Biohydrogen Production through Solid Substrate Fermentation of Organic Municipal Wastes: a Multivariable Evaluation

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ABSTRACT

Municipal solid waste (MSW) generation is a constant problem on growing cities. The organic fraction (OFMSW), ca. 60% of MSW, is being literally 'wasted' despite its applicability on energy production. Therefore, this work was focused on hydrogen production from the OFMSW with a double purpose: *(i)* to evaluate the effect of the total solids content (20.9 and 35% TS), temperature (35 and 55 ºC) and mass retention time (MRT, 21 and 14 d) on semi-continuous fermentation, and *(ii)* to test the supplementation with nutrient nitrogen in the form of waste activated sludge in batch mini-reactors.

Firstly, in the semi-continuous fermentation, it was found that factors had significant influence on hydrogen productivity in the order: total solids > MRT > temperature. Significant interactions amidst factors were only observed between TS and temperature or MRT. Indeed, best hydrogen productivity averaged up to 123 NmL $H_2/(kg_{wmr}$ d) at 20.9 %TS, 55 ºC and 21 d MRT. Secondly, in the batch fermentation, supplementation with nitrogen in the form of activated sludge did not show a significant effect. Highest results were P_{H2} = 1 983 μmol_{H2}/gVS and R_{H2} = 68.3 μmol_{H2}/(gVS h) in the mini-reactors without addition of alkalinity or sludge. No significant lag phase was observed in none of the experimental units. Microorganisms introduced through supplemented sludge might have affected fermentation, particularly boosting hydrogen consumption.

In general, variations and inhibition of hydrogen production were related to low pH and lactic acid and solvent deviation of the fermentation. This was in agreement with reports of a strong correlation between high lactic acid concentrations and inhibition of hydrogenogenesis.

1. Introduction

The incoming oil age decline has promoted over 15 years of intensive research for developing alternative energy technologies; hydrogen production is among them [1]. One of the promising technologies for hydrogen production is through the dark fermentation of biomass [2]. For the sake of process economic feasibility, it has been suggested that the substrate ought to be inexpensive and inexhaustible [1, 3]. Indeed, municipal solid wastes are a major source of biomass as organic wastes (OFMSW): in Mexico, 102 000 ton/day are produced and its the organic fraction (paper and food wastes) represents up to 60% of the total [4].

OFMSW are biosolids with 50-70% water contents, yet its application to biofuel production has been mainly studied at low solids (<10% TS) fermentation [5]. It is noteworthy that submerged culture under such conditions employs large dilution water volumes, thus generating polluted wastewaters. Although less studied, solid substrate fermentation (SSF) has advantages such as reactor volume reduction, no leachate generation and high H_2 productivities [6, 7].

Although important investigations have determined the factors influencing the process performance (such as solid contents, temperature and mass retention time), there is a lack of comprehensive experiments in the open literature embracing all these factors in such a way to find interactions and the optimal operating conditions. For instance, high solids content means more OFMSW being treated but also a possibility of osmotic stress to microorganisms. Due to the complexity of the temperature strong influence on biochemical processes and microbiological interactions, a consensus on which thermal regime is better has not yet been determined. Furthermore there is a wide variability amidst processes, inocculum and substrates employed. Moreover, comparisons of mesophilic and thermophilic regimes are still scarce in high solids semi-continuous hydrogenogenesis process, as most studies have operated bioreactors mainly in the mesophilic range of temperature [7-11].

On the other hand, the organic load or mass retention time (MRT) can significantly affect the organic matter available to microorganisms and metabolic deviations from hydrogenogenic conditions [7, 8, 12]. Despite its importance, the information on the effect of MRT on solid substrate dark fermentation is still scarce, except for recent reports from our group [5, 13].

Moreover, it has been supposed that OFMSW by itself provides all the nutrients required for dark fermentation. However, carbon to nitrogen (C/N) ratio of municipal organic wastes is usually high, up to 76 [14], yet for anaerobic fermentation the suggested C/N ratio is 25-35 [15]. This may suggest that nitrogen may be supplemented as ammonia or in the form of organic nitrogen, such as manure, waste sludges, or food wastes [16, 17].

Therefore, this work was focused on the hydrogen production from the OFMSW in SSF with a double purpose: *(i)* to evaluate the effect of the total solids content (20.9 and 35% TS), temperature (35 and 55 ºC) and mass retention time (MRT, 21 and 14 d) on semi-continuous fermentation, and *(ii)* to evaluate the supplementation with nutrient nitrogen in the form of waste activated sludge.

2. Materials and methods

2.1. Effect of total solids, temperature and mass retention time on SSF

First hydrogenogenic set of experiments was carried out in glass bioreactors containing 500 g biosolids [5]. The OFMSW consisted of dried food wastes from cafeteria (60% w/w) and waste office paper (40% w/w). It was implemented a 2^3 experimental design for evaluating the effect of the total solids content (TS, 20.9 or 35 %), temperature (35 or 55 ºC) and mass retention time (MRT, 21 and 14 d) on semi-continuous hydrogen production.

2.2. Nitrogen and alkalinity supplementation at thermophilic and mesophilic regimes

The organic nitrogen and alkalinity supplementation studies were performed on batch, solid substrate anaerobic hydrogenogenic fermentation in intermittently vented mini-reactors (SSAHF-IV) containing 80 g biosolids, according to set-up described by Muñoz-Paez *et al*. [13]. Experimental design basis was 2^3. Factors were temperature (35, 55 ºC), C/N ratio (basal 50, 30 adjusted with activated sludge), and alkalinity (none, 0.06 g $CaCO_3$/g dry substrate with phosphate salts).

2.3. Analyses

Analyses were performed to solid samples taken from OFMSW and bioreactors. Volatiles solids (VS) and total solids (TS), pH, volatile organic acids (VOA), lactic acid and solvents were analyzed as reported elsewhere [18]. Biogas production was measured by acid brine displacement [5]; gas volumes were normalized to 273 K and 101.15 kPa (reported as NmL or NL). H_2 and CH_4 contents were determined in a GOW-MAC gas chromatograph model 350 fitted with TCD and a Molecular Sieve 5A packed column. Water availability of these mixtures was determined with a thermohigrometer Thermoconstanter Humidat RTD-33/TH-2 (Novasina, Zurich, Switzerland), according to specifications in manual. Analyses were performed by triplicate.

2.4 Response variables

Acetate to butyrate ratios A/B (on COD basis) is an indirect indicator of hydrogenogenesis. Theoretically, acetic fermentation from hexose would yield four mol of hydrogen whereas butyric yields only two. If we assume that 50% of hexose is fermented to H_2 plus acetic acid whereas the other 50% is fermented to H_2 plus butyric acid, then A/B relationship would be 0.802 (on COD basis). Consequently, a value greater than 0.802 would mean that hydrogen generation coupled to acetic acid production was favored [9].

The ρ ratio indicates whether metabolism was leaned to acidogenesis or solventogenesis. This parameter is the product of the sum of the organic acids, divided by the sum of the solvents. Low rho values would be associated to solventogenesis, and consequently, related to poor hydrogen generation [19, 20]. Lactic acid is considered also an important parameter in dark hydrogen fermentation. Lactic acid fermentation is known to act as a hydrogen sink similarly to solvents [5].

3. Results and discussion

3.1 Effect of total solids, temperature and mass retention time on SSF

It was observed that hydrogen productivities (I_{H2}) were positively influenced by 20.9% TS, 21 d MRT and thermophilic regime. Indeed, I_{H2} averaged up to 123 NmL H_2/(kg$_{wmr}$ d) with factor 20.9% TS (Table 1). The I_{H2} in thermophilic regime was higher to that in mesophilic operation (Table 1). In general, the highest I_{H2} were related to the highest organic acids to solvent ratios (ρ) and the lowest production of lactic acid. According to Table 2, the factors were significant to I_{H2} in the order TS > MRT > temperature. Significant interactions ($p < 0.0554$) amidst factors only occurred between TS and temperature or MRT (Table 2). Acetate to butyrate ratios A/B (on COD basis) were 0.62 to 0.68, meaning that hydrogen fermentation with butyric acid generation was the prevailing one in all the reactors.

Effect of total solids

Total solids (TS) influence on response variables (Table 1) was very pronounced. During operation, hydrogen contents reached up to 40 - 50% in selected bioreactors working at 20.9% TS. Reactors at 35% TS were below 25% H_2. Furthermore, difference in I_{H2} amidst 21 and 35% TS was over 15 fold. Although the A/B ratios were similar, the production of VOA and solvents for each TS content was markedly different: the ΣVOA was 0.7 fold higher whereas the ΣSolvents was 3.4 fold lower. Consequently, the ρ for 20.9% TS was over 5 fold superior to that of 35% TS. The higher ρ in the 20.9% TS fermentation indicated acidogenic fermentation prevalence over solventogenic fermentation. Oppositely, the 35% TS fermentation showed a performance leaned to solventogenesis. Furthermore, the [HLac] was almost 4 fold superior for 35% TS. This was in agreement with reports of a strong correlation between high lactic acid concentrations and inhibition of hydrogenogenesis [5]. All these results clearly show the superiority of using 20.9% TS OFMSW.

Moreover, the water availability might be affecting the performance of the anaerobic fermentation, by the simple equation that the higher the TS, the lesser of water availability. The water activity (a_w) and water potential (Ψ) of the OFMSW in this work were 0.9300 (Ψ = -9.2 MPa) and 0.9550 (Ψ = -5.8 MPa) for 35 and 20.9 %TS respectively. The water available to microorganisms can decrease by the osmotic effect (interaction with solute molecules, ionic or not) and by the matric effect (*i.e.*, adsorption of water to the surfaces of solids) in SSF. Likely, both mechanisms could have contributed to low a_w and high Ψ in our OFMSW [21, 22]. Indeed, in soils an a_w of 0.90 is related to the inability of plants to withdraw water from soil (*i.e.*, permanent wilting point). Extra effort is required for a microbe to grow in a medium with low a_w because it must spend energy to maintain a high internal solute concentration in order to retain water [15]. Therefore it is likely that metabolism of most fermentative bacteria in our H-bioreactors was adversely affected by the low availability of water [23].

Table 1. Main effects of temperature, total solids and mass retention time on response variables

Factors	Levels	I_{H2} [a] (21)[b]	ΣVOA[c] (1195)[b]	ΣSolvents[d] (223)[b]	A/B [e] (0.1)[b]	ρ[f] (2.5)[b]	[HLac] [g] (1734)[b]
Thermal regime	35 ºC	55.3	11 285	1 415	0.68	8.5	12 639
	55 ºC	75.7	8 070	2 508	0.62	11.3	13 672
Mass retention time	14 d	48.1	10 154	1 957	0.62	10.5	14 470
	21 d	82.8	9 200	1 967	0.68	9.3	11 841
Total solids	20.9 %	122.9	12 186	888	0.65	16.3	5 432
	35 %	8.1	7 168	3 035	0.65	3.4	20 879

Notes: [a] Hydrogen productivity (NmL H_2/(kg_{wmr} d)); [b] standard error of the experimental design (EED = $(MSS_{error}/r)^{0.5}$); [c] Volatile organic acids accumulation (mg COD_{VOA}/kg_{wmr}); [d] Solvent accumulation (mg $COD_{solvent}/kg_{wmr}$); [e] Acetic acid to butyric acid ratio (mg $COD_{acetic\ acid}$/mg $COD_{butyric\ acid}$); [f] ΣVOA/ΣSolvents factor; [g] Lactic acid accumulation (mg $COD_{lactic\ acid}/kg_{wmr}$).

Table 2. Significance probability of the effects for the response variables in the H-stage

	I_{H2} [a]	ΣVOA[b]	ΣSolvents[c]	A/B[d]	ρ[e]	[Hlac][f]
Model	**0.0009**	**0.0013**	**< 0.0001**	0.4348	**0.0022**	**< 0.0001**
Temperature	0.1864	**0.0052**	**0.0001**	0.7423	0.1578	0.4239
MRT	**0.0396**	0.2919	0.9508	0.6387	0.5247	**0.0643**
TS	**< 0.0001**	**0.0003**	**< 0.0001**	**0.0623**	**< 0.0001**	**< 0.0001**
Temp x MRT	0.1361	0.6849	0.0630	0.7210	0.6227	0.6882
Temp x TS	0.1161	**0.0007**	**< 0.0001**	0.1871	**0.0079**	**0.0192**
MRT x TS	**0.0554**	0.4984	0.9452	0.8667	0.6397	0.7892
Temp x MRT x TS	0.1224	0.4595	0.1385	0.4849	0.5728	0.4327

Notes: Same keys as in Table 1.

Effect of Temperature of operation

The thermophilic temperature (55 ºC) had a positive but not significantly noticeable effect on I_{H2} (Table 2). Indeed, thermophilic regime had an increase of 36% in I_{H2} when compared to mesophilic regime (Table 1). Analysis of operation variables does not show a clear explanation for the difference on I_{H2}. For instance, the only significant differences amidst these thermal regimes only occurred for ΣVOA and ΣSolvents. Lactic acid concentrations in meso and thermo regimes were not significantly different. The ρ had higher but no significant different value in the thermophilic regime. A partial explanation was given by Youn and Shin [24], who compared H_2 production at thermophilic (55 ºC) and mesophilic (35 ºC) regimes in submerged dark fermentation of food waste (6.7 %). In that work they found that thermophilic operation had higher hydrogen production, and they ascribed this to the higher temperature in thermophilic reactors that helps reducing the dissolved hydrogen concentration, thus being more effective in releasing hydrogen than mesophilic reactors. Another plausible explanation might be the microbiological profiles fostered by temperature and substrate [25]. Thermophilic and mesophilic regimes exhibit different microbial communities. Two of the principal genera related to hydrogen production in thermophilic regimes are *Thermoanaerobacterium sp.* (more specifically *T. thermosaccharolyticus*) and *Clostridium thermocellum* [6, 26-29]. On the other hand mesophilic regime is more likely to show diverse hydrogen producing communities [30], which may include *Clostridium pasteurianum*, *C. acidisoli*, *C. butyricum*, *C. acetobutylicum*, [31-33].

Some authors claim the superiority of thermophilic processes over the mesophilic ones [27, 34, 35]. However, some other authors allege the mesophilic regimes to be more economically feasible process [3, 9, 36], or at least to have a larger infrastructure distribution [37, 38]. For instance, the net energy gain at both thermal regimes was negative in most hydrogenogenesis cases analyzed by Perera *et al.* [36]. As a measure to overcome negative energy balances, they proposed coupling either methanogenic process or direct electricity production via microbial fuel cells. Indeed, according to Escamilla-Alvarado *et al.* [5] the use of thermophilic regime is fully justified in coupled processes producing hydrogen and methane (H-M process) despite its higher power consumption for heating. With productivities of 202 mL $H_2/(kg_{wmr}$ d) at 21 d TRM and 55 ºC in hydrogenogenic reactor, and 2 023 NmL $CH_4/(kg_{wmr}$ d) at 55 ºC when feeding a methanogenic bioreactor with fermented solids from 14 d MRT hydrogenogenic reactor, the energetic balance proved that the net power was positive. Indeed, the net energy was twice (even higher) the energy invested in the H-M process. Moreover, the highest contribution to the energy balance corresponded to the methanogenic stage, which accounted for 95-98% of the total power potential.

Effect of Mass Retention Time

The MRT also had significant influence on I_{H2} (Table 2). The I_{H2} at 21 d MRT was 2 fold higher than hydrogen productivity at 14 d MRT (Table 1). It is known that hydrogen is produced during the exponential growth of hydrogenogenic microorganisms [39]. Although 14 d MRT regime provided a higher organic load to microorganisms, this was not reflected on a higher I_{H2}. This could have been a consequence of excessive substrate addition. For instance, [HLac] was higher in bioreactors operated at 14 d MRT, indicating a lactic deviation.

Furthermore, the high amount of metabolites produced could be used as substrate in, for example, methane production through dark fermentation (submerged or solid substrate), photoheterotrophic hydrogen production, etc. Solids remaining after metabolites extraction could also be further used. In this regard, Muñoz-Páez *et al.* [13] washed fermented solids from a first batch dark fermentation of OFMSW and subjected them to a second fermentation round (re-fermentation), thus obtaining 15% increase on total cumulative biohydrogen production (*ca.* 2.4 mmole H_2/mini-reactor). They ascribed this phenomenon to the elimination of inhibiting organic metabolites present at the end of the first batch.

Concentration of lactic acid in our bioreactors was very high compared to that reported in works in submerged fermentation [29, 31, 34, 40]. This pointed out that our SSF process showed a significant lactic deviation that probably could have impaired biohydrogen generation. At present, it is not clear if hydrogenogenesis failure was due to the lactic deviation or due to the secretion of bacteriocins by lactic acid bacteria (LAB), or both. Microorganisms such as *Lactobacillus* sp., *Enterococcus* sp. and *Bifidobacterium* spp. are identified as the main responsible for lactic acid production. LABs also produce bacteriocins, which are proteins with bactericidal activity directed against many Gram-positive bacteria including most renowned hydrogen producing microorganisms, those belonging to the *Clostridium* genus [41]. Neverthelesss, lactic acid could be extracted from the solid matrix and be used in other applications such as food, pharmaceutical, leather and cosmetic industries, and as precursor for biodegradable plastic [42].

3.2. Nitrogen and alkalinity supplementation at thermophilic and mesophilic regimes

Highest results of hydrogenogenesis performance in batch experiments in terms of cumulative hydrogen production (P_{H2}) and hydrogenogenesis rate (R_{H2}) were respectively 1 983 µmol$_{H2}$/g VS and 68.3 µmol$_{H2}$/(g VS h), which belonged to the thermophilic mini-reactor with no addition of alkalinity nor sludge (TOO treatment in Table 3). Operation of batch process was carried out for 250-450 h. Controls did not show significant hydrogen production.

One and two cycles of hydrogen production were obtained. Exponential hydrogen production from OFMSW fermentation was evident up to first 50 h (Fig. 1 and 2). This result was similar to that of Watanabe and Yoshino [40], who had its maximum production of hydrogen near the 50 h of batch glucose fermentation. Indeed, in our work 75% of the P_{H2} was reached during a similar period. It is known that the microorganisms of genera *Clostridia* produce hydrogen during its exponential growth phase. When stationary phase is reached, the metabolism is shifted towards solvent generation [25]. Once the stationary production of hydrogen was reached, the mini-reactors were sparged with N_2 gas in order to overcome the partial pressure inhibition of hydrogen, and thus initiate a new cycle production. This technique generated a new cycle of hydrogenogenesis for the thermophilic regime but did not for the mesophilic one. Other works have used this strategy, thus obtaining from 2 [13, 43] to 5 hydrogen-producing cycles [44]. Despite the lesser hydrogenogenesis cycles, our cumulative P_{H2} was comparable and higher to those with higher number of cycles (Table 4). Another strategy to increase hydrogen production is through the already mentioned re-fermentation of the fermented solids from a hydrogenogenic batch [13].

During the first cycle of operation, P_{H2} and maximum R_{H2} were higher in thermophilic regime than mesophilic one (Fig. 1 and 2). No lag phase was observed: at 14 h most mini-reactors had hydrogen contents superior to 30%. In contrast, other works presented lag times up to 350 h for heat-shock treated hydrogenogenic inocula, and 180 h for

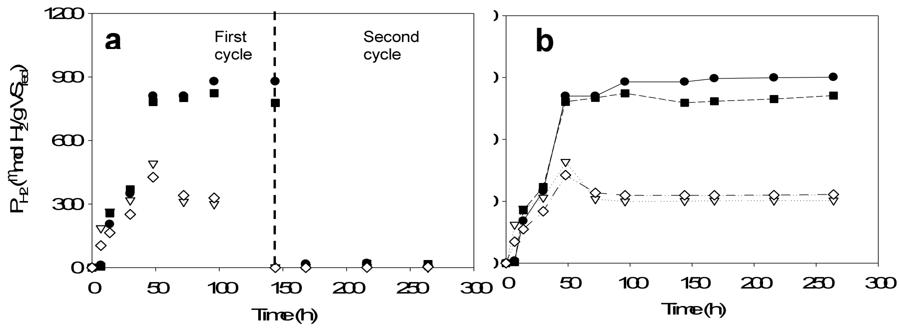

Figure. 1. Mesophilic hydrogen production: (a) cumulated per cycle, (b) cumulated sum. Keys: ●, no sludge or alkalinity addition; ▽, only sludge addition; ■, only alkalinity addition; ◇, sludge and alkalinity addition

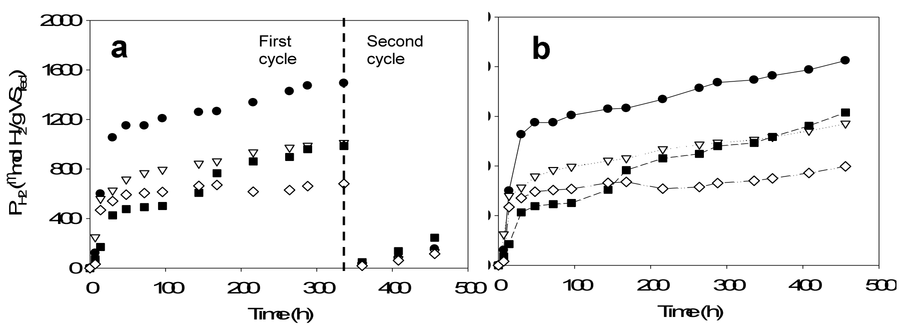

Figure. 2. Thermophilic hydrogen production: a) per cycle, b) cumulated. Keys: ●, no sludge or alkalinity addition; ▽, only sludge addition; ■, only alkalinity addition; ◇, sludge and alkalinity addition

Table 3. SSAHF-IV results

Reactors[a]	P_{H2}[b] cum (µmol H_2/g VS)	P_{H2}[b] cum (µmol H_2/reactor)	R_{H2}[c] max (µmol H_2/(g VS h))	pH_f	ΔpH	C/N
1 (MOO)	912 ± 83	14 311 ± 791	27.6 ± 3.8	5.66	2.50	44.5 ± 2.7
2 (MNO)	304 ± 35	5 253 ± 365	26.6 ± 2.4	5.87	2.02	29.3 ± 2.0
3 (MOA)	812 ± 179	12 742 ± 1 698	36.0 ± 9.2	6.07	1.34	44.8 ± 3.2
4 (MNA)	333 ± 74	5 752 ± 773	14.9 ± 2.0	6.25	1.11	31.4 ± 2.7
5 (TOO)	1 983 ± 17	30 931 ± 274	68.3 ± 10.4	6.20	1.62	47.2 ±5.0
6 (TNO)	1 488 ± 369	25 554 ± 6 337	44.2 ± 4.2	6.41	1.32	36.8 ± 6.0
7 (TOA)	1 785 ± 124	27 840 ± 1 948	16.0 ± 2.0	6.39	1.02	53.2 ±3.6
8 (TNA)	1 238 ± 130	21 265 ± 656	62.4 ± 5.9	6.86	0.39	38.9 ± 2.9
Control C	0.0 ± 0.0	0.0 ± 0.0	ND	5.71		25.7 ± 2.0
Control F	3.2 ± 0.2	50.4 ± 4.6	ND	5.38		34.6 ± 2.2

Notes: [a], M stands for mesophilic regime, T for thermophilic regime, N for sludge addition, A for alkalinity supplementation, O is no addition of sludge or alkalinity; [b] cumulated hydrogen production; [c], hydrogenogenesis rate.

Table 4. Batch hydrogenogenesis using organic waste

Operating conditions	P_{H2} cumulated (µmol H_2/g VS)	R_{H2} max (µmol H_2/(g VS h))	C/N	$pH_{initial}$	pH_{final}	Ref
OFMSW 25% TS, 55 ºC (4 cycles)	1 363	2.69	18.3	9.0	NR	[3]
OFMSW 24.5 % TS, 55 ºC	1 346	7.19	18.3	7.34[a]	NR	[3]
OFMSW 24.5 % TS, 35 ºC (2 cycles)	186	1.55	-	6.3[a]	NR	[43]
ASW 18.0 %TS, 35 ºC (5 cycles)	3 220	NR	19.0	6.65[a]	NR	[44]
OFMSW 20.9% TS, 55 ºC (2 cycles)	1 983	68.3	55.3	7.82	6.20	This work

Notes: [a] pH adjusted, NR, not reported

acetylene inhibition of methanogenic archaeas [3]. The P_{H2} in the second hydrogen producing cycle for our mini-reactors at thermophilic regime was in average, 10% of that obtained in their first cycles.

Effect of N nutrient supplementation

Apparently, the sludge supplementation affected negatively the cumulative P_{H2} ($p < 0.0008$), whereas it did not have any significant effect on R_{H2} ($p = 0.8$). The sludge addition was expected to favor the fermentative process by adjusting C/N ratio to 30 (according to literature). Negative results of sludge addition might be due to two reasons: *(i)* alkalinization of pH beyond the optimum for dark fermentation due to the release of ammonium cations, and *(ii)* contamination of culture by hydrogenotrophic microorganisms from activated sludge.

In the respect to the alkalinization explanation, it is known that excess nitrogen addition might be counterproductive since it may favor the production of ammonium in anaerobic environments [45]. Its generation does not only have the capability to increase the pH in the medium, but also to capture H^+ which could have been used for H_2 generation. For instance, glutamic amino acid hydrolysis may produce hydrogen and ammonium (Ec. 1). Lay *et al*. [46] reported hydrogen production from soluble condensed molasses (CMS), a by-product from molasses microbial fermentation, which contains high amounts of carbohydrates and amino acids as monosodium glutamate. Indeed, they ascribed their higher hydrogen production from CMS than from molasses either because CMS contained glutamate as a good substrate for hydrogen-producing bacteria, or because CMS contained other microorganisms that could convert glutamate into hydrogen. On the other hand, according to Strohm *et al*. (2007) reduction of nitrate to N_2 (denitrification) or to ammonia (nitrate ammonification) is the highest-energy-yielding process, and therefore a more favorable reaction (Eq. 2).

$$H_2NC(C_3H_5O_2)COO^- + 2H_2O \rightarrow 2CH_3COO^- + NH_4^+ + CO_2 + H_2 \quad (1)$$

$$C_6H_{12}O_6 + 3NO_3^- + 6H^+ \rightarrow 6CO_2 + 3NH_4^+ + 3H_2O \quad (\Delta G^{0'}=-1870 \text{ KJ/mol glucose}) \quad (2)$$

Regarding the second explanation, hydrogenotrophic bacteria could have been present in the sludge supplemented and thus have been introduced into our batch mini-reactors. Hydrogenogenesis, as many biological processes, is susceptible of microbial contamination and consequently to hydrogenogenic failure. For instance, Escamilla-Alvarado *et al*. [5] ascribed hydrogenogenesis unstability to lactic and solvent deviations. Moreover, at 72 h a drop on I_{H2} and presence of methane in the biogas in concentrations up to 9% v/v at 144 h, were observed on mesophilic mini-reactors supplemented with sludges (MNO and MNA treatments, Table 3). This could be due to the presence of hydrogenotrophic microorganisms in the sludge such as acetogenic and solventogenic bacteria, or even methanogenic archaea [5, 31, 32]. To overcome such problem, all the mesophilic min-reactors were subjected to a new thermal shock 93 ºC for one hour. From this pretreatment on, the hydrogen production was not re-started, yet methane production was utterly inhibited. The thermophilic mini-reactors received also the heat-shock pretreatment at 144 h just as a preventive measure. Methane production was not evident throughout their operation.

It is noteworthy that methane production at low pH is not usual. Yet, anaerobic digesters have also been reported to work at pH 4.0 - 5.3 when using acetic acid for methane production. Taconi *et al*. [47] ascribed this phenomenon to the simplicity of their feed, consisting only of acetic acid. However they did not discard the possibility of microenvironments at neutral pH, as previously proposed by de Beer *et al*. [48]. Indeed, in our case niches of high pH were likely to occur due to the physiology of the granules formed in our 20.9% TS solid substrate. Moreover, mixing in our bioreactors was poor. So according to mass transfer in biofilms and biological solids, diffusion of nutrients, salts, gas and heat diminish exponentially throughout the particles. These niches could be a protective against pH variations and excessive heat to microorganisms inside the particles [48].

Effect of thermal regime

The P_{H2} and R_{H2} were positively affected by thermophilic regime ($p < 0.0004$ and $p < 0.0025$, respectively). Mini-reactors in thermophilic regime averaged almost two-fold the I_{H2} and the R_{H2} of the mesophilic mini-reactors. In thermophilic regime after the exponential hydrogen production observed in the first cycle, a constant production rate occurred in all the mini-reactors (Fig. 2). This constant hydrogen production was kept even after gas sparging. Indeed, the exponential hydrogen production was not repeated in the second cycle. Final pH on mesophilic reactors was in the range 5.5 – 6.3. In mesophilic regime, run MOO showed the highest P_{H2} and R_{H2}, and also presented the lowest pH in mesophilic runs. Indeed, there was a correlation between pH and P_{H2}.

Regarding the methanogenic deviation already discussed, only the mesophilic mini-reactors supplemented with sludges exhibited methane production. Probably, thermophilic temperature could have served as a selective barrier that avoided hydrogenotrophic microorganisms progression as previously proposed by Youn and Shin [24].

Effect of alkalinity supplementation

Alkalinity in form of phosphate salts supplementation did not have a positive effect on P_{H2} and the R_{H2} ($p < 0.05$ and $p = 0.1$, respectively). Previous experiments from our workgroup reported that alkalinity 0.11 g CaCO3/g dry substrate on 20.9 %TS semi-continuous SSF of OFMSW had a positive influence on higher hydrogen productivity [49]. The supplementation of alkalinity performed in our study was 0.06 g $CaCO_3$/g dry substrate in order to control the pH variations. The negative effect in our work of alkalinity supplementation was probably due to an alkalinity that may have promoted an increase in osmotic pressure on the one hand, or a deviation from the optimal initial pH in the mini-reactors on the other hand, or even both [6]. It is noteworthy that pH in our mini-reactors was not adjusted previously to the beginning of fermentation. For instance, mesophilic mini-reactors had their initial pH in the range 7.36 – 8.16, whereas thermophilic ones were 7.25 – 7.82 (data not shown). This work demonstrated that heat-shocked inocula had the capacity to acidify the solid substrate thus favoring hydrogenogenesis. It was evident that mini-reactors supplemented with phosphate salts had a lower ΔpH, which was a result of buffer capacity provided by the salts. Therefore we ascribe the higher P_{H2} to a final pH closer to the optimal, *i.e.* a higher ΔpH. Nonetheless it was also likely that each thermal regime could have a different optimal pH.

4. Conclusion

In semi-continuous hydrogenogenic fermentation of OFMSW, high total solids content in the feedstock affected negatively the hydrogen productivity. The 21 d MRT promoted 2 fold higher hydrogen productivities compared to that at 14 d MRT. Thermophilic regime had higher but no significantly different productivities than mesophilic regime. It was observed that variations and inhibition of hydrogen production were mainly related to low pH, high lactic acid and solvents production.

Regarding the studies on N nutrient supplementation on batch hydrogenogenic fermentation of OFMSW, both the sludge and alkalinity supplementation did not have a positive effect on P_{H2} in any of the thermal regimes. It seems that the intrinsic C/N of OFMSW was sufficient to produce high amounts of hydrogen (P_{H2} = 1 983 µmol H_2/g VS, R_{H2} = 68.3 µmol H_2/(g VS h)). On the other hand, it could be inferred that microorganisms introduced through supplemented sludge may have affected fermentation, particularly boosting hydrogen consumption. Side exploration of the effect of temperature indicated that this factor had considerable influence on hydrogen production. Finally, despite the few or inexistent multiple cycles, the hydrogen productions in our work were comparable to those in literature of SSAHF-IV. It is very likely that there would be a maximum of hydrogen that might be harvested, independently of the cycles.

Several observations were identified from results in this research. Further hydrogen experiments should focus on increasing productivities and stability. Major efforts should be made towards:

i) Reducing/avoiding lactic deviation
ii) Increasing mixing in the bioreactors
iii) Bioaugmentation with hydrogenogenic fermentative bacteria from *Clostridium* genus

These technology improvements should be implemented in such a way that they do not compromise the economic feasibility of the process.

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Notation

ASW	agricultural solid waste
a_w	water activity
COD	chemical oxygen demand
LAB	lactic acid bacteria
MRT	mass retention time
OFMSW	organic fraction of municipal solid waste
SSAHF-IV	solid substrate anaerobic hydrogenogenic fermentetation in intermittently vented mini-reactors
SSF	solid substrate fermentation
TS	total solids
VOA	volatile organic acids
VS	volatile solids

Greek characters

ρ	VOA to solvents ratio
Ψ	water potential