Enrichment of Homoacetogens Converting H2/CO2 into Acids and Ethanol and Simultaneous Methane Production

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Research Article

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Enrichment of homoacetogens converting H_2/CO_2 into acids and ethanol and simultaneous methane production

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Abstract

An anaerobic granular sludge was enriched to utilize H_2/CO_2 in a continuous gas-fed up-flow anaerobic sludge reactor by applying operating conditions expected to produce acetic acid, butyric acid and ethanol. Three stages of fermentation were found: Stage I with acetic acid accumulation with the highest concentration of 35 mM along with a pH decrease from initial 6 to 4.5. In Stage II, H_2/CO_2 was replaced by 100% H_2 to induce solventogenesis, whereas butyric acid was produced with the highest concentration of 2.5 mM. At Stage III with 10 μM tungsten (W) addition, iso-valeric acid, valeric acid and caproic acid were produced at pH 4.5 -5.0. In the batch tests inoculated with the enriched sludge taken from the bioreactor (day 70), however, methane production occurred at pH 6. Exogenous 15 mM acetate addition enhanced both the H_2 and CO_2 consumption rate compared to exogenous 10, 30 and 45 mM acetate by the enriched sludge. Exogenous acetate failed to be converted to ethanol using H_2 as electron donor by the enriched acetogens.

Key words: H_2/CO_2 fermentation, anaerobic sludge, acetogenesis, solventogenesis, methanogenesis

1 Introduction

CO_2 fermentation to generate bio-commodities (e.g. acetic acid (Karekar et al. 2020)) or biofuels (e.g. ethanol (He et al. 2020) and methane (Liu et al. 2016) relieves the paradox of fossil fuel utilization and carbon emission reduction. CO_2 fermentation simultaneously mitigates carbon emission and generates valuable bioenergy products and hence becomes a promising economical and sustainable way of biofuel production (Bhatia et al. 2019; Gunes 2021).

H_2 and CO_2 can be converted to volatile fatty acids (VFAs) and alcohols via the Wood-Ljungdahl pathway (WLP) by autotrophic acetogens, mainly containing *Clostridium spp.* According to the two-stage fermentation theory for CO_2 bioconversion, the first stage is acetogenesis with accumulation of acetic acid, followed by solventogenesis under stress conditions such as nutrient limitation or low pH (Mohammadi et al. 2012). The mechanism of solventogenesis, however, still remains to be explored. One of the widely recognized mechanisms to induce solventogenesis is a low pH (Ganigué et al. 2016). Low pH (below 5) induces more undissociated acids that can enter the cells, which convert the acids to neutral charged ethanol to avoid their death caused by an intracellular pH drop (Padan et al. 1981). On the other hand, microorganisms are one of the key components in CO_2 autotrophic fermentation, e.g. *Clostridium autoethanogenum* (Abrini et al. 1994) and *Clostridium carboxidivorans* (Liou et al. 2005; Fernández-Naveira et al. 2017). Several pure strains have been studied, however, mixed culture fermentations are easier to implement at large scale than pure cultures with the merits of resistance to non-sterile conditions (Liu et al. 2014). The potential products converted from H_2 and CO_2 include:

$$2CO_2(g) + 4H_2(g) \rightarrow CH_3COOH(l) + 2H_2O(l) \qquad \triangle G^\theta = -75.4\ \text{kJ/mol} \qquad (1)$$

$$2CO_2(g) + 6H_2(g) \rightarrow CH_3CH_2OH(l) + 3H_2O(l) \qquad \triangle G^\theta = -96.5\ \text{kJ/mol} \qquad (2)$$

$$CO_2(g) + 4H_2(g) \rightarrow CH_4(g) + 2H_2O \qquad \triangle G^\theta = -130.4\ \text{kJ/mol} \qquad (3)$$

52 $$CH_3COOH(l) + 2H_2(g) \rightarrow CH_3CH_2OH(l) + H_2O(l) \quad \triangle G^{\theta} = -25.2\ \text{kJ/mol} \qquad (4)$$

53 Limited studies reported ethanol production from H_2/CO_2 (Modestra et al. 2020; Stoll et
54 al. 2018). The positive role of exogenous acetate on ethanol production by a *Clostridium*
55 strain has been reported using syngas as the gaseous substrate (Xu et al. 2020). However,
56 whether acetic acid with H_2 can be directly converted to ethanol by mixed cultures remains to
57 be explored. Therefore, one possible strategy for enhancing solventogenesis is to supply
58 exogenous acetate with H_2 as the electron donor under low pH by mixed cultures.

59 Tungsten (W) is an important trace element involved in the formation of enzyme activity
60 such as formate dehydrogenase (FDH), one of key enzymes in the WLP, converting CO_2 into
61 formate. It has been reported that FDH synthesis could be stimulated in its presence
62 (Yamamoto et al. 1983). The other key metalloenzyme related to W is alcohol dehydrogenase
63 (ADH) catalyzing the reduction of acetyl CoA to ethanol (Andreesen et al. 2008). Tungsten
64 can enhance ethanol production from carbon monoxide by anaerobic granular sludge
65 (Chakraborty et al. 2020).

66 This study investigated CO_2 and H_2 fermentation by heat-treated granular sludge in a
67 bioreactor with both gas and medium circulation at 25°C. It was assumed that ethanol
68 production could be enhanced by feeding 100% H_2 or tungsten from acetic acid produced by
69 homoacetogens. Acetate produced from H_2/CO_2 or pure H_2 as the gaseous substrate and
70 ethanol degradation were further investigated in batch tests by the enriched sludge taken from
71 the reactor after 70 days of operation, from which the homoacetogenesis, methanogenesis and
72 solventogenesis potential was assessed.

73 ## 2 Materials and methods

74 ### 2.1 Biomass and medium composition

75 The same inoculum anaerobic granular sludge from a wastewater treatment plant was used
76 as in our previous study on acids and alcohol production from H_2/CO_2 (He et al. 2020). The

77 total solid (TS) and volatile solid (VS) content was 42.7 (± 1.0) g/L and 24.8 (± 0.5) g/L,
78 respectively. The granular sludge was first centrifuged at 5500 rpm for 10 min to remove the
79 supernatant and the pellet was heat-treated at 90°C for 15 min to select for spore forming
80 acetogens as described by Dessì et al. (2017). The medium was prepared according to a
81 previous study (He et al. 2020).

82 **2.2 Experimental set-up**

83 2.2.1 Semi-continuous gas fed bioreactor

84 An up-flow semi-continuous gas fed reactor was set-up with a total working volume of 1
85 L (Fig. 1) and liquid flow rate was 60 mL/min by a Verdeflex pump (Utrecht, The
86 Netherland). A 10 L gas bag filled with H_2/CO_2 (80/20 v/v) was connected on the gas outlet.
87 H_2/CO_2 gas was cycled at a gas flow rate of 10 mL· min^{-1} controlled by gas tight tubes using
88 a Verdeflex pump (Utrecht, The Netherland) and a mass flow meter (FMA-1618A, Omega,
89 San Antonio, US). The temperature was controlled at 25℃ by a water jacket. The initial pH
90 was 6.0 and when the pH decreased to 4.5, the pH control system would start working to
91 prevent further pH drop by adding 1 M NaOH to stimulate solventogenesis.

92 2.2.2 Batch tests

93 Batch experiments were conducted in 120 mL serum bottles with 50 mL medium and 5%
94 enriched sludge (day 70). The bottles were sealed with rubber stoppers and capped with
95 aluminum crimp caps. All bottles were pressurized with pure H_2 or H_2/CO_2 (80/20 v/v) at an
96 initial pressure of 1.8 bar and were incubated at 150 rpm and at 25°C.

97 **2.3 Experimental design**

98 2.3.1 Semi-continuous gas fed bioreactor operation

99 The semi-continuous gas fed bioreactor operation included three stages. In stage I (0-26
100 d), the reactor was fed with H_2/CO_2 gas (80/20 v/v) with initial pH of 6.0 for acetic acid

101 production without pH control. In stage II (day 27-50), H_2/CO_2 was replaced by 100% H_2 to
102 stimulate ethanol production at a pH controlled at 4.5-5. In stage III (day 50-70), 10 μM
103 tungsten was added to the medium to stimulate solventogenesis according to the report of
104 Chakraborty et al. (2020), while the gas phase was still 100% H_2.

105 Microbial community analysis was conducted for the anaerobic granular sludge in
106 duplicate (G-a, G-b) on 10 mL bioreactor suspension samples at the end of stage I, II and III
107 (in triplicate, III-a, b and c). At the end of the stage I (day 26, the log phase of the
108 autotrophic acetogens) to sustain and further enrich the sludge, 10 mL liquid sludge from the
109 reactor was inoculated into two 120 mL batch bottles with 50 mL liquid medium (duplicate).
110 H_2/CO_2 (80/20, v/v) was used as the substrate and the initial pH was 6.0. The bottles were
111 incubated at 150 rpm and at 25℃ in a water-bath shaker.

112 2.3.2 Batch studies on different H_2/CO_2 ratio utilization by enriched sludge

113 To elucidate the conversion pathway and failure of solventogenesis in the reactor, batch
114 tests of H_2/CO_2, 15 mmoL·L^{-1} acetate+ H_2/CO_2 and 10 μM tungsten+ H_2/CO_2 were
115 conducted using the bioreactor sludge as the inoculum. The enriched sludge from gas fed
116 reactor after 70 days fermentation was used as the inoculum for the following batch tests. To
117 investigate the effect of exogenous acetate on ethanol production using H_2 as electron donor,
118 the bottles were sparged with 100% H_2 and H_2/CO_2 (v/v, 80/20) and 5% inoculum at an
119 initial pressure 1.8 bar. Acetate was added to make the final concentration of 10, 15, 30 and
120 40 mmoL·L^{-1}, respectively. To test whether acetic acid and ethanol degradation occurred in
121 the reactor, 15 mmoL·L^{-1} acetate + 5 mmoL·L^{-1} ethanol, 30 mmoL·L^{-1} acetate + 15 mmoL·L^{-1}
122 ethanol were added with H_2/CO_2 (v/v, 80/20) in the headspace in batch tests using 5%
123 enriched sludge.

2.4 Analysis

2.4.1. Gas phase

H_2, CO_2 and CH_4 concentrations were measured using a HP 6890 gas chromatograph (GC, Agilent Technologies, Palo Alto, USA) equipped with a thermal conductivity detector (TCD). The GC was fitted with a 15-m HP-PLOT Molecular Sieve 5A column (ID 0.53 mm, film thickness 50 mm). The oven temperature was kept constant at 60°C. The temperature of the injection port and the detector was maintained constant at 250°C. Helium was used as the carrier gas.

2.4.2. VFAs and solvent analysis

VFAs, ethanol and butanol concentrations were analysed for each bottle from the liquid phase (1 mL) using high performance liquid chromatography (Agilent Co., Palo Alto, USA) equipped with a refractive index detector (RID) and an Agilent Hi-Plex H column (Internal diameter × length, 7.7 ×300 mm, size 8 μm). A H_2SO_4 solution (5 mM) was used as mobile phase at a flow rate of 0.7 ml/min and with a sample injection volume of 50 μl. The column temperature was set at 60°C and the RID detector at 55 °C.

2.4.3. Microbial analysis

DNA was extracted using a DNeasy® PowerSoil Kit (QIAGEN, Germany) following the manufacturer's protocol. Approximately 0.5 g of the solids from the samples was used for DNA extraction. The extracted DNA was quantified and its quality was checked by a Nanodrop 2000c Spectrophotometer (Thermo Scientific, USA). A total of 1,103,482 sequences were obtained from all investigated samples (Table SI 1). After eliminating chimeras, a sequence identity of 70%, across at least 80% of the representative sequences, was a minimal requirement for considering reference sequences. Further processing of the operational taxonomic units (OTUs) and taxonomic assignments were performed using the QIIME software package (version 1.9.1, http://qiime.org/). Abundances of bacterial

149 taxonomic units were normalized using lineage-specific copy numbers of the relevant marker
150 genes to improve estimates (Angly et al. 2014).

151 **3 Results**

152 **3.1 Enrichment of acetogenic sludge and production of acids and ethanol in gas fed**
153 **reactor**

154 During the reactor operation, after 10 days of adaption, acetic acid started to be produced
155 and reached to 35 mM (Fig. 2a, Eq. 1). Ethanol was detected at day 11 and increased to 1.35
156 $mmoL \cdot L^{-1}$ at day 12 but it was then degraded (Fig. 2a, Eq. 2). Instead, butyric acid started to
157 be produced at day 12 when ethanol degradation occurred and increased to 0.5 $mmoL \cdot L^{-1}$ at
158 day 26. Propionic acid started to be produced at day 14 and reached to 1.82 $mmoL \cdot L^{-1}$ at day
159 26. The pH decreased along with the accumulation of acetic acid and kept at 4.5–5.0 after day
160 21 (Fig. 2b). However, ethanol production was not observed when the pH was as low as 4.5
161 from day 21 to 26 (Fig. 2a).

162 To stimulate ethanol production from acetic acid, H_2/CO_2 was replaced by 100% H_2 at
163 day 27 (Stage II, 27-50 d). Indeed, 100% H_2 addition induced ethanol production and it
164 reached to 1.2 $mmoL \cdot L^{-1}$ at day 37 (Fig. 2a, Eq. 4). Thereafter, ethanol production started to
165 decrease to a concentration of 0.5 $mmoL \cdot L^{-1}$ (Fig. 2a). Meanwhile, butyric acid accumulated
166 and reached 2.4 $mmoL \cdot L^{-1}$ at day 50 (Fig. 2a). The concentration of both acetic acid and
167 propionic acid decreased at the end of the stage II.

168 At stage III, 10 μM tungsten addition induced both acetic acid and butyric acid
169 degradation, accompanied with the production of valeric acid and caproic acid, respectively,
170 1.3 and 0.4 $mmoL \cdot L^{-1}$ at the end of incubation (Fig. 2a).

3.2 Effect of exogenous acetate and tungsten addition on H_2/CO_2 conversion by enriched sludge

When using H_2/CO_2 as the substrate (the control) for the enriched sludge (day 70), acetic acid was produced with a final concentration of 6.1 mmoL·L^{-1} (Fig. 3a, Table SI 2). Methane production was observed along with the acetic acid production and 36.6 mmoL·L^{-1} methane had accumulated at the end of the incubation (Fig. 3a). H_2 and CO_2 consumption was, respectively, 160 and 40.6 mmoL·L^{-1} at the end of the incubation (Fig. 3a).

With 15 mmoL·L^{-1} acetate addition, 0.9 mmoL·L^{-1} ethanol was produced after 144 h but it was degraded after 192 h and did not accumulate at the end of the incubation. However, methane production was observed and accumulated to 60.0 mmoL·L^{-1} at the end of the incubation. The acetic acid concentration slightly decreased from initially 15 to 13 mmoL·L^{-1} at the end of the incubation (Fig. 3b). H_2 and CO_2 consumption was, respectively, 262.0 and 73.3 mmoL·L^{-1} at the end of the incubation (Fig. 3a) and was correspondingly 1.6 fold and 1.8 fold higher than the control to which no external acetate was provided. The H_2 and CO_2 consumption rate increased to, respectively, 0.85 and 0.26 mmoL·L^{-1}·h^{-1} compared to the control of 0.59 and 0.18 mmoL·L^{-1}·h^{-1} (Table SI 2).

The addition of 10 μM tungsten enhanced the H_2 and CO_2 consumption of 285.8 and 71.1 mmoL·L^{-1}, respectively, at a H_2 and CO_2 consumption rate of 1.02 mmoL·L^{-1}·h^{-1} and 0.25 mmoL·L^{-1}·h^{-1}, respectively, compared to the control. Methane (67.6 mmoL·L^{-1}) was produced at the end of the incubation (Fig. 3c). The methane production was from acetate as the substrate since the produced acetic acid at 144 h (6.0 mmoL·L^{-1}) was almost totally consumed in the 10 μM W+H_2/CO_2 incubation upon completion of the experiment (Fig. 3c, d). Surprisingly, the pH of the control and the 10 μM tungsten incubation decreased quickly even below 4 after 192 h but methane production was still detected (Fig. 4d).

3.3 Effect of exogenous acetate on with H_2/CO_2 conversion by enriched sludge

Initially, acetate was not significantly consumed while it slightly increased at the initial concentration of 15 and 30 $mmoL \cdot L^{-1}$ acetate (Fig. 4a). Methane production reached to 14.9, 59.7, 5.2 and 14.0 $mmoL \cdot L^{-1}$ along with the increased initial 10, 15, 30 and 45 $mmoL \cdot L^{-1}$ acetate concentration. Correspondingly, CO_2 consumption was respectively, 27.7, 73.3, 19.8 and 25.4 $mmoL \cdot L^{-1}$, whereas the H_2 consumption amounted to 78.9, 261.9, 45.4 and 73.1 $mmoL \cdot L^{-1}$, respectively. Correspondingly, the pH decreased from initial 6 to 5.0-5.2 at both initial 15 and 30 $mmoL \cdot L^{-1}$ acetate due to the positive net acetic acid production (Fig. 4e). The gas pressure was decreased slowly during the incubation, because part of the gas pressure came from the methane production (Fig. 4f).

The 15 mM acetate addition reached the highest CH_4 production, CO_2 and H_2 consumption compared with 10, 30 and 45 $mmoL \cdot L^{-1}$ acetate, while the acetic acid concentration slightly decreased at the end (Fig. 5). 10 and 50 $mmoL \cdot L^{-1}$ acetate had a similar effect on the CH_4 production and H_2 and CO_2 consumption, while supplementing 30 $mmoL \cdot L^{-1}$ acetate obtained the lowest CH_4 production, CO_2 and H_2 consumption.

Further experiments demonstrated that when using 100% H_2 and in the absence of CO_2, the ethanol production process did not happen after 240 h incubation. The pH did not change during the incubation and the gas pressure did not decrease. The failure of acetate and H_2 utilization might be because the enriched acetogens were mostly autotrophic acetogens, which was further confirmed by the microbial community analysis (see below).

3.4 Acetate and ethanol conversion in the presence of H_2/CO_2 by enriched sludge

Acetate and ethanol were added to simulate the conversion process, i.e. the reverse β oxidation pathway, to further assess if longer chain VFA were produced, as observed in the reactor. With 15 HAc+5 EtOH and 30 HAc+15 EtOH, methane production was observed and

219 reached, respectively, 54.1 and 46.3 mmoL·L^{-1}. Neither ethanol nor longer chain fatty acids
220 were produced during the incubation.

221 With 15 mmoL·L^{-1} acetate and 5 mmoL·L^{-1} ethanol addition, CH_4 production and CO_2
222 and H_2 consumption were all higher compared to the incubations supplied with 30 mmoL·L^{-1}
223 acetate and 15 mmoL·L^{-1} ethanol addition (Fig. 6). Both the acetate and ethanol
224 concentration slightly decreased during the incubation (Fig. 6a, b). The pH was slightly
225 increased possibly due to the decreased dissolved CO_2 in the liquid medium induced by the
226 consumption of headspace CO_2 (Fig. 6c). The gas pressure decreased slowly and showed a
227 similar trend between the 15 HAc+5 EtOH and 30 HAc+15 EtOH groups.

228 The enriched sludge was further checked for the addition of glucose to possibly enhance
229 the biomass grow and mixotrophy. However, the ethanol production did not significantly
230 enhance compared to the solely glucose fed incubation (SI Fig 1).

231 **3.5 Microbial analysis**

232 Microbial analysis of the suspended sludge of the bioreactor showed the relative
233 abundance of acetogens related at class level *Clostridia*. On day 10, when the acetic acid
234 started to be produced, they comprised a relative abundance of 3.1%, it increased to 11.4% at
235 the stage II and 9.4% at the stage III, finally reaching about 25-26% (Fig. 6a).

236 For the *Clostridium* genus, the relative abundance with 0.1% at day 10 increased to,
237 respectively, 0.5% at stage II, 0.7% at stage III and about 3.5% at the end of each stage (Fig.
238 6b). SI Fig. 2 shows the acetic acid and ethanol production in the batch bottles inoculated
239 with enriched sludge from the bioreactor at day 70. The microbial analysis data (I-a, I-b of
240 bottle 1, 2, respectively) showed that the higher relative abundance of the *Clostridium* genus
241 compared to the reactor sludge sampled on day 10. *Clostridium* was enriched with a relative
242 abundance of 3.5% in the bioreactor (day 70) and increased to 18.5 and 22.0 % in the
243 enriched batch bottle 1 and 2 (Fig. 6b).

Fig. 6c shows the distribution of the *Clostridium* genus. The *Clostridium* genus and other acetogens belonging to the *Clostridia* class occupied above 60% at the end of the incubation (III-a, b, c) (Fig. 6c). In the *Clostridia* class, the relative abundance of the *Caproiciproducens* genus increased from 3.2% on day 10 to about 30% at the end of the incubation. The increase and enrichment of *Caproiciproducens* was corresponding to the increased caproic acid production at the end of the incubation. Small amounts of the *Ethanoligenens* genus were enriched with around 3% at the end of the incubation (triplicates, III-a, b, c), which might have contributed to the ethanol production process during the fermentation. The *Oscillibacter* genus existed during the whole fermentation process with a relative abundance of 9.8% at day 10, then decreased to 5.8% at the end of the incubation (Fig. 6c). *Oscillibacter* is known to be involved in acidogenesis during dark fermentation (Goud et al. 2017) and this microorganism might play a role in the acetic acid accumulation during the adaption stage.

4 Discussion

4.1 VFAs and ethanol production by anaerobic granular sludge in the gas fed reactor

This study showed that 100% H_2 addition induced both butyric acid and ethanol production, while 10 μM tungsten induced caproic acid production at a pH as low as 4.5-5.0. Ethanol production was observed during the H_2/CO_2 fermentation process and 100% H_2 as electron donor, but it was subsequently degraded. Considering the inoculum applied was an undefined mixed culture, ethanol has been degraded to acetic acid in the presence of CO_2 (Eq. 5) or used as the electron donor for butyric acid production (Eq. 6).

$$2CH_3CH_2OH(l) + 2CO_2(g) \rightarrow 3CH_3COOH(l) \qquad \triangle G^{\theta} = -32.2\ \text{kJ/mol} \quad (5)$$

$$CH_3CH_2OH(l) + CH_3COOH(l) \rightarrow CH_3CH_2CH_2COOH(l) + H_2O \quad (6)$$

The first ethanol degradation (day 11) was possibly due to its oxidation to acetic acid in the presence of CO_2 since butyric acid production was insignificantly observed at that time (Fig. 2a). The second ethanol decrease (day 37-50) possibly supplied butyric acid production via the reverse β oxidation pathway (Grootscholten et al. 2013), during which the butyric acid concentration increased along with the ethanol consumption (Fig. 2).

The presence of CO_2 on ethanol utilization could have induced formation of longer chain fatty acids. Roghair et al. (2018) reported butyric acid and caproic acid production via controlling the ethanol use under different CO_2 loading rates (0.5 and 2.5 $LCO_2 \cdot L^{-1} \cdot d^{-1}$) by anaerobic granular sludge. However, our previous study using the same anaerobic granular sludge demonstrated that the ethanol oxidation to acetic acid was priority over chain elongation in the presence of CO_2 at initial pH 5.7 and 6.5 by the same anaerobic granular sludge (He et al. 2022). H_2 acted as electron donor for chain elongation process, which has been reported in the literature (Baleeiro et al. 2021). Further research is required with ^{13}C NMR and labelled substrate (e.g., CO_2, ethanol and acetate) to elucidate the biochemical conversions in the sludge.

4.2 Methane was the main by-product during chain elongation process when pH increased to 6 at 25°C by enriched sludge

This study showed that, with gaseous H_2/CO_2, 15 mM acetic acid addition reached the highest methane production, CO_2 and H_2 consumption compared to the 0, 10, 30 and 45 $mmoL \cdot L^{-1}$ acetic acid addition by enriched sludge (day 70) (Fig. 3a, b, Fig. 4). Despite of the different extent of gas consumption, methane occupied the main product of the enriched sludge. An initial pH of 6 could be attributed to the methane production in batch tests by the enriched sludge while methane production was totally inhibited at pH 4.5-4.7 in the bioreactor. The inhibited methane production in the reactor could be attributed to the heat pre-treatment and the long-time operation at low pH of 4.5. However, along with the

operation, methanogens could be enriched in the inoculum although the production of methane can be inhibited at a pH of 4.5 (Fernández-Naveira et al. 2017). Although methane production can be inhibited when the pH was lower than 6, its production has been observed in a few reactors operating at low pH, especially along with increased operation time (Chakraborty et al. 2019). Another reason might be the gas feeding mode or different mass transfer rate between 1 bar gas pressure in the reactor, whereas an initial 1.8 bar in the batch bottles. Higher gas pressure induced more CO_2 dissolution in the medium and may stimulate hydrogenotrophic methanogens (Roghair et al. 2018).

4.3 CO_2 instead of exogenous acetate can be used for acetogenesis or methanogenesis by enriched sludge

This study showed that exogenous acetate with 10, 15, 30 and 45 mmoL·L^{-1} cannot be used for ethanol or methane production in the presence of 100% H_2 by the enriched sludge. Even with H_2/CO_2 as the gaseous substrate, the maximum acetate consumption occupied 13.3% (thus 2 mmoL·L^{-1}) in the 15 mmoL·L^{-1} acetate incubation. Ethanol (Eq. 7) and methane (Eq. 8) production from exogenous acetate failed using 100% H_2 as electron donor by the enriched sludge. This might be because the enriched microorganisms after 70 days incubation in the bioreactor were autotrophic acetogens, such as the *Clostridia* and *Bacilli* class using CO_2 instead of acetate as the substrate.

$$2CH_3COOH + H_2 \rightarrow CH_3CH_2CH_2COOH \quad (7)$$

$$CH_3COOH + H_2 \rightarrow CH_4 + CO_2 \quad (8)$$

5 Conclusion

Autotrophic acetogens were enriched in a H_2/CO_2 gas fed reactor for acetic acid, butyric acid and caproic acid production from heat-treated anaerobic granular sludge treating dairy

wastewater. 100% H_2 induced butyric acid and ethanol production at pH 4.5-5, but ethanol was degraded and might have contributed to the butyric acid production. 10 μM tungsten addition induced caproic acid production at pH 4.0-4.5. The *Clostridia* order was enriched at the end of the gas fed reactor and contributed to VFAs and ethanol production. The enriched sludge mainly produced methane from H_2/CO_2, exogenous acetate and ethanol in batch incubations at pH 6 and 25℃. The enriched sludge failed to convert acetate and 100% H_2 to ethanol at an initial pH of 6.

List of abbreviations

ADH: alcohol dehydrogenase

CO: carbon monoxide

CO_2: carbon dioxide

EtOH: ethanol

FDH: formate dehydrogenase

H_2: hydrogen

HAc: acetic acid

TS: total solid

VS: volatile solid

VFAs: Volatile fatty acids

W: tungsten

WLP: Wood-Ljungdahl pathway

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YH carried out all experimental incubations, data analysis, and drafted the manuscript. **CC** conceived the study, participated in its design and coordination, and reviewed the manuscript. **PL** conducted the project supervision and the manuscript revision. All authors read and approved the final manuscript.

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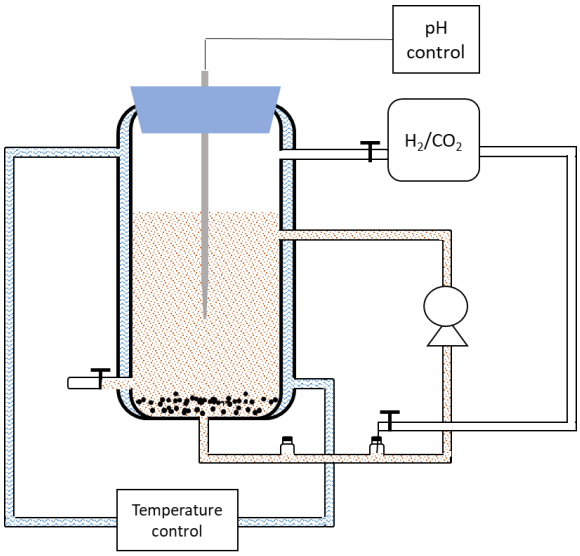
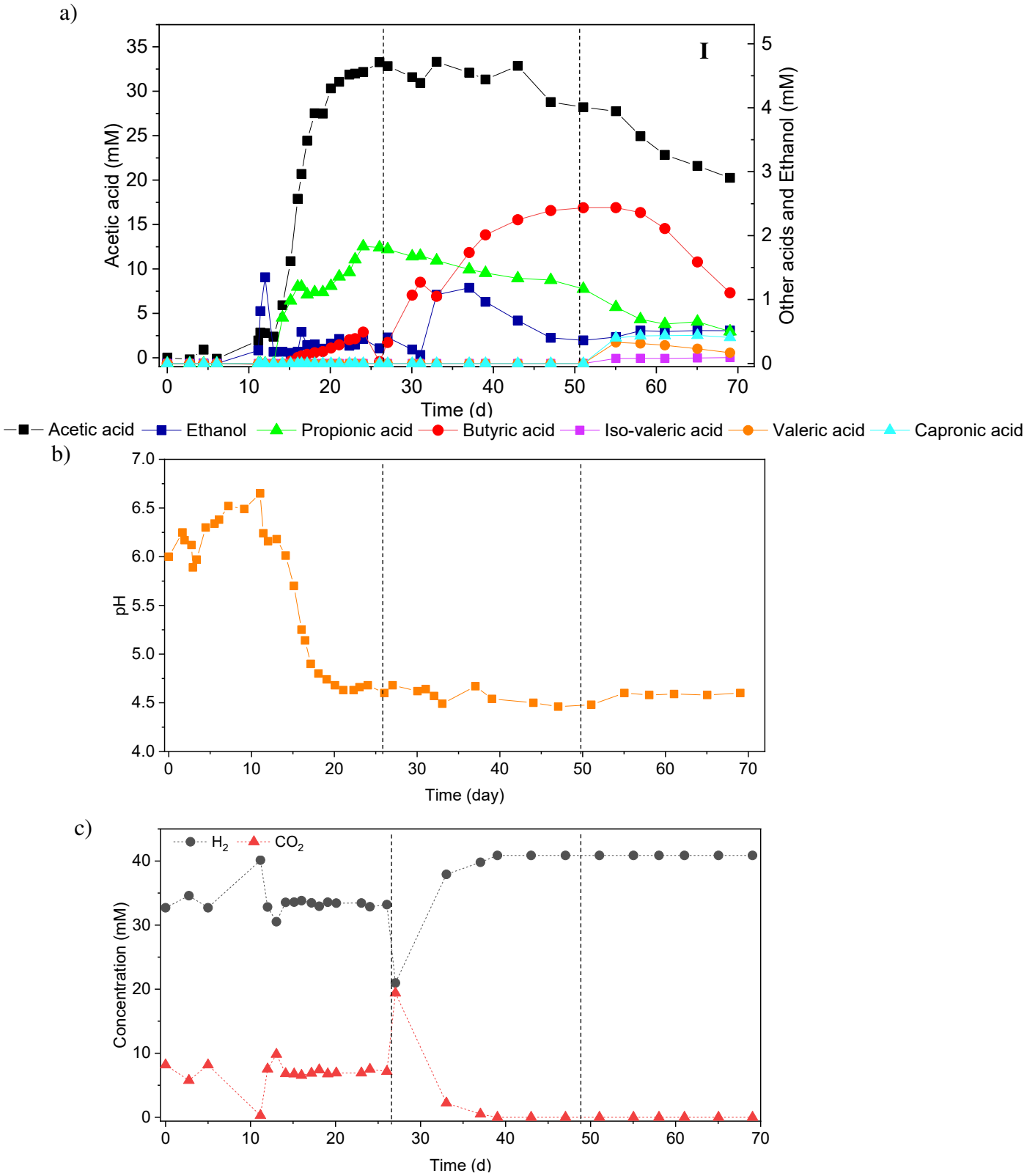


439 **Fig. 1**

440 **Fig. 1** Diagram of the up-flow gas reactor with pH control



441 **Fig. 2**

442 **Fig. 2** H_2/CO_2 fermentation in a semi-continuous gas fed reactor by anaerobic granular sludge.
443 a) acids and ethanol production, b) change of pH and c) H_2, CO_2 concentration from H_2/CO_2

or H_2 by granular sludge. The substrate of stage I, II and III are, respectively, H_2/CO_2, H_2 and H_2 + 10 µM tungsten.

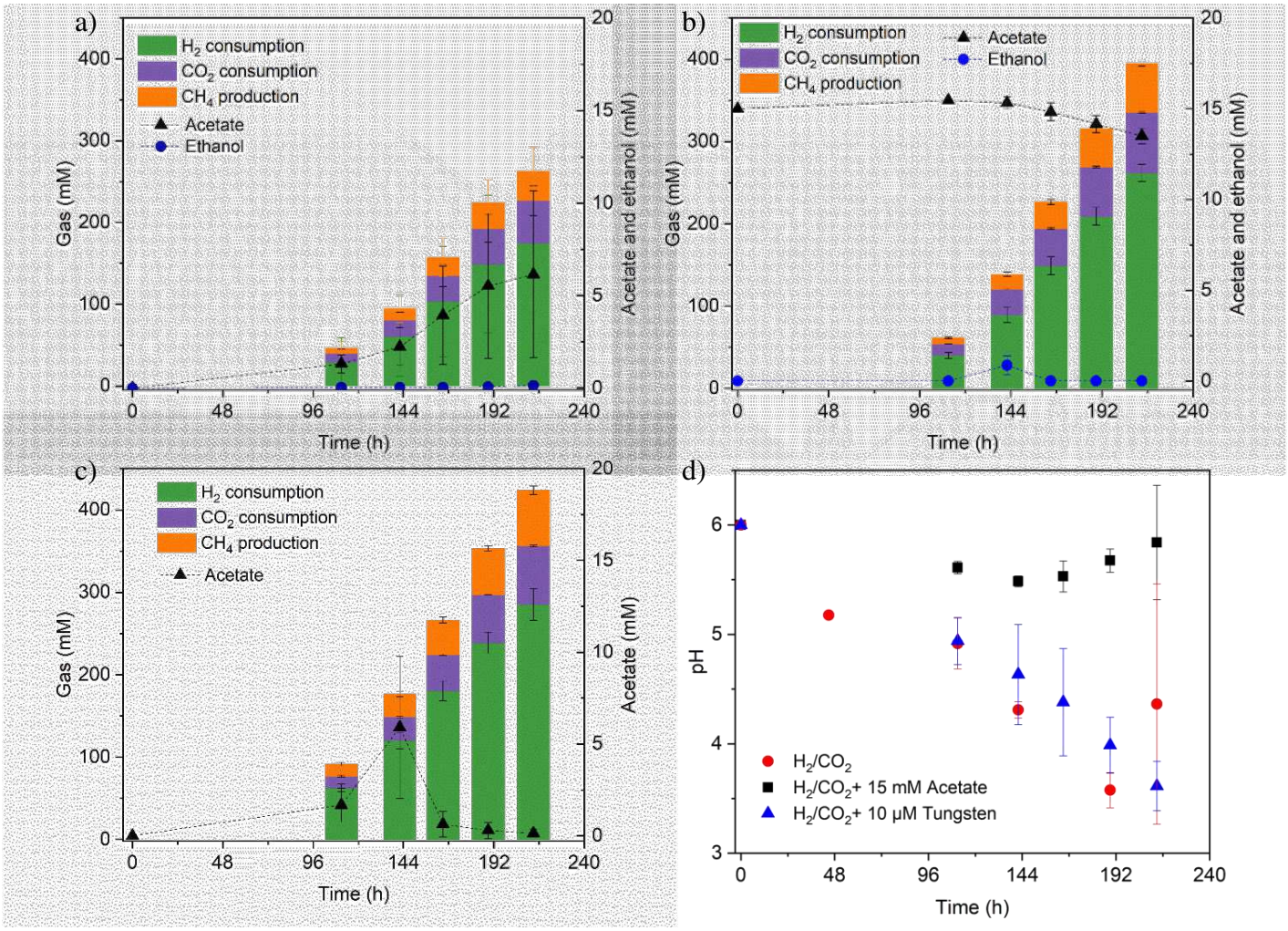


Fig. 3

Fig. 3 H_2 and CO_2 consumption and CH_4, acetate and ethanol production by enriched sludge sampled day 70 from the bioreactor using a) H_2/CO_2, b) H_2/CO_2 + 15 mM acetate and c) H_2/CO_2 + 10 µM tungsten as the substrate and d) change of pH in these incubations.

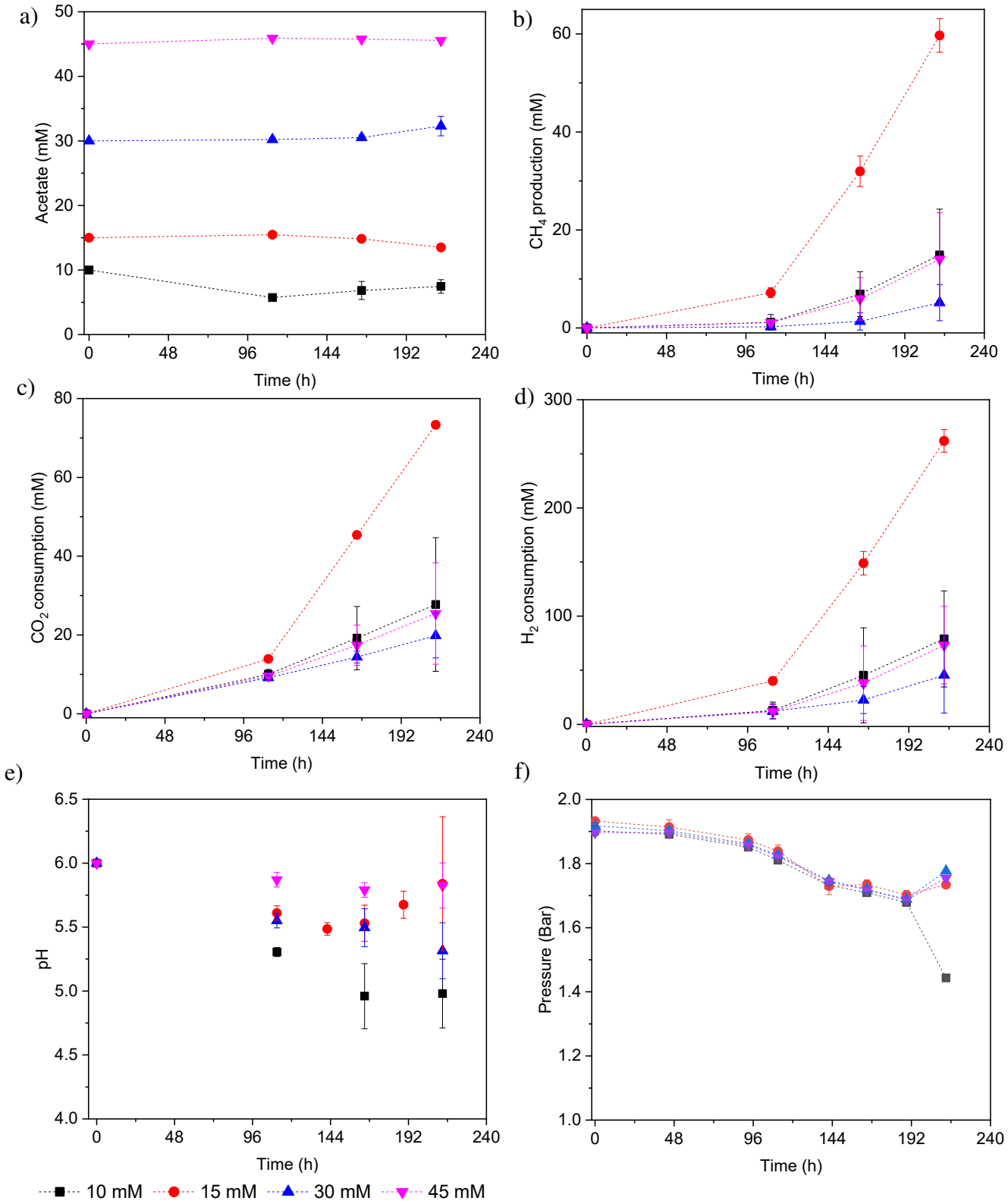

a)
Acetate (mM)
Time (h)
b)
CH_4 production (mM)
Time (h)
c)
CO_2 consumption (mM)
Time (h)
d)
H_2 consumption (mM)
Time (h)
e)
pH
Time (h)
f)
Pressure (Bar)
Time (h)
10 mM
15 mM
30 mM
45 mM

Fig. 4

Fig. 4 Effect of 10, 15, 30 and 45 mM exogenous acetate on production profiles by enriched sludge day 70 bioreactor operation using H_2/CO_2 as the substrate a) acetate concentration, b) CH_4, c) CO_2 and d) H_2 production, e) change of pH and f) gas pressure.

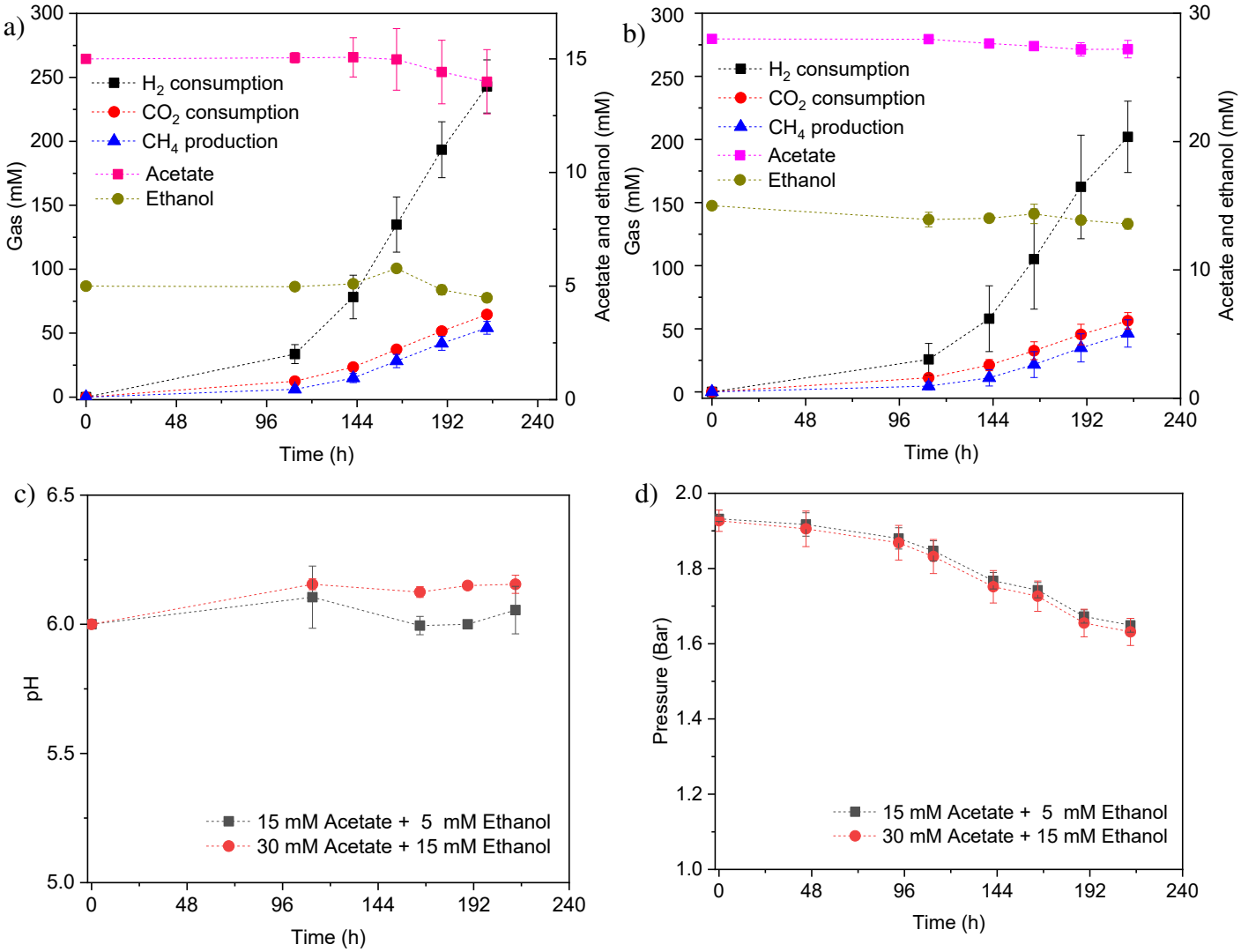
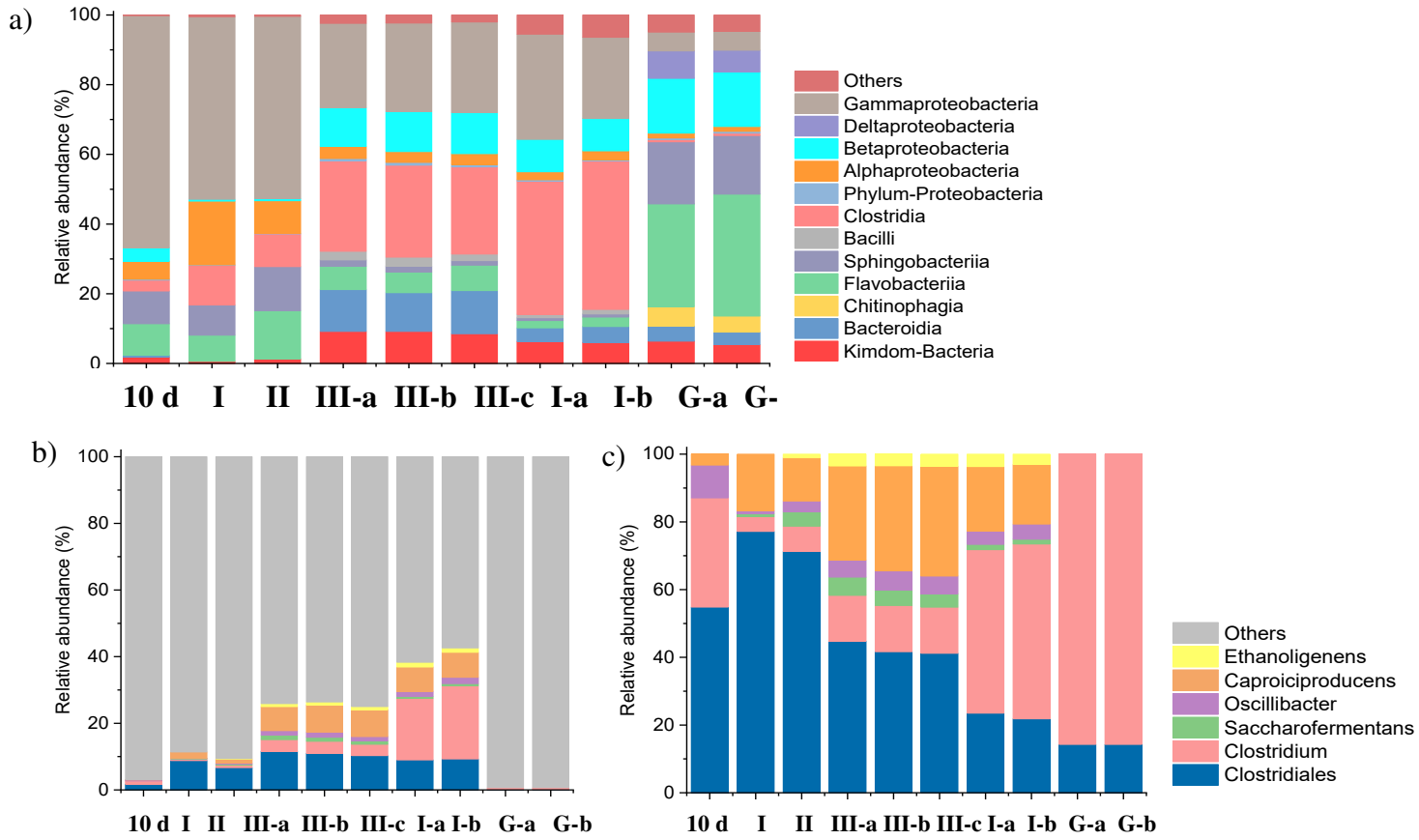


Fig. 5

Fig. 5 H_2 and CO_2 consumption and CH_4, acetate and ethanol production in the presence of a) 15 mM acetate + 5 mM Ethanol and b) 30 mM Acetate + 15 mM Ethanol, c) pH and d) gas pressure change by enriched sludge sampled day 70 using H_2/CO_2 as the substrate.



458 **Fig. 6** Relative abundance of microorganism from suspended sludge at 10 d, and the end of
459 stage I, II, III (III-a, b and c are triplicates) and the granular sludge inoculum (G-a, G-b) at
460 genus level. The two batch bottles used the sludge taken from the bioreactor as inoculum
461 (stage I, day 26) (I-a, I-b). a) Microbial analysis of all bacteria; b) Genus level in the
462 *Clostridiales* order, the relative abundance is relative to all the bacteria, and c) Genus level in
463 the *Clostridiales* order, the relative abundance is relative to the *Clostridiales* order.

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