Enrichment of homoacetogens converting \( \text{H}_2/\text{CO}_2 \) into acids and ethanol and simultaneous methane production

Yaxue He | Chiara Cassarini | Piet N.L. Lens

Abstract
An anaerobic granular sludge was enriched to utilize \( \text{H}_2/\text{CO}_2 \) 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, \( \text{H}_2/\text{CO}_2 \) was replaced by 100% \( \text{H}_2 \) to induce solventogenesis, whereas butyric acid was produced with the highest concentration of 2.5 mM. At stage III with 10 \( \mu \text{M} \) 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 \( \text{H}_2 \) and \( \text{CO}_2 \) consumption rate compared to exogenous 10, 30, and 45 mM acetate by the enriched sludge. Exogenous acetate was failed to be converted to ethanol using \( \text{H}_2 \) as electron donor by the enriched acetogens.

KEYWORDS
acetogenesis, anaerobic sludge, \( \text{H}_2/\text{CO}_2 \) fermentation, methanogenesis, solventogenesis

1 | INTRODUCTION

\( \text{CO}_2 \) fermentation to generate bio-commodities (e.g., acetic acid [1]) or biofuels (e.g., ethanol [2] and methane [3]) relieves the paradox of fossil fuel utilization and carbon emission reduction. \( \text{CO}_2 \) bioconversion mitigates carbon emission and hence becomes a promising economical and sustainable way for biofuel production [4, 5].

\( \text{CO}_2 \) can be converted to VFAs (volatile fatty acids) and alcohols via two-stage fermentation in the Wood–Ljungdahl pathway (WLP) by autotrophic acetogens including \( \text{Clostridium spp} \). The first stage is acetogenesis with accumulation of acetic acid, followed by solventogenesis under stress conditions such as nutrient limitation or low pH [6]. The mechanism of solventogenesis, however, still remains to be explored. One of the widely recognized mechanisms to induce solventogenesis is a low pH [7]. 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 [8]. On the other hand, microorganisms are one of the key components in \( \text{CO}_2 \) autotrophic fermentation, for
example, Clostridium autoethanogenum [9] and Clostridium carboxidivorans [10, 11]. Several pure strains have been studied, however, mixed culture fermentations are easier to implement at a large scale than pure cultures with the merits of resistance to non-sterile conditions [12]. The potential products converted from H\(_2\) and \(\text{CO}_2\) include:

\[
\begin{align*}
72\text{CO}_2\text{(g)} + 4\text{H}_2\text{(g)} &\rightarrow \text{CH}_3\text{COOH}\text{(l)} + 2\text{H}_2\text{O}\text{(l)} & \Delta G^\circ & = -75.4 \text{ kJ mol}^{-1} \\
2\text{CO}_2\text{(g)} + 6\text{H}_2\text{(g)} &\rightarrow \text{CH}_3\text{CH}_2\text{OH}\text{(l)} + 3\text{H}_2\text{O}\text{(l)} & \Delta G^\circ & = -96.5 \text{ kJ mol}^{-1} \\
\text{CO}_2\text{(g)} + 4\text{H}_2\text{(g)} &\rightarrow \text{CH}_4\text{(g)} + 2\text{H}_2\text{O} & \Delta G^\circ & = -130.4 \text{ kJ mol}^{-1} \\
\text{CH}_3\text{COOH}\text{(l)} + 2\text{H}_2\text{(g)} &\rightarrow \text{CH}_3\text{CH}_2\text{OH}\text{(l)} + \text{H}_2\text{O}\text{(l)} & \Delta G^\circ & = -25.2 \text{ kJ mol}^{-1}
\end{align*}
\]

Limited studies reported ethanol production from H\(_2\)/\(\text{CO}_2\) [13, 14]. The positive role of exogenous acetate on ethanol production by a Clostridium strain has been reported using syngas as the gaseous substrate [15]. However, whether acetic acid with H\(_2\) can be directly converted to ethanol by mixed cultures remains to be explored. Therefore, one possible strategy for enhancing solventogenesis is to supply exogenous acetate with H\(_2\) as the electron donor under low pH by mixed cultures.

Tungsten (W) is an important trace element involved in the formation of enzyme activity such as formate dehydrogenase (FDH), one of the key enzymes in the WLP, converting CO\(_2\) into formate. It has been reported that FDH synthesis could be stimulated in the presence of W [16]. The other key metalloenzyme related to W is alcohol dehydrogenase (ADH) catalyzing the reduction of acetyl CoA to ethanol [17]. Tungsten can enhance ethanol production from carbon monoxide (CO) by anaerobic granular sludge [18].

This study investigated CO\(_2\) and H\(_2\) fermentation by heat-treated granular sludge in a bioreactor with both gas and medium circulation at 25°C. It was assumed that ethanol production could be enhanced by feeding 100% H\(_2\) or tungsten from acetic acid produced by homoacetogens. Acetate produced from H\(_2)/\text{CO}_2\) or pure H\(_2\) as the gaseous substrate and ethanol degradation were further investigated in batch tests by the enriched sludge taken from the reactor after 70 days of operation, from which the homoacetogenesis, methanogenesis and solventogenesis potential was assessed.

### HIGHLIGHT

- H\(_2)/\text{CO}_2\) replaced by 100% H\(_2\) stimulated butyric acid and ethanol production at pH 4.5–5 by anaerobic sludge.
- 10 µM tungsten addition enhanced caproic acid production at pH 4.0–4.5.
- Methane was mainly produced from H\(_2)/\text{CO}_2\) and exogenous acetate and ethanol at pH 6 and 25°C by the enriched sludge.
- Enriched sludge failed to convert acetate and 100% H\(_2\) to ethanol.

### Practical application

CO\(_2\) fermentation simultaneously mitigates carbon emission and generates valuable bioenergy products and hence becomes a promising economical and sustainable way of biofuel production. This study investigated CO\(_2\) and H\(_2\) fermentation by heat-treated granular sludge in a bioreactor with both gas and medium circulation at 25°C. H\(_2)/\text{CO}_2\) replaced by 100% H\(_2\) stimulated butyric acid and ethanol production at pH 4.5–5 by anaerobic sludge. 10 µM tungsten addition enhanced caproic acid production at pH 4.0–4.5. Methane was mainly produced from H\(_2)/\text{CO}_2\) and exogenous acetate and ethanol at pH 6 and 25°C by the enriched sludge. 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. This study provides an attractive strategy in acetogenesis and solventogenesis in a bioreactor via 100% H\(_2\) and tungsten addition by anaerobic sludge and obtained methane production in H\(_2)/\text{CO}_2\) fermentation by the enriched sludge.

### MATERIALS AND METHODS

#### 2.1 Biomass and medium composition

The same inoculum anaerobic granular sludge from a wastewater treatment plant was used as in our previous study on acids and alcohol production from H\(_2)/\text{CO}_2\) [2]. The total solid (TS) and volatile solid (VS) content was 42.7 (± 1.0) g L\(^{-1}\) and 24.8 (± 0.5) g L\(^{-1}\), respectively. The granular sludge was first centrifuged at 5500 rpm.
2.2 | **Experimental set-up**

2.2.1 | **Semi-continuous gas fed bioreactor**

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

2.2.2 | **Batch tests**

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

2.3 | **Experimental design**

2.3.1 | **Semi-continuous gas fed bioreactor operation**

The semi-continuous gas fed bioreactor operation included three stages. In stage I (0–26 d), the reactor was fed with $H_2/CO_2$ gas (80/20 v/v) with the initial pH of 6.0 for acetic acid production without pH control. In stage II (day 27–50), $H_2/CO_2$ was replaced by 100% $H_2$ to stimulate ethanol production at a pH controlled at 4.5-5. In stage III (day 50–70), 10 μM tungsten was added to the medium to stimulate solventogenesis according to the report of Chakraborty et al. [18], while the gas phase was still 100% $H_2$.

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

2.3.2 | **Batch studies on solventogenesis using different substrates by enriched sludge**

To elucidate the conversion pathway and failure of solventogenesis in the reactor, batch tests of $H_2/CO_2$, 15 mmol L⁻¹ acetate+$H_2/CO_2$ and 10 μM tungsten+$H_2/CO_2$ were conducted using the bioreactor sludge as the inoculum. The enriched sludge from gas fed reactor after 70 days fermentation was used as the inoculum for the following batch tests. To investigate the effect of exogenous acetate on ethanol production using $H_2$ as electron donor, the bottles were sparged with 100% $H_2$ and $H_2/CO_2$ (v/v, 80/20) and 5% inoculum at an initial pressure 1.8 bar. Acetate was added to make the final concentration of 10, 15, 30, and 40 mmol L⁻¹, respectively. To test whether acetic acid and ethanol degradation occurred in the reactor, 15 mmol L⁻¹ acetate + 5 mmol L⁻¹ ethanol, 30 mmol L⁻¹ acetate + 15 mmol L⁻¹ ethanol were added with $H_2/CO_2$ (v/v, 80/20) in the headspace in batch tests using 5% 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 solvents analysis

Volatile fatty acids, ethanol, and butanol concentrations were analyzed 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 H₂SO₄ solution (5 mM) was used as mobile phase at a flow rate of 0.7 ml min⁻¹ 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 S1). 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 taxonomic units were normalized using lineage-specific copy numbers of the relevant marker genes to improve estimates [20].

3 | RESULTS

3.1 | Enrichment of acetogenic sludge and production of acids and ethanol in gas fed reactor

During the reactor operation, after 10 days of adaption, acetic acid started to be produced and reached to 35 mM (Figure 2A, Equation 1). Ethanol was detected at day 11 and increased to 1.35 mmol L⁻¹ at day 12 but it was then degraded (Figure 2A, Equation 2). Instead, butyric acid started to be produced at day 12 when ethanol degradation occurred and increased to 0.5 mmol L⁻¹ at day 26. Propionic acid started to be produced at day 14 and reached to 1.82 mmol L⁻¹ at day 26. The pH was decreased along with the accumulation of acetic acid and kept at 4.5–5.0 after day 21 (Figure 2B). However, ethanol production was not observed when the pH was as low as 4.5 from day 21 to 26 (Figure 2A).

To stimulate ethanol production from acetic acid, H₂/CO₂ was replaced by 100% H₂ at day 27 (stage II, 27–50 d). Indeed, 100% H₂ addition induced ethanol production and it reached to 1.2 mmol L⁻¹ at day 37 (Figure 2A, Equation 4). Thereafter, ethanol production started to decrease to a concentration of 0.5 mmol L⁻¹ (Figure 2A). Meanwhile, butyric acid accumulated and reached 2.4 mmol L⁻¹ (Figure 2A). After feeding 100% H₂, both ethanol and butyric acid production occurred from day 30 to 37, but butyric acid kept increasing along with the consumption of ethanol from day 37 to 50. The concentration of both acetic acid and propionic acid decreased at the end of the stage II.

At stage III, 10 µM tungsten addition induced both acetic acid and butyric acid degradation, accompanied with the production of valeric acid and caproic acid, respectively, 1.3 and 0.4 mmol L⁻¹ at the end of incubation (Figure 2A).

3.2 | Effect of exogenous acetate and tungsten on H₂/CO₂ conversion by enriched sludge

When using H₂/CO₂ 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 (Figure 3A, Table S2). 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 (Figure 3A). H₂ and CO₂ consumption was, respectively, 160 and 40.6 mmol L⁻¹ at the end of the incubation (Figure 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 (Figure 3B). H₂ and CO₂ consumption was, respectively, 262.0 and 73.3 mmol L⁻¹ at the end of the incubation (Figure 3A) and was correspondingly 1.6- and 1.8-fold higher than the control to which no external acetate was provided. The H₂ and CO₂ consumption rate increased to, respectively, 0.85
FIGURE 2  \( \text{H}_2/\text{CO}_2 \) fermentation in a semi-continuous gas fed reactor by anaerobic granular sludge. (A) acids and ethanol production, (B) change of pH and (C) \( \text{H}_2, \text{CO}_2 \) concentration from \( \text{H}_2/\text{CO}_2 \) or \( \text{H}_2 \) by granular sludge. The substrate of stages I, II and III are, respectively, \( \text{H}_2/\text{CO}_2, \text{H}_2 \) and \( \text{H}_2 + 10 \mu\text{M tungsten} \).

and 0.26 mmol L\(^{-1}\) h\(^{-1}\) compared to the control of 0.59 and 0.18 mmol L\(^{-1}\) h\(^{-1}\) (Table S2).

The addition of 10 \( \mu\text{M tungsten} \) enhanced the \( \text{H}_2 \) and \( \text{CO}_2 \) consumption of 285.8 and 71.1 mmol L\(^{-1}\), respectively, at a \( \text{H}_2 \) and \( \text{CO}_2 \) consumption rate of 1.02 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 (Figure 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 (Figure 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 (Figure 3D).

### 3.3 Effect of exogenous acetate on 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 L\(^{-1}\) acetate (Figure 4A). Methane production reached to 14.9, 59.7, 5.2, and 14.0 mmol L\(^{-1}\) along with the increased initial 10, 15, 30, and 45 mmol L\(^{-1}\) acetate concentration. Correspondingly, CO\(_2\) consumption was respectively, 27.7, 73.3, 19.8 and 25.4 mmol L\(^{-1}\), whereas the H\(_2\) consumption amounted to 78.9, 261.9, 45.4, and 73.1 mmol L\(^{-1}\), respectively. Correspondingly, the pH decreased from initial 6 to 5.0–5.2 at both initial 15 and 30 mmol L\(^{-1}\) acetate due to the positive net acetic acid production (Figure 4E). The gas pressure was decreased slowly during the incubation, because part of the gas pressure came from the methane production (Figure 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 L\(^{-1}\) acetate, while the acetic acid concentration slightly decreased at the end (Figure 4B–D). 10 and 50 mmol L\(^{-1}\) acetate had a similar effect on CH\(_4\) production and H\(_2\) and CO\(_2\) consumption, while supplementing 30 mmol L\(^{-1}\) acetate obtained the lowest CH\(_4\) production, CO\(_2\) and H\(_2\) consumption (Figure 4B–D).

Further experiments demonstrated that when using 100% H\(_2\) and in the absence of CO\(_2\), the ethanol produc-
FIGURE 4  Effect of 10, 15, 30 and 45 mM exogenous acetate on production profiles by enriched sludge day 70 bioreactor operation using H₂/CO₂ as the substrate (A) acetate concentration, (B) CH₄, (C) CO₂ and (D) H₂ production, (E) change of pH and (F) gas pressure.
tion 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 H₂ 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 H₂/CO₂ by enriched sludge

Acetate and ethanol were added to simulate the conversion process, that is, the reverse β oxidation pathway, to further assess if longer chain VFAs were produced, as observed in the reactor. With 15 HAc + 5 EtOH and 30 HAc + 15 EtOH, methane production was observed and reached, respectively, 54.1 and 46.3 mmol L⁻¹. Neither ethanol nor longer chain fatty acids were produced during the incubation.

With 15 mmol L⁻¹ acetate and 5 mmol L⁻¹ ethanol addition, CH₄ production and CO₂ and H₂ consumption were all higher compared to the incubations supplied with 30 mmol L⁻¹ acetate and 15 mmol L⁻¹ ethanol addition (Figure 5). Both the acetate and ethanol concentration slightly decreased during the incubation (Figure 5A,B). The pH was slightly increased possibly due to the decreased dissolved CO₂ in the liquid medium induced by the consumption of headspace CO₂ (Figure 5C). The gas pressure decreased slowly and showed a similar trend between the 15 HAc + 5 EtOH and 30 HAc + 15 EtOH groups.

The enriched sludge was further checked for the addition of glucose to possibly enhance the biomass growth and mixotrophy. However, the ethanol production did not significantly enhance compared to the solely glucose fed incubation (Figure S1).

3.5 | Microbial analysis

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

For the *Clostridium* genus, the relative abundance with 0.1% at day 10 increased to, respectively, 0.5% at stage II, 0.7% at stage III and about 3.5% at the end of each stage (Figure 6B). Figure S2 shows the acetic acid and ethanol production in the batch bottles inoculated with enriched sludge from the bioreactor at day 70. The microbial analysis data (I-a, I-b of bottle 1, 2, respectively) showed that the higher relative abundance of the *Clostridium* genus compared to the reactor sludge sampled on day 10. *Clostridium* was enriched with a relative abundance of 3.5% in the bioreactor (day 70) and increased to 18.5 and 22.0 % in the enriched batch bottle 1 and 2 (Figure 6B).

Figure 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) (Figure 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 (Figure 6C). *Oscillibacter* is known to be involved in acidogenesis during dark fermentation [21] 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% H₂ 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 H₂/CO₂ fermentation process and 100% H₂ 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 CO₂ (Equation 5) or used as the electron donor for butyric acid production (Equation 6).

\[
2\text{CH}_3\text{CH}_2\text{OH}(l) + 2\text{CO}_2(g) \rightarrow 3\text{CH}_3\text{COOH}(l) \\
\Delta \text{G}^\circ = -32.2 \text{ kJ mol}^{-1} \\
\text{(5)}
\]

\[
\text{CH}_3\text{CH}_2\text{OH}(l) + \text{CH}_3\text{COOH}(l) \\
\rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH}(l) + \text{H}_2\text{O} \\
\text{(6)}
\]

The first ethanol degradation (day 11) was possibly due to its oxidation to acetic acid in the presence of CO₂ since butyric acid production was insignificantly observed
at that time (Figure 2A). The second ethanol decrease (day 37–50) possibly supplied butyric acid production via the reverse β oxidation pathway [22], during which the butyric acid concentration increased along with the ethanol consumption (Figure 2).

The presence of CO2 on ethanol utilization could have induced formation of longer chain fatty acids. Roghair et al. [23] reported butyric acid and caproic acid production via controlling the ethanol use under different CO2 loading rates (0.5 and 2.5 LCO2 L−1 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 CO2 at initial pH 5.7 and 6.5 by the same anaerobic granular sludge [24]. H2 might have acted as electron donor for the chain elongation process, which has been reported in the literature [25]. Moreover, tungsten enhanced the chain elongation process at pH 4.0–4.5. Caproic acid production occurred at a pH as low as 4, which has been seldom reported since chain elongation processes generally occur at high pH value [7]. On the other hand, acetogens adding a carbon to the carbon chain could be enriched after the second ethanol degradation and further contributed to the chain elongation process. Further research with 13C NMR and labelled substrate (e.g., CO2, ethanol, and acetate) is required 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 H2/CO2, 15 mM acetic acid addition reached the highest methane production, CO2 and H2 consumption compared to the 0, 10, 30, and 45 mmol L−1 acetic acid addition by enriched sludge (day 70) (Figure 3A,B, Figure 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

**Figure 5** H2 and CO2 consumption and CH4, 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 H2/CO2 as the substrate.
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 [11]. 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 [26]. A mixed bacterial culture isolated from slurry incubations even produced methane from H₂/CO₂ at acid pH lower than 4 [27, 28]. 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 CO₂ dissolution in the medium and may stimulate hydrogenotrophic methanogens [23].

4.3 | CO₂ 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% H₂ by the enriched sludge. Even with H₂/CO₂ 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 (Equation 7) and methane (Equation 8) production from exogenous acetate failed using 100% H₂ 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 CO₂ instead of acetate as the substrate.

\[
\text{CH}_3\text{COOH} + 2\text{H}_2 \rightarrow \text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O} \quad (7)
\]

\[
\text{CH}_3\text{COOH} \rightarrow \text{CH}_4 + \text{CO}_2 \quad (8)
\]

5 | CONCLUSION

Autotrophic acetogens were enriched in a H₂/CO₂ gas fed reactor for acetic acid, butyric acid and caproic acid production from heat-treated anaerobic granular sludge treating dairy wastewater. 100% H₂ 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 H₂/CO₂, exogenous acetate and ethanol in batch incubations at pH 6 and 25°C. The enriched sludge failed to convert acetate and 100% H₂ to ethanol at an initial pH of 6.

**AUTHOR CONTRIBUTIONS**

Yaxue He: Conceptualization, methodology, formal analysis, investigation, writing – original draft, and visualization. Chiara Cassarini: Conceptualization, software, data curation, writing – review & editing. Flora Marciano, and investigation. Piet N.L. Lens: Project administration, resources, supervision, funding acquisition, and writing – review & editing.

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**CONFLICT OF INTEREST**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**DATA AVAILABILITY STATEMENT**

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

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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