4474–9. https://doi.org/10.1016/j.biortech.2010.12.106.
[27] Fernandes BS, Peixoto G, Albrecht FR, Saavedra del Aguila NK, Zaiat M. Potential to produce biohydrogen from various wastewaters. Energy Sustain Dev 2010;14:143–8. https://doi.org/10.1016/j.esd.2010.03.004.
[28] Montañés R, Solera R, Pérez M. Anaerobic co-digestion of sewage sludge and sugar beet pulp lixiviation in batch reactors: effect of temperature. Bioresour Technol 2015;180:177–84. https://doi.org/10.1016/j.biortech.2014.12.056.
[29] Zahedi S, Solera R, García-Morales JL, Ennouri H, Sales D. Evaluation of the effect of glycerol supplementation on the anaerobic digestion of real municipal solid waste in batch mode. Fuel 2017;193:15–21. https://doi.org/10.1016/j.fuel.2016.12.024.
[30] Standard methods for the examination of water and wastewater. In: APHA, AWWA, WPCF. 22th Ed Washingt Am Public Heal Assoc 2012.
[31] Zahedi S, Sales D, García-Morales JL, Solera R. Obtaining green energy from dry-thermophilic anaerobic co-digestion of municipal solid waste and biodiesel waste. Biosyst Eng 2018;170:108–16. https://doi.org/10.1016/j.biosystemseng.2018.04.005.
[32] Montero B, García-Morales JL, Sales D, Solera R. Analysis of methanogenic activity in a thermophilic-dry anaerobic reactor: use of fluorescent in situ hybridization. Waste Manage 2009;29:1144–51. https://doi.org/10.1016/j.wasman.2008.08.010.
[33] Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 1990;56:1919–25. https://doi.org/10.1128/AEM.67.1.142.
[34] Amann RI, Krumholz L, Stahl DA. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 1990;172:762–70. https://doi.org/10.1128/jb.172.2.762-770.1990.
[35] Speece RE. Anaerobic biotechnology for industrial wastewaters. Archae Press; 1996.
[36] Zahedi S, Solera R, García-Morales JL, Sales D. Effect of the addition of glycerol on hydrogen production from industrial municipal solid waste. Fuel 2016;180:343–7. https://doi.org/10.1016/j.fuel.2016.04.063.
[37] Zahedi S, Sales D, Romero LI, Solera R. Optimisation of the two-phase dry-thermophilic anaerobic digestion process of sulphate-containing municipal solid waste: population dynamics. Bioresour Technol 2013;148:443–52. https://doi.org/10.1016/j.biortech.2013.09.002.
[38] Nualsri C, Reungsang A, Plangklang P. Biochemical hydrogen and methane potential of sugarcane syrup using a two-stage anaerobic fermentation process. Ind Crops Prod 2016;82:88–99. https://doi.org/10.1016/j.indcrop.2015.12.002.
[39] Luo G, Xie L, Zou Z, Wang W, Zhou Q. Evaluation of pretreatment methods on mixed inoculum for both batch and continuous thermophilic biohydrogen production from cassava stillage. Bioresour Technol 2010;101:959–64. https://doi.org/10.1016/j.biortech.2009.08.090.
[40] Chen CC, Lin CY, Lin MC. Acid-base enrichment enhances anaerobic hydrogen production process. Appl Microbiol Biotechnol 2002;58:224–8. https://doi.org/10.1007/s002530100814.
[41] Albanez R, Lovato G, Zaiat M, Ratusznei SM, Rodrigues JAD. Optimization, metabolic pathways modeling and scale-up estimative of an AnSBBR applied to biohydrogen production by co-digestion of vinasse and molasses. Int J Hydrogen Energy 2016;41:20473–84. https://doi.org/10.1016/j.ijhydene.2016.08.145.
[42] Lavagnolo MC, Girotto F, Rafieenia R, Danieli L, Alibardi L. Two-stage anaerobic digestion of the organic fraction of municipal solid waste – effects of process conditions during batch tests. Renew Energy 2018;126:14–20. https://doi.org/10.1016/j.renene.2018.03.039.