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Microbial competition control between hydrogenotrophic methanogens and homoacetogens for selective production of acetate under hydrogen and carbon dioxide in successive batches

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Abstract:	<p>Capturing and utilising carbon dioxide (CO₂) is a major challenge for developing a low-carbon economy. In addition, the reduction of CO₂ allows the synthesis of platform molecules for the chemical and energy industry. Anaerobic mixed cultures contain homoacetogenic microorganisms (HAC) capable of reducing CO₂ to acetate. However, one of the obstacles to their use is the understanding and control of their functional diversity. In particular, managing the competition between HAC and hydrogenotrophic methanogens (HM) that convert CO₂ into methane is crucial to selectively produce acetate.</p> <p>This study contributes to bring new knowledge on the competition between HAC and HM. In this sense, mass transfer between the gas phase where the substrates are located, and the liquid phase which contains the microbial catalysts, as well as kinetic and thermodynamic aspects of biological reactions have been integrated in this work. The microbial competition between HM and HAC was studied in successive batches. The effect of temperature between 25 °C and 35 °C was investigated, as well as the time of each batch, leading to different states of mass transfer limitation in the system. A clear effect of temperature between 25 °C and 35 °C on the outcome of the competition between HM and HAC was highlighted, as well as the effect of mass transfer limitation. This study contributes to identify specific process parameters influencing the selection of HAC over HM and should help in the design of experiments depending on the target product from H₂/CO₂.</p> <p>In successive batches, lifting of mass transfer limitation, as well as lowering of the temperature to 25 °C made it possible to select HAC in mixed anaerobic cultures for acetate production by eliminating the methanogens, after four successive batches of 4 to 6 days each.</p>
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Title:

Microbial competition control between hydrogenotrophic methanogens and homoacetogens for selective production of acetate under H₂/CO₂ in successive batches

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Declaration of interests

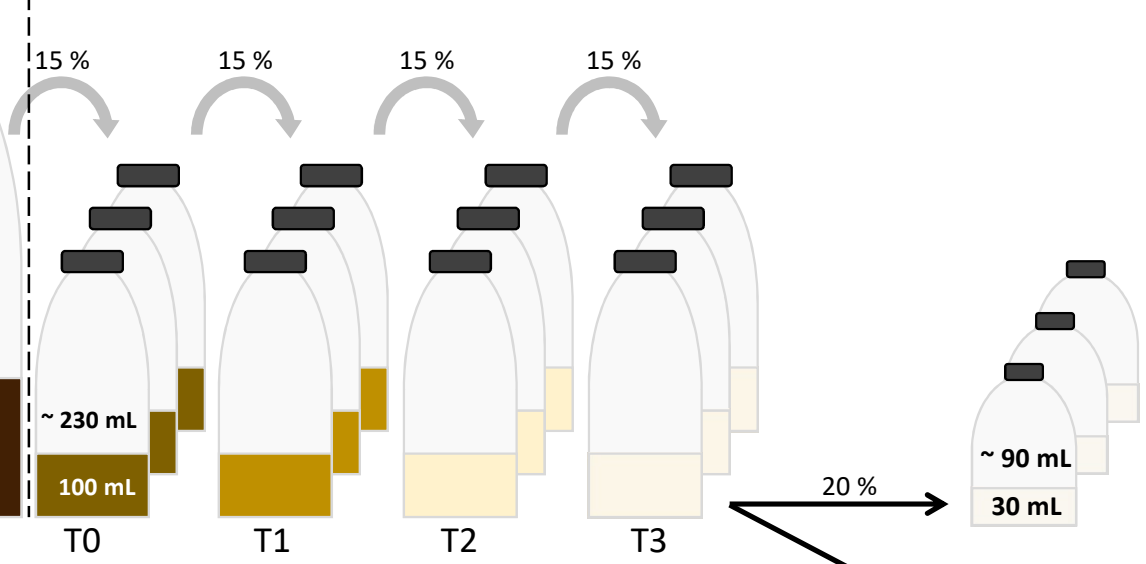
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Inoculum activation with Glucose at 35 °C



SB Enrichments under H₂/CO₂ 25 or 35 °C



Activity tests

- H₂/CO₂
- H₂/CO₂ + BES
- N₂ + Acetate

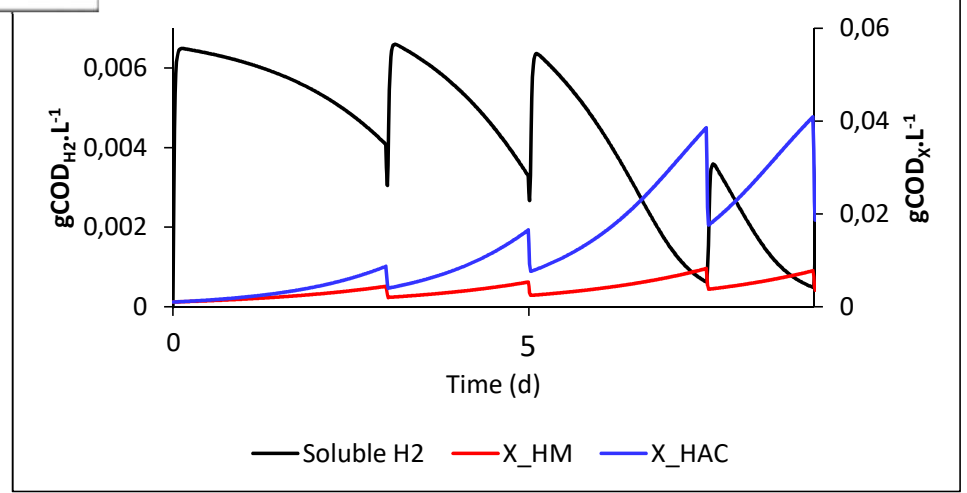
Active production routes

16S rDNA sequencing

Microbes enriched

Successful HAC selection method

Modeling of the MMC specialization in SB



Highlights:

- A homoacetogens selection method was developed from anaerobic mixed cultures
- Mass transfer limitation led to hydrogenotrophic methanogens selection
- Lower temperature from 35 °C to 25 °C benefited to homoacetogens growth

Figure 1: Main biological reactions considered in the system and discussed in this study.

Figure 1: Graphical explanation of the activities identified under the conditions of the activity tests carried out on the MMCs selected during the SB experiments. In green and black the feasible and unfeasible routes according to thermodynamics at initial state of the test respectively. Red cross represent the inhibitory effect of BES on methanogenesis. Adapted from (Navarro et al., 2016).

Figure 2: Electron balances considering acetate and methane produced over H_2 consumed during the four sets of SB carried out at 25 °C and 35 °C, under mass transfer limitation or not.

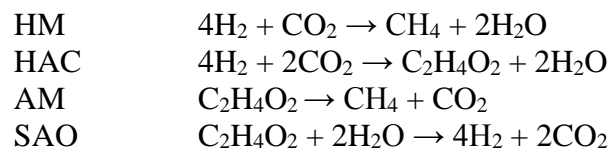
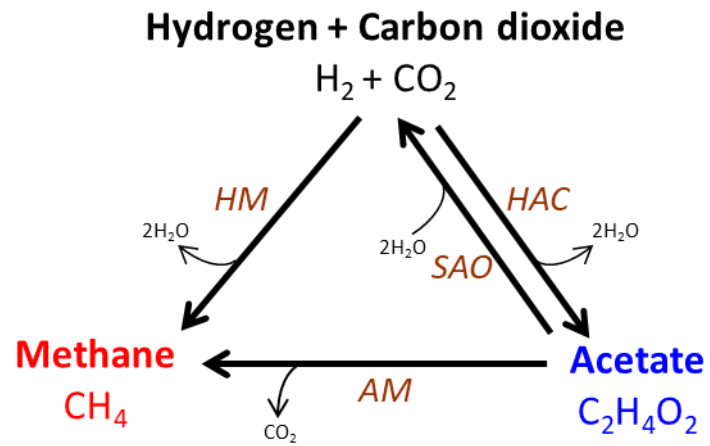
Figure 4: Results of the activity tests carried out with the MMCs enriched in SB at 35 °C under mass transfer limitation.

Figure 3: Results of the activity tests carried out with the MMCs enriched in SB at 25 °C A: under mass transfer limitation; B: avoiding mass transfer limitation.

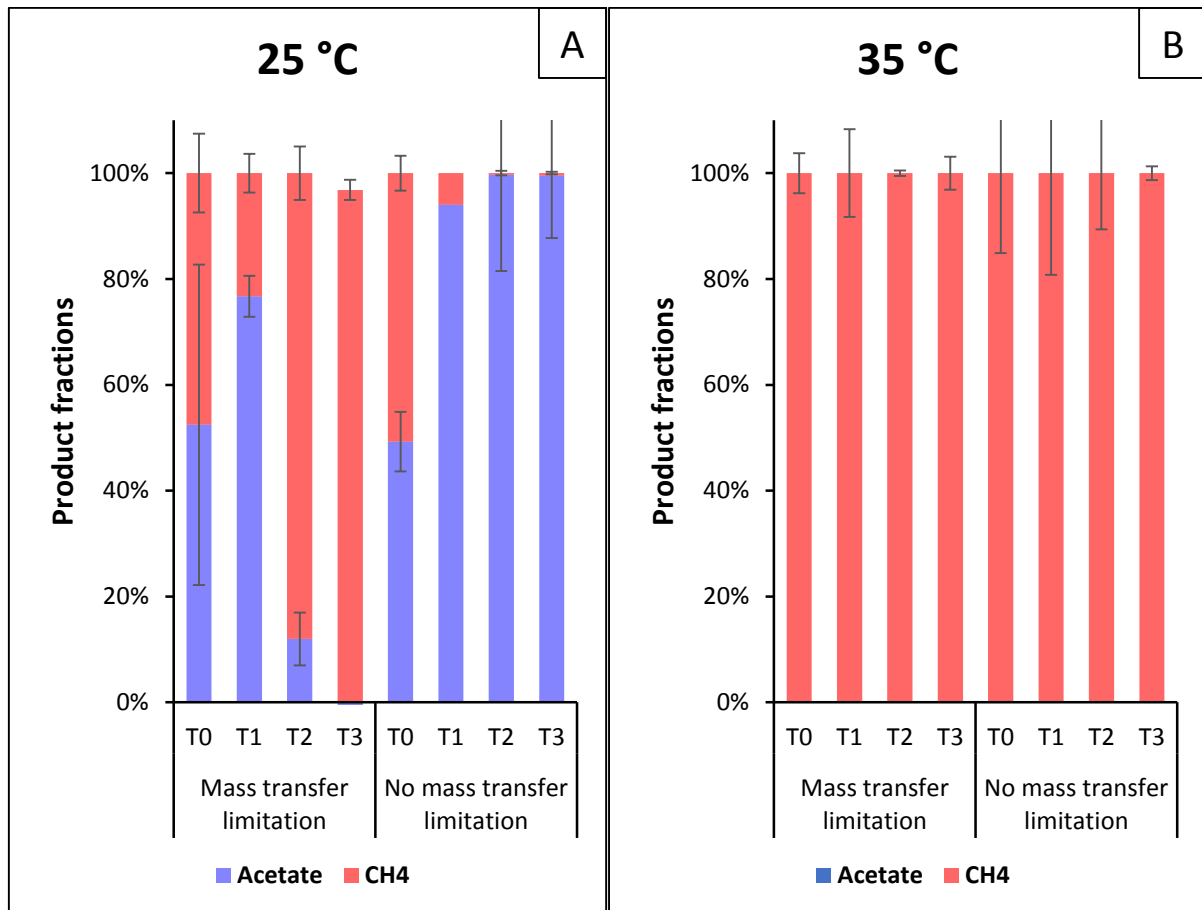
Figure 6: Growth yields calculated during the activity tests of the enriched culture in SB at 25 °C with several gas injections and late transfer. In blue is represented the activity test with BES, corresponding to HAC growth. In red is represented the activity test without BES, corresponding to HM growth. A: growth yield as a function of time (HAC: n=3; HM: n=2). B: Growth yield according to PH2.

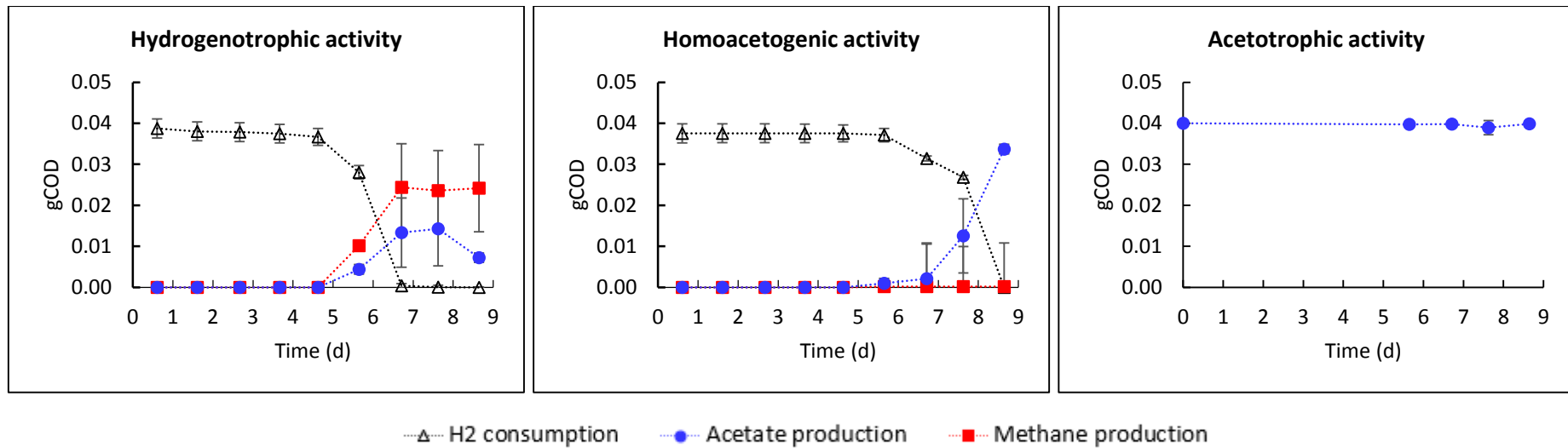
Figure 7: Relative abundance of taxa detected in the MMCs, at genus level, generated with rANOMALY package in R. MTL stands for mass transfer limitation.

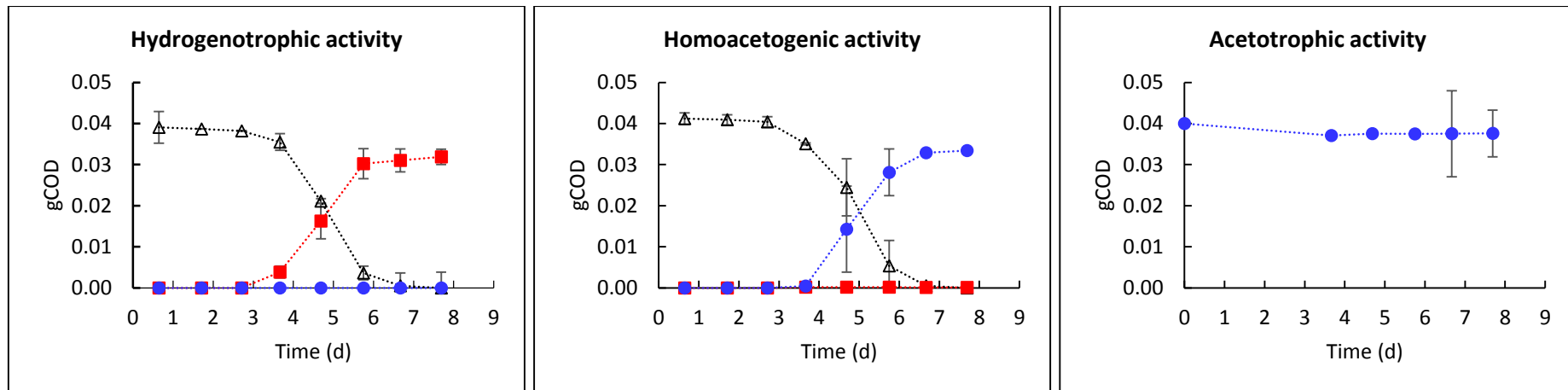
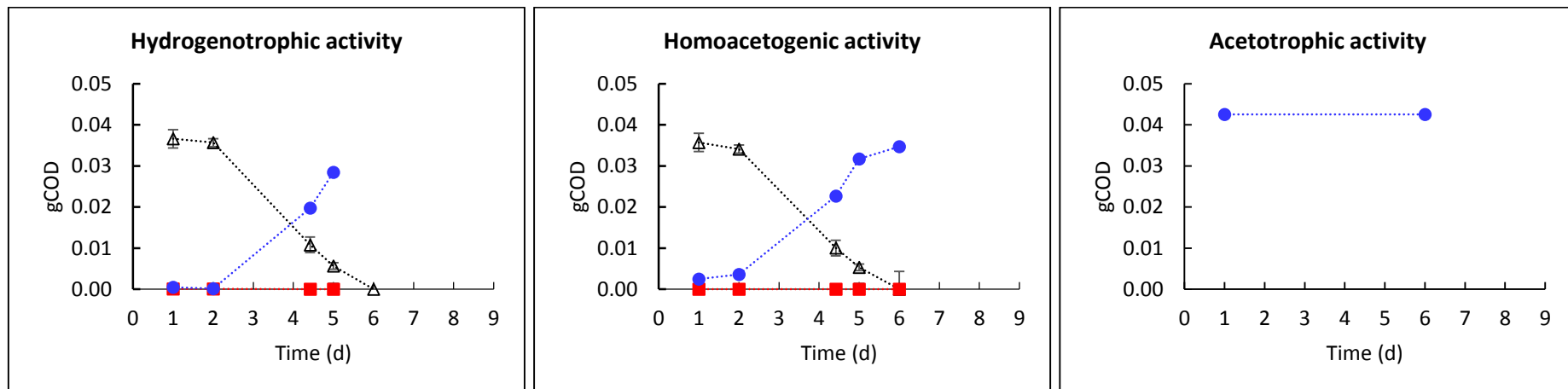
Figure 8: Simulation of microbial competition during H_2/CO_2 fermentation in batch mode. A: Hydrogen and biomasses concentrations in the liquid phase; B: Total pressure and partial pressures of the different gases. Liquid volume: 0.5 L; Gas volume: 1.5 L; kLa : 10d⁻¹; initial HM and HAC concentrations: 0.001 gCOD.L⁻¹; KH_2 (mol.L⁻¹): 0.000001 for HM and 0.00052 for HAC; k_m (gCODH₂.gCODX⁻¹.d⁻¹): 78.13 and 98.77 for HM and HAC respectively; growth yields (gCODX.gCODH₂⁻¹): 0.064 and 0.081 for HM and HAC respectively. C: ΔrG and D: FT were calculated as described in chapter2 section.



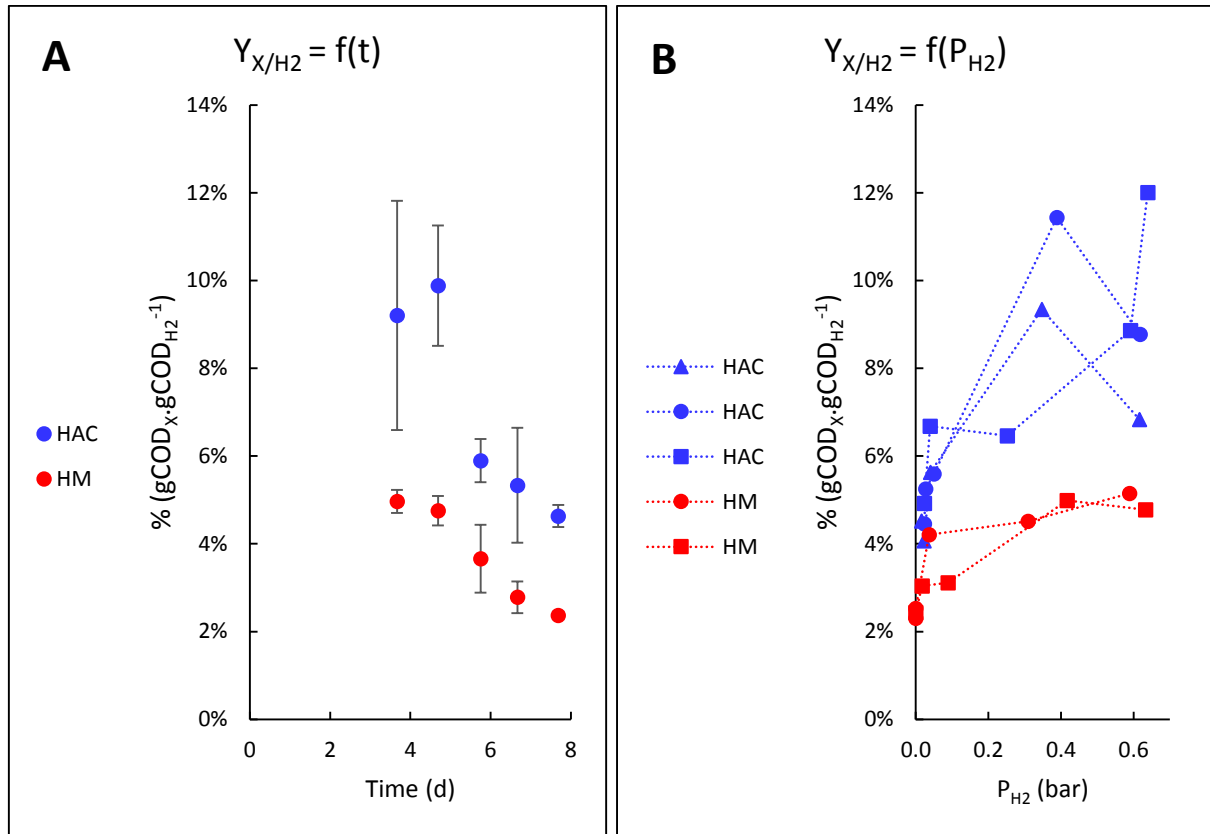
Hydrogenotrophic activity test	Homoacetogenic activity test	Acetotrophic activity test
$H_2 + CO_2$	$H_2 + CO_2 + BES$	$N_2 + Acetate$
<p>Diagram illustrating the hydrogenotrophic activity test. The reaction network shows $H_2 + CO_2$ at the top, $Methane$ (CH_4) at the bottom left, and $Acetate$ ($C_2H_4O_2$) at the bottom right. The pathways are: $H_2 + CO_2 \rightarrow Methane$ (HM), $H_2 + CO_2 \rightarrow Acetate$ (HAC), $Methane \rightarrow Acetate$ (AM), and $Acetate \rightarrow H_2 + CO_2$ (SAO).</p>	<p>Diagram illustrating the homoacetogenic activity test. The reaction network shows $H_2 + CO_2$ at the top, $Methane$ (CH_4) at the bottom left, and $Acetate$ ($C_2H_4O_2$) at the bottom right. The pathways are: $H_2 + CO_2 \rightarrow Methane$ (HM, active), $H_2 + CO_2 \rightarrow Acetate$ (HAC), $Methane \rightarrow Acetate$ (AM, inactive, marked with a red X), and $Acetate \rightarrow H_2 + CO_2$ (SAO).</p>	<p>Diagram illustrating the acetotrophic activity test. The reaction network shows $H_2 + CO_2$ at the top, $Methane$ (CH_4) at the bottom left, and $Acetate$ ($C_2H_4O_2$) at the bottom right. The pathways are: $H_2 + CO_2 \rightarrow Methane$ (HM), $H_2 + CO_2 \rightarrow Acetate$ (HAC), $Methane \rightarrow Acetate$ (AM, inactive, marked with a green X), and $Acetate \rightarrow H_2 + CO_2$ (SAO, active, marked with a green +).</p>

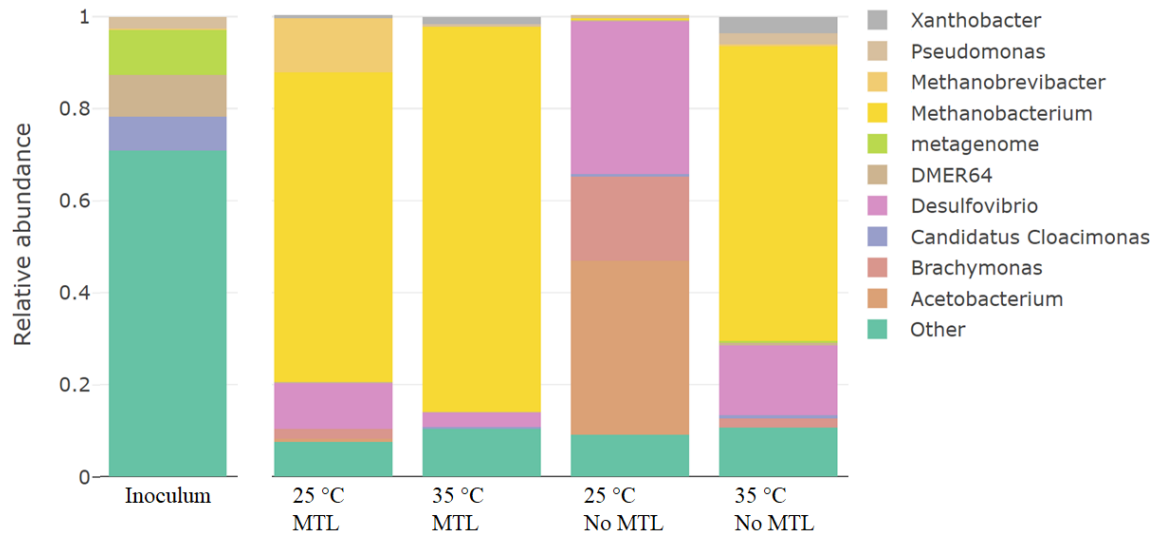


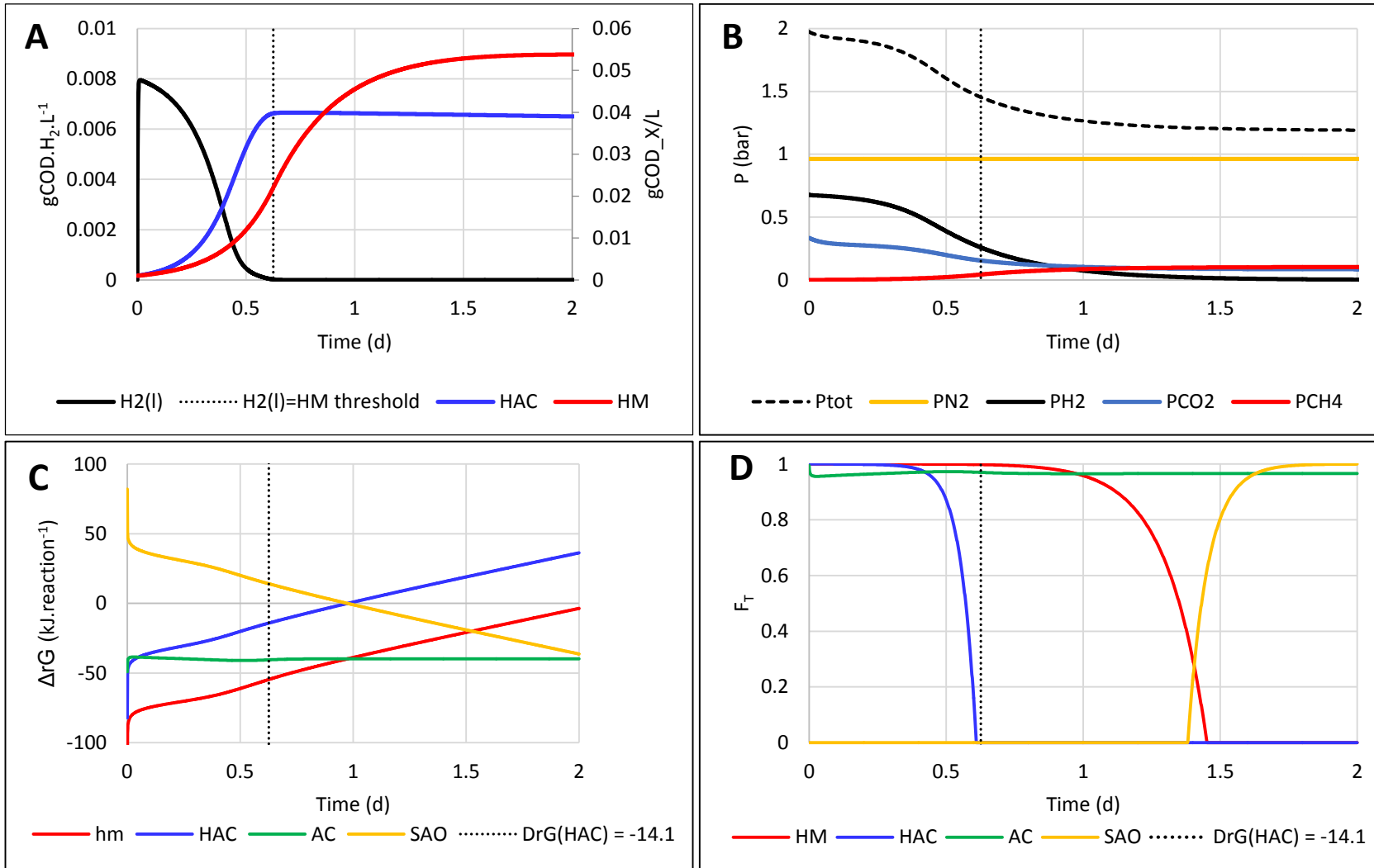
Activity tests on MMCs enriched in SB at 35 °C under mass transfer limitation:

A: Activity tests on MMCs enriched in SB at 25 °C under mass transfer limitation:**B: Activity tests on MMCs enriched in SB at 25 °C avoiding mass transfer limitation:**

---△ H₂ consumption
 ---● Acetate production
 ---■ Methane production







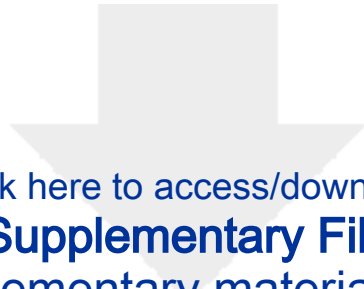
Abstract:

1
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1 **Keywords:**

2 Carbon dioxide

3 Methane

4 Acetate

5 Mixed Microbial Culture

6 Microbial selection

7 Successive batches

8 **Abbreviations:**

9 AD: Anaerobic Digestion

10 AM: Acetoclastic Methanogens

11 HAC: Homoacetogens

12 HM: Hydrogenotrophic Methanogens

13 MMC: Mixed Microbial Culture

14 MTL: gas to liquid mass transfer limitation

15 SAO: Syntrophic Acetate Oxidisers

16 SB: Successive Batch

17 TS: Total solid

18 VS: volatile solid

19 WLP: Wood Ljungdahl Pathway

20 WWT: Waste Water Treatment

21

22

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24

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27 ***1. Introduction***

28 The development of a circular carbon economy has become a major area of research. Research
29 efforts are on the rise to reuse organic agricultural and household residues as well as residual
30 streams linked to industrial activity. These wastes constitute an interesting substrate for many
31 biological processes aimed at the production of energy or high added value materials. New
32 branches of the biorefinery are appearing to reuse solid wastes, which are more difficult to
33 biodegrade. One of the ways of reusing this type of waste is gasification by thermochemical
34 treatment. The solid carbonaceous material is thus transformed into synthesis gas, that is to say
35 a mixture of gases such as CO, CO₂, H₂, and other minor gases such as H₂S or CH₄. Other
36 sources of gas to be upgraded exist, such as the CO₂ co-produced in many industrial processes,
37 and also the CO₂ contained in biogas from anaerobic digestion, which contains methane, but
38 also around 30-50 % of CO₂ (Gavala et al., 2021; Angelidaki et al., 2018).

39 Homoacetogens (HAC) are interesting candidates for the biological reduction of CO₂ to
40 chemicals using the Acetyl-CoA reductive pathway (or Wood Ljungdahl Pathway, WLP). The
41 latter is a highly energetically efficient route fixing two CO₂ into acetyl-CoA, inherited from
42 the earliest stages of life on earth. However, discovery of HAC dates from the 1930s, since
43 when new homoacetogens keep being identified, isolated, and characterized. Interestingly,
44 homoacetogenesis is not a phylogenetic trait, because HAC are found in different phyla. HAC
45 can use a wide range of electron acceptors and donors, such as nitrate, but also organic reduced
46 compounds such as ethanol. HAC use WLP as a catabolic route producing acetate during the
47 generation of ATP, as well as for biosynthesis of cell components from acetyl-CoA. Other
48 anaerobic microorganisms are encoding the WLP, including hydrogenotrophic methanogens
49 (HM) and sulphate reducers. Hence, under anaerobic and autotrophic conditions and depending
50 on the available substrates, different communities can coexist in a microbial mixed culture
51 (MMC). Under H₂/CO₂ as sole energy and carbon sources, some interactions, in particular

52 competitive interactions, exist to fix the common substrates. If the medium doesn't contain
53 other electron acceptors than CO₂ (*i.e.* Fe³⁺, NO³⁻, SO₄²⁻ or S⁰), then the communities remaining
54 in competition are HM and HAC. To favour HAC and acetate production over methane, it is
55 necessary to find a way to eliminate or inhibit methanogens.

56 To inhibit methanogenesis, different strategies may work. It is possible to apply heat treatment
57 to the microbial consortium with the aim of eliminating communities that are not able to
58 sporulate, which is the case of methanogens. A revitalization work must then be done to exploit
59 the metabolic potential of the sporulating microbial strains in MMC. This strategy has the
60 advantage of efficiently eliminating methanogenic populations in most cases, even if some
61 authors still observe the recovery of methane production after treatment (Liu et al., 2018). Some
62 authors like Omar et al. (2018), were even able to observe a methane yield of 100 % after heat
63 treatments at 70 or 90 °C for 30 min. In addition, this technique can also rule out non-
64 sporulating hydrogenotrophic bacteria which may be of interest for the desired activity. In
65 particular, *A. woodii*-like HAC are non-spore-forming bacteria (Schuchmann and Müller,
66 2014). This technique is also expensive, and energy consuming, and doesn't prevent
67 contamination later during the operations. Another strategy, widely used in laboratory-scale
68 studies, is the addition of chemical inhibitors of methanogenesis. The most used is 2-
69 bromoethanesulfonate (BES), but there are others such as mercaptoethanesulfonate (MES) or
70 lumazine (Liu et al., 2011). MES and BES mimic the methyl-CoM and thus block the enzyme
71 of the last stage of methanogenesis (HM and AM). These inhibitors therefore make it possible
72 to inhibit any type of methanogenesis. BES is used at different concentrations depending on the
73 studies, from 10 to 50 mM and even 100 mM (Wang et al., 2017; Luo et al., 2018; Omar et al.,
74 2018; Shen et al., 2018). It is interesting to note that the concentrations chosen do not seem to
75 depend on the duration of the experiment nor on the temperature, and are rarely justified or
76 discussed. However, during long cultivation times it is necessary to regularly add BES because

77 it is degraded over time in the system (Steinbusch et al., 2011; Luo et al., 2018). Chemical
78 inhibitors are convenient for specific lab experiment, though they are not an economically
79 viable solution either in the framework of the development of industrial production processes
80 (Agler et al., 2011).

81 For this reason, the present study focuses on engineering MMC in order to eliminate
82 methanogens' activity and selectively produce chemicals (*i.e.* acetate). Hence, the main
83 challenge is to understand the competition between HM and HAC and be able to favour HAC
84 rather than HM by applying specific process conditions. Different studies focused on the
85 competition between HM and HAC because it is an undesirable reaction in dark fermentation
86 for the production of H₂ for example (Fu et al., 2019; Regueira et al., 2018; Molenaar et al.,
87 2017; Annie Modestra et al., 2015; Saady, 2013; Kotsyurbenko et al., 2001). Some researchers
88 also have showed interest in HAC in the field of anaerobic digestion when it comes to operate
89 at psychrophilic temperatures (Vavilin et al., 2000; Conrad et al., 1989). In gas fermentations
90 though, when the substrate is either syngas or CO₂ and H₂, homoacetogenesis constitute a
91 significant share of microbial activity responsible for acetic acid generation, directly competing
92 with HM while it is greatly influenced by the temperature of the process (Grimalt-Alemany et
93 al., 2019).

94 In this article, the competition between HAC and HM is investigated in batch mode under
95 H₂/CO₂, by the implementation of successive batches (SB). SB were carried out at 25 °C or 35
96 °C, according to different community engineering strategies: with transfer during exponential
97 phase or during stationary phase after several gas injections. In the first case, mass transfer
98 limitation during the enrichment period was avoided, and the time to grow was shorter than in
99 the second case that aimed to increase biomass production, implying mass transfer limitation.
100 Activity tests were carried out on the enriched cultures to identify the metabolic functions
101 selected. 16S rDNA high throughput sequencing was also undertaken on the enriched cultures.

103 2. **Materials and methods**

104

105 **2.1. Mixed microbial culture origin and preparation**

106 Two different sludges have been mixed to maximize the initial biodiversity for the enrichment
107 experiments: 700 mL of a WWT plant sludge and 700 mL of a lab scale AD sludge. After
108 mixing, the inoculum was placed in two serum bottles of 2L with 5 g.L⁻¹ of glucose, flushed
109 with N₂ during 30 minutes and incubated at 35 C° without mixing. Active inoculum contained
110 19.7 g.L⁻¹ of total solids (TS) and 12.3 g.L⁻¹ of volatile solids (VS).

111

112 **2.2. Growth medium**

113 The growth medium was prepared from the four following solutions: salts (NH₄Cl, 50 g.L⁻¹;
114 NaCl 10 g.L⁻¹; MgCl₂.6H₂O, 10 g.L⁻¹; CaCl₂.2H₂O, 5 g.L⁻¹), Na₂SO₄ 50 g.L⁻¹, Vitamins (biotin,
115 2 mg.L⁻¹; folic acid, 2 mg.L⁻¹; pyridoxine-HCl, 10 mg.L⁻¹; riboflavin-HCl, 5 mg.L⁻¹; thiamine-
116 HCl, 5 mg.L⁻¹; cyanocobalamin, 0.1 mg.L⁻¹; nicotinic acid, 5 mg.L⁻¹; p-aminobenzoic acid, 5
117 mg.L⁻¹; lipoic acid, 5 mg.L⁻¹; D-pantothenic acid hemicalcium salt, 5 mg.L⁻¹), Trace metals
118 (Nitrilotriacetic acid, 2000 mg.L⁻¹; Fe(SO₄)₂(NH₄)₂.6H₂O 800 mg.L⁻¹; H₃BO₃, 10 mg.L⁻¹;
119 ZnSO₄.7H₂O, 200 mg.L⁻¹; CuCl₂, 20 mg.L⁻¹; MnSO₄, 1000 mg.L⁻¹; Na₂MoO₄.2H₂O, 20 mg.L⁻¹;
120 ¹; AlCl₃, 10 mg.L⁻¹; CoCl₂.6H₂O, 200 mg.L⁻¹; NiCl₂, 20 mg.L⁻¹; Na₂SeO₄.5H₂O, 18 mg.L⁻¹;
121 Na₂WO₄.2H₂O, 22 mg.L⁻¹). Potassium dihydrogen phosphate (KH₂PO₄ 1M) and dipotassium
122 hydrogen phosphate (K₂HPO₄ 1M) were used as buffering solutions. The medium was prepared
123 by adding 20 mL.L⁻¹ of salts solutions, 1 mL.L⁻¹ of Na₂SO₄ solution, 10 mL.L⁻¹ of vitamins
124 solutions, 10 mL.L⁻¹ of trace metals solution, 86 mL.L⁻¹ of KH₂PO₄ and 14 mL.L⁻¹ of K₂HPO₄.
125 The pH of the medium under N₂ atmosphere was 5.98 ± 0.05.

126

127 **2.3. Enrichment cultures in successive batches at 25 °C and 35 °C, with transfer**
128 **during exponential or stationary phase of growth**

129 SB were carried out in triplicates in 330 mL serum vials at 25 °C and 35 °C. The flasks were
130 filled with 85 mL of growth medium, sealed with rubber stoppers and screw plugs and flushed
131 15 min with N₂ before the addition of 15 mL of active inoculum. 75 mL of CO₂ and 150 mL of
132 H₂ were added in the flasks to reach a final relative pressure of 1 bar with H₂ and CO₂ partial
133 pressures of 0.66 ± 0.03 and 0.33 ± 0.03 bar, respectively. The pH (measured externally) was
134 6.3 ± 0.1 after inoculation with raw activated inoculum (T0), and 6.1 ± 0.0 after inoculation
135 with enriched cultures (from T1 to T3). Four successive batches were operated (T0-T3),
136 selecting the most active culture at each step to inoculate the next triplicate of batches, similarly
137 to Grimalt-Aleman et al. (2019).

138
139 **2.4. Activity tests**

140 To assess the enriched microbial activities, tests were carried out in 120 mL serum vials with
141 30 mL of liquid volume. Vials were filled with 24 mL of growth medium, sealed with rubber
142 stoppers and screw plugs and flushed 10 min with N₂ before the addition of 6 mL of enriched
143 culture T3 (20 % v/v). Triplicates of three different conditions were carried out (Figure 2). The
144 first condition was H₂/CO₂, 30 mL of CO₂ and 60 mL of H₂ were injected in the vial. The
145 second condition was H₂/CO₂ + BES, 15 mM of BES were added to the culture broth, 30 mL
146 of CO₂ and 60 mL of H₂ were injected in the vials. The third condition was N₂/Acetate, 20 mM
147 of acetate were added to the culture broth, without H₂/CO₂ injection. The vials were incubated
148 at the temperature at which the inoculum (T3) had been grown (25 °C or 35 °C).

149

150

2.5. Analytical Methods

151 Gas composition in H₂, CO₂, CH₄, N₂, O₂ was analysed using a gas chromatograph (GC 8610C,
152 SRI Instruments, USA) while liquid was analysed for volatile fatty acids and alcohols
153 concentrations determination (formic acid, acetic acid, butyric acid, valeric acid, hexanoic acid,
154 isobutyric acid, isovaleric acid, ethanol, butanol) using a High-Performance Liquid
155 Chromatograph (HPLC Shimadzu, USA). Detailed conditions of the analytical methods are
156 described in previous paper (Grimalt-Aleman et al., 2019). Microbial biomass was monitored
157 with OD measurements at 600 nm using a spectrophotometer (DR2800, Hach Lange). A
158 correlation between OD₆₀₀ and total solids (TS) have been done with 50 mL samples of a CSTR
159 culture inoculated with the same MMC at 25 °C under H₂/CO₂ (eq. K).

160

161

2.6. DNA extraction and 16S rDNA sequencing

162 Samples for genomic DNA extraction and 16S rDNA sequencing were collected from the
163 activated inoculum, from the successive batch enrichments at the end of the third transfer at 25
164 and 35 °C, and from the CSTR on day 41, 62 and 68. DNeasy PowerSoil™ DNA Isolation Kit
165 (Qiagen, Denmark) was used for the DNA extraction of the activated inoculum. DNeasy Blood
166 & Tissue™ Kit (Qiagen, Denmark) was used for the DNA extraction of all other samples. DNA
167 extracted were submitted to Macrogen Europe. Amplification of V4 and V5 regions of 16S
168 rDNA was performed with 515F-Y 5'-GTGYCAGCMGCCGCGGTAA and 926R 5'
169 CCGYCAATTYMTTTRAGTTT primers (Walters et al., 2015). Library preparation was done
170 according to Illumina NGS workflow with Illumina Miseq instrument (Vigliar et al., 2015).
171 Raw reads containing primers were trimmed using cutadapt and untrimmed reads were
172 discarded (Martin, 2011). Remaining reads were processed using the Qiime2 pipeline (Hall and
173 Beiko, 2018). Specifically, reads were filtered and denoised using DADA2 and the taxonomic

174 assignment was performed with classify-sklearn using a classifier trained on the reference
 175 database SILVA132 using a naive-bayesian classifier (Callahan et al., 2016; Quast et al., 2013;
 176 Pedregosa et al., 2011). Data visualization was processed with rANOMALY package (Theil
 177 and Rifa, 2021).

178 **2.7. Dynamic model on AQUASIM**

179 A kinetic model has been developed on Aquasim 2.1g (Reichert, 1994) in order to study and
 180 simulate the microbial competition within the consortium, according to kinetics and
 181 thermodynamics features. The model includes dynamic microbial growth in accordance with
 182 Monod equation; dynamic physical-chemical processes of weak acid dissociation, acid-base
 183 equilibria (Musvoto et al., 2000), gas to liquid mass transfer, and thermodynamic calculations.
 184 H₂, CO₂ and CH₄ mass transfer rates are described in the model based on eq (1).

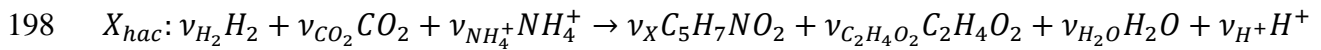
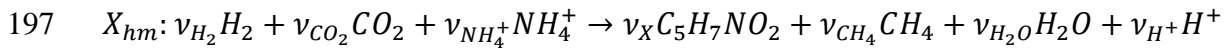
$$185 \quad T_{ri} = K_L a_i \times (K_{Hi}^{cc} \times C_{i,aq} - C_{i,g}) \quad (1)$$

186 where $C_{i,aq}$ and $C_{i,g}$ the soluble aqueous and gaseous concentrations of compound i , k_{La_i} is the
 187 volumetric transfer coefficient and K_{Hi}^{cc} is dimensionless Henry's law volatility constant
 188 expressed as the ratio of concentrations in the gas over the liquid at equilibrium state. K_{Hi}^{cc} values
 189 were taken from Sander (2015), and were corrected for temperature based on eq. (2) and (3), Δ
 190 _{sol}H being the molar dissolution enthalpy, and H_i^{cp} the Henry's law solubility, values used for
 191 the correction are detailed in S1.

$$192 \quad K_{Hi}^{cc} = \frac{1}{H_i^{cp}(T) \times RT} \quad (2)$$

$$193 \quad H_i^{cp}(T) = H_i^{cp}(T^\theta) \times \exp\left(\frac{-\Delta_{sol}H}{R} \times \left(\frac{1}{T} - \frac{1}{T^\theta}\right)\right) \quad (3)$$

194 The model includes two growth processes describing hydrogenotrophic methanogenic archaea
 195 (X_{HM}) and homoacetogenic bacteria (X_{HAC}) production rates (Table 1), and cell biomass
 196 composition is considered as C₅H₇NO₂.



199 Hydrogen is considered as the limiting substrate in any case due firstly to its low solubility and
200 secondly because CO₂ is supplied in excess compared to H₂ according to the stoichiometric
201 ratio of the reactions. Thus, only Monod term for H₂ is included in growth rates and CO₂
202 substrate limitation is neglected. The model includes two decay processes describing X_{HM} and
203 X_{HAC} biomass decomposition into composite materials X_C as it is described in ADM1 (Table
204 1), with k_{dec} set to 0.02 for HAC and HM. Note that in the framework of this project, the
205 heterotrophic growth on composite material, as well as the volume variations due to water
206 production, were considered as negligible and were not included. Table 2 shows the acid-base
207 equilibria integrated in the model, values of dissociation constant used are found in S2.

Processes ↓	State variables (mol.L ⁻¹)										Rates (mol.L ⁻¹ .d ⁻¹) ↓
	C _{Xc}	C _{Xh2}	C _{Xhac}	C _{H2,liq}	C _{CH4,liq}	C _{ac-}	C _{H2O produced}	C _{IC,liq}	C _{IN,liq}	C _{H+}	
Growth of X _{HM}		Y _{XHM/H2}		-1	1 - Y _{XHM/H2}		$\sum_{i=IC_{CO_2}, X_{HM}, CH_4} O_i v_i, X_{HM}$	$-\sum_{i=X_{HM}, CH_4} C_i v_i, X_{HM}$	- Y _{XHM/H2}	Y _{XHM/H2}	$k_{mol, HM} \times \frac{C_{H2}}{C_{H2} + K_{s, HM}} \times X_{HM}$
Growth of X _{HAC}			Y _{XHAC/H2}	-1	1 - Y _{XHAC/H2}		$\sum_{i=IC_{CO_2}, X_{HM}, CH_4} O_i v_i, X_{HAC}$	$-\sum_{i=X_{HAC}, C_2H_4O_2} C_i v_i, X_{HAC}$	- Y _{XHAC/H2}	Y _{XHAC/H2}	$k_{mol, HAC} \times \frac{C_{H2}}{C_{H2} + K_{s, HAC}} \times X_{HAC}$
Decay of X _{HM}	1	-1									$k_{dec, HM} \times X_{HM}$
Decay of X _{HAC}	1		-1								$k_{dec, HAC} \times X_{HAC}$

209 *Table 1: Biochemical rate coefficients (v_i) and kinetic rate equations for growth and decay processes included in the model.*

Processes ↓	State Variables (mol.L ⁻¹)							Rates (mol.L ⁻¹ .d ⁻¹) ↓	
	C _{acH}	C _{ac}	C _{IN}	C _{IC_co2}	C _{IC_hco3}	C _{IC_co3}	C _H		C _{oh}
f_acH_ac	-1	1					1		$Ka_{acH} \times C_{acH}$
r_ac_acH	1	-1					-1		$C_{ac-} \times C_{H+}$
f_NH4_NH3			-1				1		$Ka_{NH_3} \times C_{NH_4^+}$
r_NH3_NH4			1				-1		$C_{NH_3} \times C_{H+}$
f_CO2_HCO3				-1	1		1		$Ka_{CO_2} \times C_{CO_2}$
r_HCO3_CO2				1	-1		-1		$C_{HCO_3^-} \times C_{H+}$
f_HCO3_CO3					-1	1	1		$Ka_{HCO_3^-} \times C_{HCO_3^-}$
r_CO3_HCO3					1	-1	-1		$C_{CO_3^{2-}} \times C_{H+}$
f_H_H2O_OH							1	1	K_w
r_OH_H2O_H							-1	-1	$C_{OH^-} \times C_{H+}$

210 *Table 2: Rate coefficients and kinetic rate equations for acid-base reactions in the model.*

211 Thermodynamic considerations were included into the model to evaluate the feasibility of the biological
 212 reactions. Especially the calculations of Gibbs free energy (ΔrG) and thermodynamic factor (F_T) as
 213 described by Jin et Bethke (2007). Table 3 shows the parameters sued for the calculations.

	HM	HAC	AM	SAO
ΔrG° (kJ/reaction)	-131	-82	-49	+82
Y_ATP (molATP/reaction)	0.5	0.3	0.5	0.3
ΔG_p (kJ/reaction)	45	45	45	45
ΔG_c (kJ/reaction)	22.50	14.85	22.50	14.85
X	2	1	2	1

214 *Table 3: Thermodynamic parameters used for the calculation of the ΔrG of the different reactions*
 215 *considered in the experimental conditions (Grimalt-Aleman et al., 2020).*

216

217 **2.8. Theory/calculations**

218 Experimental growth yields in g COD cells per g COD substrate (H_2) of HM and HAC were calculated
 219 from the activity tests in which only HM and HAC functions were detected.

220
$$Y_{\frac{X}{H_2}} = \left(\frac{\Delta OD_{600} + 0.2807}{3.8255} \right) \times 1.4159 \times \frac{V_{liq}}{\Delta H_2} \text{ gCOD}_X \cdot \text{gCOD}_{H_2}^{-1} \quad (4)$$

221 With $1.4159 = \text{gCOD}_X \cdot \text{gvs}^{-1}$ if $X = C_5H_7O_2N$

222

223

224 3. Results and Discussion

225 HAC is a promising route for biotechnology development of C1 compounds (CO, CO₂, methanol etc.)
226 recovery into value added molecules. Studying the competition between HM and HAC could result in
227 a method for inoculum development that presents advantages compared to addition of inhibitors or heat
228 treatment. Grimalt-Alemany (2019) highlighted that the gas to liquid mass transfer rate was crucial in
229 the outcome of the competition between HM and HAC. Especially, the author shows that HAC only
230 grow under high H₂ availability and mesophilic conditions, due to the better capacity of HM to fix H₂
231 traces, and their much higher specific growth rates under thermophilic conditions. This indicates that
232 the competition between HM and HAC follows the resource ratio competition model. Microorganisms
233 can adopt two different strategies to have the advantage over their competitors. The first type of
234 competitors are r-strategists, meaning that they have high specific growth rates, or high specific
235 consumption rates. They will grow faster and become dominant. Conversely, K-strategists are microbes
236 with a better substrate affinity, meaning that the bioavailability of the substrate is higher for them than
237 for their competitors (Paul et al., 2021). Considering the low solubility of hydrogen, mass transfer
238 coefficient appears as a determinant parameter during gas fermentation in batch mode, as the substrate
239 is continuously delivered through gas to liquid mass transfer rate, rather than being totally available
240 from the beginning of the fermentation, as it is the case in a classical single liquid phase fermentation.
241 Indeed, mass transfer limitation will lead to a K-competition, benefiting HM in most cases. The
242 exception could be psychrophilic conditions, under which HAC could have faster H₂ fixating capacities.
243 Indeed, Conrad et al. (1989) observed that in MMC enrichments at 17 °C, HAC outcompeted HM, while
244 at 30 °C HM outcompeted HAC. The authors also identified a critical temperature determining a switch
245 in the outcome of the competition around 20-25 °C.

246 In this study, the microbial competition between HM and HAC under H₂/CO₂ was investigated in SB.
247 The effect of temperature between 25 °C and 35 °C was investigated, as well as mass transfer limitation.
248 A method for the enrichment of homoacetogens under H₂/CO₂ was developed.

249 ***3.1. Functional activities selected in SB***

250 **Error! Reference source not found.** shows the electron balances between acetate and methane
251 produced over H₂ consumed along the different SB carried out. Significant differences could be
252 observed between the different SB. First, acetate was never detected in the liquid phase of SB conducted
253 at 35 °C. On the other hand, at 25 °C, acetate could be detected, and the balance even moved towards
254 acetate as the sole product when mass transfer limitation was overcome. In SB supposed to be mass
255 transfer limited, acetate was detected in the first batches, but the balance progressively moved toward
256 methane as the sole product by the end of the experiment. The fact that acetate was detected at the
257 beginning of these SB may be due to mass transfer limitation being delayed because of higher gas
258 solubility, and also lower HM specific growth rates at 25 °C than at 35 °C.

259 These results demonstrate a strong effect of temperature on the microbial competition between HM and
260 HAC. Additionally, the way of implementing the SB with short batch times, and fast transfers might
261 have contributed to favour HAC. A combination effect of avoiding mass transfer limitation with short
262 growth times allowed to progressively eliminate methane production along the experiments. To
263 characterize the MMCs functions selected by the end of the SB, hydrogenotrophic, homoacetogenic and
264 acetotrophic activity tests were carried out (**Error! Reference source not found.**).

	$C_{H_2(aq)}$ M	$C_{CO_2(aq)}$ M	$C_{CH_4(aq)}$ M	$C_{Acetate}$ M	$\Delta rG_{25\text{ }^\circ C}$ kJ/reaction	$\Delta rG_{35\text{ }^\circ C}$ kJ/reaction
Acetic acid \rightarrow CO₂ + CH₄ (AM)	1.00E-10	1.00E-10	1.00E-10	0.023	-129	-132
Acetic acid + 2 H₂O \rightarrow 2 CO₂ + 4 H₂ (SAO)	1.00E-10	1.00E-10	1.00E-10	0.023	-164	-175

265 *Table 4: Gibbs free energy calculation of the acetate consuming reactions at the initial conditions*
266 *of activity tests with N₂/Acetate. 1.00E-10 M is used instead of 0 to do the calculations. 0.023 ±*
267 *0.003 M was the average concentration measured at initial state over all the vials carried out with*
268 *N₂/Acetate (n=18).*

269

270 **Error! Reference source not found.** shows the results of activity tests carried out on the MMC
271 enriched at 35 °C under mass transfer limitation. The results show that even if acetate was not
272 detected during the SB period, HAC as well as HM were active in the activity tests.
273 Hydrogenotrophic tests show that HAC and HM could grow, with methane being the main product.
274 At 35 °C, HM specific growth rate was likely higher than that of HAC, and also higher than at 25
275 °C. Additionally, H₂ solubility is lower at 35 °C than at 25 °C, inducing that the mass transfer
276 limitation was reached earlier, benefiting thus HM. Activity tests were also carried out on the two
277 MMCs obtained at 25 °C. According to **Error! Reference source not found.**A, in MMC enriched
278 at 25 °C under mass transfer limitation, HAC was active in the homoacetogenic tests, but couldn't
279 grow in hydrogenotrophic tests in which HM outcompeted them. This demonstrates that HAC were
280 still in the MMC even if acetate production was not detected anymore at the end of these SB.
281 However, with HM being active, acetate production was not observed. Conversely, in the MMC
282 enriched in SB at 25 °C, overcoming the mass transfer limitation, it was methane production that
283 was not detected anymore at the end of the SB. But in this case, activity tests revealed that
284 methanogenesis function was lost in the corresponding MMC. Indeed, only acetate production was
285 detected during the hydrogenotrophic test.

286

287 These results demonstrate that methanogenic function could be irreversibly eliminated from the
288 MMC by implementing a specific cultivation/enrichment method. This method consisted in
289 lowering temperature to 25 °C, and avoid mass transfer limitation by transferring from a batch to
290 another as soon as some substrate consumption was observed. In fact, the short time of each batch
291 must have been another constraint for HM. Indeed, at 25 °C, longer lag phases were observed for
292 HM than for HAC during the SB phase.

293
294 Acetate concentration and total pressure remained constant in all acetotrophic activity tests,
295 indicating that the MMCs were not able to consume acetate in these conditions. The results of these
296 activity tests show that AM and SAO were likely washed out from the MMCs along the SB, and
297 could not be reactivated afterwards when placed under favourable conditions. AM have generally
298 lower specific growth rates than HM (Batstone et al., 2002, p. 1), so if HM were washed out, it is
299 consistent that AM were too. Regarding SAO, the reaction is only possible after acetate is
300 produced, and thermodynamic calculations show that low H₂ and CO₂ partial pressures are
301 necessary. In the case of the SB transferred rapidly to overcome mass transfer limitation, H₂ and
302 CO₂ partial pressures were still significant in the gas phase, and acetate concentration was still low
303 so SAO could not be active. A discussion about thermodynamic analysis of the SB is presented in
304 section 3.3.

305 **3.2. HM and HAC growth yields**

306 The activity tests on the enriched culture in SB at 25 °C with several gas injections and late transfer
307 were used for the calculation of growth yields.

308 According to Figure 6.A, HAC had higher growth yield than HM. For both HM and HAC, the
309 growth yield was not constant along the batch and decreased. Growth yields calculated at the end
310 of the batch were 2.4 % and 4.6 % ($\text{gCOD}_X \cdot \text{gCOD}_{\text{H}_2}^{-1}$) for HM and HAC respectively. However,
311 the average growth yields were 3.7 % and 7.0 % ($\text{gCOD}_X \cdot \text{gCOD}_{\text{H}_2}^{-1}$) for HM and HAC
312 respectively. This indicates that the production of biomass represented a higher part of H_2 fixed
313 during the first stage of the batch. According to Figure 6.B, it is possible that HM and HAC growth
314 yields are P_{H_2} dependent and increase with P_{H_2} . This would imply a variable stoichiometry, which
315 has already been reported for the fermentation of glucose to acetate and butyrate (Rodríguez et al.,
316 2006). Interestingly, Figure 6.B allows to identify minimal P_{H_2} for which an activity is detected. In
317 the case of HM, this P_{H_2} was not quantifiable. However, for HAC, an average residual P_{H_2} of 0.021
318 bar was identified in the vials, corresponding to $1.65 \cdot 10^{-5} \text{ mol} \cdot \text{L}^{-1}$ of H_2 in the liquid phase at 25
319 °C. This confirms that HAC might have higher H_2 uptake thresholds than HM, and 25 °C was not
320 low enough to reverse the substrate affinity of both communities.

321 **3.3. Microbial compositions of the enriched MMCs**

322 Figure 7 shows the different genera found in the enriched MMCs after the different SB carried out.

323 HAC genus *Acetobacterium* was only selected at 25 °C and not at 35 °C, with highest abundance

324 of 38 % in the MMC enriched avoiding mass transfer limitation (MTL). Additionally, genus

325 *Methanobacterium* was selected in all enrichments except in this same MMC. However,

326 *Methanobrevibacter* was found in all samples, with the highest abundance of 12 % in the enriched

327 MMC at 25 °C under mass transfer limitation. This suggests that *Methanobrevibacter* was more

328 adapted to 25 °C than *Methanobacterium*, and that temperature also affects the competition

329 between different HMs. Finally, *Desulfovibrio* was found in all the samples, with particularly high

330 abundance of 33 % in the SB at 25 °C without mass transfer limitation. The latter are sulphate

331 reducing bacteria (SRB) capable of growing in chemolithoautotrophy on CO₂, but also in

332 heterotrophy on acetate, using the WLP or the reductive glycine pathway (Sánchez-Andrea et al.,

333 2020). Sulphate is the electron acceptor of the metabolism, and the electrons can come from

334 different monomers such as sugars, amino acids, short chain fatty acids (including acetate), ethanol,

335 and hydrogen. Consistently, they can be found in acetogenic and hydrogenogenic systems when

336 sulphate is available (Sánchez-Andrea et al., 2020; Weijma et al., 2002; Cord-Ruwisch et al., 1988;

337 Wood et al., 1986; Badziong et al., 1979). Since the mineral medium contained sulphates, these

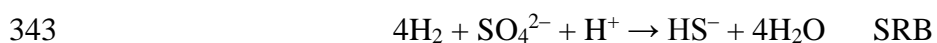
338 bacteria remained in competition with HAC at 25 °C when mass transfer limitation was overcome,

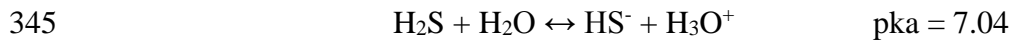
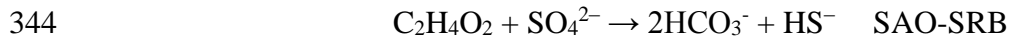
339 demonstrating that conversely to HM, they had competitive specific growth rates with HAC in

340 these conditions. SRB can use H₂ to reduce sulphate of the mineral growth medium as an anaerobic

341 respiration, they can also oxidize acetate as an anaerobic fermentation, according to the following

342 stoichiometries:





346 The initial concentration of sulphate in the medium was 0.35 mM corresponding to a maximum of
347 H_2 consumed by SRB of 1.4 mM, or 0.022 $\text{gCOD}_{\text{H}_2}\cdot\text{L}^{-1}$. As the reactions produces reduced sulphur
348 in the forms of HS^- , it could explain that HM are enriched in most of the SB despite omitting
349 sulphide addition in the mineral medium (Muyzer and Stams, 2008).

350

351 **3.4. Modelling of the microbial competition between HM and HAC**

352 The biomasses profile (**Error! Reference source not found.A**) can be divided into two phases.
353 During the first phase, soluble H_2 concentration is much higher than H_2 thresholds of HM and
354 HAC. Both communities grow at their μ_{max} , which is the parameter that governs the competition
355 (r-competition). So, in this configuration, HAC dominate, but HM can also grow and remain in the
356 reactor, leading to a cohabitation of both microbial communities. During the second phase
357 delimited by vertical dot lines, soluble H_2 is limiting ($r_{\text{H}_2} = T_{\text{H}_2}$). Microorganisms enter in a phase
358 of competition for trace amounts of H_2 , in which HM take the advantage due to their better affinity
359 to the substrate (K-competition). With these kinetic parameters, a k_{LA} increase from 10 to 50 d^{-1}
360 allows to produce twice more HAC than HM. However, further increase of the k_{LA} doesn't promote
361 anymore HAC. This is explained by the fact that increasing k_{LA} delays the time when mass transfer
362 limitation is reached. Hence, above a certain k_{LA} value, the final H_2 consumption rate remains
363 below the maximal mass transfer rate for the entire experiment, and the competition remains
364 governed by μ_{max} .

365 **Error! Reference source not found.**C and D show the calculations of ΔrG and F_T of HM, HAC,
366 AM and SAO along the simulation. It is interesting to note that the mass transfer limitation is
367 closely related to thermodynamic limitation for HAC. Indeed, when the system enters in mass
368 transfer limitation (vertical lines), the ΔrG reaches -14 kJ/reaction, which is close to the value of
369 ΔrG_c found in literature for this community (-14.85 kJ.reaction⁻¹) (Grimalt-Aleman et al., 2020).
370 Additionally, from 0.7 d, it shows that HM are also thermodynamically limited until the end.
371 Hence, the limitation term of Monod, defined with K_{H_2} is closely related to thermodynamic
372 constraints. This demonstrates that empirical parameters such as K_{H_2} , could be replaced by
373 thermodynamic limitation terms. This also show that when substrate is not limiting, the system is
374 mostly governed by kinetics, if products concentrations are not above the thermodynamic
375 threshold. When the system is mass transfer limited, then the main limitation is thermodynamic.
376 Although SAO and AM growth was not activated in the model, F_T calculations show that AM
377 reaction is feasible all along the batch. A slight thermodynamic limitation of AM during the batch
378 is due to P_{CO_2} first, and then P_{CH_4} at the end of the batch. SAO reaction is not feasible until acetate
379 is produced and P_{H_2} et P_{CO_2} become low.

380 Dynamic simulation based on Monod kinetics is consistent with the F_T profiles calculated. Hence,
381 both approaches represent well the substrate limitation phenomenon and are satisfying at a
382 macroscopic scale for H_2/CO_2 fermentation in batch mode. However, the Monod kinetics predict a
383 very low growth under H_2 limitation while F_T stops immediately the growth once the reaction is
384 no more thermodynamically feasible. This implies that according to Monod growth kinetics, the
385 competition between HM and HAC keeps going until the end of the batch. On the contrary, with
386 F_T , the competition only occurs during the first stage of the batch. Furthermore, considering the
387 difficulty to measure parameters such as K_s , the use of thermodynamic limitation terms is relevant

388 to describe anaerobic systems under other types of limitations, such as CO₂ limitation. Indeed, F_T
389 not only considers substrate availability, but also product concentrations inhibition, temperature,
390 and pH. Therefore, F_T should be more accurate to describe systems in dynamic state, or in new
391 environmental conditions for which kinetic parameters are not available.

392 According to these simulations, it should be possible to favour HAC in batch mode, as long as the
393 mass transfer rate (T_{H_2}) is higher than H₂ consumption rates (r_{H_2}). Hence, in batch mode, a key
394 process parameter is the ratio between r_{H_2} and T_{H_2} . To decrease this ratio, two main options are the
395 increase of the k_{La} of the reactor, and the decrease of the initial biomass concentration by diluting
396 the MMC. Of course, a compromise must be found between the biomass dilution, and the amount
397 of enriched MMC needed by the end of the experiment. The initial concentration of HM is also
398 important and should be minimized, for example by choosing a MMC coming from anaerobic
399 environment where methanogenic activity is low, like in psychrophilic environments. Considering
400 this, batch mode appears as an interesting cultivation strategy for MMC specialization and
401 enrichment, to provide inoculum. However, it seems not advisable for acetate production processes,
402 as the substrate limitation will be an unavoidable obstacle.

403 In batch mode, biomass accumulates, and its concentration increases. This implies that r_{H_2} reaches
404 mass transfer limitation sooner or later, as long as substrate is available. This explains why batch
405 mode in respect to the liquid phase is preferred for biological methanation, with a continuous gas
406 supply. This way, higher biomass concentrations are reached, which increases methane production
407 rate. Additionally, in the case of methanation, methane is continuously extracted in the gas phase,
408 which makes it possible to operate over long periods without product accumulation issues.

409 In the case of acetate production though, the product accumulates in the liquid, which makes
410 continuous mode on the liquid phase more interesting for this application to avoid product

411 concentration inhibition effects. Considering lab constraints to implement continuous reactors, it
412 can still be envisaged to carry out successive batches, diluting the biomasses at each new
413 inoculation step and therefore maintaining low rates and preventing mass transfer limitation. This
414 could be an interesting way to specialize and enrich a MMC with batch cultures. This strategy
415 could be a method of producing a specialized MMC and operate with higher k_{La} would allow to
416 reach higher HAC concentrations by the end of the experiment.

417 **4. Conclusions**

418 In this study, the microbial competitions were investigated in the framework of the fermentation
419 of H_2/CO_2 with MMCs. The objective was to favour the HAC and eliminate HM. SB were
420 implemented, at 25 °C and 35 °C, and with two different cultivation strategies. The first one waiting
421 for mass transfer limitation after multiple gas injections and consumption phases, and the second
422 not waiting for mass transfer limitation by transferring faster as soon as activity was detected after
423 a single gas injection.

424 First, the effect of temperature was observed, as acetate was never detected in the SB at 35 °C.
425 Then, at 25 °C and avoiding mass transfer limitation, homoacetogenesis remained as the sole
426 metabolic function identified in the activity tests. Hence, this cultivation method even allowed to
427 eliminate HM in the MMC. This was due to too low kinetic parameters values of HM, leading to
428 their loss along the SB set. In perspective of this study, it will be interesting to evaluate the
429 reproducibility of this cultivation method with different starting anaerobic MMCs to prove its
430 robustness and applicability to provide HAC specialized MMCs.

431 The temperature effect between 25 °C and 35 °C on the outcome of the competition was clearly
432 demonstrated, with *Methanobacterium* HM outcompeting HAC at 35 °C, while *Acetobacterium*

433 HAC coexisted with *Methanobacterium* and *Methanobrevibacter* HMs at 25 °C with simultaneous
434 methane and acetate production observed. This behaviour indicates that the competition between
435 HM and HAC follows the resource ratio model, and that under mass transfer limitation HM
436 outcompete HAC because of their lower H₂ consumption threshold. Conversely, when mass
437 transfer limitation is overcome, both HM and HAC can grow. As HAC have higher growth rates,
438 HM can be washed out the system by implementing transfers before mass transfer limitation is
439 reached.

440 AM and SAO were not observed in the activity tests. At 35 °C, this was mostly because of
441 thermodynamic limitation of the reaction. However, at 25 °C, AM was feasible but still not
442 detected. According to literature, AM have lower specific growth rates than HM, explaining that
443 they were not capable of maintaining themselves in the SB, especially because their substrate,
444 acetate, was produced along the batches, and so decreasing their potential time for growth.
445 However, the SRB *Desulfovibrio* was detected at significant relative abundance in the MMC,
446 because of the addition of sulphate instead of sulphide in the mineral medium. The promotion of
447 SRB provided reduced sulphur in significant amounts as sulphate is the electron acceptor of these
448 bacteria. Hence, HM were not sulphur limited during the tests. The consideration of SRB will be
449 of interest for further optimizations of the microbial selection method developed in this work, to
450 provide acetate and other VFA producing anaerobic MMCs.

451

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455

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- Agler, M.T., Wrenn, B.A., Zinder, S.H., Angenent, L.T., 2011. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. *Trends in Biotechnology* 29, 70–78. <https://doi.org/10.1016/j.tibtech.2010.11.006>
- Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., Kougias, P.G., 2018. Biogas upgrading and utilization: Current status and perspectives. *Biotechnology Advances* 36, 452–466. <https://doi.org/10.1016/j.biotechadv.2018.01.011>
- Annie Modestra, J., Navaneeth, B., Venkata Mohan, S., 2015. Bio-electrocatalytic reduction of CO₂: Enrichment of homoacetogens and pH optimization towards enhancement of carboxylic acids biosynthesis. *Journal of CO₂ Utilization* 10, 78–87. <https://doi.org/10.1016/j.jcou.2015.04.001>
- Badziong, W., Ditter, B., Thauer, R.K., 1979. Acetate and carbon dioxide assimilation by *Desulfovibrio vulgaris* (Marburg), growing on hydrogen and sulfate as sole energy source. *Arch. Microbiol.* 123, 301–305. <https://doi.org/10.1007/BF00406665>
- Batstone, D.J., Keller, J., Angelidaki, I., Kalyuzhnyi, S.V., Pavlostathis, S.G., Rozzi, A., Sanders, W.T.M., Siegrist, H., Vavilin, V.A., 2002. The IWA Anaerobic Digestion Model No 1 (ADM1). *Water Sci Technol* 45, 65–73. <https://doi.org/10.2166/wst.2002.0292>
- Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* 13, 581–583. <https://doi.org/10.1038/nmeth.3869>
- Conrad, R., Bak, F., Seitz, H.J., Thebrath, B., Mayer, H.P., Schütz, H., 1989. Hydrogen turnover by psychrotrophic homoacetogenic and mesophilic methanogenic bacteria in anoxic paddy soil and lake sediment. *FEMS Microbiology Ecology* 5, 285–293. <https://doi.org/10.1111/j.1574-6968.1989.tb03382.x>
- Cord-Ruwisch, R., Seitz, H.-J., Conrad, R., 1988. The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. *Arch Microbiol* 149, 350–357. <https://doi.org/10.1007/BF00411655>
- Fu, B., Jin, X., Conrad, R., Liu, Hongbo, Liu, He, 2019. Competition Between Chemolithotrophic Acetogenesis and Hydrogenotrophic Methanogenesis for Exogenous H₂/CO₂ in Anaerobically Digested Sludge: Impact of Temperature. *Front. Microbiol.* 10. <https://doi.org/10.3389/fmicb.2019.02418>
- Gavala, H.N., Grimalt-Alemany, A., Asimakopoulos, K., Skiadas, I.V., 2021. Gas Biological Conversions: The Potential of Syngas and Carbon Dioxide as Production Platforms. *Waste Biomass Valor.* <https://doi.org/10.1007/s12649-020-01332-7>
- Grimalt-Alemany, A., 2019. Syngas Fermentation to Biofuels: Evaluation of the Interplay of Kinetics and Thermodynamics for Directing Bioconversions based on Mixed Microbial Communities.
- Grimalt-Alemany, A., Asimakopoulos, K., Skiadas, I.V., Gavala, H.N., 2020. Modeling of syngas biomethanation and catabolic route control in mesophilic and thermophilic mixed microbial consortia. *Applied Energy* 262, 114502. <https://doi.org/10.1016/j.apenergy.2020.114502>

- Grimalt-Alemany, A., Łężyk, M., Kennes-Veiga, D.M., Skiadas, I.V., Gavala, H.N., 2019. Enrichment of Mesophilic and Thermophilic Mixed Microbial Consortia for Syngas Biomethanation: The Role of Kinetic and Thermodynamic Competition. *Waste Biomass Valor.* <https://doi.org/10.1007/s12649-019-00595-z>
- Hall, M., Beiko, R.G., 2018. 16S rRNA Gene Analysis with QIIME2, in: Beiko, R.G., Hsiao, W., Parkinson, J. (Eds.), *Microbiome Analysis: Methods and Protocols*, Methods in Molecular Biology. Springer, New York, NY, pp. 113–129. https://doi.org/10.1007/978-1-4939-8728-3_8
- Jin, Q., Bethke, C.M., 2007. The thermodynamics and kinetics of microbial metabolism. *Am J Sci* 307, 643–677. <https://doi.org/10.2475/04.2007.01>
- Kotsyurbenko, O.R., Glagolev, M.V., Nozhevnikova, A.N., Conrad, R., 2001. Competition between homoacetogenic bacteria and methanogenic archaea for hydrogen at low temperature. *FEMS Microbiol Ecol* 38, 153–159. <https://doi.org/10.1111/j.1574-6941.2001.tb00893.x>
- Liu, F., Monroe, E., Davis, R.W., 2018. Engineering Microbial Consortia for Bioconversion of Multisubstrate Biomass Streams to Biofuels. *Biofuels - Challenges and opportunities.* <https://doi.org/10.5772/intechopen.80534>
- Liu, H., Wang, J., Wang, A., Chen, J., 2011. Chemical inhibitors of methanogenesis and putative applications. *Appl Microbiol Biotechnol* 89, 1333–1340. <https://doi.org/10.1007/s00253-010-3066-5>
- Luo, G., Jing, Y., Lin, Y., Zhang, S., An, D., 2018. A novel concept for syngas biomethanation by two-stage process: Focusing on the selective conversion of syngas to acetate. *Science of The Total Environment* 645, 1194–1200. <https://doi.org/10.1016/j.scitotenv.2018.07.263>
- Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* 17, 10–12. <https://doi.org/10.14806/ej.17.1.200>
- Molenaar, S.D., Saha, P., Mol, A.R., Sleutels, T.H.J.A., ter Heijne, A., Buisman, C.J.N., 2017. Competition between Methanogens and Acetogens in Biocathodes: A Comparison between Potentiostatic and Galvanostatic Control. *Int J Mol Sci* 18. <https://doi.org/10.3390/ijms18010204>
- Musvoto, E.V., Wentzel, M.C., Loewenthal, R.E., Ekama, G.A., 2000. Integrated chemical–physical processes modelling—I. Development of a kinetic-based model for mixed weak acid/base systems. *Water Research* 34, 1857–1867. [https://doi.org/10.1016/S0043-1354\(99\)00334-6](https://doi.org/10.1016/S0043-1354(99)00334-6)
- Muyzer, G., Stams, A.J.M., 2008. The ecology and biotechnology of sulphate-reducing bacteria. *Nat Rev Microbiol* 6, 441–454. <https://doi.org/10.1038/nrmicro1892>
- Omar, B., Abou-Shanab, R., El-Gammal, M., Fotidis, I.A., Kougias, P.G., Zhang, Y., Angelidaki, I., 2018. Simultaneous biogas upgrading and biochemicals production using anaerobic bacterial mixed cultures. *Water Research* 142, 86–95. <https://doi.org/10.1016/j.watres.2018.05.049>
- Paul, E., Bessièrè, Y., Dumas, C., Girbal-Neuhauser, E., 2021. Biopolymers Production from Wastes and Wastewaters by Mixed Microbial Cultures: Strategies for Microbial Selection. *Waste Biomass Valor* 12, 4213–4237. <https://doi.org/10.1007/s12649-020-01252-6>
- Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., Blondel, M., Prettenhofer, P., Weiss, R., Dubourg, V., Vanderplas, J., Passos, A., Cournapeau, D., 2011. Scikit-learn: Machine Learning in Python. *MACHINE LEARNING IN PYTHON.*

- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* 41, D590–D596. <https://doi.org/10.1093/nar/gks1219>
- Regueira, A., González-Cabaleiro, R., Ofiteiru, I.D., Rodríguez, J., Lema, J.M., 2018. Electron bifurcation mechanism and homoacetogenesis explain products yields in mixed culture anaerobic fermentations. *Water Research* 141, 349–356. <https://doi.org/10.1016/j.watres.2018.05.013>
- Reichert, P., 1994. AQUASIM – A TOOL FOR SIMULATION AND DATA ANALYSIS OF AQUATIC SYSTEMS. *Water Science and Technology* 30, 21–30. <https://doi.org/10.2166/wst.1994.0025>
- Rodríguez, J., Kleerebezem, R., Lema, J.M., Loosdrecht, M.C.M. van, 2006. Modeling product formation in anaerobic mixed culture fermentations. *Biotechnology and Bioengineering* 93, 592–606. <https://doi.org/10.1002/bit.20765>
- Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. *International Journal of Hydrogen Energy* 38, 13172–13191. <https://doi.org/10.1016/j.ijhydene.2013.07.122>
- Sánchez-Andrea, I., Guedes, I.A., Hornung, B., Boeren, S., Lawson, C.E., Sousa, D.Z., Bar-Even, A., Claassens, N.J., Stams, A.J.M., 2020. The reductive glycine pathway allows autotrophic growth of *Desulfovibrio desulfuricans*. *Nat Commun* 11, 5090. <https://doi.org/10.1038/s41467-020-18906-7>
- Sander, R., 2015. Compilation of Henry's law constants (version 4.0) for water as solvent. *Atmos. Chem. Phys.* 15, 4399–4981. <https://doi.org/10.5194/acp-15-4399-2015>
- Schuchmann, K., Müller, V., 2014. Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria. *Nature Reviews Microbiology* 12, 809–821. <https://doi.org/10.1038/nrmicro3365>
- Shen, N., Dai, K., Xia, X.-Y., Zeng, R.J., Zhang, F., 2018. Conversion of syngas (CO and H₂) to biochemicals by mixed culture fermentation in mesophilic and thermophilic hollow-fiber membrane biofilm reactors. *Journal of Cleaner Production* 202, 536–542. <https://doi.org/10.1016/j.jclepro.2018.08.162>
- Steinbusch, K.J.J., Hamelers, H.V.M., Plugge, C.M., Buisman, C.J.N., 2011. Biological formation of caproate and caprylate from acetate: fuel and chemical production from low grade biomass. *Energy Environ. Sci.* 4, 216–224. <https://doi.org/10.1039/C0EE00282H>
- Theil, S., Rifa, E., 2021. rANOMALY: Amplicon workflow for Microbial community Analysis. *F1000Res* 10, 7. <https://doi.org/10.12688/f1000research.27268.1>
- Vavilin, V.A., Lokshina, L.Ya., Rytov, S.V., Kotsyurbenko, O.R., Nozhevnikova, A.N., 2000. Description of two-step kinetics in methane formation during psychrophilic H₂/CO₂ and mesophilic glucose conversions. *Bioresource Technology* 71, 195–209. <https://doi.org/p>
- Vigliar, E., Malapelle, U., de Luca, C., Bellevicine, C., Troncone, G., 2015. Challenges and opportunities of next-generation sequencing: a cytopathologist's perspective. *Cytopathology* 26, 271–283. <https://doi.org/10.1111/cyt.12265>
- Walters, W., Hyde, E.R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., Gilbert, J.A., Jansson, J.K., Caporaso, J.G., Fuhrman, J.A., Apprill, A., Knight, R., 2015. Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene

- Primers for Microbial Community Surveys. *mSystems* 1, e00009-15. <https://doi.org/10.1128/mSystems.00009-15>
- Wang, Y.-Q., Yu, S.-J., Zhang, F., Xia, X.-Y., Zeng, R.J., 2017. Enhancement of acetate productivity in a thermophilic (55 °C) hollow-fiber membrane biofilm reactor with mixed culture syngas (H₂/CO₂) fermentation. *Appl Microbiol Biotechnol* 101, 2619–2627. <https://doi.org/10.1007/s00253-017-8124-9>
- Weijma, J., Gubbels, F., Hulshoff Pol, L.W., Stams, A.J.M., Lens, P., Lettinga, G., 2002. Competition for H₂ between sulfate reducers, methanogens and homoacetogens in a gas-lift reactor. *Water Sci Technol* 45, 75–80. <https://doi.org/10.2166/wst.2002.0294>
- Wood, H.G., Ragsdale, S.W., Pezacka, E., 1986. The acetyl-CoA pathway of autotrophic growth. *FEMS Microbiology Reviews* 2, 345–362. <https://doi.org/10.1111/j.1574-6968.1986.tb01865.x>