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Homoacetogenesis and solventogenesis from H_2/CO_2 by granular sludge at 25, 37 and 55 °C

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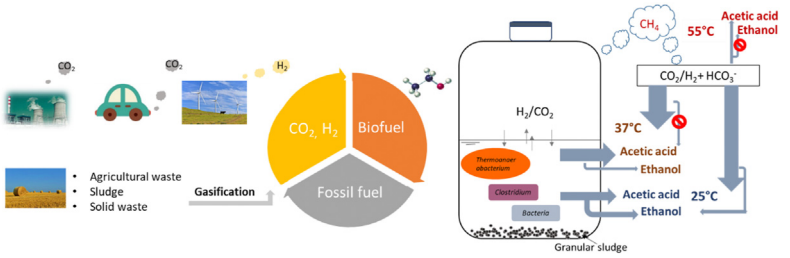
HIGHLIGHTS

- Highest ethanol concentration obtained at 25 °C was 5-fold higher than at 37 °C.
- Ethanol starts to be produced when pH is decreased to 4.7 at both 25 and 37 °C.
- Bicarbonate addition enhanced acetic acid, but inhibited ethanol production.
- *Clostridium* sp. were enriched and contributed to acetic acid and ethanol production from H_2/CO_2 at 25 and 37 °C.

GRAPHICAL ABSTRACT



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ABSTRACT

CO_2 fermentation is a promising process to produce biofuels like ethanol. It can be integrated in third generation biofuel production processes to substitute traditional sugar fermentation when supplied with cheap electron donors, e.g. hydrogen derived from wind energy or as surplus gas in electrolysis. In this study, granular sludge from an industrial wastewater treatment plant was tested as inoculum for ethanol production from H_2/CO_2 via non-phototropic fermentation at submesophilic (25 °C), mesophilic (37 °C) and thermophilic (55 °C) conditions. The highest ethanol concentration (17.11 mM) was obtained at 25 °C and was 5-fold higher than at 37 °C (3.36 mM), which was attributed to the fact that the undissociated acid (non-ionized acetic acid) accumulation rate constant (0.145 h^{-1}) was 1.39 fold higher than at 25 °C (0.104 h^{-1}). Methane was mainly produced at 55 °C, while neither acetic acid nor ethanol were formed. Ethanol production was linked to acetic acid production with the highest ethanol to acetic acid ratio of 0.514 at 25 °C. The carbon recovery was 115.7%, 131.2% and 117.1%, while the electron balance was almost closed (97.1%, 110.1% and 109.1%) at 25 °C, 37 °C and 55 °C, respectively. The addition of bicarbonate inhibited ethanol production both at 25 °C and 37 °C. *Clostridium* sp. were the prevalent species at both 25 and 37 °C at the end of the incubation, which possibly contributed to the ethanol production.

1. Introduction

Carbon dioxide (CO_2) is the first largest contributor to human-induced global warming. Renewable energy sources such as wind and solar power are facing the challenges of balancing power production and demand. One promising approach is to convert the

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excess power to H_2 gas, which is a good alternative for fossil fuels. However, the storage and safety of H_2 are big challenges (Pereira 2013). Using H_2 in CO_2 or syngas (mainly containing CO, H_2 and CO_2) fermentation to generate biocommodities (e.g. acetic acid) or biofuel (e.g. ethanol or butanol) is a future trend with economical and sustainable advantages (Burk et al., 2010), such as CO_2 valorization (Bajón Fernández et al., 2017; Sadhukhan et al., 2016), compared to traditional corn (Mohammadi et al., 2011; Eisentraut 2010) or cellulosic biomass (Naik et al., 2010) fermentation.

Acetic acid and ethanol production from CO_2 occur via the Wood-Ljungdahl pathway (WLP), which is the most effective non-photosynthetic carbon fixation pathway by acetogens (Charubin et al., 2019). The known acetogens comprise more than 100 bacterial species, with *Clostridium* sp. as an omnipresent species (Fast and Papoutsakis 2012). In the WLP, 2 mol of CO_2 are reduced, using H_2 as electron donor, to form 1 mol of acetyl-CoA (Ragsdale 1997). Several species such as *Clostridium autoethanogenum, Clostridium ljungdahlii* and *Clostridium carboxidivorans* convert syngas to acetate and ethanol in pure culture fermentation via, respectively, homoacetogenesis and solventogenesis (Abubackar et al., 2012; Guo et al., 2010). However, mixed cultures have advantages over pure cultures, such as no contamination problems, higher flexibility and resistance to changes in operation conditions. Homoacetogenesis (Eq. 1), solventogenesis (Eq. 2) and methanogenesis (Eq. 3) from H_2/CO_2 are shown in the following reactions:

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O\ \Delta G_r^{\theta} = -75.4\ kJ/mol \quad (1)$$

$$2CO_2 + 6H_2 \rightarrow CH_3CH_2OH + 3H_2O\ \Delta G_r^{\theta} = -96.5\ kJ/mol \quad (2)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O\ \Delta G_r^{\theta} = -130.4\ kJ/mol \quad (3)$$

$$CH_3COOH \rightarrow CH_4 + CO_2\ \Delta G_r^{\theta} = -55\ kJ/mol \quad (4)$$

Anaerobic sludge from wastewater treatment processes has been studied as inoculum to produce valuable chemicals, such as volatile fatty acids (VFAs) (Dogan et al., 2005), H_2 (Liu and Fang 2003) and ethanol (Steinbusch et al., 2008). Pretreatment methods, including heat (Dessì et al., 2017), acid (Agu et al., 1997) and alkali (Zhu et al., 2006) treatment have been used to eliminate the methanogens and select for spore-performing bacteria, e.g. *Clostridium* sp. Heat treatment has been applied to many inocula, such as sewage sludge (Lay et al., 2012), cow dung sludge (Lin and Hung, 2008) and anaerobic sludge.

Temperature is an important factor influencing fermentation, for example, mesophilic conditions (30–37 °C) are the optimum temperature range for homoacidogenic *Clostridium* sp. and has been extensively applied in syngas bioconversion (Stoll et al., 2018; Sun et al., 2019). The CO_2 to H_2 ratio influences the conversion of acetic acid and ethanol (Eq. 1, 2). Bicarbonate addition will thus increase the carbon concentration and act as buffer to avoid sharp pH drops. Furthermore, the effect of bicarbonate on fermentation processes has been reported for hydrogen production, while seldom for acetic acid or ethanol production (Pancha et al., 2015). The biological reduction of gaseous inorganic carbon compounds like CO_2 and CO to alcohols has been described for the synthesis of valuable chemicals, such as ethanol and butanol (Kundiyana et al., 2011; Richter et al., 2016; Gao et al., 2013; Phillips et al., 2015). Limited studies, however, focused on the comparison of homoacetogenesis and solventogenesis at varied temperatures by mixed cultures (Singla et al., 2014; Liu et al., 2018). Considering the important role of temperature on the microbial community composition during fermentation, the present study investigated ethanol production using CO_2 as the sole carbon source and H_2 as the sole electron donor by mixed cultures at submesophilic (25 °C), mesophilic (37 °C) and thermophilic (55 °C) conditions.

2. Materials and methods

2.1. Biomass and medium composition

The anaerobic sludge was obtained from a 200 m^3 upflow anaerobic sludge bed (UASB) reactor producing methane from dairy industry effluent at 20 °C and a hydraulic retention time (HRT) of 9–12 h. The total solid (TS) and volatile solid (VS) content was 42.7 (±1.0) g/L and 24.8 (±0.5) g/L, respectively. The granular sludge was first centrifuged at 5500 rpm for 10 min to remove the supernatant and the solid was heat treated at 90 °C for 15 min as described by Dessì et al. (2017).

1 L medium was prepared according to Stams et al. (1993) and modified as follows: 408 mg/L KH_2PO_4, 534 mg/L $Na_2HPO_4 \cdot 2H_2O$, 300 mg/L NH_4Cl, 300 mg/L NaCl, 100 mg/L $MgCl_2 \cdot 6H_2O$, 110 mg/L $CaCl_2 \cdot 2H_2O$; 1 mL trace metal and 1 mL vitamin stock solution (Stams et al., 1993). 1 L medium (except for $CaCl_2 \cdot 2H_2O$ and vitamins) was prepared and brought to boiling in order to remove O_2, cooled down to room temperature under an oxygen-free N_2 flow, then $CaCl_2 \cdot 2H_2O$ and the vitamins were added as well as Na_2S (0.24 g) was added as reducing agent.

2.2. Batch experimental set-up

Batch tests were conducted in 125 mL serum bottles with 50 mL medium (gas: liquid ratio of 3:2) and granular sludge with an initial VS concentration of 1.0 g/L. The bottles were sealed with rubber inlets and capped with aluminum crimp caps. A H_2/CO_2 (v/v, 80/20) gas mixture was injected by a gas exchanger system to an initial pressure of 1.8 (±0.15) bar ($P_{H2} = 1.44$ bar, $P_{CO2} = 0.36$ bar), in which 124.4 mL of the gas mixture was compressed in the 75 mL headspace. Control bottles were set up with H_2/CO_2 (v/v, 80/20) without the granular sludge and N_2 (100%) with the granular sludge with initial VS concentration of 1.0 g/L.

Hydrogen was in excess for acetic acid production (Eq. 1), for which a H_2/CO_2 ratio of less than 4/1 is required in the substrate gas. In order to enhance the carbon to hydrogen ratio, 2.1 g/L $NaHCO_3$ (1.25 mmol of carbon) was added in 50 mL medium, which altered the H_2/CO_2 ratio to 64/36 (v/v). 1 mL of 1 M HCl was added in order to correct the pH increase upon the addition of $NaHCO_3$. At the start of experiments, the gas pressure was measured every 24 h. The headspace was vacuumed and then H_2/CO_2 was injected again by a gas exchange system after 96 h when the gas pressure decreased below 1 bar. Then, the gas was injected every 48 h till the end of the incubation (408 h). All experiments were performed in triplicates.

1 mL of headspace and 1 mL of liquid sample were withdrawn from each bottle every 24 h to analyze the gas and liquid phases. The liquid sample was then centrifuged at 8000×*g* for 5 min and the supernatant was used to analyze the ethanol and acetic acid concentrations.

2.3. Analytical methods

2.3.1. Gas phase

H_2 and CO_2 concentrations were measured using a HP 6890 gas chromatograph (GC, Agilent Technologies, 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 μm). The oven temperature was kept constant at 60 °C. The temperature of the injection port and the detector were maintained constant at 250 °C. Helium was used as the carrier gas.

2.3.2. VFAs and solvent analysis

Ethanol and butanol concentrations were analyzed for each bottle from the liquid phase (1 mL) using high performance liquid chromatography (Agilent Co., USA) equipped with a refractive index detector (RID) and an Agilent Hi-Plex H column (7.7 × 300 nm, 8 μm). A 5 mM H_2SO_4 solution 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 and refractive index detector (RID) temperatures were, respectively, set at 60 °C and 55 °C. TS and VS were measured according to the EPA 2001 methods (Telliard, 2001).

2.3.3. Microbial analysis

DNA was extracted using a DNeasy® PowerSoil Kit (QIAGEN, Germany) following the manufacturer's protocol. Approximately 0.5 g of the solids was used for DNA extraction at the end of the incubations at 25 °C, 37 °C and 55 °C. The extracted DNA was quantified and its quality was checked by a Nanodrop 2000c Spectrophotometer (Thermo Scientific, USA). A total of 1,540,359 sequences were obtained from all investigated samples (SI Table 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 taxonomic units were normalized using lineage-specific copy numbers of the relevant marker genes to improve estimates (Angly FE, 2014).

2.4. Thermodynamic calculations

The biological reduction of H_2/CO_2 to either acetic acid, ethanol or methane, the production of ethanol from acetic acid and H_2 and the degradation of ethanol release energy at standard conditions (Schink 1997; Thauer et al., 1977). The reaction Gibbs free energy for acetic acid production from H_2/CO_2 and for ethanol production from acetic acid was defined by Eq. 5 and Eq. 6, respectively (For derivation see Supporting Information):

$$\Delta G_r' = \Delta G_r^0 + RTln\frac{[Acid]}{[p_{CO_2}]^2[p_{H_2}]^4} + RTln\frac{K_a[H^+]}{K_a + [H^+]} \quad (5)$$

$$\Delta G_r' = \Delta G_r^0 + RTln\frac{[CH_3CH_2OH]}{[Acid][p_{H_2}]^2} + RTln\frac{K_a + [H^+]}{K_a[H^+]} \quad (6)$$

2.5. Carbon balance and electron balance calculation

The change of the total amount of carbon was defined as the value at time 0 compared to time t. The change of the total amount of carbon of the substrate equals the sum of the total amount of carbon of the products and biomass (Eq. 7), where C_{s_i} is the substrate, C_{p_j} the products and C_b the biomass. Carbon recovery α was calculated by the ratio between the total amount of carbon of the products and the substrates (Eq. 8):

$$\sum_{i=1}^{m} C_{s_i}(0) - \sum_{i=1}^{m} C_{s_i}(t) = \sum_{j=1}^{m} C_{p_j}(t) + C_b(t) \quad (7)$$

$$\alpha = \frac{\sum \Delta C_{p_j}}{\sum \Delta C_{s_i}} \times 100\% \quad (8)$$

The total amount of electron equivalents (e_{total}) is defined as the sum of the electrons given per reactant (e_{r_i}), per product p_j (e_{p_j}) and per biomass (e_b) (Eq. 9) (Steinbusch et al., 2008). The electron equivalents of the reactants and products were 12 mol e^-/mol ethanol, 8 mol e^-/mol acetic acid, 0 mol e^-/mol CO_2, 8 mol e^-/mol CH_4 and 2 mol e^-/mol H_2 (For derivation see Supporting Information). The electron recovery (β) is defined in Eq. 10:

$$e_{total} = \sum_{i=1}^{m} e_{r_i}(t) + \sum_{j=1}^{n} e_{p_j}(t) + e_b(t) \quad (9)$$

$$\beta = \frac{\sum_{j=1}^{n} e_{p_j}(t)}{\sum_{i=1}^{m} e_{r_i}(t)} \times 100\% \quad (10)$$

2.6. Dissociated and undissociated acids calculation

Considering the ionization of acetic acid (Eq. 11), the free acid concentrations were calculated according to the Henderson-Hasselbalch buffer equation (Maddox et al., 2000) (Eq. 12):

$$CH_3COOH \leftrightharpoons CH_3COO^- + H^+ \quad pK_a = 4.757 \text{ at } 25°C \quad (11)$$

$$[HA] = \frac{10^{-pH} \times C_{Total\ acid}}{10^{-pK_a} + 10^{-pH}} \quad (12)$$

[HA]: concentration of undissociated acid.
[A^-]: concentration of dissociated acid.
$C_{total\ acid}$: concentration of total acid
pK_a: negative decadic logarithm of the acid dissociation constant.

Table 1
Ethanol fermentation using H_2/CO_2 or $H_2/CO_2/CO$ as the substrate by mixed and pure cultures in batch experiments.

Inoculum	Substrate (v/v)	Batch bottles V Liquid/Total (mL)	T/ °C	pH	Time (d)	Gas pressure	Ethanol production (mM)	Reference
Manure samples from cattle farm	CO_2/H_2 (80/20)	100/250	37	7	30 d	1 atm daily flushed	5.49	Xu et al. (2015)
Clostridium ljungdahlii	CO_2/H_2 (80/20)	4000	37	5.9	90 h	1 bar 4 bar 7 bar	13 4 <2	Stoll et al. (2018)
Clostridium ljungdahlii	$CO/CO_2/H_2/N_2$ (20/20/5/55)	200/250	37	6.8	24 h	NA	2.5 without and 6.7 with nanoparticles	Kim et al. (2014)
Clostridium ljungdahlii	$CO/CO_2/H_2/N_2$ (20/20/5/55)	200/250	37	6.8	60 h	NA	10.9 with $CoFe_2O_4$	Kim and Lee (2016)
Granular sludge	50 mM acetic $H_2$100%	37.5/120	30		21 d	0.5 bar	3.69	Steinbusch et al. (2008)
Granular sludge	CO_2/H_2 (80/20)	50/120	25		408 h	1.8 bar	17.1	This study

2.7. Statistical analysis

The correlations between the acetic acid and ethanol concentration and between the acetic acid and methane production were analyzed by the Pearson correlation test. The statistical analyses were conducted using the SPSS v24 statistical package for Windows (LEAD Technologies Inc., USA). Significance levels were established for p-values ≤ 0.05.

3. Results and discussion

3.1. Acetic acid and ethanol production at 25, 37 and 55 °C from H_2/CO_2 by granular sludge

At 25 °C and 37 °C, the acetic acid concentration constantly increased during the incubation period, reaching a maximum average concentration of 50.06 and 63.25 mM, respectively (Fig. 1a and b). Ethanol started to be produced after 88 h at 25 °C, while after 64 h at 37 °C. Ethanol reached the highest concentration of 17.1 mM after 240 h of incubation at 25 °C, corresponding to an acetic acid concentration of 33.3 mM (Fig. 1a). After 240 h of incubation, the ethanol concentration did not increase, while the acetic acid concentration kept increasing to 83 mM until the end of the incubation (Fig. 1a). The highest average concentration of ethanol reached a maximum of 3.36 mM ethanol at 37 °C (Fig. 1b). Despite differences in absolute values, the ethanol production and product ratios of each experiment showed that more ethanol is produced at lower temperature by granular sludge from H_2/CO_2. The highest ethanol concentration in these batch tests without pH control is comparable with previous work (Table 1). The highest ethanol concentration of these studies varied from 3.69 to 13 mM, which is lower than in this study. Besides, the duration of the lag phase in our study (17 d) was shorter than in other studies using granular sludge (21 d) or manure (30 d) as inoculum (Table 1).

The highest average ethanol concentration at 25 °C was 5-fold higher than at 37 °C due to the fast acid accumulation at 37 °C (Fig. 2). The high undissociated acid accumulation possibly contributed to the premature termination of ethanol production at 37 °C. The undissociated acid concentrations increased linearly with time at 25 °C and 37 °C (Fig. 2) and the accumulation rate constant was 0.145 h^{-1} at 37 °C, 1.2-fold higher than at 25 °C (0.104 h^{-1}) (Fig. 2). Mohammadi et al. (2014) reported that ethanol production was prevented when the undissociated acid concentration was, respectively, 34.5 and 33.16 mM using 9 and 11 g/L fructose as the substrate by *Clostridium ljungdahlii*. Maddox et al. (2000) investigated acetone-butanol-ethanol (ABE) fermentation from glucose by *Clostridium beijerinckii* NRRL at 34 °C and found that when the undissociated acid concentration did not exceed 50 mM, the ABE production was high. Ramió et al. (2015) performed batch tests using syngas (CO:H_2:CO_2 [32:32:8]) at 25 °C and 37 °C by *Clostridium carboxidivorans* P7 and concluded that ethanol and butanol were produced at 25 °C, but not at 37 °C because of an 'acid crash' at pH values below 4.8, where the microbes lose their ability to convert the acids to solvents.

CH_4 production was still observed at 37 °C at the end of the incubation (6.7 mM) (Fig. 1b), whereas no CH_4 production was observed at 25 °C (Fig. 1a), which was possibly because the heat-pretreatment did not fully eliminate methanogens, which could regrow during the longtime incubation, though the pH of the medium was below 5. Besides, the high H_2 partial pressure in this study (1.44 bar) might be in favor of the hydrogenotrophic mesophilic methanogens. Hydrogenotrophic methanogens in anaerobic sludge have a higher capacity of H_2 consumption than homoacetogens at high H_2 partial pressure, neutral pH and mesophilic temperatures (Liu et al., 2016a; Yasin et al., 2015).

3.2. Threshold pH and acetic acid concentration for ethanol production

The fast acetic acid accumulation rate caused a quick decrease in pH from initially 6 to 4.5 after 144 h of incubation at 25 °C and 37 °C (Fig. 1d), while the pH at 37 °C decreased faster than at 25 °C. After 144 h, the pH at both incubation temperatures (25 and 37 °C) decreased slowly to 4.1 until the end of the incubation. A minimum pH (~4.75) and undissociated acid (with an average concentration of 15 mM) seemed to be required for solventogenesis. Along with the decrease in pH, acetic acid accumulation leveled off, in accordance with the thermodynamics (Eq. 5). When ethanol started to be produced ($\geq$1 mM), the acetic acid and pH in each bottle were 15.2 mM and 4.68, 19.5 mM and 4.53, 12.5 mM and 4.86, respectively (SI Fig. 1). This is a large difference among the respective triplicates, which is illustrated by the large error bars (standard deviation) (Fig. 1). Considering the differences in the triplicates, SI Fig. 1 plotted the acetic acid concentration and pH as a function of the ethanol concentration for each triplicate bottle. The difference in acetic acid and ethanol production among the triplicates at 25 °C might be attributed to inhomogeneities among the inocula used. This might have resulted from the heat treatment, which caused differences in the population size of the acetogens in the inoculum at each bottle (Fig. 5). Indeed, there was a large difference in the relative abundance of *Clostridium* sp. in the three bottles (SI Fig. 2), which was 1.5%, 30.3% and 59.4%, respectively (SI Table 2).

Solventogenesis occurs at a pH ranging from 4.5 to 5.5, varying according to the different strains (Table 2). Acetic acid production is thermodynamically more feasible with an increase in H_2/CO_2 partial pressure and pH (Eq. 5, SI Fig. 3a), whereas ethanol production is thermodynamically more favorable at elevated acetic acid concentrations and high hydrogen partial pressure (higher than 0.1 bar) and low pH at 25 °C (Eq. 5, SI Fig. 3b). Undissociated acetic acid can cross the cytoplasmic membrane by diffusion, reduce the intracellular pH and disrupt the transmembrane proton motive force for ATP formation (Herrero et al., 1985). To avoid inhibition or death of the cells due to the protons released by dissociated acids and prevent a further pH decrease, the cells start to convert acids to neutrally charged alcohols (Liu et al., 2014). Gottwald and Gottschalk (1985) reported that the internal pH needed to stay above 5.5 in cultures of *Clostridium acetobutylicum* for the shift from acid to solvent formation. The *Clostridium* sp. present in the sludge (Fig. 5) were unable to keep a constant pH inside the cells when grown in a phosphate-limited synthetic medium.

3.3. Methane production at 55 °C from H_2/CO_2 by granular sludge

At 55 °C, acetic acid increased slowly to 4.07 mM after 134 h of incubation after which it kept stable until the end of the incubation (Fig. 1c). Ethanol production was not observed at 55 °C. Methane accumulated constantly with a final concentration of 126.05 mM (Fig. 1c). The pH increased to 6.3 and then kept constant at 6.0–6.5 (Fig. 1d). The heat pretreatment of the inoculum at 90 °C for 15 min did not inhibit methanogenesis and thermoanaerobacteria including *Thermoanaerobacteraceae* and *Theranaerobacterales* were enriched at 55 °C (SI Fig. 4). Wang et al. (2017) studied acetate production under thermophilic conditions by acetogens, but required addition of bromoethane sulfonate (BES) to eliminate CH_4 production. Besides, the pH was above 6 (6.0–6.5) during the 55 °C incubation, which is favorable for methane production since usually methane production is inhibited only at pH values below 6 (Chakraborty et al., 2019). The lack of acetic acid and ethanol production was due to the activity of hydrogen utilizing methanogens in the thermophilic incubation. The ratio of the consumed H_2 and CO_2 in the gas phase was kept at 4 after 96 h at 55 °C (SI Fig. 5),

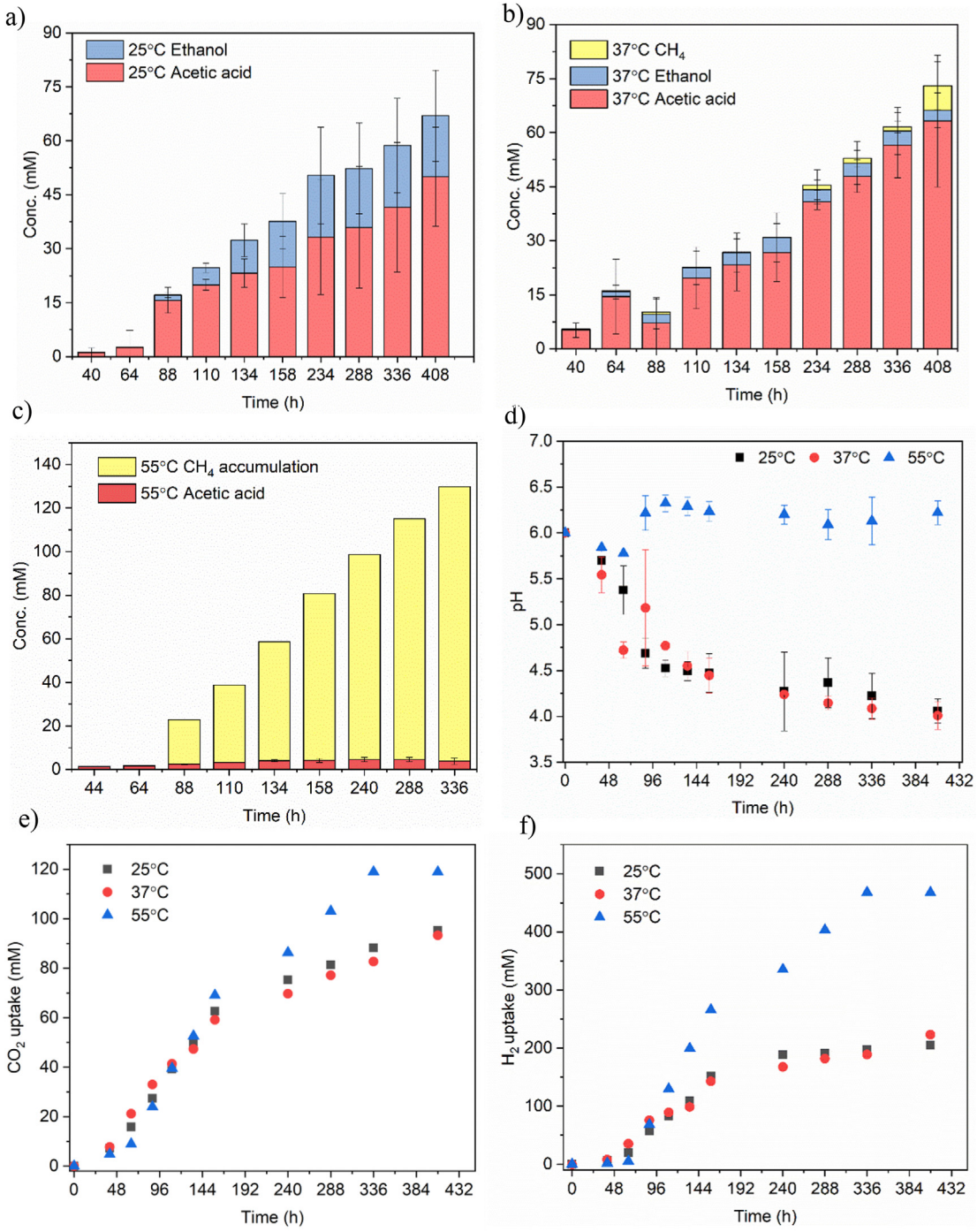

Fig. 1. Acetic acid and ethanol yield a) at 25 °C, b) at 37 °C, c) at 55 °C, d) pH, e) CO_2 uptake and f) H_2 uptake for the incubations with heat-treated granular sludge at 25, 37, 55 °C. Every point shows the average of three independent batch cultures, error bars indicate the standard deviation of the triplicates. H_2/CO_2 was injected at every time point.

which is conform to the theoretical H_2/CO_2 ratio for CH_4 production (Eq. 3). Besides, the low acetic acid concentration during fermentation supports that methane was not produced from acetate via H_2/CO_2 acetogenesis (Eq. 4). Indeed, H_2/CO_2 methanogenesis is about 3 times more exergonic than H_2/CO_2 acetogenesis (Breznak and Kane 1990). Moreover, the H_2 utilization threshold for methanogens is 10–100 times lower than that of acetogens. For example, the acetogen *Acetobacterium woodii*, when in a co-culture with a H_2-utilizing methanogen, using fructose as the carbon source transfers fermentatively the generated H_2 to the methanogens, instead of using it for acetogenesis as it would do in pure culture (Lovley and Klug, 1983). Development of solventogenic communities at thermophilic conditions might thus require the deactivation of the methanogens in the inoculum, as also required for dark (Dessì et al., 2017) and syngas (Wang et al., 2017) fermentation.

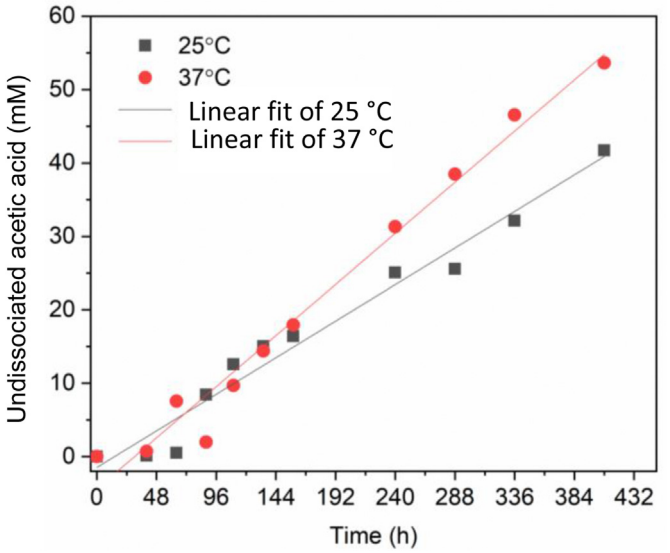

Fig. 2. Undissociated acetic acid concentrations and the linear fit at 25 °C and 37 °C using H_2/CO_2 as substrate by heat-treated granular sludge (0.104 h^{-1} at 25 °C, $R^2 = 0.97$; 0.145 h^{-1} at 37 °C, $R^2 = 0.98$).

3.4. Gas consumption and production rates

The amount of CO_2 and H_2 taken up at 25 °C was first lower than at 37 °C till 110 h and then higher till 336 h (Fig. 1 e, f), while H_2 taken up at 37 °C was higher than 25 °C at the end of incubation, which might be attributed to the offset of the methane production at 37 °C with a higher H_2/CO_2 consuming ratio than acetic acid and ethanol production (Eqs. 1-3). However, the amount of both CO_2 and H_2 taken up is the highest at 55 °C (Fig. 1 e, f). The highest CO_2 consumption rate reached at 55 °C was 0.779 mmol L^{-1} h^{-1}, corresponding to the highest H_2 consumption rate of 3.153 mmol L^{-1} h^{-1} (Table 3). The average CO_2 and H_2 consumption rates in the batches producing methane at 55 °C are also higher than in the batches producing acetic acid and ethanol at 25 °C and 37 °C (Table 3).

Considering both gas consumption (i.e. H_2 and CO_2) and product formation (i.e. acetic acid, ethanol and CH_4), the incubations can be divided in three phases: an adaption (0–88 h), an accumulation (88–240 h) and a stable (240–408 h) phase. The consumption rate of H_2 and CO_2 and the production rate of CH_4, acetic acid and ethanol also demonstrate this (SI Fig. 6). The highest acetic acid and ethanol production rates at 25 °C were 0.545 mmol L^{-1} h^{-1} and 0.201 mmol L^{-1} h^{-1}, respectively (Table 3). It should be noted that at 25 °C the highest ethanol production rate was reached later (at 134 h) than the one of acetic acid (at 64 h) (SI Fig. 6a). At 25 °C, the

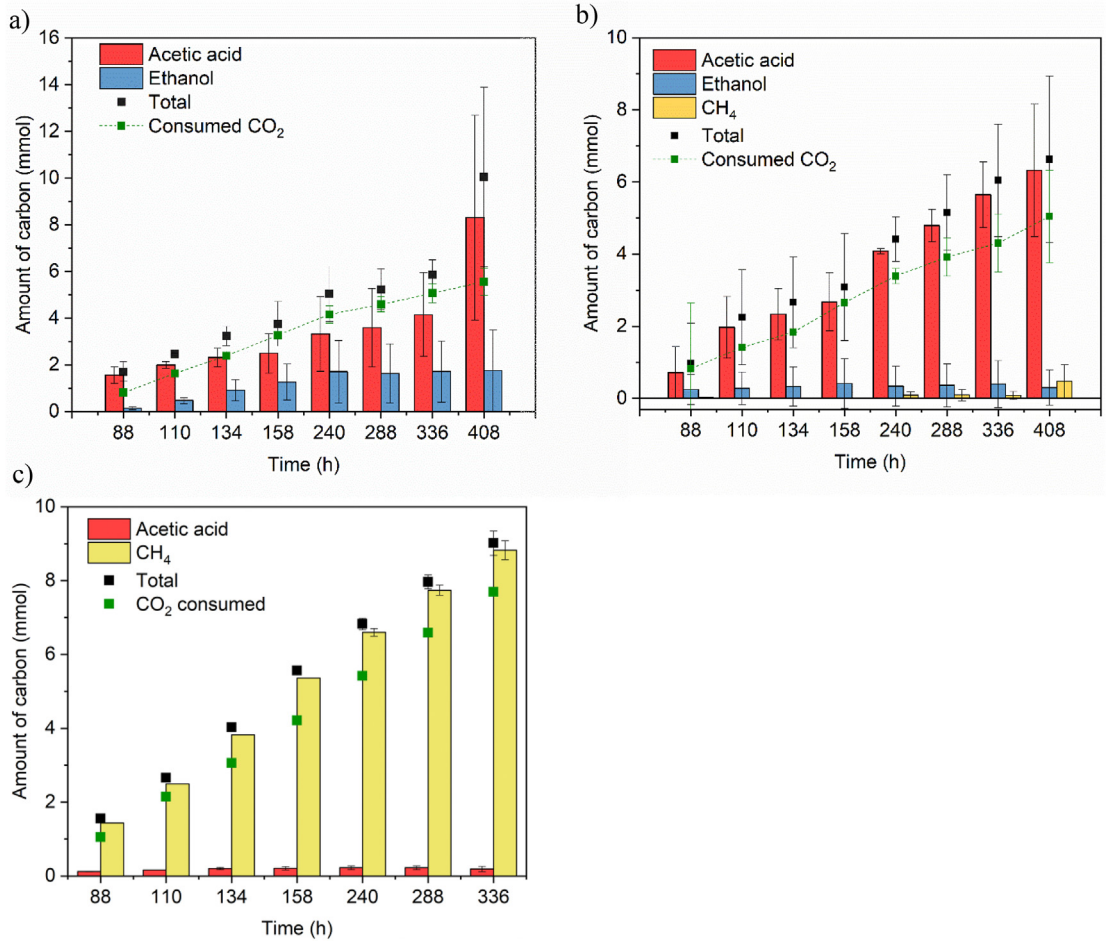

Fig. 3. Carbon distribution at the end of each batch culture (each time injecting gas means a batch culture at a) 25 °C, b) 37 °C and c) 55 °C. The columns refer to the mmol of carbon found in the different metabolites at the end of every batch culture and the black dots represent their sum. The green dots with dash line represent the mmol carbon of consumed CO_2. Every column or point shown in the graphs is calculated as the average of three independent batch cultures, error bars indicate the standard deviation of the triplicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

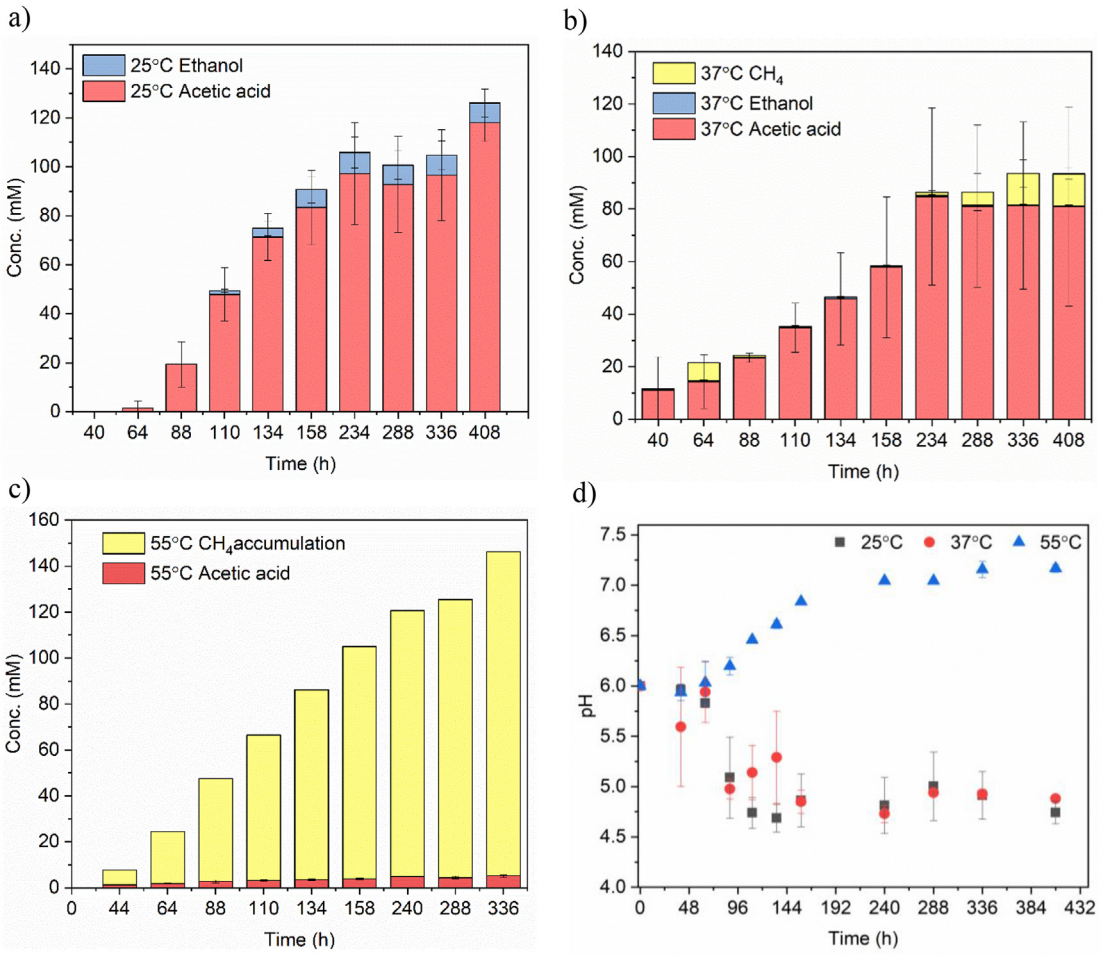

Fig. 4. Acetic acid, ethanol and CH_4 yield at a) 25 °C, b) 37 °C, c) 55 °C and d) pH in incubations with the heat-treated granular sludge at 25, 37, 55 °C in the presence of HCO_3^-. Every point shown in the graphs is calculated as the average of three independent batch cultures, error bars indicate the standard deviation of the triplicates.

acetic acid production rate was lower, while the ethanol production rate was higher than at 37 °C (SI Fig. 6a and b).

3.5. Carbon and electron balance

Bioconversion of H_2/CO_2 by granular sludge at different temperatures resulted in the production of different amounts of acetic acid, ethanol and CH_4 (Fig. 1). Carbon from CO_2 was converted to acetic acid and ethanol at 25 °C and 37 °C, while CH_4 was the main product at 55 °C. The total carbon of acetic acid, ethanol and CH_4 production is higher than the amount of carbon from CO_2 consumption after 88 h till the end of incubation at 25, 37 and 55 °C (Fig. 3). The mass balance based on carbon (mmol) was almost closed, while the carbon recovery was higher: 115.7%, 131.2% and 117.1 at 25 °C, 37 °C and 55 °C, respectively (Table 3). The carbon released from calcium carbonate precipitates, upon a pH decrease, can cause a positive carbon balance (Liu et al., 2016b). In the control experiment with 100% N_2, acetic acid was still detected at the highest concentration of 1.48, 0.39 and 2.9 mM at, respectively, 25 °C, 37 °C and 55 °C. Along with incubation time and temperature increase, the difference between carbon consumption and production increased (Fig. 3). The electron balance was 97.1%, 110.1% and 109.1% at 25 °C, 37 °C and 55 °C, respectively (Table 3), which was almost closed and unaffected by carbonate. Ethanol recovery reached 31.6% at 25 °C, much higher than at 37 °C (5.9%) using H_2/CO_2 as the substrate.

3.6. HCO_3^- enhanced acetic acid production and inhibited ethanol production

At 25 °C and 37 °C, the acetic acid concentration constantly increased during the whole fermentation process, reaching a maximum average concentration of 118.17 and 81.03 mM, respectively (Fig. 4a and b). The highest average ethanol concentration was 7.31 mM at 25 °C and 0.50 mM at 37 °C. The highest acetic acid production rate was 3.62 at 25 °C, compared to 2.51 at 37 °C (Table 3). Ethanol production was not observed at 55 °C. Acetic acid increased slowly to 5 mM at the end of the incubation. The gas phase methane accumulated constantly till a final concentration of 141.10 mM (Fig. 4c). The pH of both incubations at 25 °C and 37 °C decreased to 4.75 after 144 h of incubation and then varied between 4.5 and 5. At 55 °C, the pH increased to 7.0 after 144 h of incubation and subsequently varied between 7.0 and 7.5 until the end of the incubation (Fig. 4b).

The ethanol concentration was lower in the presence of HCO_3^- than without HCO_3^- at both 25 °C and 37 °C (Fig. 4). Bicarbonate is

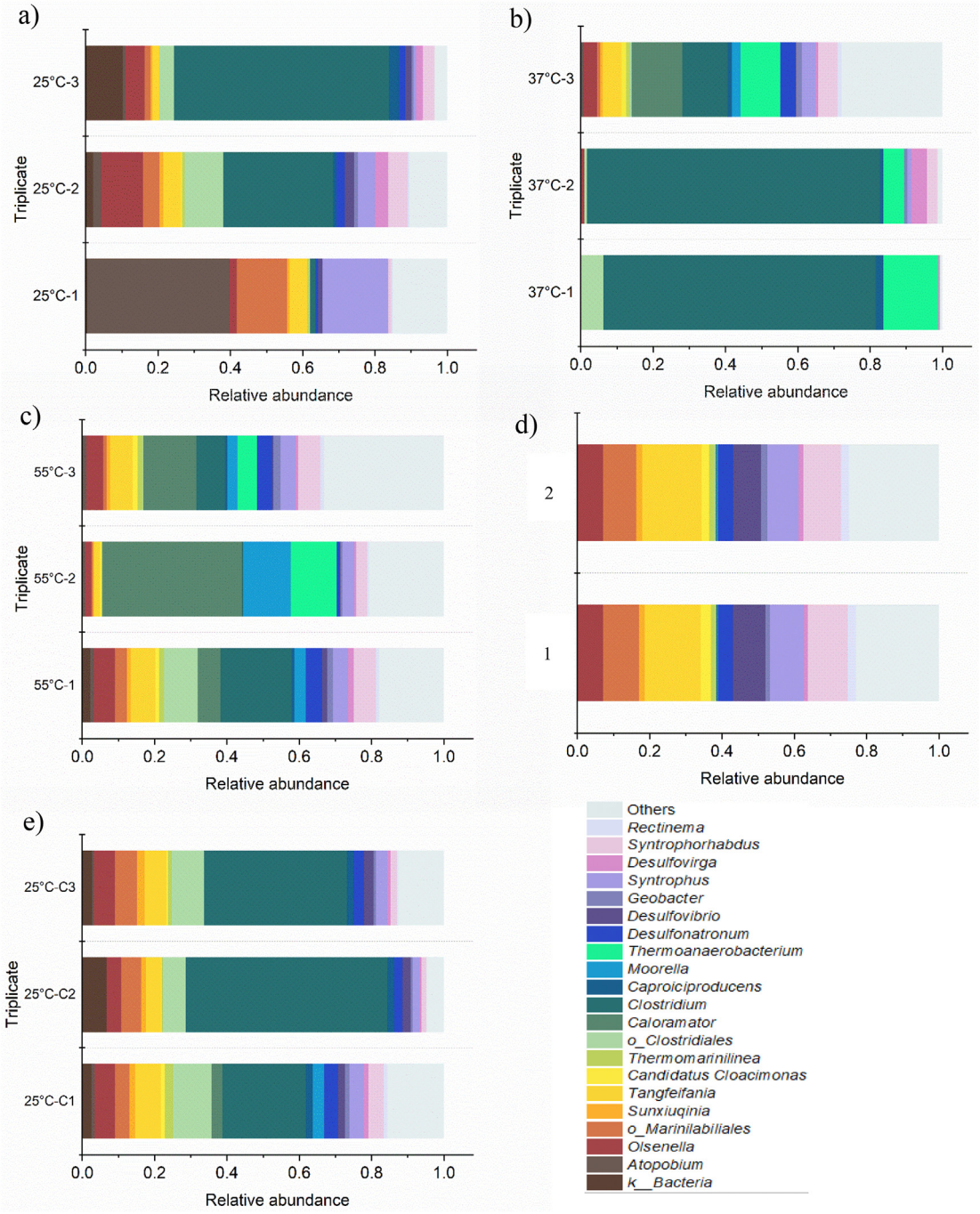

Fig. 5. Relative taxonomic abundance of the batch cultures at genus level with heated treated granular sludge as inoculum at the end of incubation at a) 25, b) 37 and c) 55 °C using H_2/CO_2 as substrate and d) untreated granular sludge; e) 25 °C using H_2/CO_2 + HCO_3^- as substrate.

utilized by bacteria as the carbon source, since CO_2 dissolves from the gas phase to the liquid phase as bicarbonate (Zhu et al., 2017). Similarly, the carbon dioxide addition induced an increased growth of acetogenic bacteria during syngas fermentation (Heiskanen et al., 2007). Additionally, the H_2:CO_2 ratio of the 4:1 of feeding gas is stoichiometrically higher than that (3:1) for acetic acid production (Eq. 1). The relative abundance of *Clostridium* sp. was 39.5%, similar to the value obtained for the batch without HCO_3^- (30.4%) at 25 °C (SI Table 2), which shows that the addition of HCO_3^- did not change the bacterial community composition compared to without HCO_3^- at the genus level. However, in the presence of HCO_3^-, the highest acetic acid concentration at 25 and 37 °C was, respectively, 2.9 and 1.5-fold higher than without the addition of HCO_3^- (Fig. 4). The enhanced acetic acid production upon HCO_3^- addition might thus cause the accumulation of undissociated acid, thus inhibiting ethanol production (Fig. 4a and b). Indeed, the pH of both incubations varied between 4.5 and 5 at both 25 °C and 37 °C after 144 h of incubation (Fig. 4a and b). The strong enhancement of the

Table 2
Shift pH from acetic acid to ethanol of *Clostridium* sp. or anaerobic sludge at various temperatures using H_2 or syngas as the substrate.

Strain	Substrate (v/v)	Shift pH	T (°C)	Reference
Clostridium autoethanogenum	CO/CO_2 (95/5)	4.74	37	Guo et al. (2010)
	CO	4.75	30	Abubackar et al. (2015)
	$H_2/CO_2/N_2$ (65/23/9)	5	37	Mock et al. (2015)
Clostridium carboxidivorans	$CO/H_2/CO_2/N_2$ (20/5/15/60)	4.5–5.5	37	Shen et al. (2017)
	CO	4.75	30	Abubackar et al. (2012)
	CO	4.75	33	Fernández-Naveira et al. (2016)
Clostridium ljungdahlii	$H_2/CO_2/N_2$ (53.3/26.7/20)	NA	37	Stoll et al. (2018)
Anaerobic sludge	CO (100%)	4.9	33	Chakraborty et al. (2019)
	$CO/H_2/CO_2/N_2$ (20/20/15/45)	4.7	37	Singla et al. (2014)
	H_2/CO_2 (80/20)	4.7	25	This study

Table 3
Molar concentration changes of products with H_2/CO_2 as the carbon source at the end of the incubation.

Conditions	Substrate					
	H_2/CO_2			$H_2/CO_2+HCO_3^-$		
	25 °C	37 °C	55 °C	25 °C	37 °C	55 °C
Products (mM)						
Acetic acid	41.5 ± 18.0	56.4 ± 9.1	4.3 ± 1.0	118.2 ± 7.7	84.8 ± 33.7	4.5 ± 1.0
Ethanol	17.1 ± 13.4	4.1 ± 6.8	0	8.6 ± 6.4	0.5 ± 0.3	0
Methane	0.2 ± 0.2	1.3 ± 2.3	126.0	0.3	38.4	141.1
CO_2	−131.1[a]	−134.1[a]	−119.5[a]	−95.9[a]	−93.26[a]	−118.9[a]
H_2	−524.3[a]	−536.4[a]	−478.1[a]	−205.1[a]	−223.08[a]	−467.4[a]
Highest rates (mmol L^{-1} h^{-1})						
H_2 consumption	1.840	1.909	3.153	2.088	1.155	4.047
CO_2 consumption	0.593	0.511	0.779	–[b]	–[b]	–[b]
CH_4	0.005	0.020	0.955	0.002	0.289	0.929
Acetic acid	0.545	1.228	0.185	3.624	2.515	0.167
Ethanol	0.201	0.179	0	0.318	0.023	0
Highest ethanol/acetic ratio	0.514	0.346	0	0.088	0.022	0
H_2 consumption (%)	39.1	41.6	97.8	49.5	52.3	98.6
CO_2 consumption (%)	72.7	69.6	99.5	–[b]	–[b]	–[b]
Carbon recovery (%)	115.7	131.2	117.1	–[b]	–[b]	–[b]
Electron recovery (%)	97.1	110.0	109.1	117	121.3	115.9
Recovery in ethanol (%)	31.6	5.9	0	–[b]	–[b]	–[b]

[a] Negative value indicate an overall consumption of component during the experiment.
[b] Data not known since carbon is excess.

acetic acid concentration by HCO_3^- addition provides a new strategy for enhancing acetic acid production, which can support a high ethanol production yield when using a two stage fermentation process (Richter et al., 2013).

3.7. Microbial community analysis

The analysis of the microbial community composition at the end of the batch incubations at genus level revealed significant differences between the inoculum and the different fermentation conditions. The initial granular sludge was dominated by the genera *Tangfeifania* (15.9%), *Desulfonatronum* (8.4%), and *Syntrophus* (9.1%) and the order *Marinilabilialeswhile* (9.6%), whereas other genera like *Clostridium* amounted to less than 1% of the whole microbial community (Fig. 5). However, at the end of the incubation the microbial composition had shifted to different dominant genera. *Clostridium* sp. and *Olsenella* were the prevalent species at 25 °C at the end of the incubation, with a relative abundance of, respectively, 30.4% and 6.4%. Samples from 37 °C incubations exhibited a lower diversity at genus level and represented a high *Clostridium* abundance with an average of 56.3% sequence reads and *Thermoanaerobacterium* with 10.6%. *Caloramator* sp. (19.8%) and *Thermoanaerobacterium* sp. (6.0%) were the dominant bacteria in the 55 °C incubation (Fig. 5). The average relative abundance of *Thermoanaerobacterium*, including *Thermoanaerobacteraceae* and *Theranaerobacterales*, was 24.5% at 55 °C (SI Fig. 4), which may have contributed to the methane production at 55 °C.

4. Conclusions

A fermentation process that converts CO_2 to ethanol using H_2 as electron donor and anaerobic granular sludge as inoculum was studied at submesophilic (25 °C), mesophilic (37 °C) and thermophilic (55 °C) temperatures. Heat pre-treatment and fermentation at 25 °C efficiently inhibited methanogens and achieved the highest ethanol production (17.1 mM). Ethanol production occurred when both the pH decreased to 4.7 and acetic acid accumulated to 15 mM at 25 °C by granular sludge using H_2/CO_2 as the substrate. The addition of HCO_3^- promoted homoacetogenic acetate production both at 25 °C and 37 °C. Microbial community analysis showed that the addition of H_2/CO_2 and different fermentation temperatures induced changes in the microbial community composition, with *Clostridium* being the functional microorganism at genus level at both 25 and 37 °C. Methane was produced from H_2/CO_2 at 55 °C.

Credit author statement

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Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.128649.

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