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1 **“Biomethanation of Syngas by Enriched Mixed Anaerobic Consortium in Pressurized**
2 **agitated column”**

3
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8

9 **Abstract**

10 In a circular economy approach, heterogeneous wastes can be upgraded to energy in the
11 form of syngas via pyrogasification, and then to methane via biomethanation. Working at
12 high pressure is a promising approach to intensify the process and to reduce gas-liquid
13 transfer limitations. However, raising the pressure could lead to reaching the CO inhibition
14 threshold of the microorganisms involved in syngas-biomethanation. To investigate the
15 impact on pressure on the process, a 10L continuous stirred tank reactor working at 4 bars
16 and 55°C was implemented. Syngas (40% CO, 40% H₂, 20% CO₂) biomethanation was
17 performed successfully and methane productivity as high as 6.8 mmol_{CH₄}/L_{reactor}/h with
18 almost full conversion of CO (97%) and H₂ (98%) was achieved. CO inhibition was
19 investigated and carboxydrotrophs appeared less resistant to high CO exposition than
20 methanogens.

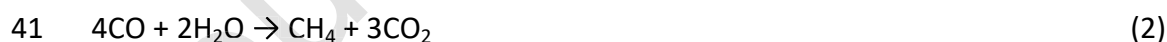
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22 **Keywords:** Biomethanation, syngas, fermentation, carbon monoxide conversion, biological
23 water-gas shift

24

25 1 Introduction

26 As the world population grows, waste management becomes an increasing issue. More and
27 more heterogenous wastes including plastic are produced each year which leads to issues
28 regarding health or environment. Waste management can then come at high cost for
29 industries or public actors. In a circular economy perspective, gasification can help reducing
30 overall waste and convert it to energy in the form of syngas, a mixture of N_2 , H_2 , CO , CO_2 ,
31 and other minor compounds. Promising studies show that heterogenous wastes including
32 plastics can be converted to syngas with gasification (Arena, 2012; Perkins, 2020). However,
33 syngas has a relatively low calorific value. Its conversion into methane would then
34 represent an interesting upgrade, considering the natural gas grid extent and storage
35 infrastructure already in place in Europe. Moreover, syngas methanation features in the
36 study "A 100% renewable gas mix in 2050?" conducted by ADEME (French Agency for
37 Ecological Transition), which explores the conditions of the technical and economic
38 feasibility of a gas system in 2050 based on 100% renewable gas in France. Thus, syngas
39 methanation is a promising technology at the center of renewable energy transition plans.



43 Syngas conversion to methane can be performed via catalytic methanation (Eq. (1)) or
44 biological methanation (Eq. (2) and (3)). Catalytic methanation of CO and CO_2 is a more
45 mature process, however its higher sensitivity to impurities, especially H_2S and tar, might
46 make it less relevant regarding syngas methanation. Biological methanation could be more

47 resilient to impurities in the feed gas and can be achieved under mild operating conditions.
48 Indeed, it can be operated at ambient pressure and temperatures around 35-75°C, whereas
49 catalytic methanation requires higher pressure and temperatures above 250°C (Grimalt-
50 Alemany et al., 2018). Moreover, biomethanation can convert CO and H₂ independently, as
51 different biological routes are involved as discussed below. This allows biomethanation to
52 convert syngas independently from the CO/H₂ ratio.

53 Syngas biological methanation is generally performed by the syntrophic association of
54 anaerobic microorganisms, whether using an association of pure cultures or an adapted
55 mixed consortium. The consortium uses syngas as both carbon and energy source to
56 support their growth and synthesize methane and carbon dioxide. Complex biochemical
57 reactions are involved, carried out by different microbial groups. They can be identified
58 using specific inhibitors such as bromoethane sulfonate (BES) for methanogens and
59 vancomycin for bacteria (Oremland and Capone, 1988), implementing specific activity tests
60 with CO, H₂/CO₂ or acetate as sole substrates and characterizing the microbial population.

61 Regarding syngas-biomethanation, the main observed reactions are carboxydrotrophic
62 hydrogenogenesis (water-gas shift reaction), carboxydrotrophic acetogenesis,
63 homoacetogenesis, hydrogenotrophic methanogenesis and acetoclastic methanogenesis
64 (Grimalt-Alemany et al., 2019) as described in **Fig. 1**. In addition, Li et al. (2020) have
65 suggested that syntrophic acetate oxidation (SAO) could also occur in syngas-
66 biomethanation processes. Direct CO methanogenesis (Eq.(2)) could theoretically be
67 another conversion route, as Sipma et al. (2004) have suggested its occurrence in CO-
68 biomethanation experiments. However, to our knowledge, it has not been observed in a
69 syngas-biomethanation process to date.

70 The biological mechanisms involved in syngas conversion by a mixed microbial consortium
71 strongly depend on the operating temperature. Grimalt-Alemany et al. (2019) have adapted
72 the same inoculum to mesophilic or thermophilic conditions. Based on activity tests and
73 microbial consortia analysis, they suggested that in mesophilic conditions, acetate seemed
74 to be the main intermediate for CO conversion paired with H₂/CO₂ as second intermediates
75 through water-gas shift. In addition, homoacetogens appeared to be active and in
76 competition with methanogens for H₂/CO₂. This makes sense with regards to the kinetic
77 parameters of the populations involved: known hydrogenotrophic methanogens have
78 smaller μ_{\max} in mesophilic conditions (0.02–2.6 day⁻¹) compared to homoacetogens (1.20–
79 4.68 day⁻¹) (Rafrafi et al., 2020). On the other hand, in thermophilic conditions, Grimalt-
80 Alemany et al. (2019) suggested that H₂/CO₂ seemed to be the main intermediate through
81 water-gas shift reaction, with no acetogenic nor acetotrophic activity detected. These
82 results are in accordance with other findings from CO-biomethanation studies, with acetate
83 being the main intermediate for CO conversion in mesophilic conditions and H₂/CO₂ in
84 thermophilic conditions (Guiot et al., 2011; Sipma et al., 2003).

85 In addition, the operating temperature also has an impact on the methane productivity,
86 which was found to be generally more than twice higher in thermophilic conditions
87 compared to mesophilic conditions (Asimakopoulos et al., 2020; Grimalt-Alemany et al.,
88 2019; Youngsukkasem et al., 2015). Bu et al. (2018) also observed that higher CO and H₂
89 conversion efficiencies were achieved in thermophilic conditions (97.2% and 100% for CO
90 and H₂, respectively) compared to extreme-thermophilic conditions (83.7% and 96.2% for
91 CO and H₂, respectively). This indicates that thermophilic conditions could be more
92 promising regarding the economic viability of the process.

93 Biomethanation of CO as sole substrate has been studied both in batch (Alves et al., 2013;
94 Arantes et al., 2018; Sancho Navarro et al., 2016; Sipma et al., 2003) and continuous mode
95 (Guiot et al., 2011, 2010; Luo et al., 2013). As it gives important insight on CO conversion
96 routes, one should note that when reviewing literature, CO-biomethanation should be
97 distinguished from syngas-biomethanation, as different microorganisms seem to be
98 involved. Indeed, Alves (2013) observed that the same sludge enriched with either CO or
99 syngas would adapt differently and the microbial composition would be different, as well as
100 the observed reaction products.

101 Moreover, the enrichment strategy could have an impact on the capacity of the inoculum
102 to adapt to syngas-biomethanation. Grimalt-Alemany et al. (2019) hypothesized that,
103 compared to other publications (Alves et al., 2013), their success into carrying an
104 enrichment with a partial pressure of CO of 0.4 atm using successive batch transfers was
105 due to starting right away at the final CO pressure instead of gradually increasing it. This
106 makes sense, as the enrichment culture technique appears to specialized a consortium
107 after the 2nd to 3rd transfer (Beck, 1971). This tends to indicate that the most efficient
108 enrichment strategy would be to start at the target partial pressures.

109 Nevertheless, the main limiting step of biomethanation is the necessity to transfer the
110 gaseous substrates to the biological catalyst which is in aqueous phase (Asimakopoulos et
111 al., 2018; Klasson et al., 1991).

$$112 \quad Rt_i = k_L a_i (H_{i, cp} * P_i - C_{i, L}) \quad (4)$$

113 The mass transfer rate of a specific substrate Rt_i [mol/(m³.s)] can be described by Eq.(4). It is
114 linked to the mass transfer coefficient $k_L a_i$ [1/s], which can be influenced by reactor type
115 and operating parameters. It is also correlated to the concentration gradient ($H_{i, cp} * P_i - C_{i, L}$),

116 with $H_{i, cp}$ [mol/(L.bar)] being the Henry's law constant, P_i [bar] the partial pressure of the
117 gas and $C_{i, L}$ [mol/L] its concentration in the liquid phase. It appears that increasing the
118 partial pressure of the substrates by increasing the overall pressure of the process can lead
119 to better transfer performances. Therefore, a higher pressurized process can lead to
120 intensified methane production, which is a significant step towards an industrial
121 application.

122 However, with an intensified pressurized process, another limiting step of syngas
123 biomethanation could be CO inhibition. An increase in CO partial pressure and thus in CO
124 transfer rate can lead to high soluble CO concentrations. These high soluble CO
125 concentrations could be close to inhibition level for the different microbial groups involved
126 into biomethanation reactions. CO inhibition has mainly been studied with CO-
127 biomethanation experiments (Guiot et al., 2011; Sancho Navarro et al., 2016) and methane
128 production has been achieved using CO-biomethanation with CO partial pressures as high
129 as 1.8 bar (Sipma et al., 2003). However, considering syngas-biomethanation, the highest
130 CO partial pressure tested with successful methane production has been 0.56 bar
131 (Westman et al., 2016; Youngsukkasem et al., 2015). As specializing a mixed consortium to
132 syngas or CO could lead to different microbial communities, the CO inhibition limits could
133 vary with the different species present and thus be different for syngas-biomethanation
134 compared to CO-biomethanation. Therefore, information on the capacity of syngas-
135 biomethanation to operate at higher CO partial pressure without inhibition is lacking.

136 Therefore, the aim of the present study was to operate a continuous lab-scale pilot at high
137 pressure in thermophilic conditions, using a mixed microbial consortium. A pressurized
138 agitated column has been chosen for this study. The achieved methane productivity was

139 compared to previous studies of different continuous operated reactors. Since CO
140 inhibition is a central matter of syngas-biomethanation, this study aimed to explore high CO
141 exposition starting with initial partial pressure as high as 1.6 bar to investigate the
142 resilience and adaptability of the consortium.

143 **2 Materials and Methods**

144 *2.1 Reactor setup*

145 The experimental setup is described in **Fig. 2**. The reactor (height 588 mm; inner diameter
146 161.5 mm) was a stainless-steel gastight tank with a water jacket for thermal regulation.
147 The total inner volume was about 12 L, and the working volume was about 10 L. The
148 reactor was stirred by an electric motor with an integrated magnetic coupling and three
149 Rushton turbines (Büchi AG, Switzerland). A temperature-controlled thermostat (Labelians,
150 France) maintained the temperature inside the reactor at $55.0 \pm 0.1^\circ\text{C}$ by circulating hot
151 water in the water jacket.

152 The pressure in the reactor was regulated at 4.000 ± 0.001 bar with a pressure controller
153 (Brooks Instrument, USA). The outlet gas was then analyzed by a Fusion micro gas
154 chromatography (Inficon, Switzerland) and a drum gas meter (Ritter, Germany) measured
155 the outlet flow rate.

156 CO (>99%) and CO₂ (>99.7%) were supplied with gas bottles (Air Liquide, France) whereas
157 H₂ (>99.9999%) was obtained using a H₂ generator (Claind srl, Italy). All gases were supplied
158 continuously, and flow rates were regulated for each gas using mass flow controllers
159 (Brooks Instrument, USA).

160 The reactor was depressurized three times a week to perform liquid sampling and
161 injections of nutrient solution, with nitrogen source. The liquid level was computed from
162 the pressure difference between the top and the bottom of the tank, which was measured
163 by a pressure transmitter (Keller, Switzerland).

$$164 \quad k_L a_i = k_L a_{O_2} \left(\frac{D_i}{D_{O_2}} \right)^{0.5} \quad (5)$$

165 Mass transfer coefficients ($k_L a$) were characterized for oxygen with the reoxygenation
166 method by measuring dissolved oxygen concentration in clean water as described by He et
167 al. (2003), using a EasySense O₂ 21 probe (Mettler Toledo, Switzerland). The coefficients
168 corresponding to the operational parameters (55°C – 1000 rpm – 7.5 NL/h – 4 bars) in clean
169 water were estimated to be $27.8 \pm 1.4 \text{ h}^{-1}$ for O₂. Using the diffusivities ratio D_i/D_{O_2}
170 according to Eq. 5, the mass transfer coefficients were then computed to be $35.2 \pm 1.8 \text{ h}^{-1}$
171 and $27.0 \pm 1.3 \text{ h}^{-1}$ for H₂ and CO, respectively.

172 2.2 Inoculation and operating conditions

173 The reactor was inoculated with a mesophilic anaerobic sludge sampled from the sludge
174 digester of the municipal wastewater treatment plant of La Feyssine, Lyon, France. The
175 inoculum was diluted to final concentrations of $11.8 \pm 0.2 \text{ g/L}$ for total solids (TS) and $8.0 \pm$
176 0.2 g/L for volatile solids (VS). Thermophilic conditions were chosen due to the higher
177 methane productivity expected under those conditions as discussed previously and the
178 reactor was heated at 55°C. The stirring rate was set at $1000 \pm 1 \text{ rpm}$. Once the reactor was
179 inoculated, the experiment started directly with a continuous gas feed of 7.500 ± 0.003
180 NL/h total flow rate with H₂/CO/CO₂ (molar ratio of 40/40/20 %). The total pressure was set

181 at 4 bar to apply a high CO partial pressure (1.6 ± 0.2 bar) right from the start, in order to
182 adapt the consortium to the target pressure as discussed above.

183 The reactor was at first operated continuously for 50 days (Phase 1) and then had to be
184 stopped due to lockdown measures and laboratory closure in March 2020. The adapted
185 consortium was stored at 4°C for 78 days and then restarted for 72 days straight (Phase 2).
186 It was decided to restart it at atmospheric pressure to check if the biological activity was
187 still present, and the pressure was then gradually increased up to 4 bars. The two phases of
188 continuous operations will be referred to in this paper as Phase 1 and Phase 2.

189 Nutrients and liquid level were regulated by adding digestate obtained by centrifugation at
190 5000g of the same mesophilic sludge kept at 4°C. It was analyzed for several nutrients
191 concentrations and presented the following compositions (concentrations in mg per liter,
192 5% uncertainty): 0.143 B, 0.008 Co, 0.186 Cu, 0.192 Fe, 127 K, 22.6 Mg, 0.041 Ni, 5.09 S,
193 0.176 Zn, 714 NH_4^+ . pH was maintained above 6.0 ± 0.1 by addition of NaOH (100 mg/L) or
194 NH_4OH (25%) depending on the nitrogen requirements, with an average addition of 2 mL/d.
195 Ammonia nitrogen –N- NH_4^+ concentration in the reactor was regularly measured according
196 to the method described below.

197 From day 9 of Phase 2, a solution of $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ (30 g/L) was supplied to the reactor. It was
198 first added manually 3 times a week using a 20 mL syringe. Then from day 51, it was
199 injected semi continuously using a dosing pump (GrundFos, Denmark). The amount added
200 was between 5 and 10 mL/d.

201 *2.3 Analytical Methods*

202 Online measures included pH, temperature, pressure, inlet and outlet flows, and gas
203 composition. The pH inside the reactor was acquired using EasySense 31 probe (Mettler
204 Toledo, Switzerland) and temperature using a thermocouple sensor (TC Ltd, France). The
205 composition of the outlet gas was analyzed every 15 minutes with a Fusion micro gas
206 chromatography (Inficon, Switzerland). H₂, CO, N₂, O₂, and CH₄ were measured with a
207 molecular sieve column and CO₂, H₂O, and H₂S with a RT-Q-Bond column. A calibration gas
208 bottle (Air Liquide, France) was used to regularly calibrate the gas analyzer, with the
209 following composition: 5% H₂; 5% CO; 500ppm H₂S, 60% CO₂ and CH₄ balance.

210 Liquid analyses included Volatile Fatty Acid (VFA), NH₄⁺, trace elements, TS and VS, and
211 water-soluble chemical oxygen demand (COD). All analyses excepting TS and VS were
212 performed after centrifugation and filtration at 0.45 μm.

213 VFA were measured by ion chromatography (Shimadzu Corporation, Japan) equipped with
214 a AS11-HC-4 μm, 2*250mm (Thermo Fisher, USA), using H₂ as carrier gas and a flame
215 ionization detector. Water-soluble COD was measured with a LT200 mineralizer and a
216 DR1900 HACH spectrometer. N-NH₃ were analyzed with Hack LCK303 tubes and DR1900
217 HACH spectrometer. Trace elements concentrations were measured by ICP-OES Ultima 2
218 (HORIBA Jobin Yvon, Japan) according to the NF EN ISO 11885 (1998) AFNOR NF T 90-136
219 Method.

220 The TS concentration was determined through 105°C drying for 24h and VS concentration
221 after heating the sample at 550°C during 2h, following the ASTM standard methods
222 recommendations.

223 *2.4 Mass Balance Calculation Method*

224 Mass balances calculations were done during steady-state operation phases with constant
 225 gas composition. A particular attention was paid to H₂ and CO. In the calculation, we
 226 assumed a simplified metabolic route, with CO being converted to H₂ and H₂ to CH₄. This
 227 simplification was chosen because the amount of other intermediate products was very
 228 small (See 3.1). It should be noted that due to reactions stoichiometry, the path through
 229 which secondary products are produced does not change the mass balance results. Acetate
 230 and cell production were neglected, as we computed that they represented less than 1% of
 231 the total products of reaction.

232 Mass balance calculations were done on specific time ranges Δt . Volatile suspended solids
 233 variations during Δt are referred to as Δm_{VSS} (g_{VSS}).

234 When computing H₂ conversion rates, H₂ produced from CO was taken into account in
 235 addition to injected H₂. This means that we assumed that all the CO consumed was
 236 converted into H₂ through the water gas shift reaction. This methodology allows to express
 237 the effective capability of microorganisms to convert H₂ to CH₄.

238 In order to study reactions kinetics, the following values were considered. \dot{n}_i , the molar flux
 239 of component i (mmol/h), is computed with the measured gas compositions and the
 240 measured total outlet flow rate.

241 • Production rates $\left[\frac{\text{mmol}}{\text{h}}\right]$:

242
$$R_{CO} = \dot{n}_{CO, in} - \dot{n}_{CO, out} \quad (6.a)$$

243
$$R_{H_2} = [\text{Consumed injected } H_2] + [\text{Consumed } H_2 \text{ produced from CO}]$$

244
$$R_{H_2} = [\dot{n}_{H_2, in} - \dot{n}_{H_2, out}] + [\dot{n}_{CO, in} - \dot{n}_{CO, out}] \quad (6.b)$$

245
$$R_{CH_4} = \dot{n}_{CH_4, out} \quad (6.c)$$

246 Production rates r_i (mmol/L_R/h) were then computed by dividing these rates by the reactor
 247 volume, and similarly specific production rates $r_{x,i}$ (mmol/g_{VSS}/h) by dividing them by Δm_{VSS} .

248 • Global biomass yield:

$$249 \quad Y_X = \frac{\text{Growth rate}}{\text{Converted energy}} = \frac{\Delta m_{VSS}/\Delta t}{\dot{n}_{CO,in} - \dot{n}_{CO,out} + \dot{n}_{H_2,in} - \dot{n}_{H_2,out}} \left[\frac{g_{VSS}}{\text{mol}_{\text{substrate}}} \right] \quad (7)$$

250 Mass transfer rate is highly influenced by the gas partial pressure (Eq. 4). However, as the
 251 gas is converted throughout the reactor, its partial pressure varies and cannot be assumed
 252 equal to the applied partial pressure. For instance, when stable methanogenesis occurred,
 253 CO partial pressure was equal to 1.6 ± 0.2 bar at the inlet of the reactor, and to 0.1 ± 0.2
 254 bar at the outlet. Hence, the logarithmic mean of the partial pressure between the
 255 entrance (P_i^{in}) and the exit (P_i^{out}) of the reactor was used to estimate the average partial
 256 pressure experienced by the microorganisms (Doran, 2013):

$$257 \quad P_i^{log} = \frac{P_i^{in} - P_i^{out}}{\ln(P_i^{in}) - \ln(P_i^{out})} [bar] \quad (8)$$

258 3 Results and Discussion

259 3.1 Water gas shift as the main intermediate reaction for methane production at 4 bars.

260 The results of Phase 1, obtained before the lockdown, are shown in Fig. 3. Phase 1 began at
 261 4 bars with an early start of biological activity, starting with a decrease in CO outlet flow
 262 rate correlated with an increase in H₂ and CO₂ flow rates, indicating CO conversion into H₂
 263 (days 1 – 5). It is more likely that CO is converted through the water-gas shift reaction ($\Delta G =$
 264 20 kJ/mol), which has been observed by other authors in thermophilic conditions (Grimalt-
 265 Alemany et al., 2019; Guiot et al., 2011; Sipma et al., 2003). It was then followed by a
 266 decrease in H₂ and CO₂ flow rates and an increase in CH₄ flow rate, suggesting
 267 hydrogenotrophic methanogenesis (days 5 – 20). Then we observed conversion of both H₂

268 and CO for a short period of time and CH₄ and CO₂ production (days 24 – 26). However, the
269 reactor was unstable probably due to nutrients deficiency, and the results of Phase 1 were
270 not straightforward. Indeed, no Na₂S.9H₂O was added during Phase 1, and it has been
271 suggested that it is a necessary nutrient for methanogenesis (Strübing et al., 2017).

272 Phase 2 started after an interruption of 78 days due to the sanitary lockdown. The
273 biological activity was restored within the first days after restart at atmospheric pressure as
274 described previously. At day 5 and under 1 bar, the conversion efficiency of CO and H₂
275 reached 87.8 ± 1.2 % and 91.0 ± 0.9 %, respectively. This result showed the high resilience
276 of the mixed consortium, even after a long storage period at low temperature [Results not
277 shown]. With an appropriate supply of nutrients (trace elements, sulfur, and ammonium
278 sources) the reactor recovered a stable methane production with a full conversion of all
279 gaseous substrates around day 10 of Phase 2. The reactor was then pressurized at 4 bars at
280 day 17 and methane production remained stable.

281 **Fig. 4** shows the evolution of flow rates during stable methane production periods in Phase
282 2 (days 30 -73). During this phase, the reactor reached stable conversion efficiencies of 99.0
283 ± 0.4 % and 97.6 ± 1.0 % for H₂ and CO, respectively.

284 The supply of sulfur was stopped on day 40 in order to stop methane production and
285 identify intermediate mechanisms. Methane production decreased after a few days, the
286 time for sulfur stored in the reactor to be consumed. We observed that both H₂ and CO₂
287 outlet flow rates increased compared to inlet flow rates, and that CO conversion still took
288 place. This indicates that the water-gas shift reaction still occurred during this period. We
289 had observed the same phenomenon during Phase 1. This means that H₂/CO₂ are the main
290 products of CO conversion under these conditions. This result is consistent with previous

291 studies, showing that in thermophilic conditions, CO would be mainly converted via H_2/CO_2
292 whereas in mesophilic conditions the main intermediate seemed to be acetate (Grimalt-
293 Alemany et al., 2019).

294 3.2 Acetate production during water gas shift phases

295 As we can see in **Fig. 4**, the water gas-shift phase (days 44 – 57) is correlated with pH
296 decrease (from 6 – 6.5 when methanogenesis was occurring to 5.6 during the water-gas
297 shift phase). This could be due either to the higher dissolved CO_2 concentration that
298 increased as more CO_2 is produced from water-gas shift, or to the increased acetate
299 concentration.

300 Indeed, during the water-gas shift phase, we also observed an increase in acetate
301 concentration in the reactor, from around 2 g/L (day 40) up to 6 g/L during water-gas shift
302 (day 53). The same phenomenon was observed during Phase 1, with concentrations going
303 from about 3.5 g/L during methanogenesis up to about 8.5 g/L during water-gas shift (Fig.
304 3.). However, the molar balance revealed that the amount of acetate accumulated during
305 this phase represented only 0.75% of the COD consumed from CO conversion. This could
306 suggest that the acetate production is only a secondary metabolic route happening at the
307 same time as the water-gas shift reaction.

308 However, it is unclear in our case from which mechanism acetate is produced, as it can be
309 obtained either from CO (carboxydrotrophic acetogenesis) or from H_2/CO_2
310 (homoacetogenesis) (**Fig. 1**). Indeed, acetate production was also observed in H_2/CO_2
311 biomethanation processes by other authors: high H_2 concentrations can lead to increased
312 acetate concentration via homoacetogenesis, in thermophilic (Kougias et al., 2017; Strübing
313 et al., 2017) and in mesophilic conditions (Agneessens et al., 2018). For instance, Liu et al.

314 (2016) reported that at a H₂ partial pressure of 0.96 bar, 40% of hydrogen was consumed
315 by homoacetogens in mesophilic conditions. Nevertheless, even if acetate production from
316 H₂ has been observed both in thermophilic and mesophilic conditions, kinetic parameters
317 suggest that mesophilic conditions would be more favorable to homoacetogens (Rafrafi et
318 al., 2020).

319 However, in the case of mixed culture syngas-biomethanation, different microorganisms
320 could be involved. Acetate could thus be produced from various metabolic routes.
321 Moreover, thermophilic hydrogenotrophic carboxydrotrophs such as *Carboxydotherrmus*
322 *hydrogenoformans* can shift their metabolism from hydrogenogenic to acetogenic (Henstra
323 and Stams, 2011). Taking this into account, Grimalt-Alemany et al. (2020) when modelling
324 syngas biomethanation, hypothesized that this shift takes place when CO conversion to
325 H₂/CO₂ is thermodynamically limited, which occurs at high H₂ partial pressure.

326 In the case of thermophilic syngas-biomethanation, some elements lead to favor the
327 hypothesis of acetate production via carboxydrotrophic route rather than by
328 homoacetogenesis. Indeed, Grimalt-Alemany et al. (2019) used activity tests with either CO,
329 H₂/CO₂ and acetate as sole substrates and bromoethane sulfonate (BES) as inhibitory agent.
330 They concluded that in their enrichment experiment no homoacetogenesis would occur in
331 thermophilic conditions, whereas it would occur in mesophilic conditions. This could be in
332 accordance with the better substrate affinity and higher maximum specific growth rate of
333 hydrogenotrophic methanogens over homoacetogens in thermophilic conditions (Rafrafi et
334 al., 2020), indicating higher competitiveness. In contrast, in mesophilic conditions, the
335 maximum growth rates of both groups are in the same range and homoacetogens seem to
336 be able to outcompete hydrogenotrophic methanogens.

337 More experiments are required to determine if the acetate production observed in our
338 study correlated with higher H₂ partial pressure comes from homoacetogenesis as usually
339 observed in H₂/CO₂-biomethanation, or if it comes from a shift in CO conversion
340 metabolism as observed in other syngas-biomethanation experiments (Diender et al., 2018;
341 Grimalt-Alemanly et al., 2019).

342 It should also be noted that acetate concentrations decreased after methanation restart
343 (**Fig. 4**) around day 60, indicating acetotrophic activity. This is not in accordance with other
344 findings that observed no acetotrophic activity in thermophilic conditions for syngas-
345 biomethanation (Asimakopoulos et al., 2020; Grimalt-Alemanly et al., 2019). However,
346 Westman et al. (2016) when studying syngas-biomethanation with co-substrate addition
347 containing acetate, observed an acetate conversion, indicating the presence of
348 acetotrophic activity in thermophilic conditions. Thus, presence or not of acetotrophic
349 activity in regard to thermophilic syngas-biomethanation requires further research.

350 *3.3 Kinetics and productivity of syngas-biomethanation*

351 From our results, we selected 6 periods of stable gas production to perform mass balances
352 and to assess the conversion kinetics (they are listed **Table 1**). These periods were selected
353 because they were associated to three different observed phenomena: methane
354 production with conversion of all gaseous substrates (period I, III and VI), CO conversion to
355 H₂/CO₂ with limited methane production (period IV and V, also called “water gas shift
356 periods”), and methane production from H₂/CO₂ with limited CO conversion (period II). The
357 fact that either CO conversion or methane production were limited during certain periods
358 was attributed to CO inhibition (See 3.4) and nutrients deficiency (See 3.1), respectively.

359 The results from mass balance calculations are listed in **Table 2** . As not all gaseous
360 substrates are converted, we hypothesized that the reactor was limited by gas-liquid mass
361 transfer. Optimizing the mass transfer was not the purpose of this study. However, it could
362 be improved by adding baffles to the tank (Cabaret et al., 2008). Regarding
363 biomethanation, different reactor configurations have been studied to improve the gas-
364 liquid mass transfer (Asimakopoulos et al., 2018), and hollow fiber membrane bioreactors
365 appear to be the most efficient (Orgill et al., 2013; Yasin et al., 2019).

366 During the three methanation periods (I, III, VI), good conversion efficiencies have been
367 observed as high as $97.6 \pm 1.7 \%$ and $98.6 \pm 0.4 \%$ for CO and H₂, respectively. H₂ conversion
368 efficiency was always slightly higher than CO conversion efficiency, which could be
369 explained by higher mass transfer coefficients for H₂ ($35.2 \pm 1.8 \text{ h}^{-1}$) than for CO (27.0 ± 1.3
370 h^{-1}).

371 The CO volumetric conversion rates appeared to be similar around $13 \text{ mmol}/(\text{L}_R \cdot \text{h})$ during
372 all three methanation periods. However, the CO specific rates decreased from 1.72 ± 0.11
373 to $1.20 \pm 0.02 \text{ mmol}/(\text{g}_{\text{VSS}} \cdot \text{h})$ with time. This could be explained by the growth of non
374 carboxydotrophs microorganisms, such as methanogens for instance, leading to the overall
375 increase of VSS and to the decrease of CO specific rate.

376 Furthermore, methane volumetric productivity increased over time, from 5.49 ± 3.51
377 during Period I to $6.80 \pm 0.50 \text{ mmol}/(\text{L}_R \cdot \text{h})$ during Period VI. This is probably because the
378 VSS concentration in the reactor had increased from 7.9 ± 0.2 to $10.9 \pm 0.2 \text{ gVSS/L}$. Indeed,
379 the specific methane production activity was rather constant around $0.7 \text{ mmol}/(\text{g}_{\text{VSS}} \cdot \text{h})$ over
380 the three methanation periods. Those elements could also suggest a growth of

381 methanogenic populations, leading to an overall increase of the VSS concentration and of
382 the methane volumetric productivity.

383 The global biomass yields over the three methanation periods was $0.20 \text{ g}_{\text{VSS}}/\text{mol}$ substrate
384 in average. This is relatively low compared to the batch experiment conducted by Grimalt-
385 Alemany et al. (2019), where they obtained $0.66 \text{ g}_{\text{VSS}}/\text{mol}$ substrate.

386 During period II, limited CO conversion ($10.9 \pm 8.0\%$) took place but methanogenesis from
387 H_2 occurred. We can see that specific rates for H_2 conversion and CH_4 production are lower
388 (2.25 ± 0.65 and $0.50 \pm 0.07 \text{ mmol}/(\text{g}_{\text{VSS}}\cdot\text{h})$, respectively) than those obtained during the
389 methanation periods(I, III, VI) where H_2 and CH_4 specific rates were around 2.43-3.19 and
390 0.63-0.88 $\text{mmol}/(\text{g}_{\text{VSS}}\cdot\text{h})$ respectively. This makes sense, as during period II carboxydrotrophs
391 appeared to be inhibited (See 3.4). Therefore, a portion of the overall biomass is unactive,
392 which decreases the specific activity. Volumetric rates are also almost twice lower during
393 Period II compared to the three methanation periods (H_2 and CH_4 volumetric rates around
394 13.65 ± 3.27 and $3.03 \pm 0.26 \text{ mmol}/(\text{L}_\text{R}\cdot\text{h})$, respectively compared to 24.58-26.07 and 5.49-
395 6.80 for the methanation periods). This is makes sense as CO conversion to H_2 is limited.

396 Regarding water-gas shift periods, it can be observed that CO conversion efficiency is lower
397 in periods IV and V when only CO conversion occurs, compared to the periods where both
398 CO and H_2 are converted (around 91-93% versus 97-98%). However, CO mass transfer rates
399 should be higher as less CO is converted, leading to higher CO partial pressure in the
400 headspace. This result seems to indicate rather a thermodynamic limitation than a mass
401 transfer issue: as H_2 is not converted, it accumulates and might limit CO conversion to H_2 .
402 Thermodynamic limitations of CO conversion to H_2 when H_2 accumulates in the headspace
403 have already been suggested by other studies (Grimalt-Alemany et al., 2020).

404 An overview of some literature data of syngas-biomethanation in continuous reactors is
405 presented in **Table 3**. When needed, the methane production rates have been converted to
406 different units to be able to compare the results. Most of the time, the selected data
407 correspond to the highest conversion efficiencies of the cited reference and not the highest
408 methane productivity. It should be noted that in the biomethanation process, the
409 performance of a given system is generally a compromise between a high methane
410 productivity and good conversion efficiencies (Asimakopoulos et al., 2020).

411 To date, syngas-biomethanation research is rather new and there are very few studies with
412 continuous processes. Moreover, the operational parameters in the existing research are
413 highly different: reactor configuration, syngas composition, inlet syngas flow rate or
414 microbial consortium. All these parameters have an influence on methane productivity and
415 conversion efficiencies, hence making difficult to compare the results. Still, this literature
416 review in Table 3 gives a comprehensive view of the current state of the art. Some trends
417 can be noticed: for instance, higher H₂ proportion in the syngas gives higher methane
418 production rates. This is consistent with the stoichiometry of reactions 1 and 2, which
419 indicates that increasing H₂ proportion will increase CH₄ proportion of the produced gas
420 within the limit of the stoichiometry and if H₂ is limiting. Moreover, this theoretical
421 assertion has been supported by the experimental results of Li et al. (2019).

422 The results of the current study are promising, with methane productivity of 6.80 ± 0.50
423 mmolCH₄/L/h paired with good conversion efficiencies of $96.6 \pm 0.3\%$ and $98.1 \pm 0.2\%$ for
424 CO and H₂, respectively. Steady state operation was achieved within a few days after start-
425 up (with a mesophilic non-adapted inoculum) and restart (after the COVID sanitary
426 lockdown and inoculum storage at 4°C), which shows the good versatility of the process.

427 The production rate 6.80 ± 0.50 mmolCH₄/L/h is rather high compared to results published
428 so far. For example, Asimakopoulos et al. (2020) measured 3.80 mmolCH₄/L_R/h at similar
429 conversion efficiencies and similar inlet flow rates in relation to reactor size (0.75
430 NL_{syngas}/L_R/h for this study and about 0.67 L_{syngas}/L_R/h for theirs). The difference in
431 productivity could be explained by the slightly lower syngas flow rate, and perhaps by the
432 difference in concentration and composition between the two consortia. In a more recent
433 study, by increasing the inlet flow rate up to 3 L_{syngas}/L_R/h and improving the mass transfer,
434 Asimakopoulos et al. (2021) reached 17.6 mmolCH₄/L_R/h.

435 By working at 4 bars, our reactor reached good conversion efficiencies and good
436 productivity in regard to literature. Hence, working at high pressure seems a promising
437 approach to reach high conversion efficiencies with satisfying methane productivity.
438 However, this relies on the condition that working at higher pressure and therefore at
439 higher CO partial pressure will not lead to CO inhibition.

440 3.4 CO inhibition of carboxidotrophs

441 The microbial inhibition by a given compound is generally due to the concentration
442 experienced by the microorganisms. In the case of CO, the dissolved concentration results
443 from gas to liquid mass transfer and from CO uptake by the microorganisms. In our case, as
444 gases are converted, P_{co} varies in the reactor from the bottom where the gas is injected
445 (P_{co} = 1.6 bar) to the top where it is vented out. Taking this into account, the logarithmic
446 mean of the CO partial pressure in the reactor according to Eq. 8 was used to be able to
447 study the response of the consortium to CO.

448 In our experiments, the mass transfer coefficient in the reactor should not vary for constant
449 operating parameters (total gas flow rate). However, the CO partial pressure in the

450 headspace can vary during transient states as the gas composition varies with the start-up
451 of biological reactions. A transient state is represented in **Fig. 5**, with the restart of the
452 reactor after a maintenance break during Phase 2.

453 After the restart at 4 bars, we see that the first reaction was methanogenesis from H₂ (from
454 day 22 to day 24). At the same time, CO conversion did not occur. As methanogenesis from
455 H₂ decreases the quantity of moles of gas, and with no CO conversion, the proportion of CO
456 in the headspace even increased. It was then decided to reduce the total operating
457 pressure at 1 bar on day 25. This reduction of the CO partial pressure resulted in a recovery
458 of the carboxydrotrophic activity in less than 24h. Our hypothesis is that high CO partial
459 pressure could inhibit the carboxydrotrophic microorganisms. From day 22 to 25, the partial
460 pressure experienced by the microorganisms P_{CO}^{log} probably increased up to the inhibitory
461 limit, before CO conversion could occur and participate in decreasing P_{CO}^{log} . The reduction
462 of the total pressure probably stopped this inhibition. This demonstrates the impact of
463 P_{CO}^{log} on CO conversion kinetics.

464 This was the first time that we observed this phenomenon from the startup of the
465 experiment. Indeed, during previous startups at 4 bar the reactor was nutrient limited
466 (sulfur) and H₂ was only partially converted to CH₄. Hence, P_{CO}^{log} remained below 1.6 bars
467 from the start, allowing for CO conversion to slowly take place, resulting in a decrease of
468 P_{CO}^{log} due to CO consumption. This raises questions regarding the startup strategies of a
469 syngas-biomethanation process with an adapted consortium. In our case, this observation
470 led to a strategy following maintenance breaks consisting of a first step at atmospheric
471 pressure until the substrates conversions were stabilized, then followed by a pressure
472 increase up to 4 bar.

473 **Fig. 5** shows that with an adapted consortium, methanogenesis from H₂ occurred under
474 P_{CO}^{log} up to 2 bars, with H₂ conversion efficiency up to 92%. To our knowledge no previous
475 work has studied methanogenesis from H₂ with such high P_{CO} exposure. However, at such
476 high P_{CO}, CO conversion did not take place.

477 So, it appears on **Fig. 5** that after a short adaptation, methanogenesis from H₂ started
478 quickly at high pressure compared to CO conversion. This in accordance with the findings
479 reported by Li et al. (2020), who also observed that methanogenesis from H₂ started before
480 CO conversion with initial 0.25 atm CO and 0.75 atm H₂. However, the authors suggested
481 that it was due to the lower solubility of CO compared to H₂, thus inducing lower CO
482 conversion rates. Yet, in our case, at day 22 of Phase 2, maximum mass transfer rates are
483 estimated to 28.1 ± 4.7 mmol/(L.h) and 36.3 ± 7.9 mmol/(L.h) for CO and H₂ respectively.
484 Those values are rather close to each other, which probably excludes CO mass transfer
485 limitations to explain the quicker start of methanogenesis from H₂.

486 However, other authors studying CO-biomethanation in thermophilic conditions observed
487 the opposite, with methanogenesis starting only after CO was fully converted (Guiot et al.,
488 2010; Sipma et al., 2003). This seemed to indicate that CO was inhibitory to
489 methanogenesis and carboxydrotrophic hydrogenogenic activity was useful to “clean” the
490 headspace from CO, allowing for methanogenesis to start. Yet, CO-biomethanation results
491 and syngas-biomethanation results should be compared with caution, as it has been
492 observed that the two enrichments methods performed on the same initial inoculum can
493 lead to different microbial compositions (Alves et al., 2013), and therefore perhaps to
494 different inhibition thresholds.

495 So, it can be deduced that, after adaptation, the microorganisms operating methanogenesis
496 from H₂ are more resistant to CO inhibition than carboxydrotrophic hydrogenogens. More
497 experiments should be performed to determine more accurately the inhibition limit for
498 carboxydrotrophs, as we only know for now that it is with at P_{CO} at least higher than 2 bars.

499 **Conclusions**

500 This study has demonstrated, for the first time, the possibility for syngas-biomethanation to
501 be operated in continuous mode at a pressure of 4 bars. The performances obtained were
502 very promising, with methane productivity of 6.8 mmol/L_R/h and high conversion of CO
503 (97%) and H₂ (98%). CO inhibition of carboxydrotrophs was suspected in some conditions
504 and was investigated for the first-time regarding syngas-biomethanation. Carboxydrotrophs
505 appeared to be more sensitive to CO than methanogens and to be inhibited at CO partial
506 pressure higher than 2 bars. Overall, this study demonstrated that syngas-biomethanation
507 in a pressurized reactor is a promising approach to industrialization.

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514

515

516 **Figure Captions**

517 **Fig. 1.** Simplified representation of biological mechanisms involved in syngas
518 biomethanation by a mixed consortium (Rafrafi et al., 2020). SAO: Syntrophic Acetate
519 Oxidation.

520 **Fig. 2.** Simplified scheme of the reactor system. (1) tank, (2) stirring system, (3) pressurized
521 sulfide circuit, (4) thermostat, (5) CO gas bottle, (6) CO mass flow controller, (7) CO₂ gas
522 bottle, (8) CO₂ mass flow controller, (9) H₂ generator, (10) H₂ mass flow controller, (11)
523 liquid addition or withdraw, (12) pressure controller, (13) gas analyzer, (14) drum gas
524 counter.

525 **Fig. 3.** Outlet flow rates (A) pH, and acetate, propionate and volatile solids concentrations
526 (B) during operational days 0-50 of Phase 1. Data acquisition of outlet flow rates was
527 interrupted due to technical difficulties from day 13 to 15, 18 to 19 and 48 to 50.

528 **Fig. 4.** Outlet flow rates (A) pH, acetate, propionate and volatile solids concentrations (B)
529 during operational days 30-73 of Phase 2. Data acquisition of outlet flow rates was
530 interrupted due to technical difficulties from day 57 to 60.

531 **Fig. 5.** Effect of P_{CO}^{log} during Phase 2, after a maintenance break of the reactor at day 22.

532

Tables and Figures

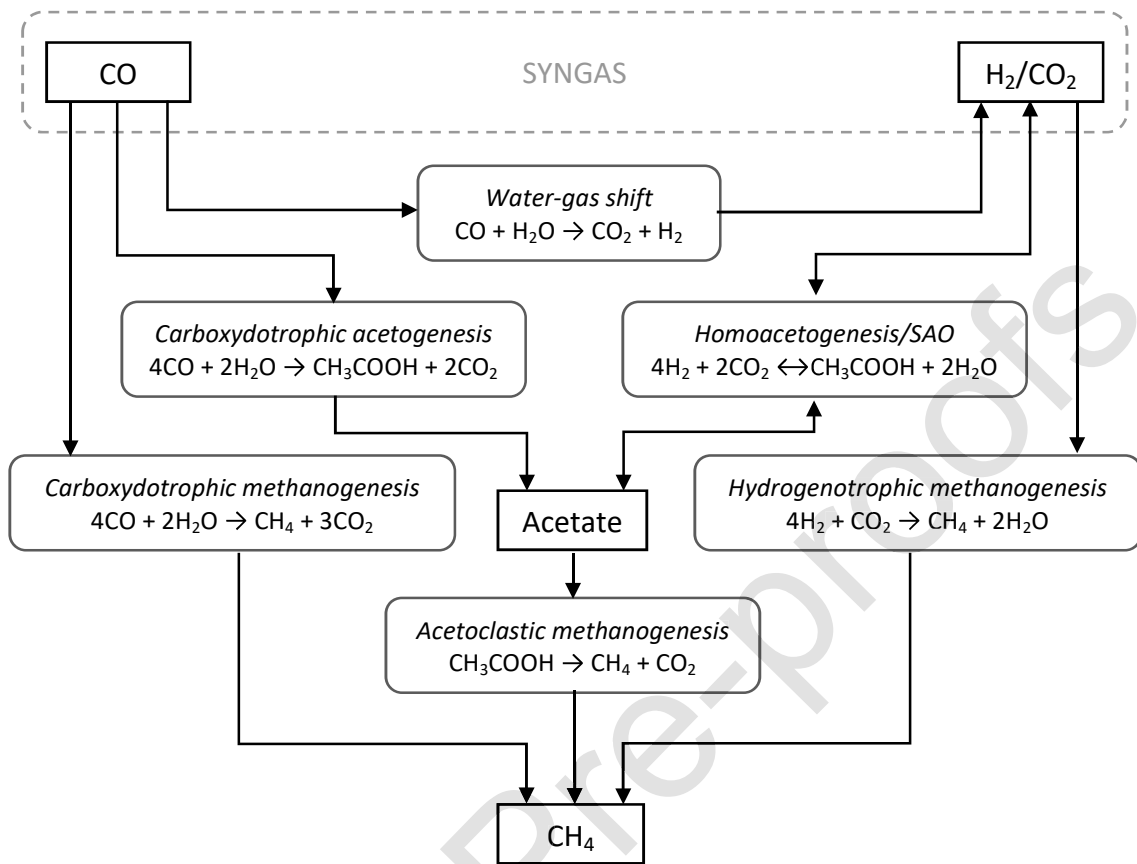
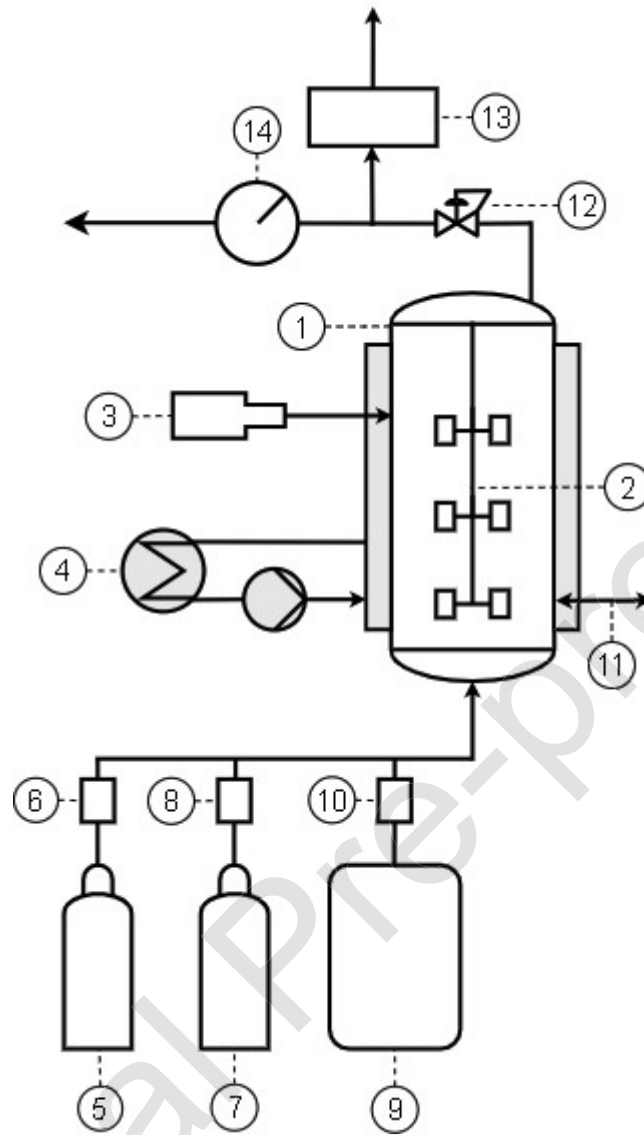
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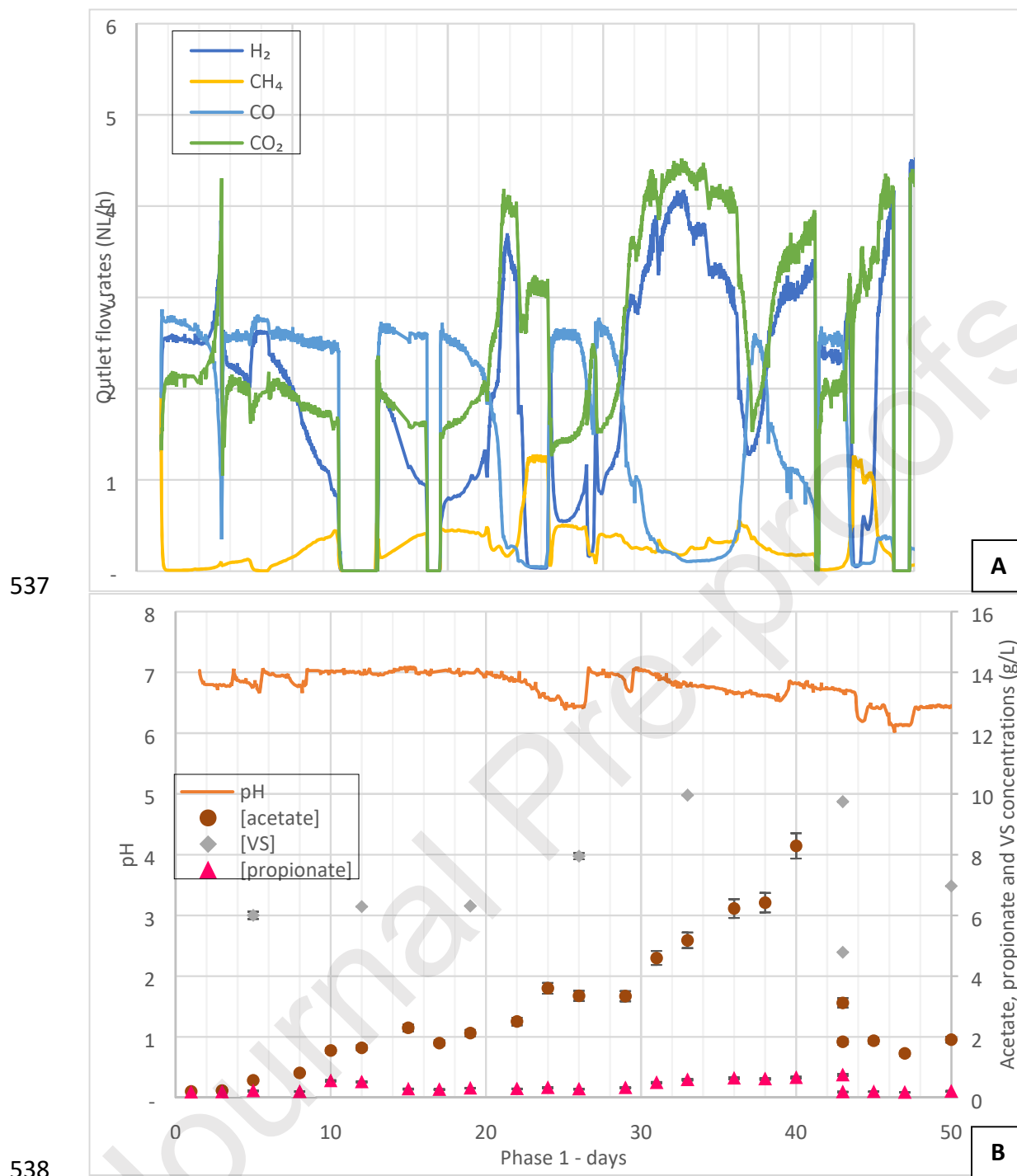
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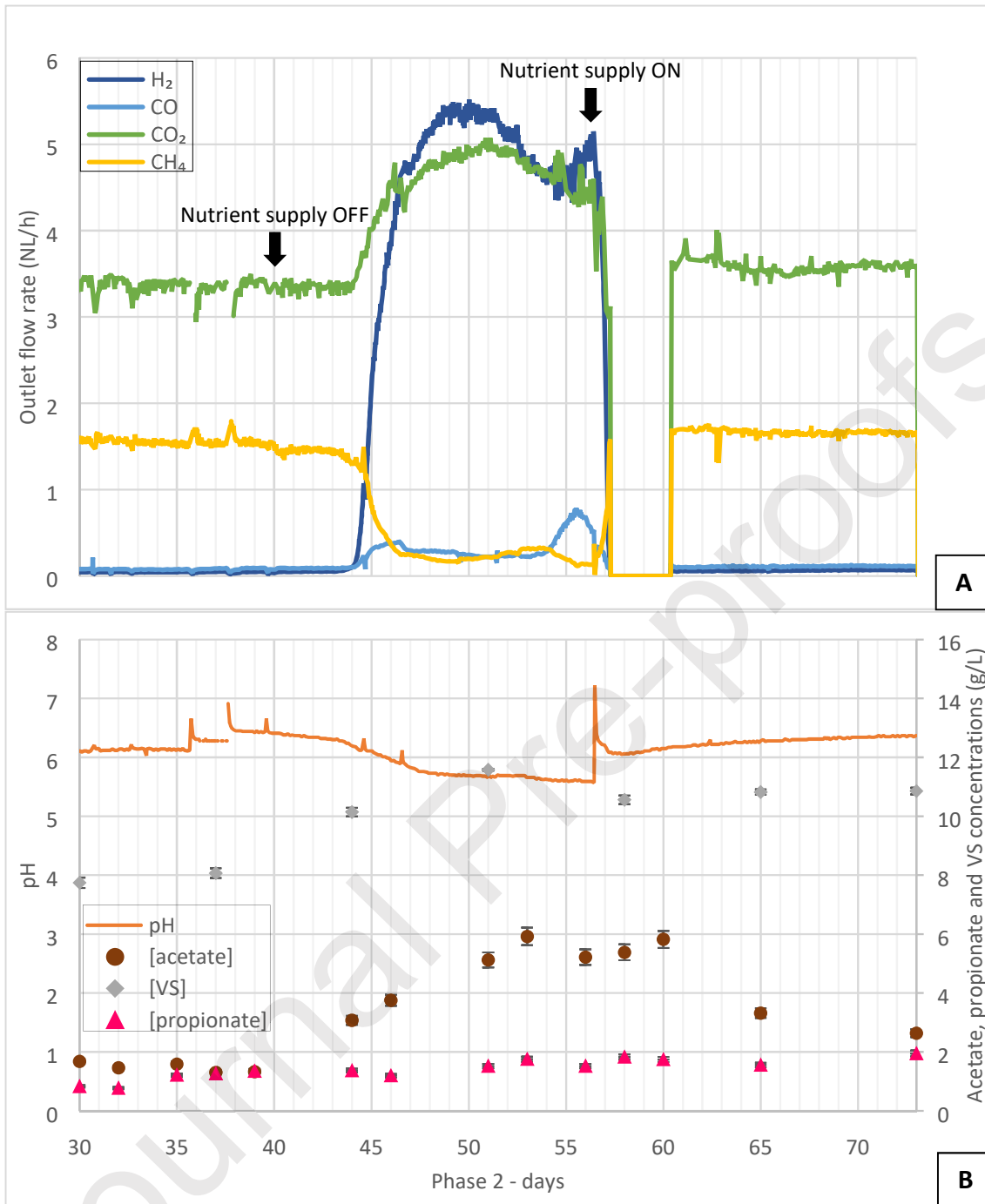


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Figure 2





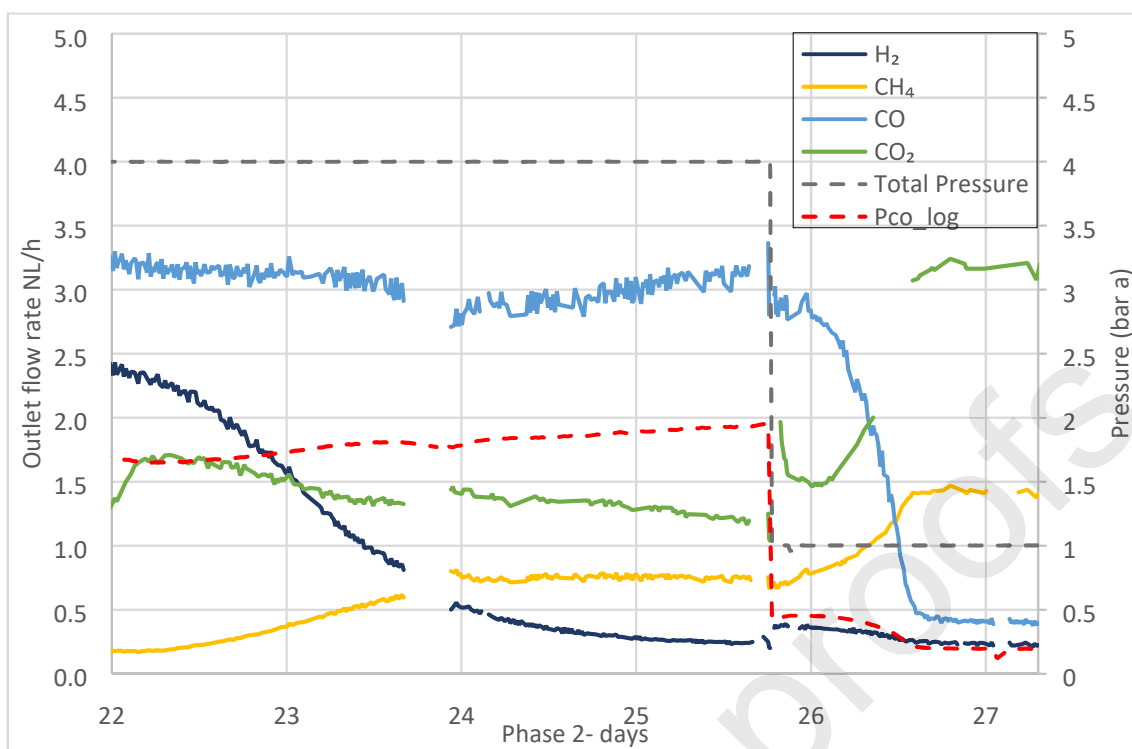
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Figure 4



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Figure 5

549

550 **Table 1** : Selected steady-state periods for mass balance calculations.

N°	Date	reaction description
I	Phase 1 - day 24-26	Methanogenesis from H ₂ /CO ₂ with CO conversion
II	Phase 2 – day 24-25	Methanogenesis from H ₂ /CO ₂ – limited CO conversion
III	Phase 2– day 30-39	Methanogenesis from H ₂ /CO ₂ with CO conversion
IV	Phase 2– day 47-49	WgS : CO conversion to H ₂ /CO ₂ – limited methane production
V	Phase 2– day 49-53	WgS : CO conversion to H ₂ /CO ₂ – limited methane production
VI	Phase 2– day 60-73	Methanogenesis from H ₂ /CO ₂ with CO conversion

551 WgS: water gas shift

552

553 **Table 2** : Mass balance and specific rates during steady-state periods.

	Water Gas Shift periods		Limited CO conversion period	Methanation periods		
	IV	V	II	I	III	VI
Conversion efficiencies (%)						
X_{CO}	91.3% ± 0.6%	92.9% ± 0.6%	10.9% ± 8.0%	97.8% ± 1.7%	97.6% ± 1.0%	96.6% ± 0.3%
X_{H_2}	-56.9% ± 7.3%	-55.2% ± 7.9%	90.8% ± 2.2%	89.2% ± 19.1%	98.6% ± 0.4%	98.1% ± 0.2%
Volumetric rates [mmol/(L_R.h)]						
r_{CO}	12.22 ± 0.21	12.43 ± 0.21	1.46 ± 2.66	12.85 ± 0.47	12.98 ± 0.26	12.94 ± 0.10
r_{H_2}	4.60 ± 2.58	5.04 ± 2.80	13.65 ± 3.27	24.58 ± 6.15	26.10 ± 0.41	26.07 ± 0.17
r_{CH_4}	0.80 ± 0.15	1.01 ± 0.24	3.03 ± 0.26	5.49 ± 3.51	6.62 ± 2.07	6.80 ± 0.50
Specific rates [mmol/(g_{VSS}.h)]						
$r_{X, CO}$	1.15 ± 0.04	1.11 ± 0.02	0.24 ± 0.45	1.67 ± 0.11	1.58 ± 0.12	1.20 ± 0.02
r_{X, H_2}	0.43 ± 0.25	0.45 ± 0.25	2.25 ± 0.65	3.19 ± 0.89	3.18 ± 0.23	2.43 ± 0.04
r_{X, CH_4}	0.08 ± 0.02	0.09 ± 0.02	0.50 ± 0.07	0.71 ± 0.48	0.88 ± 0.28	0.63 ± 0.05

Table 3 : Overview of existing continuous reactors in literature. Most production rates have been converted to unify the units.
V: working volume, T: temperature, X_{CO} and X_{H_2} : conversion efficiencies of CO and H₂ respectively, r^{CH_4} : volumetric methane production rate, r^{X, CH_4} : specific methane production rate, CSTR: Continuous Stirred Tank Reactor, NM: not mentioned.

	Reactor type	Pressure	Microbial inoculum	V (L)	T (°C)	Syngas composition	X_{CO} (%)	X_{H_2} (%)	r^{CH_4} [mmol/(L _g .h)]
Current work	Pressurized agitated column	4 bars (a)	Mixed Microbial Consortia	10	55	40% H ₂ , 20% CO ₂ , 40% CO	97	98	6.8
Asimakopoulos et al. (2020)	Trickle bed reactor	NM	Mixed Microbial Consortia	0.18	60	45% H ₂ , 25% CO ₂ , 20% CO, 10% N ₂	73	89	8.49
Asimakopoulos et al. (2020)	Trickle bed reactor	NM	Mixed Microbial Consortia	0.18	60	45% H ₂ , 25% CO ₂ , 20% CO, 10% N ₂	96	98	3.80
Asimakopoulos et al. (2021)	Trickle bed reactor	1 bar	Mixed Microbial Consortia	5	60	45% H ₂ , 25% CO ₂ , 20% CO, 10% N ₂	98	100	10.6
Asimakopoulos et al. (2021)	Trickle bed reactor	1 bar	Mixed Microbial Consortia	5	60	45% H ₂ , 25% CO ₂ , 20% CO, 10% N ₂	76.3	96.7	17.6
Chandolias et al. (2019)	Floating membrane reactor	NM	Mixed Microbial Consortia	1.7	55	20% H ₂ , 55% CO, 10% CO ₂ , 15% N ₂ , acetate	16	19	1.43
Diender et al., (2018)	CSTR	1 bar	Coculture	0.75	65	66.6% H ₂ , 33.3% CO	93	97	7.44*
Westman et al. (2016)	Reverse membrane bioreactor	NM	Mixed Microbial Consortia	0.6	55	20% H ₂ , 55% CO, 10% CO ₂ , 15% N ₂	100**	100**	0.2*
Westman et al. (2016)	Free cells bioreactor	NM	Mixed Microbial Consortia	0.6	55	20% H ₂ , 55% CO, 10% CO ₂ , 15% N ₂	100**	100**	0.2
Sun et al. (2020)	In situ biomethanation	NM	Mixed Microbial Consortia	37.5	37	50% H ₂ , 50% CO	94	100	0.46***
Kimmel et al. (1991)	Trickle bed reactor	1 bar	Triculture	1.05	37	14.82% Ar, 9.67% CO ₂ , 55.62% CO, 19.68% H ₂	70	NM	3.00
Kimmel et al. (1991)	Trickle bed reactor	1 bar	Triculture	1.05	37	14.74% Ar, 9.72% CO ₂ , 54.42% CO, 21.11% H ₂	38	NM	0.12

*This result is higher than the maximum theoretical stoichiometric value.

**Full conversion has probably been achieved because of gas recirculation.

***Methane produced from syngas only was considered, not from added glucose

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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:

- A continuous pressurized bioreactor has been run for syngas biomethanation.
- A high methane productivity and high CO and H₂ conversions were achieved.
- CO inhibition on carboxydrotrophs was a possible cause of limitation.