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Biomass hydrolysis inhibition at high hydrogen partial pressure in solid-state anaerobic digestion

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Abstract

In solid-state anaerobic digestion, so-called ss-AD, biogas production is inhibited at high total solids contents. Such inhibition is likely caused by a slow diffusion of dissolved reaction intermediates that locally accumulate. In this study, we investigated the effect of H₂ and CO₂ partial pressure on ss-AD. Partial pressure of H₂ and/or CO₂ was artificially fixed, from 0 to 1 557 mbars for H₂ and from 0 to 427 mbars for CO₂. High partial pressure of H₂ showed a significant effect on methanogenesis, while CO₂ had no impact. At high P_{H₂}, the overall substrate degradation decreased with no accumulation of metabolites from acidogenic bacteria, indicating that the hydrolytic activity was specifically impacted. Interestingly, such inhibition did not occur when CO₂ was added with H₂. This result suggests that CO₂ gas transfer is probably a key factor in ss-AD from biomass.

Keywords

Dry anaerobic digestion; gas transfer; hydrogen; hydrolysis; lignocellulosic residues.

1 Introduction

Over the past decade, solid-state anaerobic digestion (ss-AD) also called dry anaerobic digestion or high-solids AD has gained a wide interest in Europe. Agricultural waste and organic fraction of municipal waste had been particularly used as substrates (Baere et al., 2010). In ss-AD, organic matter contained in the waste is biologically converted by anaerobic bacteria into a biogas composed of CH₄ (50-70%) and CO₂ (30-50%). Remaining organic matter called digestate can be further reused on land as fertilizer with several sanitary and environmental restrictions. The biogas produced by AD can be converted into electricity and heat by cogeneration or, after purification, can be used as biofuel (95% of CH₄) or directly injected in natural gas pipelines (Weiland, 2010).

Anaerobic digestion is composed of four microbial steps: first, organic matter (proteins, lipids and polysaccharides) is hydrolyzed into soluble molecules by extracellular enzymes excreted by hydrolytic microorganisms (Montero et al., 2008). Hydrolysis is mostly the limiting step of AD when solid organic matter is used as substrate (Pavlostathis and Giraldo-Gomez, 1991). The second step corresponds to acidogenesis: amino-acids, saccharides and fatty acids are transformed into volatile fatty acids (VFAs) such acetate, butyrate, propionate, or into others organics acids, such as lactate, or in alcohols, i.e. ethanol, butanol, in presence of fermentative

microorganisms such as *Clostridium* sp. (Fritsch et al., 2008). The third step is called acetogenesis where all types of VFAs are transformed into acetate, CO₂ and H₂ by two types of microorganisms: (1) syntrophic acetogens, e.g. *Syntrophobacter wolinii* or *Syntrophomonas wolfei*, also called Obligate Hydrogen Producing Bacteria (OHPB), are converting VFAs, alcohols and fatty acids to H₂, CO₂ and acetate (Amani et al., 2010). These microorganisms are syntrophs of methanogens since all acetogenic reactions are thermodynamically not favorable and end-products accumulation can inhibit their own production, in particular hydrogen (2) non syntrophic homoacetogens, e.g. *Clostridium aceticum*, are using H₂ and CO₂ to produce acetate (Amani et al., 2010). In this case, the reaction is thermodynamically favorable and does not require the presence of methanogenic *Archaea*. Acidogenesis is also impacted by H₂ and CO₂ in wet AD and more specifically the production of propionate, butyrate and caproate (Arslan et al., 2012). Acetate and butyrate are concomitantly produced with H₂ and the consumption of these VFAs may be inhibited by high P_{H₂} (Ahring and Westermann, 1988; Ding et al., 2010; Hallenbeck, 2005). The last step corresponds to methanogenesis. Acetate, H₂ and CO₂ are transformed into CH₄ by two types of microorganisms: (1) acetotrophic methanogens using acetate as substrate and producing 70% of CH₄ in AD (Pavlostathis and Giraldo-Gomez, 1991) such as *Methanosaeta concilii* or *Methanosarcina acetivorans* (Amani et al., 2010), (2) hydrogenotrophic methanogens using CO₂ and H₂ as substrates, such as *Methanobacterium bryantii* or *Methanobrevibacter arboriphilus* (Amani et al., 2010).

Two main types of technologies have been developed to convert the solid organic matter in anaerobic digestion : wet AD and solid-state AD (ss-AD), with a Total Solids (TS) content below or higher than 15%, respectively (Baere et al., 2010). ss-AD presents several advantages such as reducing the water demand and lowering the costs related to water management, with a subsequent reduction of reactor size and less energy requirements for heating. These advantages contributed to a recent and large industrial development of ss-AD, with a rapid emergence of full-scale plants (Baere et al., 2010). Industrial digesters usually use agricultural, green or solid organic waste, under a TS content ranging from 15 to 30%. Since many industrial installations have been empirically developed, recent research has been devoted to optimize ss-AD processes through a better understanding of their limitations (Abbassi-Guendouz et al., 2012; Motte et al., 2013).

The key parameter driving the microbial processes in ss-AD is low water content since microbial end-products can locally accumulate and inhibit methanogenesis (Abbassi-

Guendouz et al., 2012). In such systems, water availability presents two distinct forms: (1) “free water” that can act as a solvent for salts and soluble compounds (Pommier and Chenu, 2007), (2) “bound water” that has more structural bonding (chemical and physical interactions) than liquid or free water and thus, is unable to act as a solvent. The form of water depends mainly on the structure and composition of the organic matter structure. Moreover, when the TS content increases, the quantity of free water decreases (García-Bernet et al., 2011) and the transport of soluble content within the substrate can become a limiting factor (Bollon et al., 2013). Microbial activity can be impacted by water availability and methane yields varies significantly between wet and ss-AD, according to the nature of the substrate. Brown et al. (2012) reported that CH₄ yields of paper decreased from 19.2 L/kg TS to 8.7 L/kg TS in wet and ss-AD, respectively. In contrast, no CH₄ yields variation was observed with wheat straw, i.e. 11.6 L/kg TS (wet AD) and 12 L/kg TS (ss-AD). Overall, by increasing the TS content, substrate degradation and consequently the biogas production are reduced (Abbassi-Guendouz et al., 2012; García-Bernet et al., 2011; Motte et al., 2013). As an illustration, a decrease of organic waste degradation by 17% was observed when changing the TS content from 20 to 30% (Fernández et al., 2008).

When the water content decreases, the rheological behavior of the substrate is also modified (García-Bernet et al., 2011). Therefore, the diffusion of metabolic compounds within the substrate at a macroscopic level is lowered (Bollon et al., 2013). The limitation of ss-AD might be explained by the slow diffusion of dissolved inhibitory products inside the organic matrix generating local accumulation at microbial scale (Martin, 2001; Staley et al., 2011). In a recent study, Abbassi-Guendouz et al. (2012) suggested that methanogenesis inhibition at high solids content was mainly caused by gas transfer limitation and more particularly by a local accumulation of gases (H₂ and CO₂) leading to VFAs accumulation. H₂ has a well-described inhibitory effect on wet AD but the exact effect of H₂ on ss-AD remains unknown.

The objective of this study was to investigate the effect of dissolved gases on ss-AD. The accumulation of H₂ and CO₂, as by-products of acidogenesis, was investigated in particular since local accumulation of these gases may impact the overall ss-AD process. Experiments were designed and carried out to evaluate the effect of the partial pressure of H₂ alone or mixed with CO₂ on the overall reaction of ss-AD.

2 Materials and methods

2.1 Substrate preparation

Wheat straw (*Triticum aestivum*) was used as model substrate representing lignocellulosic agricultural waste for its well-known composition in hemicelluloses, cellulose and lignin (Vassilev et al., 2012). Particles were fractionated in a cutting miller through a 1 mm grid, and further sieved between grids of 1 mm and 400 μm . Wheat straw particles had a TS content of 95%.

2.2 Operating conditions of the batch tests

Granular sludge originated from an industrial UASB reactor treating sugar factory effluents was used to inoculate the batch reactors. The sludge was mixed during 24 h at 35°C to break the granules, and then centrifuged at 7 841 g (20 min, 4°C) to obtain a microbial anaerobic inoculum with a TS content ranging between 10 to 13 %. Final pH of the inoculum was 7.8.

A substrate/inoculum biomass (S/X) ratio of 3 (in volatile solids basis) was used as suggested elsewhere (Liew et al., 2012). The initial TS content of the flask was fixed at 25 % where no inhibition of AD was reported (Abbassi-Guendouz et al., 2012; Motte et al., 2013). Wheat straw and inoculum were added in a S/X ratio of 3 and TS content was adjusted with the buffer solution after addition of trace elements. At this TS content, A solution of trace elements (FeCl_2 2g/L, CoCl_2 0.5g/L, MnCl_2 0.1 g/L, NiCl_2 0.1 g/L, ZnCl_2 0.05 g/L, H_3BO_3 0.05g/L, Na_2SeO_3 0.05g/L, CuCl_2 0.04 g/L, Na_2MoO_4 0.01g/L) was added with a volume of 0.2 mL by flask. A buffer solution of sodium bicarbonate (0.0026 g NaHCO_3 /g substrate) was added at the beginning to keep the pH around 7-8.

First, the medium (wheat straw, trace elements, buffer and inoculum) was introduced into a 3 L reactor operated during 10 days at 35°C. This reactor was flushed with N_2 to avoid the presence of O_2 . This pre-culture was performed to reach an active phase of methanogenesis and homogenize the substrate. Then, 20 g of the pre-culture was distributed in a thin layer (<1 cm) at the bottom of a 600 mL flask. Such thin layer was used to minimize the influence of the gas diffusion within the medium. The flasks were flushed with N_2 before the addition of other gases (H_2 alone or mixed with CO_2). Whatever the gas added, the initial pressure was around 1.5 bars. The flasks were then incubated during 13 days at 35°C to evaluate the effect of the partial pressure of H_2 and/or CO_2 on the initial degradation of wheat straw in ss-AD.

Experiments were conducted in five separate runs, with three to four replicates for each condition. An experimental design plan was performed in order to investigate the partial pressure of H₂ (P_{H2}) alone or mixed with CO₂ (P_{CO2}) (Table 1). When H₂ was added alone, initial P_{H2} ranged from 0 to 1 557 mbars. CO₂ and H₂ were also added simultaneously in a mixture corresponding to partial pressures of H₂ and CO₂ ranging from 0 to 1 060 mbars and 0 to 427 mbars, respectively (Table 1). For each run, a control (only N₂ in headspace) was carried out.

2.3 Analytical methods

Gas pressure and composition were measured periodically with an automated multiplexed micro-gas chromatograph (μ GC R3000, SRA instrument) using 12 to 20 flasks at once. It was composed of an injector and two capillary columns heated at 90°C and 80°C, respectively. One column corresponded to a MolSieve 5Å (with a length of 10 m and a diameter of 0.32 mm) for analyzing CO₂ with argon as carrier gas, at a pressure of 30 psi. The second column corresponded to a PLOT Q with a length of 8 m and a diameter of 0.32 mm, and was used for analysis of the O₂, H₂, N₂ and CH₄ with helium as carrier gas at a pressure of 20 psi. The detection was ensured by a thermal conductivity detector.

At the end of the experiment, 5 g of the medium were diluted in 20 g of deionized water, mixed during 30 minutes, and centrifuged at 39 121 g during 20 min at 4°C. The supernatant was then filtrated at 0.2 μ m with a nylon membrane, and was then used to measure VFAs, others microbial metabolites (formate, succinate, lactate, ethanol) and residual soluble sugars.

Volatile fatty acids (VFAs) were measured at start and at the end of each experiment. VFA concentrations were determined with a gas chromatograph Perkin Clarus 580 with an Elite-FFAP crossbond®carbowax® 15 m column connected to a flame ionization detector at 280°C and N₂ at 6 mL.min⁻¹ as carrier gas, as described elsewhere (Motte et al., 2013).

Others metabolites and soluble sugars were measured at start and at the end of each experiment. These compounds were quantified using high performance liquid chromatograph. This chromatograph was composed of an automatic sampler (Water 717), a pre-column to filter residues (Micro guard cation H refill cartridge, Bio-rad) and a Aminex HPX-87H column (300 mm on 7,8 mm, Bio-rad). The carrier liquid was sulfuric acid at 0.005 M at 0.4 mL.min⁻¹, as described elsewhere (Motte et al., 2013).

The quantity of microorganisms (*Bacteria* and *Archaea*) was quantified using quantitative PCR (Polymerase Chain Reaction) according to the protocol of Braun et al. (2011) and Bru et al. (2012). The community structures of *Bacteria* and *Archaea* were obtained using Capillary Electrophoresis Single Strand Conformation Polymorphism (CE-SSCP) using the protocol described by Wéry et al., (2008). The microbial community composition (Archaea and Bacteria) was evaluated by sequencing after purification of the PCR products, using the Illumina MiSeq System with 2x300 bp paired-end chemistry at the GenoToul sequencing center (www.genotoul.fr). An average of 81 707 high quality sequences per sample for Archaea and 453 382 high quality sequences per sample for Bacteria were retained after assembly, demultiplexing and cleaning steps in Mothur version 1.33.2 (Schloss et al., 2009). SILVA release 102 was used for alignment and as taxonomic outline.

2.4 Data analysis

Statistical analyses of the data obtained were performed using R software (version 2.15.2) with the package Rcmdr (version 1.8-4). Non-significant P-values were fixed >0.05 and significant P-values were estimated when <0.05; <0.01 and <0.001.

Total substrate degradation was assessed with Chemical Oxygen Demand (COD) mass balance between the start and the end of the experiment. To avoid a high experimental error when determining the COD value from solids, wheat straw COD was not considered and all calculations were expressed according to the initial TS content. Total substrate degradation was estimated theoretically according to the end-products accumulation in the gas and liquid phases, as follows:

Total Substrate Degradation (Final State-Initial State)=

$$\frac{A_{H_2,f} + A_{CH_4,f} + A_{met,f} + A_{GC}}{g\ TS} - \frac{A_{H_2,i} + A_{met,i}}{g\ TS} \quad (\text{Equation 1})$$

where, $A_{H_2,f}$ is the final amount of H_2 remaining in headspace, $A_{CH_4,f}$ the final amount of CH_4 accumulated in headspace, $A_{met,f}$ the final amount of metabolites, A_{GC} the total amount of gas (H_2 and CH_4) sampled for analyses, $A_{H_2,i}$ the amount of H_2 initially added and $A_{met,i}$ the initial amount of metabolites in the medium.

3 Results and discussion

2.1 Impact of high P_{H_2} on methanogenesis

Figure 1-a shows the cumulative CH_4 production at the end of the experiment (i.e. 13 days) according to the initial H_2 partial pressure. In this figure, results from four different runs (Runs 1-4) inoculated with distinct pre-cultures are presented. Since the pre-cultures were not exactly from the same origin, cumulated CH_4 production in the control samples ($P_{H_2}=0$) showed a high variability. This was likely due to a slightly longer lag phase for CH_4 production that was observed in the controls, although the pre-culture was active in all runs (see Supplementary material). A difference of few days in the initial lag phase could have resulted in variability of cumulated CH_4 after 13 days of experiment. Adding H_2 in headspace at start of the experiment accelerated the initial CH_4 production and the results between the assays showed a lower variability. When initial P_{H_2} ranged from 0 to 600 mbars, a slight increase of CH_4 production was observed from 23 ± 4 mL CH_4 /g TS to 28 ± 6.1 mL CH_4 /g TS, respectively.. This increase was due to a partial consumption of the H_2 initially added. At higher initial P_{H_2} , cumulative production of CH_4 decreased from 28 ± 6.1 mL of CH_4 /g TS down to 9 ± 1.1 mL CH_4 /g TS at $P_{H_2} = 1555$ mbars. Interestingly, at P_{H_2} higher than 800-900 mbars, a strong decrease of methanogenesis was observed.

Figure 1-b shows the overall substrate degradation expressed in gCOD per g of initial TS, after 13 days of experiment. The substrate degradation corresponded to the total amount of COD produced by AD degradation of wheat straw. Interestingly, substrate degradation decreased with the increase of initial P_{H_2} , from 0.09 ± 0.01 g COD/g TS in the control samples to 0.02 ± 0.01 g COD/g TS at a $P_{H_2} = 1555$ mbars. Since total amount of metabolic end products such as methane and acidogenic VFAs decreased with the increasing of P_{H_2} , this result suggests that a strong inhibition of the initial steps of hydrolysis and/or acidogenesis occurred at initial P_{H_2} higher than 742 mbars. An analysis of variance (ANOVA) showed that when H_2 was introduced alone (1) the substrate degradation was not significantly different from controls at P_{H_2} lower than 781 mbars, and (2) the substrate degradation was significantly different at 1050 mbars of H_2 with a P-value < 0.0001 (see more information in Supplementary material).

In addition, a low pH resulting from VFA accumulation could have a strong impact on methanogens. Here, since the medium was buffered, the pH remained the same between 8 and 9 all along the experiments.

Figure 2 shows the cumulated amount of VFAs expressed in g COD / g TS at the end of the experiment according to initial P_{H_2} . VFAs accumulation increased from 20 ± 4 to 60 ± 2 mg COD/g TS between P_{H_2} of 600 mbars and 1 000 mbars, respectively, and decreased at higher P_{H_2} . Acetate was the unique VFAs changing with initial P_{H_2} . The amount of acetate increased from 8 ± 5 mg COD/g TS in the control to 23 ± 1 mg COD/g TS at 600 mbars. The slight increase of acetate production at P_{H_2} higher than 603 mbars was likely due to the consumption of H_2 and CO_2 by homoacetogens (Montero et al., 2009). A maximum amount of 50 ± 3 mg COD/g TS was observed at 745 mbars, and the amount of acetate decreased at P_{H_2} of 1 350 mbars with 24 ± 2 mg COD/g TS.

In this study, no substantial VFAs accumulation that could explain methanogenesis inhibition was observed. Inhibition of methanogenesis by H_2 partial pressure is well-known process in wet AD, since acetogenesis is thermodynamically unfavorable when the P_{H_2} is too high (1.01×10^{-3} mbars) resulting in VFA accumulation and changes in VFA distribution (Deublein and Steinhauser, 2008). According to the Henry's law, the dissolved H_2 concentration at $35^\circ C$ ranged from 1.14×10^{-3} mg/L to 3.64×10^{-2} mg/L at 52 mbars of H_2 and 1 545 mbars of H_2 , respectively. In AD, H_2 is continuously produced by fermentation from sugars and acetogenic bacteria from VFAs and ethanol. Since the latter reactions are endergonic, complete AD requires an immediate and rapid H_2 consumption by methanogens to produce CH_4 (Amani et al., 2010). In wet AD, methanogenic conversion of fermentative end-products generated by acidogenic and acetogenic bacteria can decrease when H_2 accumulates with subsequent increase of VFAs. The P_{H_2} value at which VFAs accumulate and methanogenesis is inhibited in wet AD is very low when compared to the present results. As an illustration, butyrate consumption by acetogens was reported as inhibited at a P_{H_2} of 20.3 mbars, at $25^\circ C$ and pH 7, using glucose as substrate (Ahring and Westermann, 1988). However, acetogenesis and inhibition leads to a change in VFA distribution that was not observed in this study (Figure 2). When considering the amount of water contained in the medium, the maximal VFAs concentration was always lower than 17.7 ± 0.5 g VFAs/L that was even below the inhibitory limit of methanogenesis 20 g/L. This inhibitory limit of methanogenesis, but not acidogenesis was reported in wet AD by Siegert and Banks (2005) using glucose and cellulose as substrates. Arslan et al. (2012) reported that H_2 up to 2 bars has no effect on fermentative acetate production from carbohydrates, proteins and lipids rich waste. Additionally, Abbassi-Guendouz et al (2012) reported that VFAs could accumulate under similar conditions at concentrations higher than 40 g/L at high TS value.

It was therefore concluded that high P_{H_2} had a strong inhibitory impact on hydrolysis rather than acidogenesis.

In addition, the final partial pressure of CO_2 and H_2 remaining in headspace at the end of the experiment and according to initial P_{H_2} are shown in Figure 3. The remaining P_{H_2} was always lower than the amount of H_2 introduced at start-up of the experiment, showing that added H_2 was partially or totally consumed and no H_2 was overproduced. At low initial P_{H_2} , H_2 was fully consumed during the time of the experiments. H_2 was likely used to produce acetate and CH_4 at P_{H_2} lower than 745 mbars. At a P_{H_2} ranging between 745 and 1 000 mbars, H_2 was partially consumed to produce acetate by homoacetogenesis (Montero et al., 2009).

Interestingly, H_2 consumption was lower at high P_{H_2} since H_2 remained in the headspace at the end of the experiment with 26 ± 23.1 to 547 ± 55.6 mbars of H_2 / g TS for initial P_{H_2} of 745 mbars and 1 550 mbars, respectively. More precisely, H_2 consumption was rapid during the first days of experiments and then slowed down rapidly according to the initial P_{H_2} . As an illustration, at 1 418 mbars, H_2 was rapidly consumed during the first 3 days, with a consumption rate of 206 ± 112 mbars of H_2 per g TS and per day. H_2 consumption rate was much slower the following days with only 21.3 ± 16.2 mbars H_2 per g TS and per day.

Meanwhile, the remaining CO_2 in headspace was also monitored (Figure 3). The apparent remaining CO_2 corresponds to a balance between CO_2 production due to microbial degradation of wheat straw, and CO_2 consumption to produce either CH_4 by methanogens or acetate by homoacetogens, both in combination with H_2 . From 0 to 745 mbars, CO_2 was overproduced due to microbial activity on substrate. The remaining P_{CO_2} decreased 221 ± 47.8 to 67 ± 55 mbar of CO_2 /g TS in the control and at a P_{H_2} of 745 mbars, respectively. At higher P_{H_2} , no remaining CO_2 was found in headspace. Since the increase of the remaining amount of H_2 in headspace was correlated to the decrease of remaining CO_2 , it was concluded that CO_2 production was the limiting factor for H_2 consumption and, by consequence, H_2 inhibition on the hydrolytic activity (Florin and Harris, 2008). Since both CO_2 and H_2 are required to produce acetate or methane, the decrease of acetate/methane production at higher P_{H_2} may be explained by a lack of remaining CO_2 in headspace.

This assumption was supported by microbial community analysis. The archaeal community of methanogens was composed of 94.6 ± 0.6 % of *Methanobacteriales* and only 0.5 ± 0.6 % of *Methanosarcinae* at 0 and 1 020 mbars, as shown in Figure 4. *Methanobacteriales* are strict hydrogenotrophs, while members of the *Methanosarcinae* order are rather acetotrophs with some members exhibiting both activities. In absence of acetotrophs, acetate oxidation could

occur in AD, and has even been reported as main methanogenic pathway by Karakashev et al. (2006). However, acetate oxidation is thermodynamically unfavorable at high P_{H_2} and could only occur in presence of active hydrogenotrophs. Since CO_2 was a limiting factor, leading to subsequent P_{H_2} accumulation by limiting the hydrogenotrophic activity, acetate was not oxidized and accumulated in the medium and lower production of methane. The concentration of 16S rRNA genes copies of *Archaea* or *Bacteria* per g of digestate measured by quantitative PCR did not change according to the initial P_{H_2} , likely because of the limited time period of 13 days. Therefore, archaeal and bacterial community structures, evaluated by CE-SSCP, showed no significant differences between the assays and it was concluded that the initial P_{H_2} had no effect on either communities or growth of the anaerobic consortium, but rather had an impact at a cellular or physiological level (data shown in supplementary material section).

2.2 Effect of adding CO_2 with H_2 on solid state AD

A second set of experiments was carried out to evaluate the effect of CO_2 that is concomitantly produced with H_2 during acidogenesis. Abbassi-Guendouz et al. (2012) showed methanogenesis inhibition at high solids content (>30%) and suggested that such inhibition was due to local accumulation of H_2 and CO_2 leading subsequently to VFAs accumulation. To determine the exact effect of both H_2 and CO_2 on AD, a mixture of both gases were initially introduced in headspace.

As control, CO_2 was added alone at different initial P_{CO_2} , but no significant effect on substrate degradation was observed (see supplementary material).

Figure 5 shows the cumulative production of CH_4 and VFAs as well as the remaining CO_2 at the end of the experiment, according to the initial P_{CO_2} and P_{H_2} . First, CH_4 production increased when CO_2 was initially added with H_2 from 0.46 ± 0.06 mmol CH_4 /g TS to 3.4 ± 0.51 mmol CH_4 /g TS in the control with no CO_2 and with CO_2 at 1 040-1 060 mbars of H_2 , respectively. The remaining CO_2 increased when CO_2 and H_2 were added together, from 0 with only H_2 added to 1.25 ± 0.3 mmol CO_2 /g TS and 1.13 ± 0.4 mmol CO_2 /g TS at 787 mbars and 1 040-1 060 mbars of H_2 , respectively. Such values of CO_2 indicated an overproduction of CO_2 during the time of experiment. Interestingly, H_2 was entirely

consumed at the end of the experiment, when mixed with CO₂ (after 2-4 days). In contrast, remaining H₂ was present when H₂ was added alone, from 0.4 ±0.4 to 1 ±0.2 mmol H₂/g TS at the end of the experiment for 781 and 1 060 mbars of H₂ respectively. In conclusion, microorganisms likely used both CO₂ and H₂ to produce CH₄ by methanogenesis and/or acetate by homoacetogenesis rapidly transformed to methane. Hydrolysis inhibition did not occur since the P_{H2} decreased rapidly in the headspace when CO₂ was added initially.

Furthermore, the total amount of VFAs was higher when H₂ was added alone (0.03 ± 0.006 g VFAs/ g TS for both P_{H2}) than for the mixture (0.003 ± 0.002 g VFAs/ g TS for both P_{H2}/ P_{CO2}). This result suggests that methanogenesis occurred preferentially than homoacetogenesis when both CO₂ and H₂ were added, which is different than in conventional wet AD (Pavlostathis and Giraldo-Gomez, 1991). This observation was confirmed by acetate concentration which was higher when only H₂ was present in headspace, with 3.48 x 10⁻⁴ mol acetate/g TS (for 781 mbars of H₂ alone) than for a mixture of H₂ and CO₂, with 5.27 x 10⁻⁵ mol acetate/g TS (with 787 mbars of H₂/427 mbars of CO₂). Moreover, the overall substrate degradation increased from 7.28 x 10⁻³ g COD/g TS at 1 060 mbars of H₂ with no CO₂, to 0.14 g COD/g TS at similar 1 040 mbars of H₂ with 324 mbars of CO₂ (Table 2). The overall substrate degradation was 5 to 19 times higher when CO₂ was added than with only H₂. Statistically, substrate degradation was similar in control and when CO₂ and H₂ were added initially in headspace. In this case, hydrolysis inhibition did not occur, since H₂ was consumed rapidly with CO₂ by the methanogens to produce CH₄, and P_{H2} decreased rapidly under inhibitory P_{H2}. By adding CO₂ in headspace, it was confirmed that CO₂ was a limiting factor for CH₄ production when P_{H2} was high. Hydrolysis inhibition by H₂ could therefore not occur when CO₂ was present in headspace, since they were both consumed to produce acetate or CH₄.

All these experiments were performed with a thin layer of substrate to reduce gas transfer limitations and investigate local impact of gas partial pressure. To extend these observations to ss-AD reactor, gas transfer must also be considered. In high solids reactors, anaerobic digestion is inhibited at high TS contents (Abbassi-Guendouz et al., 2012; Motte et al., 2013). Diffusion coefficients of H₂ and CO₂ are also lower in ss-AD (Bollon et al., 2013). With high solids, H₂ and CO₂ may accumulate in the matrix and local P_{H2} likely increases with subsequent inhibition of hydrolysis. Diffusion of CO₂ may also be slowed, and the overall inhibition observed at high solids content may result from a low amount of CO₂ available to reduce hydrolysis inhibition by H₂.

Moreover, hydrolysis is already known to be a limiting factor in ss-AD (Pavlostathis and Giraldo-Gomez, 1991) and the composition of the substrate (morphological and chemical) has a strong effect on this step (Motte et al., 2014; Triolo et al., 2011). The actual mechanisms of hydrolysis are not well understood, but many models are including low hydrolysis rates (Vavilin et al., 2008). In ADM1 model, two mechanisms for the extracellular enzymes are proposed: (1) the microorganisms secrete enzymes into the liquid phase of the digestate and the enzymes have an effect directly on the substrate or (2) the microorganisms attach themselves on the substrate and produce enzymes (Batstone et al., 2002). As shown for the first time in our study, H_2 produced during fast hydrolysis of readily accessible carbohydrates, could act as a strong inhibitor of the hydrolytic activity of microorganisms, either on production or secretion of extracellular enzymes, or on attachment and physiological cellular activity.

In previous study, modeling of ss-AD showed that a structuration of the microorganisms could occur, dependent of VFAs concentration and pH (Martin, 2001). Since H_2 and CO_2 are produced by acidogenic and acetogenic bacteria, dissolved gases may accumulate transitory in high solids reactors, with low availability to methanogens growing at a higher pH of 7-8. Thus, at high solids content, local accumulation of gases may result in inhibition of the overall process of methanogenesis as suggested in Abbassi-Guendouz et al. (2012). The authors reported a strong inhibition of CH_4 production at TS content higher than 30%, with an increase of VFAs concentration up to 40 g/L and a decrease of pH from 7 to 6. By modeling the process, it was shown that gas transfer was probably a limiting factor for the production of CH_4 since H_2 and CO_2 may be used locally to produce acetate by homoacetogenic bacteria and since acidogens and acetogens are able to grow in same conditions rather unfavorable to methanogens, i.e. at low pH (Diekert and Wohlfarth, 1994).

4 Conclusion

This study provides new insight about an inhibitory effect of H_2 on organic matter hydrolysis in ss-AD, using wheat straw as substrate. By adding CO_2 , hydrolysis inhibition was suppressed, indicating that CO_2 was the limiting factor to avoid H_2 inhibition in ss-AD. These results were obtained with a thin layer of substrate to avoid the effect of the gas transfer. Even though the strong inhibitory effect of H_2 was only observed at a local scale, inhibition of AD at high solids content may result from similar mechanisms when low gas diffusion rates create high local H_2 accumulation.

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Figures Captions

Figure 1: Anaerobic digestion performances on wheat straw at 25% of TS according to the initial H_2 partial pressure in headspace (in mbars), expressed in terms of (1.A) cumulative CH_4 production (in mL CH_4 / g $TS_{initial}$), or (1.B) substrate degradation (in g COD /g $TS_{initial}$)

Figure 2 : Volatile Fatty Acids accumulation at the end of the experiment (in g COD / g $TS_{initial}$) according to initial partial pressure of H_2 , at 25% TS, 35°C and pH 8.

Figure 3 : Final partial pressure of CO_2 (P_{CO_2}) and H_2 (P_{H_2}) according to the initial partial pressure of H_2 (in mbars), at 25% TS, 35°C and pH 8. Final P_{CO_2} and P_{H_2} in the headspace are represented with white circles and dark circles, respectively.

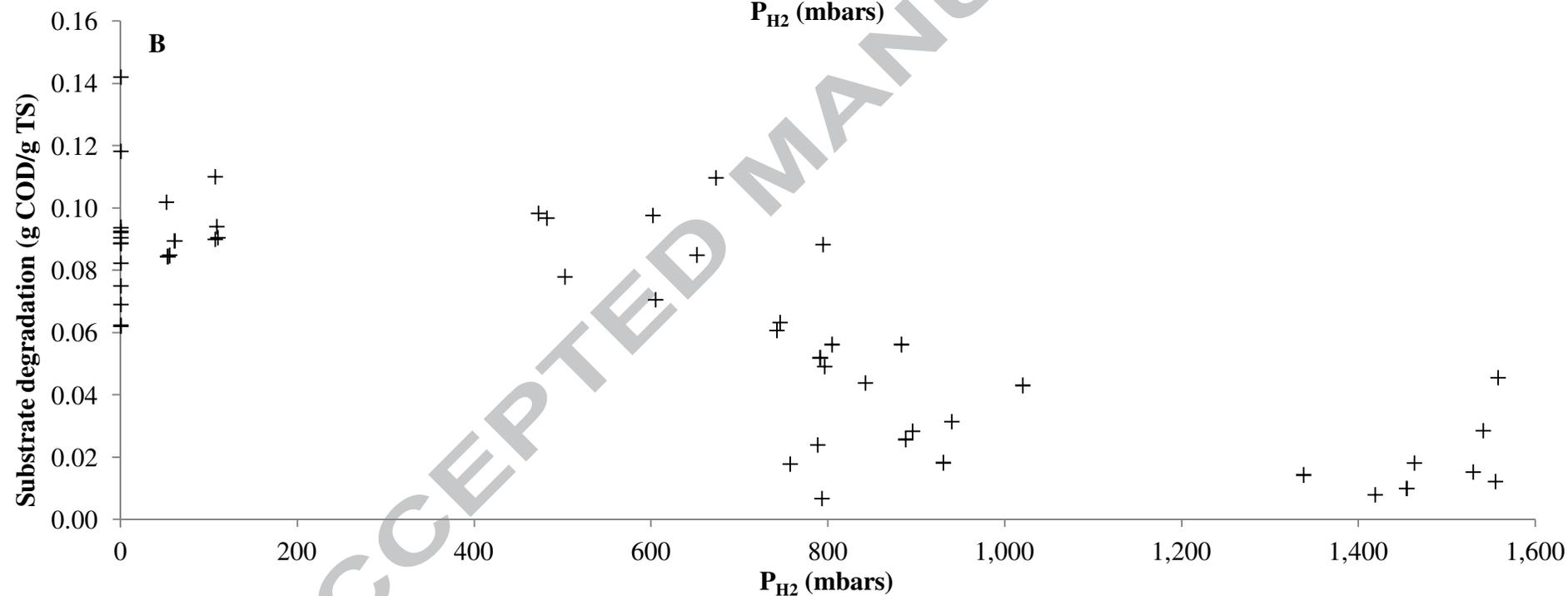
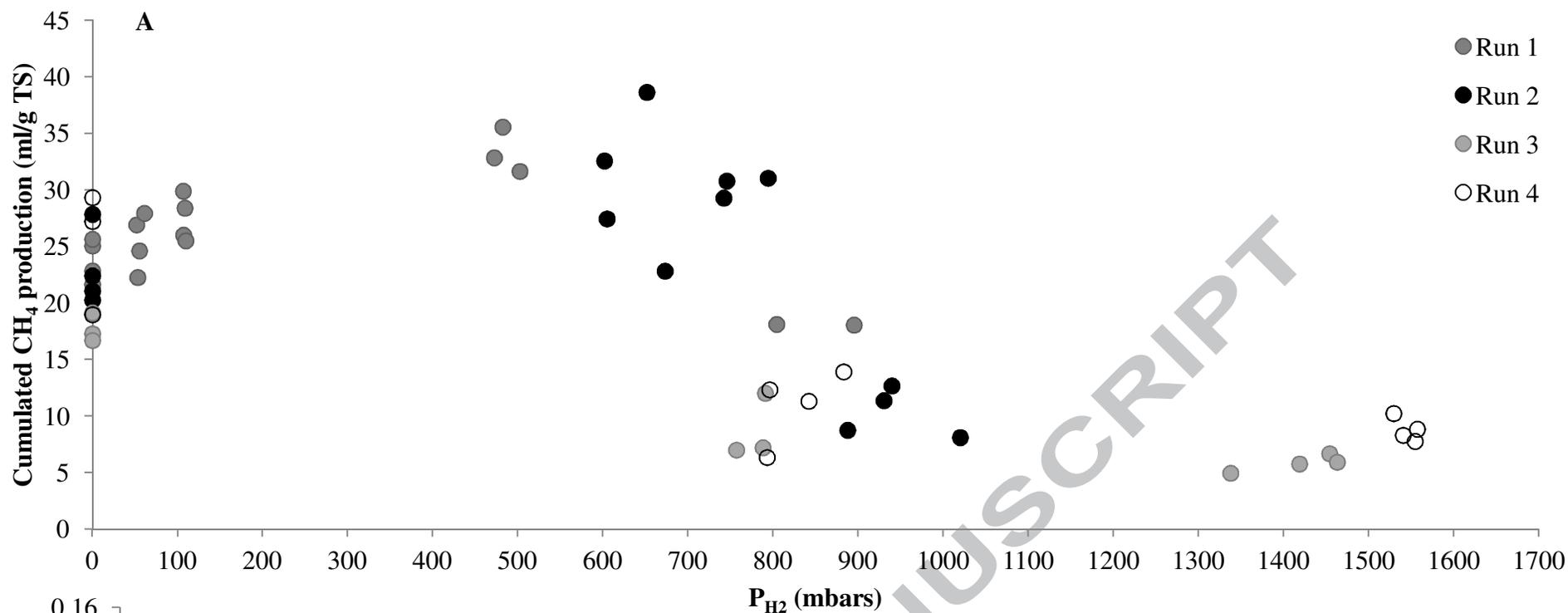
Figure 4: Microbial community composition at Order level of the archaeal community, in the inoculum (Initial), in the control (0mbars) and at 1 020 mbars of H_2 , with 25% of totals solids, 35°C and pH 8.

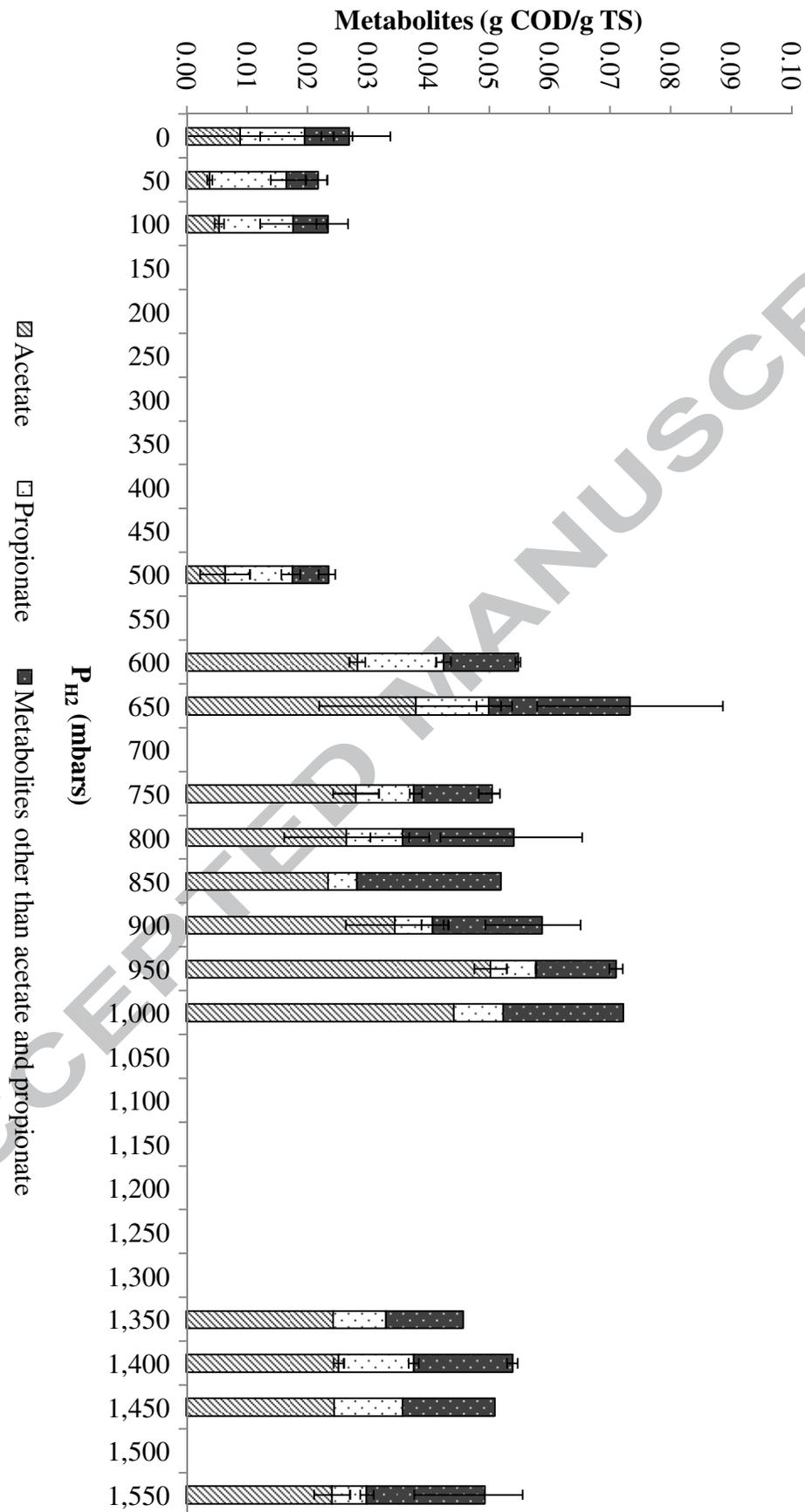
Figure 5 : Final product accumulation (gas and metabolites) (in mol per g of initial TS) according to the initial partial pressure of H_2 (on the first line) and CO_2 (on the second line) (in mbars) at 25% TS, 35°C and pH 8.

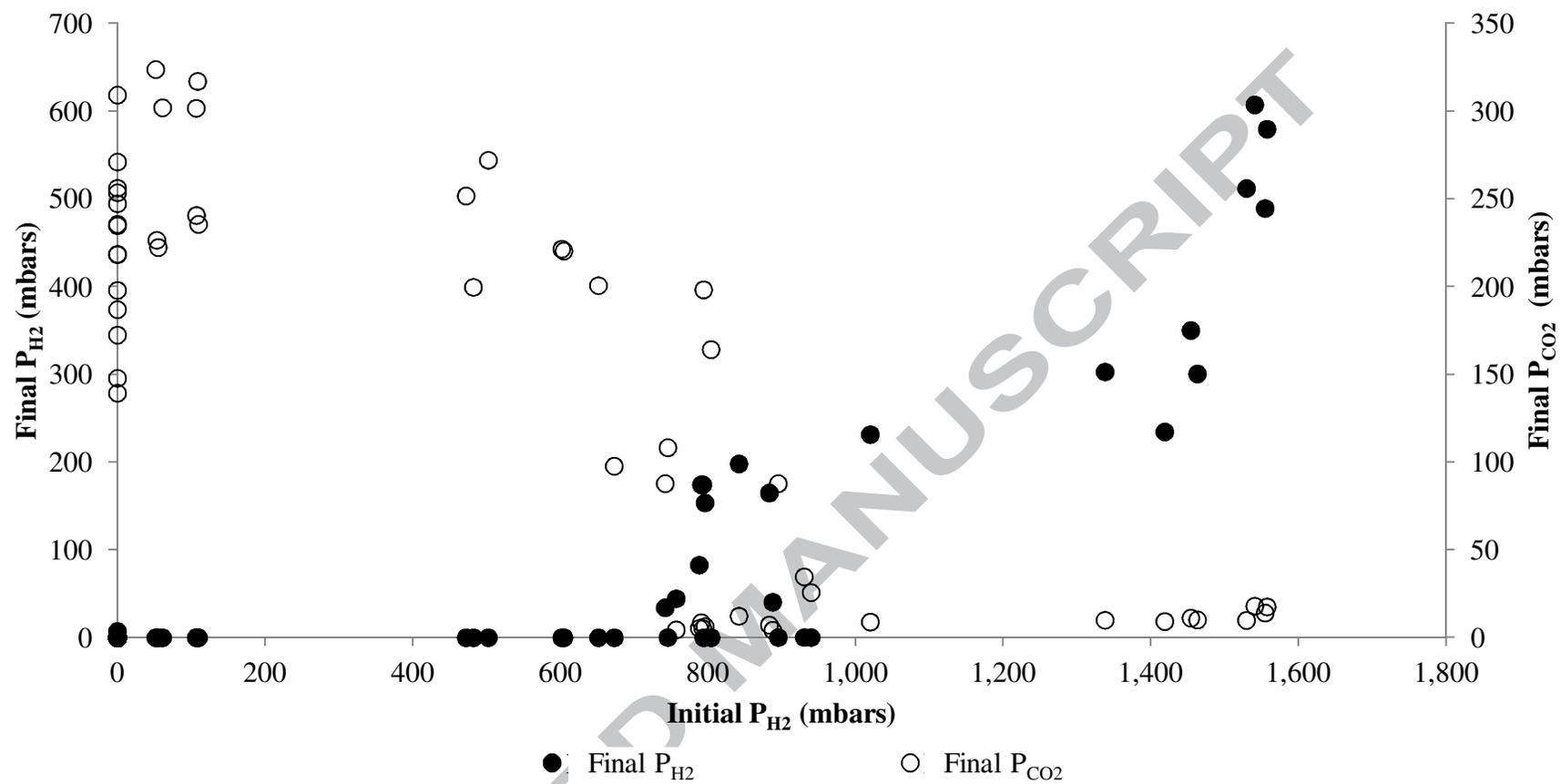
Tables Captions

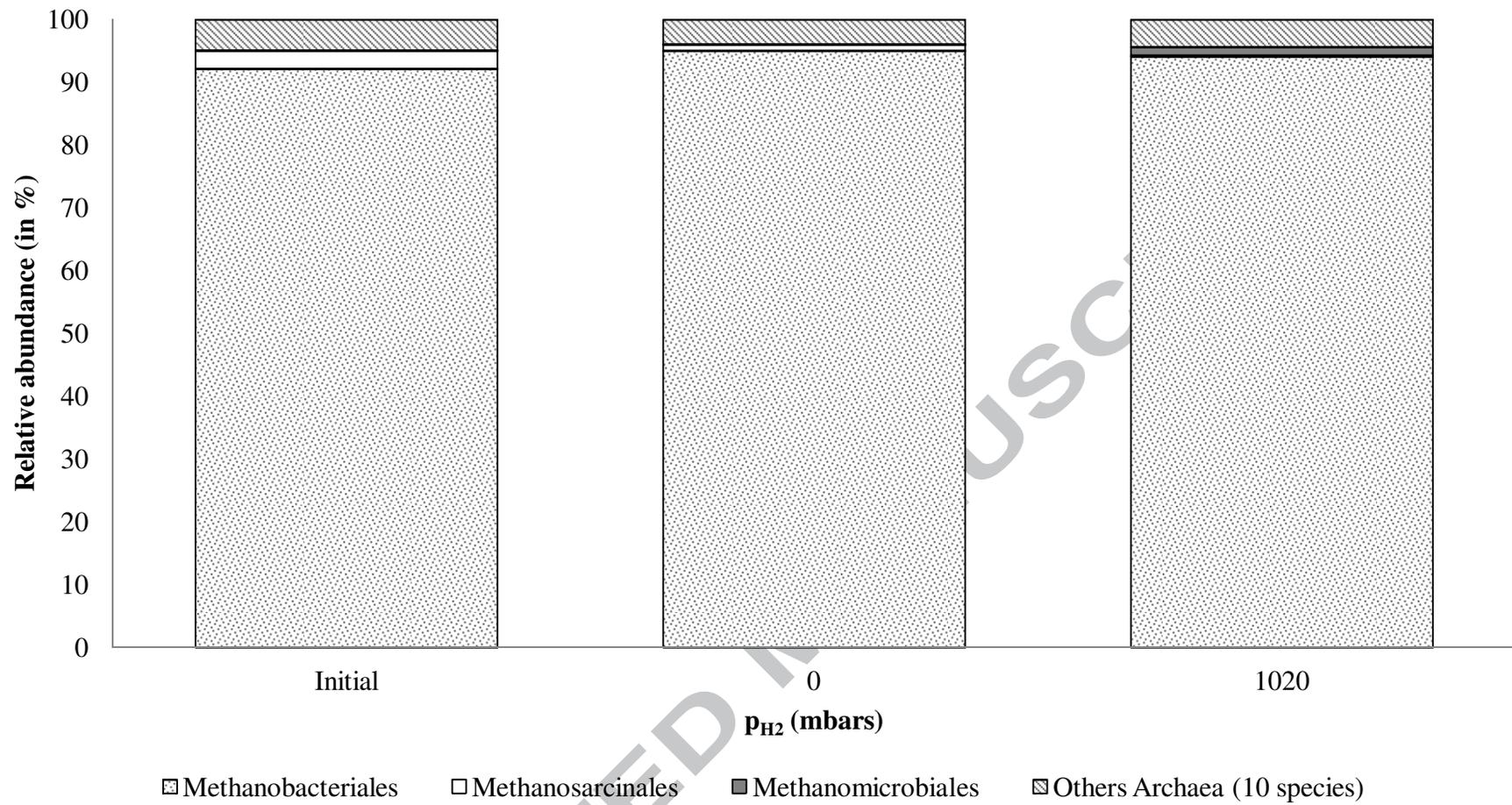
Table 1 : Gas partial pressure investigated in different runs.

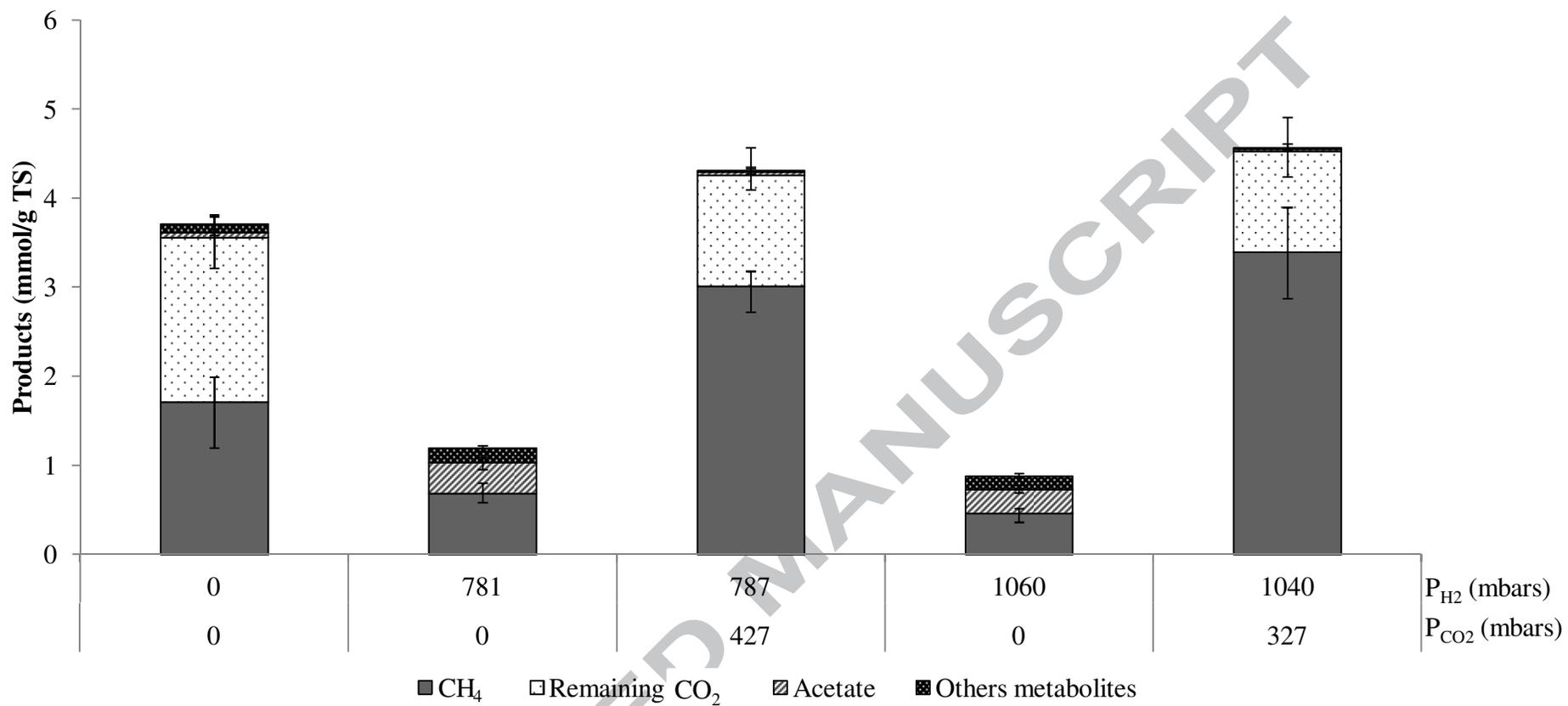
Table 2 : Final substrate degradation (in g COD per g of initial TS) according to the initial partial pressure of H_2 and CO_2 (in mbars). Similarity was determined by ANOVA (analysis of variance) test between the control (0 mbars of CO_2 and 0 mbars of H_2) and when H_2 and/or CO_2 were added. Non-significant P-value are >0.05 (Similar) and significant P-values are <0.05 ; <0.01 and <0.001 (Different).











Runs	Partial pressure of H₂ (mbars)	Partial pressure of CO₂ (mbars)
1	0, 55, 105, 485 and 895	0
2	0, 650, 750, 932 and 1 020	0
3	0, 778 and 1 418	0
4	0, 828 and 1 545	0
5	0, 780 and 1 060	0, 327 and 427

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Initial partial pressure of H₂ (mbars)	Initial partial pressure of CO₂ (mbars)	Substrate degradation (g COD /g TS)
0	0	0.11±0.018
781	0	0.03 ± 0.01 (Different)
787	427	0.13 ± 0.01 (Similar)
1 060	0	0.01 ± 0.01 (Different)
1 040	327	0.14 ± 0.03 (Similar)

Highlights

- H_2 partial pressure inhibits specifically hydrolysis in mesophilic SS-AD.
- CO_2 is a limiting factor for CH_4 production in SS-AD through H_2 consumption.
- Microbial community is not impacted by H_2 inhibition

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