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Inhibition by the ionic strength of hydrogen production from the Organic Fraction of Municipal Solid Waste

Florian Paillet[1,2,3*], Carole Barrau[1], Renaud Escudié[2], Eric Trably[2]

[1] TRIFYL, Route de Sieurac, 81300 Labessiere-Candeil, France

[2] LBE, Univ Montpellier, INRA, 102 avenue des Etangs, 11100, Narbonne, France

[3] Present address : Suez, Centre International de Recherche Sur l'Eau et l'Environnement (CIRSEE), 38 rue du Président Wilson, Le Pecq, France

[*]Corresponding author: florian.paillet@hotmail.fr

Abstract

Composition of the Organic Fraction of Municipal Solid Waste (OFMSW) in organic compounds and inorganic ions is highly variable and might impact the microbial activity in dark fermentation processes. In this study, the effect of the total amount of inorganic ions on fermentative hydrogen production was investigated. Batch experiments were carried out at pH 6 and under a temperature of 37°C. A freshly reconstituted organic fraction of municipal solid waste (OFMSW) was used as model substrate. At low concentrations in ammonium or chloride ions (2.9 - 5.1 $g.L^{-1}$, respectively), the hydrogen yield reached a maximum of 40.8±0.5. $mLH_2.gVS^{-1}$ and 25.1±5.6 $mLH_2.gVS^{-1}$. In contrast, at high total ionic concentrations of ammonium and chloride (11.1 - 35.5 $g.L^{-1}$ respectively), a strong inhibition of the fermentative microbial activity and more particularly hydrogen production, was observed. When considering the ionic strength of each ion, the effects of ammonia, chloride or a mixture of different ions (Na^+, K^+, H^+, Li^+, NH_4^+, Mn^{2+}, NH_4^+, Mg^{2+}, Cl^-, PO_4^{3-}, Br^-, I^-, SO_4^{2-}) showed very similar inhibitory trends regardless the type of ion or the composition of the ionic mixture. A threshold inhibitory value of the ionic strength was estimated at 0.75±0.13 M with a substantial impact on the fermentative activity from 0.81±0.12 M, with hydrogen yields of 18.1±3.3 and 6.2±4.1 $mLH_2.gVS^{-1}$, respectively.

Microbial community composition was also significantly impacted with a specific decrease in relative abundance of hydrogen-producing bacteria from the genus *Clostridium* sp. at high ionic strength.

Keywords: Biohydrogen; Dark fermentation; Total ammoniacal nitrogen; Ionic strength

1. Introduction

Nowadays, fossil fuels are the main resources in primary energy, and their usages have important effects on global warming. Due to a rapid decrease of their stocks and a constant increase of the energy demand, it is now necessary to find new alternatives that are not only sustainable and environment-friendly but also widely available. In this context, organic biomasses, including the organic fraction of municipal solid waste (OFMSW) are well adapted resources. In 2012, Hoornweg and Bhada-Tata [1] reported that urban cities generated about 1.3 billion tons of solid waste per year worldwide. This amount is expected to increase up to 2.2 billion tons in 2025. In addition, the organic fraction of municipal solid waste (OFMSW) has a high calorific value making the OFMSW highly suitable for bioenergy production [2,3]. Historically, methane has been the main gas biologically produced from OFMSW [3], due to the maturity of the technology in terms of performances and conversion efficiency with advanced automation and modelling [4]. Indeed, Anaerobic Digestion (AD) is a well-established biological process used to convert organic matter into a biogas that can further used as renewable energy. AD-based waste treatment plants are promoted by the European Parliament [5] to use “the waste as a source of energy”. In more recent years, hydrogen production by dark fermentation has gained an

50 increasing interest since H_2 is considered as an important energy carrier for next-generation
51 technologies towards decarbonation of the industrial sector [6]. Indeed, H_2 has a higher heat
52 of combustion (119.93 $kJ.g^{-1}$) than methane (50.02 $kJ.g^{-1}$) [7] and water is the only end-
53 product generated by its combustion or its use in fuel cells [8]. Hydrogen can also be used
54 as a fuel for vehicles and as a reactant for many chemical reactions (crack hydrocarbons,
55 sulfur or nitrogen compounds removing) [9]. The biological production of hydrogen by
56 dark fermentation is an intermediate step of AD and could be implemented in AD-based
57 plants treating OFMSW. During the dark fermentation process, hydrolytic bacteria convert
58 complex organic substrate into monomers which can be easily assimilated by fermentative
59 hydrogen producing bacteria (HPB), such as *Clostridium* sp. [10,11]. Many studies have
60 already shown the possibility to produce hydrogen from OFMSW, by using mixed culture
61 in continuous stirred tank or batch reactor, for a H_2 yield ranging from 20 to 70.1
62 $mLH_2.gVS^{-1}$ [10,12]. In AD treatment plants, hydrogen and methane can be both produced
63 by decoupling dark fermentation and methanogenesis in a two-stage AD process [14]. By
64 separating hydrolysis/acidogenesis and methanogenesis, each process can be optimized
65 with adequate operating conditions for improving the conversion of OFMSW into energy
66 [15]. In the first stage, 7.5 to 15% of the energy contained in the organic substrate can be
67 converted into hydrogen and co-metabolites, such as volatile fatty acids (VFAs) [16]. The
68 remaining organic matter and the VFAs can then be converted into methane in the second
69 stage in order to transform a maximum of anaerobically biodegradable organic matter into
70 energy.
71 However, hydrogen accumulation in a dark fermenter is often limited by the presence of
72 hydrogen-consuming anaerobes such as methanogens [17] or the accumulation of inhibitors

such as VFAs, chloride and total ammonia nitrogen (TAN) [18,19]. When using OFMSW as organic substrate, ammonia nitrogen and chloride ions are often found in high concentrations [20] due to, respectively, the decomposition of proteins and amino acids [21] and the addition of sodium chloride (salt) during food preparation. Ammonia nitrogen, defined as the sum of ammoniac gas (NH_3) and ammonium ions (NH_4^+), was already reported as an inhibitor of the microbial activity, especially under its non ionic form (NH_3) [22]. Ammonia inhibition has also been suggested on fermentative hydrogen production, but the effects are not yet well described. As an illustration, Salerno et al. [23] showed a negative effect on hydrogen production at ammonia nitrogen concentrations higher than 1.6 $gN.L^{-1}$, i.e. 0.4 $mgNH_3.L^{-1}$, in a continuous reactor. In contrast, a neutral effect on hydrogen yields in batch test was observed in the same study, even at higher concentration of ammonia nitrogen (1.3 and 13 $mgNH_3.L^{-1}$). Cavinato et al. [24] reported an inhibitory effect on hydrogen production with a change on metabolic pathways when ammonia nitrogen concentration increased from 0.97 to 1.98 $gN.L^{-1}$ in a two stage process treating food waste.

Halophilic conditions can also be detrimental to hydrogen production in particular in OFMSW rich in chloride ions. Pierra et al. [25] showed inhibition of the hydrogen production at NaCl concentrations higher than 9 $gNaCl.L^{-1}$ in the medium, using glucose as substrate.

To better understand the effect of these ions on the microbial activity in dark fermentation, the impact of ion addition on hydrogen production from OFMSW was investigated by decoupling the effect of a specific ion and the global ionic strength of the medium. The aim of this study was therefore (a) to evaluate the individual impact of ammonia and chloride

ion concentrations on hydrogen production, and (b) to investigate the impact of the total ionic strength on the fermentative metabolism with different types of ions.

2. Materials and Methods

2.1. Feedstock and inoculum preparation

A reconstituted organic fraction of MSW was freshly prepared and its composition was representative of the average composition of OFMSW collected in France on a yearly basis, according to the national characterization of MSW (MODECOM™, 1993). The proportions of each component are described in Table 1.

Table 1 : Average composition of the reconstituted OFMSW. Data are expressed in % of wet weight (% w/w).

Category	Components	% w/w
Food waste	Meat	7.0
	Coffee grounds	3.9
	Rice	4.3
	Potatoes	20.9
	Bread	5.1
	Yogurt	2.0
Garden waste	Grass	5.0
Papers	Papers	35.1
Cardboards	Cardboards	16.7

Meat, rice, potatoes and coffee grounds were first cooked and mixed with yogurt and bread. Garden waste, paper and cardboards were shredded and sieved at 1 cm. Total solid (TS) and volatile solid (VS) contents of the reconstituted OFMSW were 0.74±0.01 $gTS.g^{-1}$ and 0.63±0.01 $gVS.g^{-1}$, respectively.

113 Microbial anaerobic inoculum corresponded to a sample of an anaerobic lagoon treating
114 leachates from methanogenic storage cells in a real MSW landfill (TRIFYL). The initial pH
115 was 7.64, with a TS and VS concentration of 20.5±0.3 $gTS.L^{-1}$ and 7.4±0.2 $gVS.L^{-1}$,
116 respectively. Since the leachate initially contained a high quantity of ammonium ions
117 (4.02±0.79 $gN.L^{-1}$), a stripping pretreatment was carried out prior to experimentation.
118 Stripping corresponded to a flush of the liquid phase of the leachate with air during 3 hours
119 at pH 9, at a temperature of 90°C. Nitrogen removal efficiency was 95 %. Prior stripping,
120 the microbial community was removed by centrifugation (8000 rpm during 30 min) to not
121 be affected by the stripping treatment of the leachate. At the end of the stripping treatment,
122 the temperature was stabilized at 25°C, the pH adjusted at 6 and the microbial community
123 was reintroduced.
124
125 2.2. Experimental procedure
126
127 The batch reaction was carried out in a 500 mL flask with a working volume of 400 mL.
128 Each experiment was carried out at 37°C. Culture medium contained 9.25 gVS of OFMSW
129 inoculated with 136 g of stripping-pretreated leachate diluted with 249.4 g of water to reach
130 a S/X (substrate/biomass) ratio of 10 (on VS basis) [26]. pH was adjusted at 6 with NaOH
131 (1 M). Batch reactors were then flushed with N_2 before experiment to remove oxygen
132 traces. For each experiment, different solutions of ions were added to obtain a large range
133 of ionic concentrations in the reactors, as described in the next section. Samples of 2 mL
134 were collected and stored at -20°C at the beginning and the end of each experiment for

135 metabolite and microbial analyses. A total of 152 batch assays was performed, with
136 triplicates for each condition tested.
137
138 2.2.1. Impact of ammonium and chloride ions on hydrogen production
139
140 The first part of the study was carried out with three different types of ionic solutions. The
141 first experiment consisted in adding NH_4Cl only (Ammonium-Chloride solution), under a
142 concentration ranging from 0 to 18.5 $gN.L^{-1}$ for ammonia ions and from 0 to 42 $gCl^{-}.L^{-1}$ for
143 chloride ions. In the second experiment, a mixture of different anions coupled to NH_4^+
144 (NH_4Cl, $NH_4H_2PO_4$, $(NH_4)_2SO_4$) was added to avoid the effect of chloride ion. The final
145 concentration in ammonium ions ranged from 0 to 22.8 $gN.L^{-1}$ (Ammonium-X solution).
146 Finally, in the last experiment, a mixture of different cations coupled to Cl^- ($CaCl_2$, KCl,
147 NaCl) (Chloride-X solution) was evaluated at a concentration range of 0 - 40 $gCl^{-}.L^{-1}$.
148
149 2.2.2. Impact of ionic strength on hydrogen production
150
151 The second part of the study was carried out to evaluate the impact of the total ionic
152 strength regardless the type of ions and minimize the individual concentration. Two
153 different solutions composed of seven and six components were used, corresponding to the
154 main ions found in OFMSW leachate [20] : solution M1 (NaCl, KH_2PO_4, LiBr, KI,
155 $(NH_4)_2SO_4$, $MnCl_2$, $MgSO_4$) and solution M2 (NaCl, KH_2PO_4, LiBr, KI, $(NH_4)_2SO_4$, LiCl).
156 A range of concentration from 0 to 1.9 M in term of ionic strength was tested. For M1 and
157 M2, each components were added at the same concentrations depending of the fixed ionic
158 strength (i.e. for M1 at ionic strength of 0.161 M, a concentration of 0.016 $mol.L^{-1}$ of each

159 component was added meaning a concentration of 0.9, 2.2, 1.4, 2.7, 2.1, 3.2 and 4.0 $g.L^{-1}$
160 for NaCl, KH_2PO_4, LiBr, KI, $(NH_4)_2SO_4$, $MnCl_2$, $MgSO_4$ respectively). In this mixture,
161 ammonium and chloride concentrations did not exceed 1.4 $gN.L^{-1}$ and 8.4 $gCl^-.L^{-1}$ (i.e. 0.08
162 and 0.24 M respectively).
163
164 2.3. Analytical methods
165 Gas pressure and composition were periodically measured (every four hours) with an
166 automated multiplexed micro-gas chromatograph (μGC R3000, SRA instrument), as
167 described by Cazier et al. [27]. Cumulated hydrogen was estimated by automatic and
168 periodic gas pressure measurement.
169 Volatile fatty acids (VFAs) were measured at the beginning and at the end of each
170 experiment. Individual VFA concentrations were determined with a gas chromatograph
171 Perkin Clarus 580 with an Elite-FFAP crossbond®carbowax® 15 m column connected to a
172 flame ionization detector at 280°C and N_2 at $6mL.min^{-1}$ as carrier gas, as previously
173 described by Motte et al. [28].
174 For microbial analyses, samples were collected at the end of each experiment. DNA was
175 extracted and purified with the Fast DNA SPIN kit for soil in accordance with
176 manufacturer's instructions (MP Biomedicals). DNA quantity and purity were assessed by
177 spectrophotometry (Infinite NanoQuant M200, Tecan). The V4 and V5 regions of the 16S
178 rRNA genes were then amplified and sequenced by Illumina MiSeq (get.genotoul.fr) and
179 sequences were analysed as described elsewhere [29].
180
181

182 2.4. Data analysis and calculation

183

184 A modified Gompertz equation based model was fitted to the cumulative hydrogen
185 production curve to assess the three following kinetic parameters: the hydrogen yield, the
186 maximal hydrogen production rate and the lag phase [29].

187

188 $H(t)=H \exp\{-\exp[Re/H (\lambda-t)+1]\}$ (1)

189

190 where H(t) is cumulative hydrogen amount (mL) at time t, H is the hydrogen potential
191 (mL), R is maximal hydrogen production rate ($mL.h^{-1}$), λ is the lag-phase and t the time
192 (hours).
193 The ionic strength was calculated according to the dimensional equation as described by
194 Solomon [30].

195

196 $I= (1/2) \times \sum (C_i Z_i^2)$ (2)

197

198 where I is the ionic strength of a solution ($mol.l^{-1}$), Ci the concentration of each individual
199 ion ($mol.l^{-1}$) and Zi the charge state of each ion.

200

201 **3. Results and discussion**

202

203 3.1. Effect of ammonium and chloride ions on hydrogen production

In all experiments, no methane production was observed confirming that initial heat shock pretreatment of the inoculum was efficient to suppress methanogens. Hydrogen production from OFMSW was first evaluated at different concentrations of NH_4Cl (Ammonium-Chloride) from 0 to 18.5 $gN.L^{-1}$ (Fig. 1(a)). Overall, apart from one sample (containing 2.0 $gN.L^{-1}$), a good fitting of the data to the Gompertz model was observed on the hydrogen response (details are presented in supplementary material).

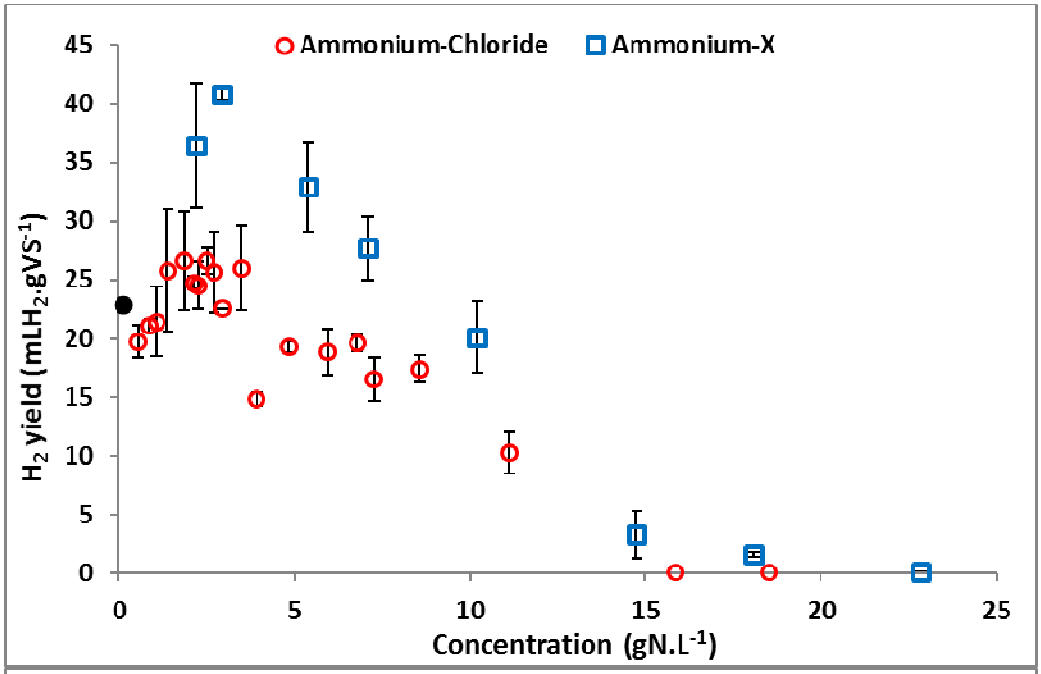
In the control where no ion was added, the hydrogen yield reached 22.9±0.1 $mLH_2.gVS^{-1}$ with a lag phase of 5.9±0.1 hours and a productivity of 3.29±0.12 $mLH_2.gVS^{-1}.h^{-1}$. Consistently, Favaro et al. [13] reported a similar hydrogen yield of 23.4±2.9 $mLH_2.gVS^{-1}$ using real OFMSW in a reactor operated at mesophilic temperature with a pH buffered at 7. However, this value is lower than the yields reported by Pan et al. [31] or Elbeshbishy et al. [32] who used batch reactors with no heat shock treatment or pH regulation (39 and 40 $mLH_2.gVS^{-1}$ respectively). In these study, more fermentescible food waste was used as substrate which probably explains these differences in hydrogen production. As shown in Fig. 1, when NH_4Cl was added at low concentration (from 1.4 to 3.5 $gN.L^{-1}$) and in comparison with the control, a slight but significant increase of 8 % (p-value < 0.001) was observed with an average yield of 24.9±2.5 $mLH_2.gVS^{-1}$. The lag phase was also shortened to 5.2±0.5 hours. A further increase of the ammonia nitrogen concentration between 3.5 and 8.6 $gN.L^{-1}$ led to a slight decrease of the hydrogen production (p-value < 0.001) to reach a yield of 17.8±1.5 $mLH_2.gVS^{-1}$ at 8.6 $gN.L^{-1}$. For concentrations higher than 11.1 $gN.L^{-1}$, a substantial decrease of the hydrogen yield down to 1.9±2.8 $mLH_2.gVS^{-1}$, and a significant increase of the hydrogen lag phase up to 44.9±9.3 hours were observed. Finally,

hydrogen was not detected for concentrations above 15.9 $gN.L^{-1}$ suggesting a strong impact of the NH_4Cl concentration on hydrogen-producing microbial activity.

Thereafter, different forms of ammonia nitrogen (Ammonium-X) were added (Fig. (1a)) to avoid the effect of the anion. Similarly, a positive effect on hydrogen production was observed for concentrations up to 7.1 $gN.L^{-1}$, with a maximum at 2.9 $gN.L^{-1}$ (40.8±0.5. $mLH_2.gVS^{-1}$). However, the lag phase was longer (12.8±4.7 hours) and the productivity lower (1.65±0.39 $mLH_2.gVS^{-1}.h^{-1}$) than in the control. At higher values (10.2 $gN.L^{-1}$,), the hydrogen yield decreased to a value lower than in the control (20.1±3 $mLH_2.gVS^{-1}$). The decrease of hydrogen yield from 10.2 $gN.L^{-1}$ was concomitant with an increase of the lag phase (28.9±9.6 hours) and a decrease of the hydrogen productivity (0.39±0.08 $mLH_2.gVS^{-1}.h^{-1}$), suggesting an impact on the microbial activity. At higher concentrations, a drastic drop of the hydrogen production was observed with a trend similar to the experiments supplemented with Ammonium-Chloride. These observations support the fact that the ions added to the medium had a strong impact on the microbial activity for concentrations above a threshold value of 8.6 $gN.L^{-1}$ for the Ammonium-Chloride solutions, and 10.2 $gN.L^{-1}$ for Ammonium-X solutions. The relative standard deviation was calculated on the hydrogen yield to evaluate the reproducibility of the triplicates (supplementary material). For NH_4Cl experiments, 57 % of the triplicates had a relative standard deviation below 10 %, while 90 % of the triplicates were below 20 %, showing a high reproducibility. For Ammonium-X, 25 % of the samples had a relative standard deviation below 10 %, and 63 % below 20 %. During this experiment, higher variability between the triplicates was observed, in particular when ammonia nitrogen was added at low concentration. Interestingly variability

248 decreased with the increase of ammonia nitrogen concentration with a good reproducibility

249 of the impact of ammonia-chloride addition on hydrogen production.



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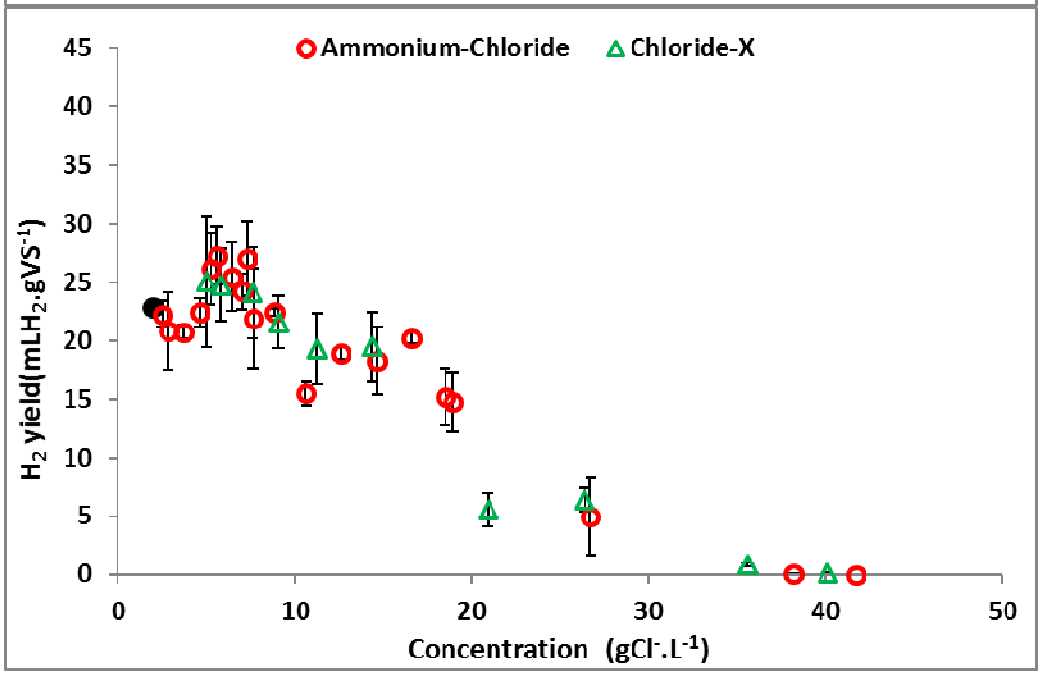

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Figure 1: Hydrogen yields estimated by Gompertz model in batch reactors operated at (a) increasing total ammonia nitrogen concentrations in the forms of [NH_4Cl] only (O) or [NH_4Cl, $NH_4H_2PO_4$, $(NH_4)_2SO_4$] (□) and (b) increasing chloride concentrations in the forms of [NH_4Cl] only (O) or [$CaCl_2$, KCl, and NaCl] (Δ), control (•).

Similar experiments were then performed by adding a mixture of $CaCl_2$, KCl, NaCl (Chloride-X) to assess the impact of chloride ions (Fig. (1b)). The trend of the inhibition curve was statistically similar (p-value < 0.05) to the experiment where chloride ion was added in the form of NH_4Cl only (Ammonium-Chloride experiments). Indeed, hydrogen production was not affected between 0 to 10.2 $gCl^-.L^{-1}$ (24.8±4.5 $mLH_2.gVS^{-1}$) with an average lag phase and a productivity of 9.9±2.1 hours and 1.56 $mLH_2.gVS^{-1}.h^{-1}$, respectively. From 20 $gCl^-.L^{-1}$ to 40 $gCl^-.L^{-1}$ hydrogen production decreased and a total inhibition was observed at 35.5 $gCl^-.L^{-1}$. By assessing the relative standard deviation of the Chloride-X experiments, 100% of the triplicates had a relative standard deviation below 20 %, showing a high reproducibility between the triplicates.

When combined to the results of the experiments carried out with Ammonium-Chloride solutions, it was concluded a strong inhibition of both ammonia nitrogen and chloride ions on hydrogen pathway. Several studies already reported that the non-ionic form of ammonia nitrogen (NH_3) can act as an inhibitor on the hydrogen production due to its high permeability to bacterial cell membrane [33]. In our study, a strong inhibitory effect was observed at an ammonia concentration of 8.6 $gN.L^{-1}$, equivalent to 11 $mgNH_3.L^{-1}$. Comparatively, Salerno et al. [23] reported that the maximum hydrogen production rate decreased from 56 $mLH_2.h^{-1}$ at 2 $gN.L^{-1}$ to 16 $mLH_2.h^{-1}$ at 10 $gN.L^{-1}$ (i.e. 13 $mgNH_3.L^{-1}$),

275 respectively, in a batch reactor operated at pH 6.2, supplement with NH_4Cl and fed with
276 glucose. However, no impact on hydrogen yield was observed (1.0±0.04 $molH_2.mol_{glucose}^{-1}$,
277 i.e. 120 $mLH_2.gVS^{-1}$). Wang et al. [34] showed an inhibitory effect on hydrogen production
278 at a concentration of ammonia nitrogen of 5 $gN.L^{-1}$ (64 $mgNH_3.L^{-1}$) using glucose as
279 substrate, with an initial pH of 7 and a reactor operated at mesophilic temperature.

280 In addition, Pan et al. [22] showed an improvement of the hydrogen yield at concentrations
281 of ammonia nitrogen as low as 1.5 $gN.L^{-1}$ and 3.5 $gN.L^{-1}$, in a batch reactor using food
282 waste as substrate. In the present study, similar improvement was observed at 3.5 $gN.L^{-1}$
283 showing the benefic effect of ammonia nitrogen on microbial activity. When different
284 forms of ammonium (Ammonium-X) were added, a higher positive effect on hydrogen
285 production was observed suggesting that the molecules added ($NH_4H_2PO_4$, $(NH_4)_2SO_4$)
286 were probably lacking in the OFMSW. Such observation confirms the positive effect of
287 phosphorus and nitrogen on hydrogen producing bacteria activity at low concentration as
288 already shown by Wang and Wan [35].
289 In comparison, no positive effect of chloride ion addition was observed in this study. Such
290 negative impact on hydrogen production of the Chloride-X solutions is consistent with the
291 literature where chloride ions are described as strong inhibitors of the microbial activity in
292 anaerobic bioreactors. Lefebvre et al. [36] showed an inhibitory effect in anaerobic
293 digestion at a concentration of NaCl of 20.0 $g.L^{-1}$ (i.e. a chloride concentration of 12.1 gCl^-
294 $.L^{-1}$), using ethanol as carbon source. In fermentation, Pierra et al. [25] observed a decrease
295 of the hydrogen production at NaCl concentrations as low as 9 $gNaCl.L^{-1}$ (i.e. 5.4 $gCl^-.L^{-1}$).

296 In comparison, our study showed an inhibitory effect on hydrogen production from 10.2
297 $gCl^{-}.L^{-1}$.
298
299 In conclusion, the comparison of the three experiments clearly shows that ammonia and
300 chloride ions had the same inhibition trend in terms of hydrogen yield, hydrogen lag phase
301 and hydrogen productivity. Indeed the hydrogen yield decreased of about 92% and 89% for
302 the Ammonium-Chloride and Ammonium-X experiments respectively, when increasing the
303 ammonium concentration from [0-5] $gN.L^{-1}$ to >10 $gN.L^{-1}$. A very similar trend was
304 observed with the chloride ions, with a decrease of the hydrogen yield by 89% from the low
305 [0-10] $gCl^{-}.L^{-1}$ to the high concentrations (up to 20 $gCl^{-}.L^{-1}$) of chloride, in the Chloride-X
306 experiments.
307 Consistently, an increase of the ammonium and chloride ion concentration led to an
308 increase of the lag phase for all the tested solutions. A very similar impact was shown on
309 the hydrogen productivity, regardless the type of ion added, with a maximum of 3.04±0.71,
310 1.48±0.52 and 1.56±0.81 $mLH_2.gVS^{-1}.h^{-1}$ observed at low concentrations for Ammonium-
311 Chloride, Ammonium-X and Chloride-X solutions, respectively, i.e. within the range of [0-
312 5] $gN.L^{-1}$ and [0-10] $gCl^{-}.L^{-1}$.
313 These results suggest that a global metabolic inhibition could have occurred and might be
314 responsible of the negative impact on microbial fermentation, independently of the nature
315 of the ion.
316
317 3.2. Impact of the ionic strength on dark fermentation
318 3.2.1. Impact of the ionic strength on hydrogen production

In light of these results, an ionic strength index was calculated to evaluate the impact of the total ionic content rather than the effect of individual ion on the fermentative activity. The total ionic strength was estimated based on each individual ionic concentration and their number of charges. By revisiting the data, the impact of the ionic strength on hydrogen production is shown in Fig. 2, for Ammonium-Chloride, Ammonium-X and Chloride-X experiments.

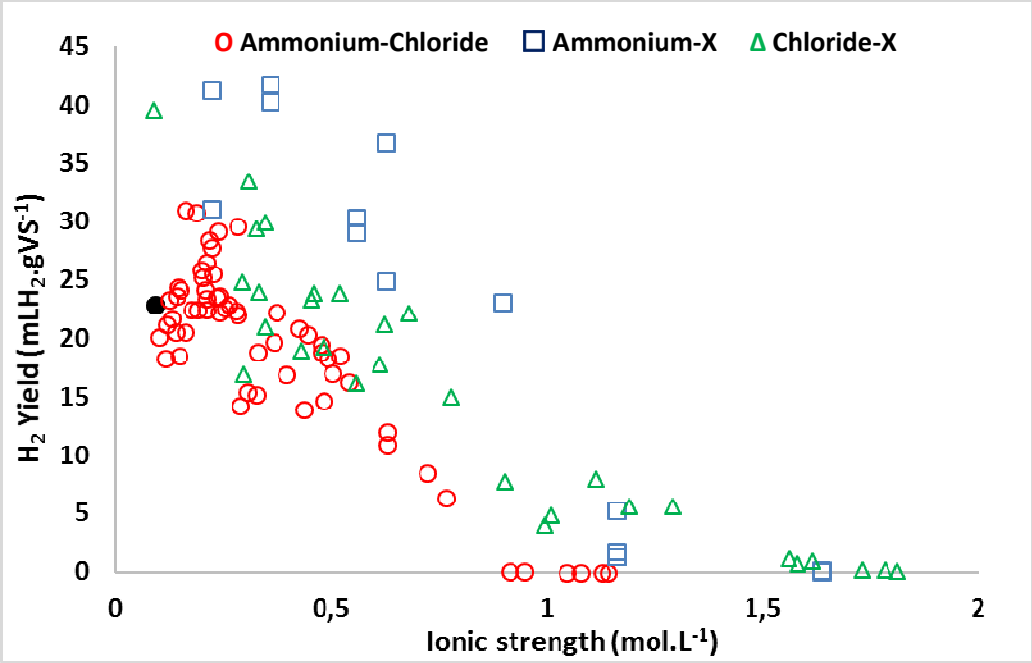

Figure 2: Effect of ionic strength on biohydrogen production in dark fermentation reactor for Ammonium-Chloride (O), Ammonium-X (□), Chloride-X (Δ) experiments and the control (●)

As noticed previously, an improvement of the hydrogen production was observed at low ionic strength, for the Ammonium-Chloride, Ammonium-X and Chloride-X experiments. Such slight positive effect confirms the benefic effect of the molecules added on hydrogen production at low concentration of ammonium. A similar inhibitory trend was observed in all the experiments according to the value of the ionic strength. A threshold value of this

334 inhibition was estimated at 0.75±0.13 M, for an average H_2 yield of 18.1±3.3 $mLH_2.gVS^{-1}$,
335 whatever the ions considered. The inhibition was significant at ionic strengths higher than
336 0.81±0.12 M with an average H_2 yield of 6.2±4.1 $mLH_2.gVS^{-1}$. Total inhibition of the
337 hydrogen production was then observed at an ionic strength of 1.04, 1.63 and 1.73 M for
338 Ammonium-Chloride, Ammonium-X and Chloride-X experiments, respectively, suggesting
339 here an effect of the type of ions.
340 To better understand the impact of the ions regardless their nature, the total ionic strength
341 was modified by changing the composition of the ionic solutions and considering several
342 anions and cations, as follows: solution M1 was composed of Na^+, K^+, H^+, Li^+, NH_4^+, Cl^-,
343 PO_4^{3-}, Br^-, I^-, SO_4^{2-} , while solution M2 was composed of Na^+, K^+, H^+, Li^+, NH_4^+, Mn^{2+},
344 Mg^{2+}, Cl^-, PO_4^{3-}, Br^-, I^-, SO_4^{2-} (Fig. 3). By increasing the number of ion types, the
345 concentration of each individual element remained low (< 0.18 M) while the ionic strength
346 increased from 0.18 to 1.9 M.
347

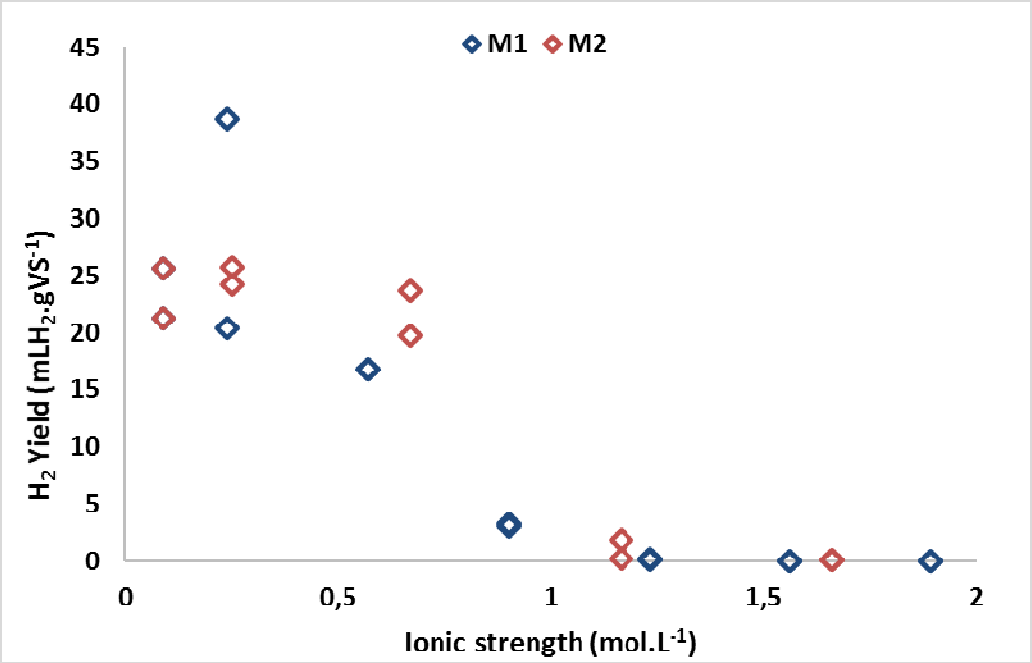

Figure 3: Effect of ionic strength on biohydrogen production for M1: NaCl, KH_2PO_4, LiBr, KI, $(NH_4)_2SO_4$, $MnCl_2.4H_2O$, $MgSO_4.7H_2O$ (◊) and M2: NaCl, KH_2PO_4, LiBr, KI, $(NH_4)_2SO_4$, LiCl (◊) experiments.

As shown in Fig. 3, the trend was similar than previously observed with an improvement of the hydrogen production at the lowest ionic strengths of 0.09 and 0.25 M with an average yield of 26.5±6.1 and 25.0±0.7 $mLH_2.gVS^{-1}$ for M1 and M2, respectively. Inhibition of the hydrogen production was significant after values of ionic strength of 0.57 and 0.67 M for the M1 and M2 solutions, which is similar to the previous observations made with Ammonium-Chloride, Ammonium-X and Chloride-X solutions (0.75±0.13 M).

No hydrogen production was observed for ionic strength higher than 1.56 and 1.66 M for M1 and M2, respectively. The concentration of ammonia nitrogen and chloride ions at 1.56 M for M1 and at 1.66 M for M2 were 1.7 and 1.1 $gN.L^{-1}$ and 13.1 and 6.5 $gCl^{-}.L^{-1}$, respectively. Interestingly, these concentrations in individual components corresponded to

363 the concentrations found at the highest performances of hydrogen production in the
364 Ammonium-Chloride experiments. These observations clearly suggest that hydrogen
365 inhibition was not due to the individual concentration in ammonium and chloride ions, but
366 to the overall ionic strength.
367
368 3.2.2. Impact of ionic strength on by-products production
369 Furthermore, soluble fermentation co-products were analyzed at the end of the batch tests
370 to estimate the impact of the ionic solutions on the global fermentation activity. Total
371 amount of metabolic co-products was expressed in mgCOD.gVS^{-1} (Table 2).
372 In the control experiment, i.e. without addition of ions, the main accumulated metabolites
373 were acetate and butyrate at a concentration of 104.2±8.3 mgCOD.gVS^{-1} (2.5±0.2 gCOD.L^{-1})
374 and 133.3±8.3 mgCOD.gVS^{-1} (3.2±0.2 gCOD.L^{-1}) representing 36 % and 46 % of the
375 total COD, respectively.
376 Theoretically acetate and butyrate are correlated to a high hydrogen yield. Indeed, two
377 moles of acetate and one mole of butyrate leads to four and two mole of hydrogen
378 respectively (Eq. 3 and 4) [37].
379 $$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (3)$$
380 $$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (4)$$
381 Many studies show these metabolites as major end product during dark fermentation
382 reaction [39,25]. Cavinato et al. [24] reported the same range of acetate and butyrate
383 concentration in a thermophilic dark fermentation using food waste (3.29±1.64 and
384 4.32±1.48 gCOD.L^{-1} respectively) which is slightly higher than our results due to

385 thermophilic application which is known to improve the microbial activity [39]. These
386 results suggest that the conditions in the control were favorable for hydrogen producing
387 pathways. Indeed, the maximum H_2 yield is related to acetate as by-product. However,
388 from an experimental point of view, high H_2 yields are usually associated to butyrate in
389 mixed culture [40].
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397 Table 2 : Final concentration of acetate, propionate, butyrate, valerate and caproate during all the experiments (Ammonium-Chloride,
398 Ammonium-X and Chloride-X) at different ionic strength and estimation of the homoacetogenic activity in all experiments. For technical
399 reasons, M1 solutions precipitates in the liquid fraction made the analyses no possible without damaging the analyzer.
400

Experiments		Metabolite ($mgCOD.gVS^{-1}$)						Homoacetogenesis (mol/mol)
	Ionic strength ($mol.L^{-1}$)	Acetate	Propionate	Butyrate	Valerate	Caproate	Ethanol	
Control	0.09	104.2±8.3	9.6±0.8	133.3±8.3	4.6±0.8	3.3±2.5	17.5±3.7	39.9 %
Ammonium-Chloride	0.25	100.0±33.3	14.6±2.9	145.8±58.3	3.7±0.8	4.6±2.9	7.5±1.4	39.7 %
	0.35	79.2±16.7	17.5±14.2	120.8±20.8	20.8±25.0	6.7±8.3	5.0±10.0	42.1 %
	0.59	58.3±4.2	39.6±9.6	62.5±16.7	0.8±1.7	17.9±0.8	40.0±35.0	31.6 %
	1.02	14.2±2.5	1.7±0.4	-	-	-	-	32.2 %
Ammonium-X	0.28	45.8±8.3	10.0±2.9	116.7±25.0	-	-	16.7±33.3	26.3 %
	0.65	33.3±8.3	11.7±8.7	75.0±20.8	-	-	3.3±6.7	22.7 %
	1.02	23.7±2.5	10.4±8.7	25.4±22.5	-	-	27.5±16.7	21.6 %
	1.39	8.3±2.1	-	-	-	-	-	24.6 %
	1.76	1.7±2.1	-	-	-	-	-	30.1 %
Chloride-X	0.35	145.8±12.5	22.9±7.5	212.5±29.2	1.2±1.7	6.2±10.0	18.3±19.0	42.7 %
	0.55	133.3±12.5	14.2±3.7	233.3±20.8	1.2±1.7	20.0±21.7	19.2±26.2	47.2 %
	0.70	79.2±16.7	8.7±1.2	133.3±25.0	-	-	-	42.8 %
	1.10	83.3±62.5	16.7±12.1	45.8±16.7	-	-	7.9±15.8	35.6 %
	1.71	12.1±5.0	-	19.6±13.7	-	-	13.3±16.7	52.3 %
M2	0.24	66.7±4.2	10.0±0.4	116.7±8.3	5.0±4.2	-	-	39.0 %
	0.57	41.7±4.2	12.9±5.0	120.8±8.3	6.2±4.0	-	-	50.4 %
	0.90	41.2±1.2	9.6±0.4	17.5±1.2	5.0±4.1	-	-	33.3 %
	1.23	31.2±0.4	10.4±0.4	15.0±0.4	5.0±4.2	-	-	36.3 %

401 Overall, the increase of ionic strength led to a decrease of the total accumulation in
402 metabolites regardless the solutions added. Concerning the Ammonium-Chloride
403 experiments, from 0.25 to 1.02 M, acetate and butyrate concentrations drastically dropped
404 to nearly no production with concomitant decrease of hydrogen production. Propionate
405 concentration reached a maximum value of 14.6±2.9 mgCOD.gVS^{-1} at 0.25 M, and
406 decreased to 1.7±0.4 mgCOD.gVS^{-1} at 1.02 M. Valerate and caproate were only present in
407 trace amounts up to 0.59 M and were not detected at higher ionic strengths. A similar trend
408 was observed for Ammonium-X and M2 solutions, with a decrease of the metabolite
409 production according to the increase of the ionic strength. At low ionic strength of 0.28 and
410 0.24 M, a maximal production of metabolites of 189±69 and 198±13 mgCOD.gVS^{-1},
411 respectively, were observed. The total metabolite concentration reached a low value
412 (100±30 and 60±10 mgCOD.gVS^{-1}) at higher ionic strengths of 1.02 and 1.23 M,
413 respectively. Similar trends were observed for the Chloride-X experiments with a
414 maximum of metabolite production (407±80 mgCOD.gVS^{-1}) at low ionic strength (0.35 M)
415 with acetate and butyrate as main metabolites (145±12 and 212±29 mgCOD.gVS^{-1}
416 respectively). A decrease of the total concentration was observed with a low metabolite
417 production of 61±47 mgCOD.gVS^{-1} at 1.71 M.
418 The consumption of hydrogen by homoacetogenesis to produce acetate was estimated and
419 is presented in Table 2. The acetate produced by homoacetogenesis can be estimated by the
420 following two equations (Eq. 5 and 6), as already described by Arooj et al. [41] :

421 $$Total\ HAc = HAc_a + HAc_b \quad (5)$$

422 $$H_2\ Yield = 2 \times (HBu) - (HPr) + 2 \times (HAc_a) - 4\ x\ (HAc_b) \quad (6)$$

423 Where Total HAc is the acetate concentration; HAca and Hacb are the acetate
424 concentration from the H_2-producing (HAC_a) and H_2-consuming (HAC_b) pathways; HBu
425 the butyrate concentration and HPr the propionate concentration.
426 Indeed, hydrogen yield is theoretically associated to the stoichiometry of 2 mol of hydrogen
427 generated per mole of acetate and butyrate accumulated, while propionate consumes 1 mol
428 of hydrogen. Eq. (5) considers the production of acetate from two distinct pathways
429 corresponding to H_2-producing (HAC_a) and H_2-consuming (HAC_b) homoacetogenic
430 pathway. Considering Eq.(5) and Eq. (6), the amount of acetate produced by
431 homoacetogenesis was estimated according to molar balances and the results are presented
432 in Table 2. The estimated amount of acetate produced by homoacetogenesis pathway in the
433 control represented about 39.9% of the total COD. Consistently, Castelló et al. [42]
434 reported that 33 to 57% of the acetate was produced by homoacetogenesis using a
435 continuous stirred tank reactor fed with cheese whey. As already reported by Saady [43],
436 homoacetogenesis is associated to fast growing strict anaerobes forming spores such as
437 *Clostridium* sp. that can consume between 11% and 43% of the hydrogen produced in
438 batch. In our experiments, the homoacetogenesis ratio is stable regardless the ionic strength
439 concentration. The percentage of acetate issued from the homoacetogenesis pathway was
440 estimated at 36.4±5.3, 25.1±3.3, 44.1±6 and 39.8±7.5% for Ammonium-Chloride,
441 Ammonium-X, Chloride-X and M2 solutions whatever the ionic strength. Therefore, the
442 decrease of hydrogen performances according to the increasing ionic strengths was not
443 attributed to an increase of hydrogen consumption by homoacetogenic bacteria. Only the
444 Ammonium-X solution showed a lower amount (25.1±3.3%) resulting from a high
445 hydrogen yield observed at low ionic strength (41.1±0.8. $mLH_2.gVS^{-1}$at 0.31±0.08 $mol.L^{-1}$).

446 Overall, the analyses of metabolites showed the same impact of the ionic strength than
447 previously observed with hydrogen production, i.e. a global decrease of the metabolite
448 production when the concentration of ions in the medium increased, regardless the
449 solutions added. Further increase of the ionic strength drastically affected the acetate and
450 butyrate pathways which are the two metabolic pathways generating hydrogen [37]. Similar
451 observations were reported by Cavinato et al. [24] who showed a decrease by 72 % of the
452 acetate and butyrate concentrations when a nitrogen accumulated from 970 to 1976 $mgN.L^{-1}$
453 in dark fermentation, using municipal solid waste as substrate. Zheng et al. [44] observed
454 a similar decrease of the butyrate and H_2 yields by adding NaCl from 0 to 500 mM and
455 using glucose as substrate. In addition, our results showed that the increase of ionic strength
456 did not change the metabolic pathways since acetate and butyrate remain the main
457 metabolites in each experiments, with no accumulation of lactate or other by-products that
458 can be produced during microbial stress [45]. Our observations rather suggest that the
459 global microbial activity was strongly impacted by the increase of the ionic strength.
460
461 3.2.3. Impact of the ionic strength on the microbial community
462 Microbial characterization using phylogenetic affiliation of bacterial 16S rDNA was
463 performed on the Ammonium-Chloride experiment, as model experiment, at different ionic
464 strengths. The results are presented in Table 3. Only sequence having affiliations above
465 98% of similarity with sequences of known species are presented.
466

467 Table 3 : Phylogenetic affiliation of bacterial 16S rDNA from experiments Ammonium-Chloride at
468 different ionic strength (mol.L^{-1}) using BLAST algorithm.

Closely related to	Sequence similarity (%)	Relative abundance of species in each ecosystem (%)							
		0.25	0.35	0.53	0.63	0.72	0.95	1.02	1.13
Clostridium butyricum	**100**	32	44	26	7	-	-	-	-
Clostridium tertium	**99**	3	3	4	6	9	-	-	-
Enterococcus saccharolyticus	**99**	32	23	48	39	16	-	-	-
Enterococcus faecalis	**100**	-	7	5	5	15	11	-	-
Pseudomonas caeni	**99**	5	5	4	5	2	2	1	1
Pseudomonas pertucinogena	**99**	-	-	-	-	-	20	18	22
Marinocpirillum minutulum	**100**	-	-	-	-	-	11	3	3
Atopostipes suicloacalis	**99**	-	-	-	-	-	-	28	1
Others (Clostridiales NC)		10	3	5	27	44	10	7	12
Others (Oceanospirillales NC)		-	-	-	-	-	10	12	15
Others (NC)		12	15	8	11	14	36	38	56

469

470 At low ionic strength (0.25, 0.35 and 0.53 M) the most abundant bacteria were assigned to
471 known hydrogen producing bacteria, i.e. *Clostridium butyricum* and *Enterococcus*
472 *saccharolyticus*. Liu et al. [46] reported high hydrogen production using *Clostridium*
473 *butyricum* in a fermentative batch reactor at pH 6 with glucose as substrate (1.83
474 mmol/mmol$_{glucose}$). The ability of *Enterococcus saccharolyticus* to hydrolyze cellobiose and
475 produce hydrogen was previously described [47]. These observations are consistent with
476 our experiment where freshly reconstituted OFMSW was composed of 51.8 % of paper and
477 cardboard. *Clostridium tertium* was found in lower abundance in the experiment, and was
478 also described as hydrogen producing bacteria [48]. The overall microbial community
479 characterization suggests that the environment at low ionic strength was ideal for the
480 development of hydrogen-producing bacteria.

481 A neutral impact of the ionic strength was shown at low concentration (from 0.25 to 0.53
482 M) on the microbial community composition, which is consistent with macroscopic
483 observations of high hydrogen production.
484 At intermediate ionic strength (0.63 and 0.72 M), a modification of the microbial
485 community was observed with a decrease in abundance of *Clostridium butyricum* (7 and
486 0% respectively) and an increase in unclassified *Clostridiales* (27 and 44 % respectively).
487 This correlates with the variability of hydrogen yields found in this range of ionic strength
488 suggesting that the population structure was disturbed. At higher ionic strength (above 0.72
489 M), the dominant *Clostridium butyricum, Clostridium tertium* and *Enterococcus*
490 *saccharolyticus* were not detected and the relative abundance in unclassified *Clostridiales*
491 drastically decreased to 10%, 7% and 12% at 0.95, 1.02, 1.13 $mol.L^{-1}$, respectively. This
492 observation is consistent with an absence of hydrogen production in these experiments. At
493 high ionic strength, the presence of other microbial populations was detected such as
494 *Pseudomonas pertucinogena*, *Marinocpirillum minutulum* and *Atopostipes suicloacalis*
495 which are not associated with hydrogen production. Therefore, the increase of ionic
496 strength seemed to have highly impacted hydrogen producing bacteria that are not
497 particularly adapted to high ionic environments. As reported by Van Niel et al. [49], an
498 accumulation of ions in the medium (above 175 mM) can cause a cell lysis in *Clostridium*
499 *saccharolyticus*. More generally, high concentrations of ions cause high osmolarity across
500 the bacterial that can lead to an inhibition of the microbial activity, as shown with
501 *Clostridium butyricum* [50].
502
503

4. Conclusion

This study provides new aspects on fermentative hydrogen production using freshly reconstituted OFMSW. During dark fermentation, the presence of ions (chloride or ammonia) at low concentrations did not impact hydrogen performances and was even beneficial with the best performances found when 2.9 $gN.L^{-1}$ was added (40.8±0.5. $mLH_2.gVS^{-1}$). However, at higher concentration of ions and whatever the ions added, a very similar trend of inhibition on hydrogen production was observed. For all experiments, the inhibition started after a similar threshold value of ionic strength (0.75±0.13 M) whatever the ions considered from 18.1±3.3 $mLH_2.gVS^{-1}$ to 6.2±4.1 $mLH_2.gVS^{-1}$ at 0.81±0.12 M. Such inhibition was also observed on the overall microbial activity with a decrease of metabolite production. Moreover, the accumulation of ions caused a stressful environment as shown by microbial community changes with a particular impact on dominant H_2-producing bacteria (*Clostridium butyricum, Clostridium tertium and Enterococcus saccharolyticus*). In the context of process upscaling and implementation of this technology for waste energy recovery, the ionic strength should be carefully monitored to avoid reaching the critical threshold value and thus, reduce the overall efficiency of the process.

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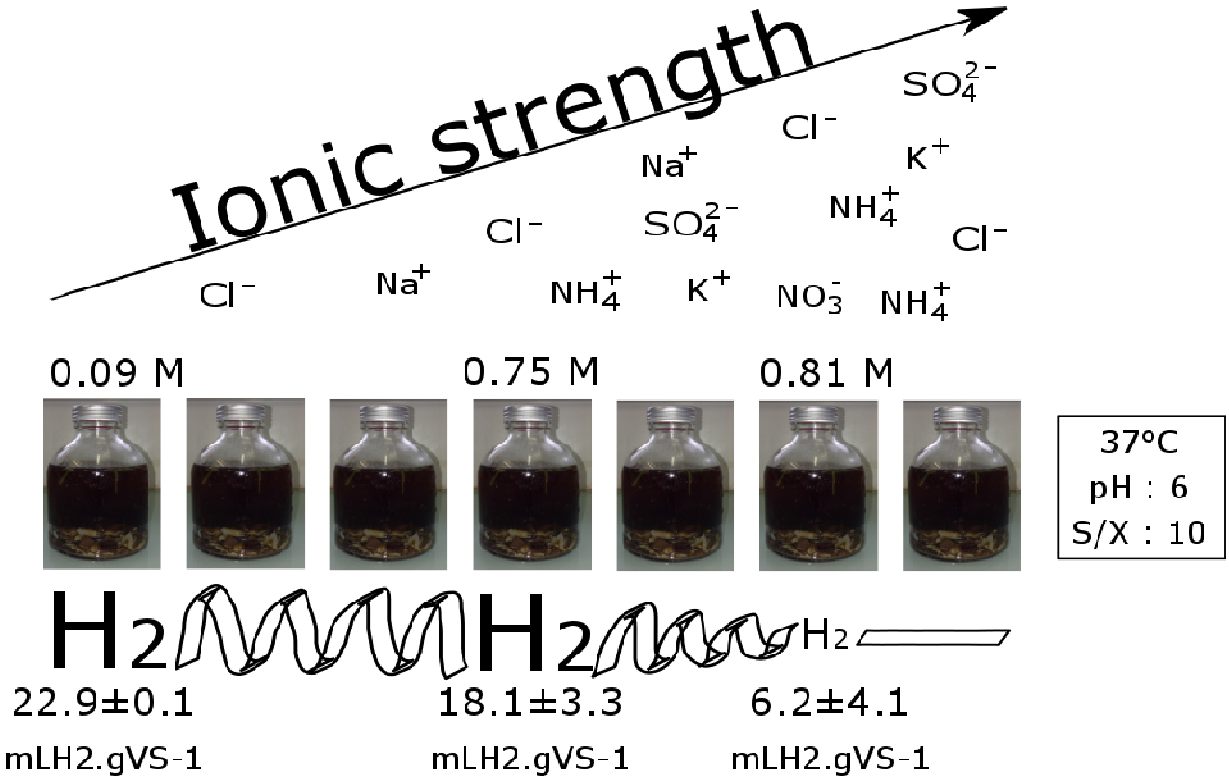
Ionic strength
Cl⁻
Na⁺
Cl⁻
NH4+
Na⁺
SO4 2-
K⁺
Cl⁻
NO3-
Cl⁻
NH4+
K⁺
SO4 2-
NH4+
0.09 M
0.75 M
0.81 M
37°C
pH : 6
S/X : 10
H2
H2
H2
22.9±0.1
mLH2.gVS-1
18.1±3.3
mLH2.gVS-1
6.2±4.1
mLH2.gVS-1