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Selective butanol production from carbon monoxide by an enriched anaerobic culture

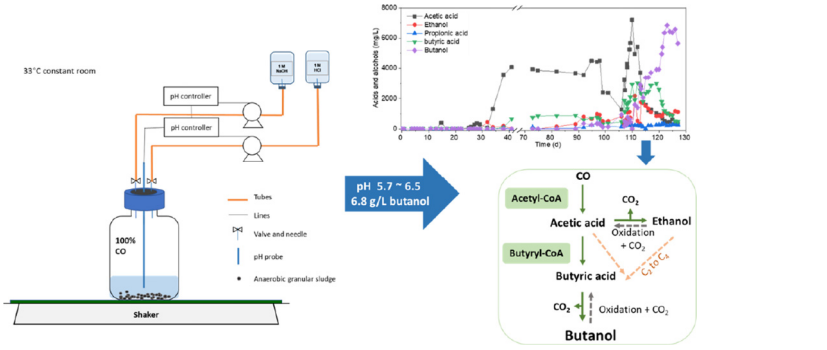
Yaxue He [a,b], Piet N.L. Lens [b], María C. Veiga [a], Christian Kennes [a,*]

[a] Chemical Engineering Laboratory, Faculty of Sciences and Center for Advanced Scientific Research (CICA), BIOENGIN Group, University of La Coruña (UDC), E-15008 La Coruña, Spain
[b] National University of Ireland Galway, H91 TK33 Galway, Ireland

HIGHLIGHTS

- Butanol-Ethanol producing acetogens were enriched in CO fed-batch incubations.
- CO fermentation by the enrichment yielded as high as 6.8 g/L butanol.
- Ethanol and butanol could be oxidized back to acetic acid and butyric acid with CO_2.
- pH 6.2 stimulated acidogenesis and pH 5.7 stimulated ethanol and butanol production.

GRAPHICAL ABSTRACT



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ABSTRACT

An anaerobic mixed culture able to grow on pure carbon monoxide (CO) as well as syngas (CO, CO_2 and H_2), that produced unusual high concentrations of butanol, was enriched in a bioreactor with intermittent CO gas feeding. At pH 6.2, it mainly produced acids, generally acetic and butyric acid. After adaptation, under stress conditions of CO exposure at a partial pressure of 1.8 bar and low pH (e.g., 5.7), the enrichment accumulated ethanol, but also high amounts of butanol, up to 6.8 g/L, never reported before, with a high butanol/butyric acid molar ratio of 12.6, highlighting the high level of acid to alcohol conversion. At the end of the assay, both the acetic acid and ethanol concentrations decreased, with concomitant butyric acid production, suggesting C_2 to C_4 acid bioconversion, though this was not a dominant bioconversion process. The reverse reaction of ethanol oxidation to acetic acid was observed in the presence of CO_2 produced during CO fermentation. Interestingly, butanol oxidation with simultaneous butyric acid production occurred upon production of CO_2 from CO, which has to the best of our knowledge never been reported. Although the sludge inoculum contained a few known solventogenic *Clostridia*, the relative taxonomic abundance of the enriched sludge was diverse in *Clostridia* and *Bacilli* classes, containing known solventogens, e.g., *Clostridium ljungdhalii*, *Clostridium ragsdalei* and *Clostridium coskatii*, confirming their efficient enrichment. The relative abundance of unassigned *Clostridium* species amounted to 27% with presumably novel ethanol/butanol producers.

1. Introduction

Biofuels such as ethanol and butanol are commercially produced from sugars, corn starch and lignocellulosic biomass (Munasinghe and Khanal, 2010). Alternatively, these feedstocks can be gasified to syngas,

* Corresponding author at: Chemical Engineering Laboratory, Faculty of Sciences and Center for Advanced Scientific Research (CICA), BIOENGIN Group, University of La Coruña (UDC), E-15008 La Coruña, Spain.
E-mail address: Kennes@udc.es (C. Kennes).

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a mixture of CO, CO_2 and H_2, which can then also be converted to ethanol, butanol or other valuable chemicals by biocatalysis (Kennes et al., 2016; Wang et al., 2014). Syngas fermentation has raised attention recently, among others for its advantage of using non-food feedstock (Devarapalli and Atiyeh, 2015). In addition, in biomass gasification the whole feedstock (cellulose, hemicellulose and lignin) is converted to syngas, while fermentation processes only use the cellulose and hemicellulose fractions of the lignocellulosic biomass. Also, syngas is an off gas of the steel industry, thus this cheap gas substrate can make syngas-based butanol production more economical (Yu et al., 2015). CO is one of the main components of syngas. Therefore, its biological conversion to biofuels such as ethanol and butanol has become a promising approach (Fernández-Naveira et al., 2017a). Butanol has a similar energy content as gasoline and a higher commercial value than ethanol. It can potentially replace and reduce fossil fuel consumption (Fast et al., 2015).

The low energy density and toxicity of CO limit its use in biological processes, but some acetogens such as *Clostridium carboxidivorans*, *Clostridium autoethanogenum*, *C. ljungdahlii* and *Clostridium aceticum* can convert CO to acids, ethanol (Arslan et al., 2019; Devarapalli et al., 2016; Richter et al., 2016) and, occasionally, butanol (Phillips et al., 2015; Fernández-Naveira et al., 2016a). Acetogens possess the key enzyme carbon monoxide dehydrogenase to convert CO to CO_2, with acetyl-CoA as main intermediate, following the Wood-Ljungdahl metabolic pathway (WLP) (Fernández-Naveira et al., 2017a). The production of alcohols from CO takes place in two stages, i.e. acids (e.g. acetic acid and butyric acid) are produced first (acetogenesis, Eqs. (1), (2)) along with cell growth, followed by the production of alcohols such as ethanol and butanol (solventogenesis, Eqs. (3), (4)):

$$CO + 2H_2O \rightarrow CH_3COOH + CO_2 \Delta G^{\theta} = -154.6\ \text{kJ/mol} \quad (1)$$

$$10CO + 4H_2O \rightarrow CH_3CH_2CH_2COOH + 6CO_2 \Delta G^{\theta} = -420.8\ \text{kJ/mol} \quad (2)$$

$$4CO + 3H_2O \rightarrow CH_3CH_2OH + 2CO_2 \Delta G^{\theta} = -271.4\ \text{kJ/mol} \quad (3)$$

$$12CO + 5H_2O \rightarrow CH_3CH_2CH_2CH_2OH + 8CO_2 \Delta G^{\theta} = -486.4\ \text{kJ/mol} \quad (4)$$

Solventogenesis has been shown to be triggered by stress conditions, such as high CO partial pressure, nutrient limitation or low pH (Benomar et al., 2015; Mohammadi et al., 2012). For example, *C. autoethanogenum* and *C. ljungdahlii* produced ethanol when the pH of the medium was around 4.75 to 5 under mesophilic conditions, while acids were produced at higher pH values (Guo et al., 2010; Fernández-Naveira et al., 2016a; Stoll et al., 2018). Besides *Clostridium* spp., some other bacteria have occasionally been reported to produce alcohols. For example, the alkaliphilic species *Alkalibaculum bacchi* grows at pH 6.5–10.5 and also produces ethanol and acetate from CO/CO_2 (Allen et al., 2010).

Limited studies have reported mixed culture C_1-gas fermentation for the production of alcohols, such as butanol, from 100% CO. Only some *Clostridium* strains, such as pure cultures of *C. carboxidivorans* (Fernández-Naveira et al., 2016a, 2016b, 2017b) and co-cultures of *C. autoethanogenum* and *Clostridium kluyveri* (Diender et al., 2016) produce these alcohols from 100% CO. From a practical point of view, however, mixed culture fermentations are easier to implement at large scale compared to pure cultures, as they do not require sterile bioreactor conditions compared to pure cultures (Charubin and Papoutsakis, 2019). Moreover, they are more resistant to unfavorable environmental conditions, such as low pH, which enables easier implementation at large scale compared to pure cultures (Liu et al., 2014). On the other hand, the production of butanol from CO by mixed cultures has been scarcely studied (Fernández-Naveira et al., 2017a; Humphreys and Minton, 2018).

Anaerobic sludges from wastewater treatment plants are a source of microbial species capable of CO to alcohol conversion (Arantes Ana et al., 2020). CO can be metabolized by a variety of trophic groups present in these anaerobic sludges such as methanogens, hydrogenogens and acetogens (Li et al., 2020). Heat pretreatment is an effective way to inhibit methanogens and select spore-forming acetogens, converting CO into acids and solvents (Cai et al., 2004; Monlau et al., 2013). To date, studies that reached high butanol concentrations from C_1 gases are scarce. Concentrations exceeding 2.7 g/L have never been reported from syngas or pure CO bioconversion (Fernández-Naveira et al., 2016a). Therefore, this study aimed at obtaining higher and selective ethanol and butanol production using CO as the sole carbon source with an anaerobic granular sludge as inoculum in an intermittent gas-fed incubation to enrich for efficient CO converting solventogenic acetogens from anaerobic granular sludge. In addition, a pH shift from 6.2 to 5.7 was applied for inducing solventogenesis from CO. The conversion pathway for selective butanol production by the enriched sludge in CO fed batch reactor was elucidated.

2. Materials and methods

2.1. Biomass

Anaerobic granular sludge was obtained from a 200 m^3 upflow anaerobic sludge bed reactor producing methane from dairy industry

Table 1
The operational conditions and production profile, highest alcohol to acids ratio and highest concentration of acids and alcohols in period I (0–99 d) and II (100–127 d) in FB1 using CO as the sole substrate by the heat treated enriched anaerobic culture.

	Duration	Period I (0–99 d)			Period II (100–127 d)		
		Stage I (15–33 d)	Stage II (34–40 d)	Stage III (41–99 d) Storage at 4 °C (40–70 d)	Stage IV (100–106 d)	Stage V (107–110 d)	Stage VI (111–127 d)
Operational conditions	Initial pH	6.2	5.7	5.7	5.7	6.2	5.7
	Main products	H_2	Acetic acid	Ethanol Butanol	Ethanol Butanol	Acetic acid Butyric acid	Butanol
	Yeast extract		0		150 mL fresh medium replacement and 0.5 g/L YE addition at 100 d		
Highest concentrations (g/L) (Period I or Period II)	Acetic acid		4.5			7.2	
	Ethanol		1.0			2.2	
	Propionic acid		0.4			0.4	
	Butyric acid		0.9			3.0	
	Butanol		0.4			6.8	
	CO_2 accumulation		151.9			261.0	
	pH		3.8–6.2			4.8–6.5	
Highest ratios (Period I or Period II)	Ethanol/acetic acid		5.1			3.6	
	Butanol/butyric acid		0.6			12.6	
CO conversion efficiency to acids and alcohols (%)			30.3			21.4	
Carbon balance (%)			91.0			68.2	

effluent (He et al., 2020) at 20 °C and a hydraulic retention time of 9–12 h (He et al., 2020). The total solids (TS) and volatile solids (VS) content were 42.7 (±1.0) g/L and 24.8 (±0.5) g/L, respectively. The anaerobic sludge was first centrifuged at 5500 rpm for 10 min to remove the supernatant and then heat treated at 90 °C for 15 min as described by Dessì et al. (2017).

2.2. Medium composition

The culture medium was prepared according to Stams et al. (1993) with some modifications as follows (/L): 408 mg KH_2PO_4, 534 mg $Na_2HPO_4 \cdot 2H_2O$, 300 mg NH_4Cl, 300 mg NaCl, 100 mg $MgCl_2 \cdot 6H_2O$, 110 mg $CaCl_2 \cdot 2H_2O$; 1 mL trace metal and 1 mL vitamin stock solution (Stams et al., 1993). Once prepared, medium (except for $CaCl_2 \cdot 2H_2O$ and vitamins) was brought to boiling to remove O_2, then cooled down to room temperature under an oxygen-free N_2 flow. $CaCl_2 \cdot 2H_2O$ and the vitamins were subsequently added, as well as Na_2S (0.24 g) as the reducing agent. To enhance the biomass growth, 0.5 g/L yeast extract was used from day 70 onwards in the fed batch reactor and in all batch experiments.

2.3. Experimental set-up

2.3.1. Gas-fed enrichment

Intermittent gas-fed enrichment experiments were carried out in two 1 L serum bottles (Fisherbrand, FB-800-1100, Waltham, U.S., fed batch reactors) with 300 mL medium and heat-treated anaerobic granular sludge at an initial VS concentration of 1.0 g/L. The first fed batch reactor was set-up with no pH control for 127 days fermentation (FB1). Enriched sludge was obtained after 127 days fermentation in FB1 and used as the inoculum for a second, pH controlled, CO fed batch reactor (FB2, see Section 2.4.1.2) with pH controlled at 6.2 and 5.7 for 35 days fermentation, as well as several batch tests (see Section 2.4.2) to study its metabolic properties.

The fed batch reactors were sealed with a gas tight septum fitted with a pH probe (9,5 × 300 mm, VWR) in the middle. The pH probe was connected to a pH controller (Cole-Parmer 300, Cambridgeshire, UK) and pH was adjusted using either 1 M NaOH or HCl solutions by two pumps (Verdeflex, The Netherland). The fed batch reactors were agitated at 150 rpm by a shaker (Infors AG CH-4103, Bottmingen, Switzerland) at 33 °C in a thermostatic chamber. Considering the positive role of CO partial pressure of 1.7 or 2.5 bar on solventogenesis (Hurst and Lewis, 2010; Lanzillo et al., 2020) and for consistency with the gas pressure of 1.8 bar of our previous study (He et al., 2020), an initial CO pressure of 1.8 bar was used in this study. CO was supplied to the headspace of the reactor as the sole carbon source and electron donor to reach an initial gas pressure of 1.8 bar. When the gas pressure decreased below 1 bar, as a result of bacterial CO gas consumption (corresponding to one CO feeding), the reactor was flushed with fresh pure CO for about 5 min, until reaching again a gas pressure of 1.8 bar.

In FB1, CO was added 19 times in total. An initial pH of 6.2 was applied at stages I and V and an initial pH of 5.7 was applied at stage II, III, IV and VI (Table 1). The pH was adjusted at the beginning of each CO feeding since the pH was not controlled automatically (Table 1).

2.3.2. Batch experiments

Batch experiments of conversion pathway elucidation were conducted in 500 mL serum bottles with 100 mL medium and 10% enriched sludge taken from the first CO fed bioreactor operating for 127 days. Batch experiments of CO and syngas fermentation were conducted in

Table 2
Maximum ethanol and butanol concentrations achieved during syngas and CO fermentation by pure and mixed cultures in batch and continuous bioreactor systems.

Microorganism	Reactor configuration	Gas composition	Working volume (L)	Time/d	Temperature/°C	pH	Maximum alcohols (g/L)		Reference
							Ethanol	Butanol	
Alkalibaculum bacchi CP15	CSTR	$CO/CO_2/H_2/N_2$ (20/15/5/60)	3.3/7	51	37	8.0	6.0	1.1	Liu et al., 2014
C. carboxidivorans P7	Bubble column	$CO/CO_2/N_2$ (25/15/60)	4.5/6.2	10	37	5.3–5.75	1.6	0.6	Rajagopalan et al., 2002
	HFR	$CO/CO_2/H_2/N_2$ (20/15/5/60)	8	15	37	6	24.0	NA	Shen et al., 2014
	CSTR	$CO/CO_2/H_2/N_2$ (20/15/5/60)	3/7.5	11	37	5.7	1.5	0.5	Ukpong et al., 2012
	CSTR	100% CO	1.2/2	21	33	5.75, 4.75	5.55	2.66	Fernández-Naveira et al., 2016a, 2016b
	CSTR	$CO/CO_2/H_2/N_2$ (30/10/20/40)	1.2/2	14	33	6.2, 5.2	5.9	2.1	Fernández-Naveira et al., 2019
	Batch	$CO/CO_2/H_2/Ar$ (56/20/9/15)	0.03/0.125	5	37	NA	3.64	1.35	Shen et al., 2020
	Batch	$CO/CO_2/H_2$ (70/20/10)	0.03/0.282	25	37	No control	3.0	1.0	Phillips et al., 2015
	Batch	$CO/CO_2/H_2$ (40/30/30)	0.045/0.25	15	37	5.0–7.0	3.6	1.0	Sun et al., 2018
C. autoethanogenum	CSTR	100% CO	1.2/2	7	30	6.0, 4.75	0.9	NA	Abubackar et al., 2015
	Batch	100% CO	0.075/0.2	NA	30	5.75, 4.75	0.65	NA	Abubackar et al., 2012
C. ljungdahlii	CSTR Bubble column (BC)	$CO/CO_2/H_2$ (60/5/35)	1/2 (CSTR) 4/6 (BC)	83	35	5.5 (CSTR) 4.3–4.8 (BC)	20.7	NA	Richter et al., 2016
C. aceticum	CSTR	$CO/CO_2/H_2/N_2$ (30/5/15/50)	1.2/2	52	30	6.98	5.6	NA	Arslan et al., 2019
C. ragsdalei	Trickling bed reactor	$CO/CO_2/H_2/N_2$ (38/28.5/28.5/5)	1	70	37	5.8–4.6	5.7	NA	Devarapalli et al., 2016
Clostridium strain P11	CSTR	$CO/CO_2/H_2/N_2$ (20/15/5/60)	3.5/7.5	15	37	6.1	5.0	0.6	Maddipati et al., 2011
Anaerobic sludge (Industrial wastewater)	CSTR	100% CO	1.2/2	42	33	6.2, 4.9	11.1	1.8	Chakraborty et al., 2019
Anaerobic sludge (Dairy wastewater)	Fed batch	100% CO	0.2/1	97	33	4.95–6.45	2.2	6.8	This study

125 mL serum bottles with 30 mL medium and 10% enriched sludge. The bottles were sealed with rubber stoppers and capped with aluminum crimp caps. All bottles were pressurized with CO or syngas at an initial pressure of 1.8 bar and were incubated at 150 rpm and at 33 °C. Enriched sludge in this study refers to the sludge taken from the CO fed FB1 after 127 days of operation.

2.4. Experimental design

2.4.1. Fed-batch reactor operation

2.4.1.1. Biomass enrichment and selective butanol production in FB1. The operation for the enrichment in FB1 had two periods. Period I lasted for 99 days and was comprised of several stages, i.e. H_2 production (stage I, 15–33 d), acetic acid accumulation (stage II, 34–40 d) and ethanol and butanol production (stage III, 41–100 d) stage. Along with acetic acid accumulation, the FB1 reactor pH was adjusted back to pH 4.8–5.2 at day 36–40 to avoid the pH decreasing further and stimulate ethanol production (Ganigué et al., 2016) and the pH was sustained at 4.8–5.2 till 69 d. At day 40 (the beginning of stage III), the reactor was maintained at 4 °C in the fridge for 30 days and later put back at 33 °C. Therefore, a 10 days adaptation period was required from day 70 to 80 (Fig. 2).

After 99 days CO feeding (the start of Period II), considering only limited activity of acid and alcohol production was detected and exhaustion of some nutrients was expected, 150 mL medium was removed and replaced by 150 mL fresh medium with initial pH of 6.2. 0.5 g/L yeast extract (YE) was added to the medium in order to obtain a higher biomass concentration and reduce the adaptation period, as YE is well known to stimulate CO converting bacterial growth (Diender et al., 2016). Period II comprised of an acetic acid accumulation (stage IV, 100–106 days), butyric acid production (stage V, 107–110 d) and butanol accumulation (stage VI, 111–127 d) stage.

2.4.1.2. Controlled pH at 6.2 and 5.7 in FB2. To better understand the effect of pH on ethanol and butanol production, FB2 was operated with pH control at high pH 6.2 to obtain acetic acid and butyric acid production and low pH 5.7 to stimulated solventogenesis. FB2 was inoculated with 10% enriched sludge from FB1. In total, 12 times CO was added to this bioreactor, which operated for 35 days.

2.4.2. Batch tests

2.4.2.1. Metabolic pathway elucidation in enrichment. Experiments were performed to test if some of the observed bioconversions with the enriched sludge (sampled day 127 d in FB1) follow the reverse β-oxidation pathway and to try to elucidate why ethanol and butanol concentrations occasionally decreased during the fermentation process in case of CO_2 accumulation. Four experimental assays were conducted with 2.2 g/L acetic acid and 6 g/L ethanol and with either 100% N_2 or 100% CO_2 at initial pH 5.7 and 6.2, using 10% enriched sludge as inoculum.

To investigate the effect of accumulated CO_2 on butanol oxidation during the fermentation process, a batch experiment was set up with 3.8 g/L butanol and with CO_2 in the gas phase, at either initial pH 6.5 or 5.7 with 10% enriched sludge (sampled on day 127) as inoculum to investigate possible butanol oxidation to butyric acid by CO_2.

2.4.2.2. Ethanol and butanol production from CO/syngas by enriched sludge in batch tests. The enriched sludge taken from FB1 (127 d) was tested for syngas bioconversion in 125 mL serum bottles with 10% inoculum and 30 mL culture medium with 0.5 g/L yeast extract addition. The headspace was flushed with syngas, i.e. $CO/CO_2/H_2/N_2$ (*v*/v, 20/20/10/50) or 100% CO to an initial pressure of 1.8 bar. Control bottles were set up with 100% N_2 at the initial pressure of 1.8 bar and 10% enriched sludge as inoculum. All experiments were performed in duplicate.

2.4.3. Sampling

In FB1 and FB2, the gas pressure was measured daily. 1 mL of liquid sample was withdrawn daily for measuring the cell concentration (OD_{600}) and pH. It was then centrifuged at 8000 ×*g* for 5 min and the supernatant was used to analyze the short chain volatile fatty acids,

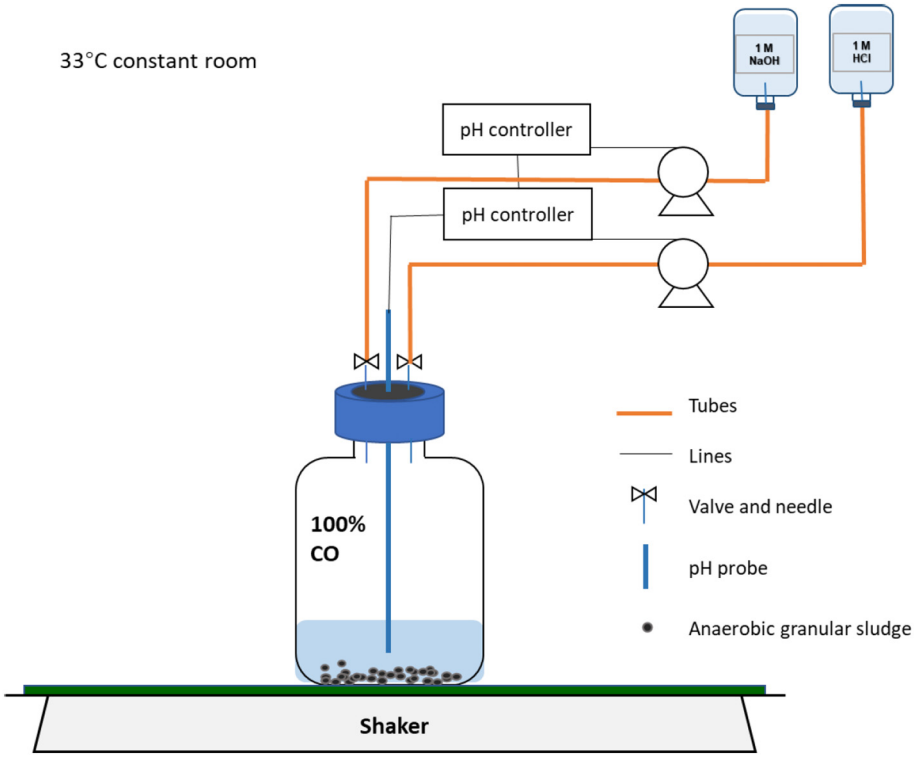

Fig. 1. Schematic diagram of the CO fed batch reactor set-up.

ethanol and butanol concentrations. 1 mL gas sample was taken at the end of each CO feeding to determine the H_2, CO, CO_2 and CH_4 concentrations in FB1. In the batch tests, the cell concentration, pH, short chain volatile fatty acids, ethanol and butanol concentrations were analyzed every two days after cell growth was observed.

2.5. Carbon balance calculation

The change of the total amount of carbon was defined as the carbon concentration 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. (5)). The carbon recovery α was calculated by the ratio between the total amount of carbon of the products and the substrates (Eq. (6)):

$$\sum_{i=1}^{m} C_{s_i}(0) - \sum_{i=1}^{m} C_{s_i}(t) = \sum_{j=1}^{m} C_{p_j}(t) + C_b(t) \quad (5)$$

$$\alpha = \frac{\sum \Delta C_{p_j}}{\sum \Delta C_{s_i}} \times 100\% \quad (6)$$

where

C_{s_i} is the substrate carbon, C_{p_j} the product carbon and C_b the carbon concentration of the biomass.

2.6. Microbial analysis

DNA was extracted using a DNeasy® PowerSoil Kit (QIAGEN, Germany) following the manufacturer's protocol. 10 mL enriched sludge taken from the CO fed FB1 after 127 days of operation was used for DNA extraction. The extracted DNA was quantified and its quality was checked by a Nanodrop 2000c Spectrophotometer (Thermo Scientific, Waltham, USA). The extracted DNA was analyzed by Metagenomics-Seq (Illumina PE150, Q30 ≥ 80%) (Novogene, UK). Taxonomic annotation analysis involved comparing metagenomic reads to the database of taxonomically informative gene families (NR database)

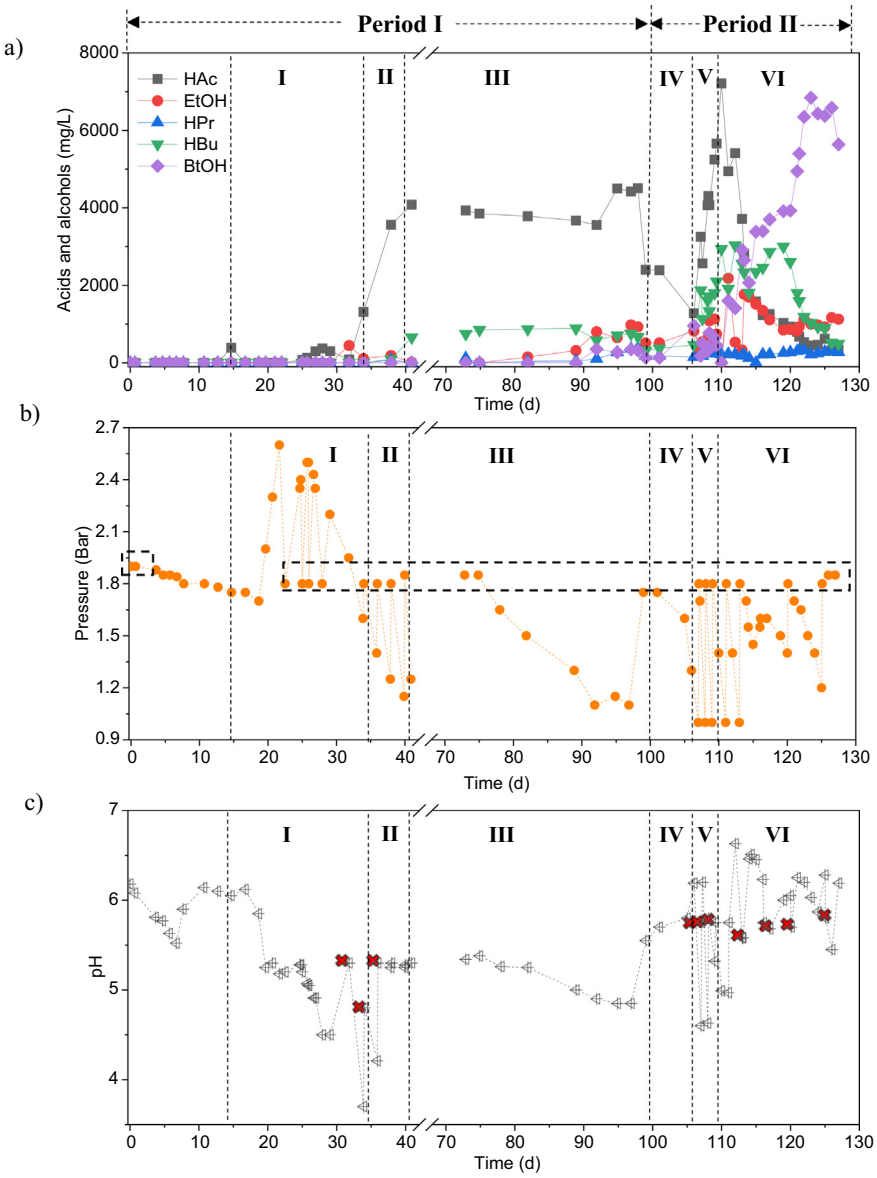

Fig. 2. CO bioconversion for ethanol and butanol production by heat-treated granular sludge in an intermittent gas-fed bioreactor (FB1) with initial CO gas pressure of 1.8 bar. a) Production of acetic acid (HAc), propionic acid (HPr), butyric acid (HBu), ethanol (EtOH) and butanol (BtOH), b) gas pressure, c) pH, d) CO_2 and H_2 production and CO consumption and e) CO_2 and H_2 production and CO consumption (mmol L^{-1} d^{-1}) at each CO feeding and consumptiom feeding using CO as sole carbon source. At 40 d (the beginning of stage III), the reactor was maintained at 4 °C in the fridge for 30 days and later put back again at 33 °C. The orange dots inside the dash box in panel represent CO feeding. The red cross mark in panel c) represents pH adjustment to 5.7 each time CO was added. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

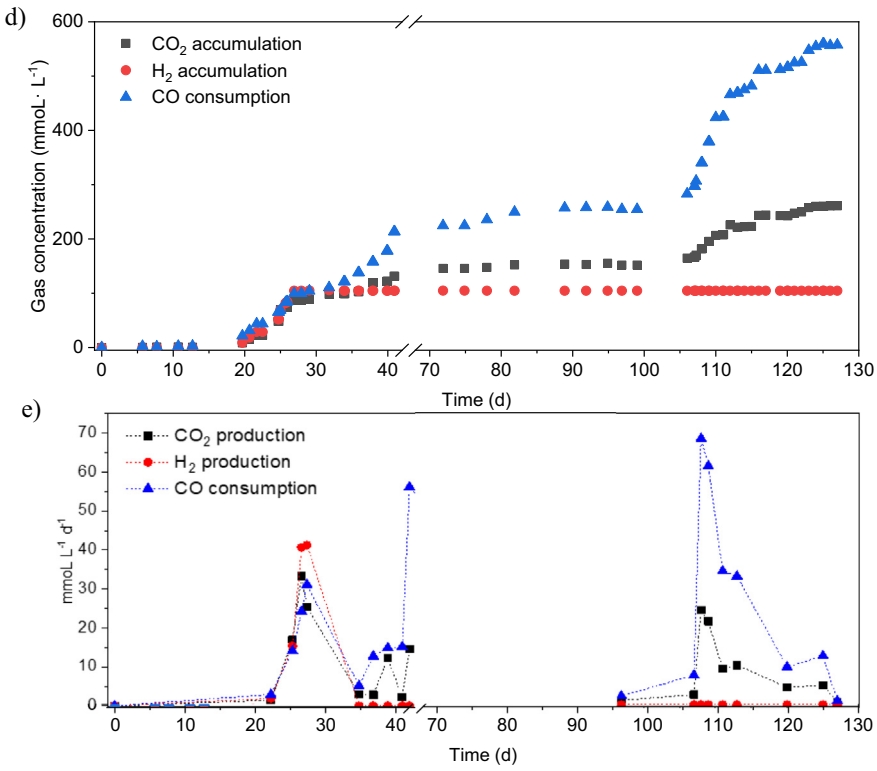

Fig. 2 (*continued*).

to annotate each metagenomic homolog (Mende et al., 2012). Taxonomic diversity involves identifying those reads that are marker gene homologs to a database of taxonomically informative gene families, using sequence or phylogenetic similarity to the database sequences (NR database; Buchfink et al., 2015) to taxonomically annotate each metagenomic homolog (MEGAN; Huson et al., 2011). According to the abundance table of each taxonomic level, various analyses were performed including Krona analysis, bar plot for abundant species and heatmap of abundance (Ondov et al., 2011).

2.7. Analytical methods

Gas pressure was measured by a pressure gauge (LEO1, Keller, Winterthur, Switzerland). Acetic, propionic and butyric acid as well as ethanol and butanol were determined by high performance liquid chromatography (HPLC, HP1100, Agilent Co., Palo Alto, USA) equipped with a refractive index detector and Agilent Hi-Plex H Column (300 × 7.7 mm) as described by Arslan et al. (2019). A 5 mmoL·L^{-1} H_2SO_4 solution was used as the mobile phase at a flow rate of 0.80 mL/min. The sample injection volume was 20 μL and the column temperature 45 °C. The cell concentration was determined with a spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Spain) at a wavelength of 600 nm (Arslan et al., 2019).

H_2 and CO were determined on a HP 6890 gas chromatograph (GC, Agilent Technologies, Madrid, Spain) equipped with a thermal conductivity detector (TCD) and a 15-m HP-PLOT Molecular Sieve 5A column (ID 0.53 mm; film thickness 50 μm) as described by Arslan et al. (2019). The initial oven temperature was kept constant at 50 °C for 5 min and then raised by 20 °C/min for 2 min, to reach a final temperature of 90 °C. The temperature of the injection port and the detector were maintained constant at 150 °C. Helium was used as the carrier gas at a flow rate of 2 mL/min. CO_2 and CH_4 were measured on an HP 5890 gas chromatograph (GC, Agilent Technologies, Spain) equipped with a TCD (Arslan et al., 2019). The injection, oven, and detection temperatures were maintained at 90, 25, and 100 °C, respectively. The area obtained from the GC was correlated with the concentration of the gases as described by Chakraborty et al. (2019).

3. Results

3.1. Enrichment and selective butanol production by anaerobic granular sludge in FB1

3.1.1. Acetic acid and butyric acid production from CO at initial pH 6.2 (Period I)

During Stage I (15–33 d) of Period I, H_2 production was initially observed, with a transient accumulation of 0.39 g/L acetic acid after two weeks (Fig. 2a). H_2 started being produced concomitantly with CO consumption and this lasted till 33 d (Fig. 2d), suggesting an initial enrichment of H_2 producing bacteria. After that, hydrogen accumulation leveled off with a simultaneous boost in acetic acid production (Fig. 2a). The gas pressure increased from 1.8 bar initially to a maximum of 2.6 bar due to H_2 and CO_2 production (Fig. 2b). The pH of the enrichment medium decreased from an initial pH 6 to pH 5.3 when H_2 started being produced (Fig. 2c). The CO consumption rate increased from 2.89 to 31.12 mmoL·L^{-1}·d^{-1} and both the H_2 and CO_2 production rate increased, respectively, from 1.89 to 41.27 mmoL·L^{-1}·d^{-1} and 1.48 and 25.37 mmoL·L^{-1}·d^{-1} from 21 to 27 d (Fig. 2e). When H_2 production leveled off, the accumulation of H_2 and CO_2 reached, respectively, 66.7 and 66.6 mmoL·L^{-1} and the amount of CO consumed reached 88.8 mmoL·L^{-1} (Fig. 2d).

Stage II (34–40 d) was dominated by acetic acid accumulation. The highest concentration of acetic acid reached 4.2 g/L at 40 d, together with 0.66 g/L butyric acid. A small peak of 0.45 g/L ethanol was found at day 32, but then gradually decreased to reach 0.01 g/L on day 40. The CO consumption rate decreased at 27–33 d, but then increased from 5.14 to 15.15 mmoL·L^{-1}·d^{-1} at 33–40 d (Fig. 2e).

Stage III (41–99 d) was characterized by ethanol and butanol production after a 10 days adaptation period from day 70 to 80, subsequent to the 30 day storage of the sludge at 4 °C (Fig. 2a). The highest ethanol,

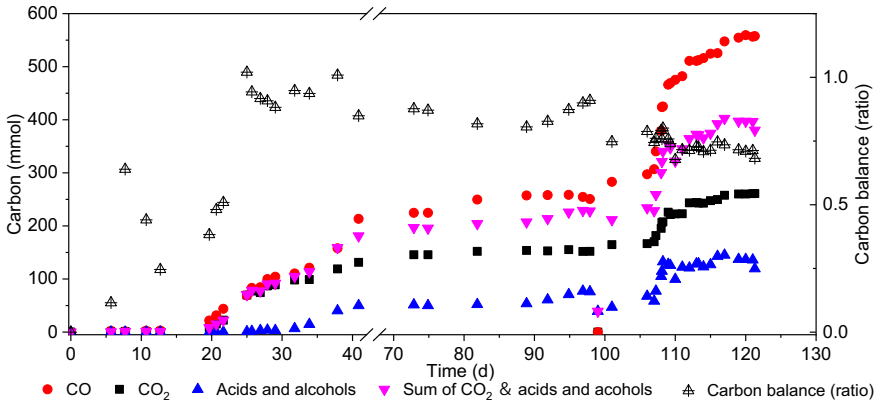

Fig. 3. Carbon balance for ethanol and butanol production using CO as the sole carbon source by heat-treated granular sludge in an intermittent gas-fed bioreactor (FB1). The input mmol carbon of CO consumption and distributed as CO_2 and acids and alcohols production, produced carbon as sum of CO_2, acids and alcohols.

butanol and butyric acid concentrations were, respectively, 0.98, 0.35 and 0.89 g/L (Fig. 2a). Acetic acid showed a slight decrease (3.79 to 3.56 g/L), while ethanol increased from 0.15 g/L to 0.80 g/L from 81 to 91 d (Fig. 2a). Thereafter, the acetic acid concentration increased from 3.56 g/L at 61 d to 4.50 g/L at 94 d during which the CO pressure slowly decreased (Fig. 2d). The ethanol concentration increased to 0.98 g/L at 96 d (Fig. 2a, c). After day 96, another stable phase of acetic acid and ethanol production established till the end of Period I (day 99).

3.1.2. Selective butanol production from CO at pH 5.7–6.5 (Period II)

After one week incubation in a culture medium with initial pH of 5.7 (stage IV, 100–106 days), the acetic acid concentration decreased from

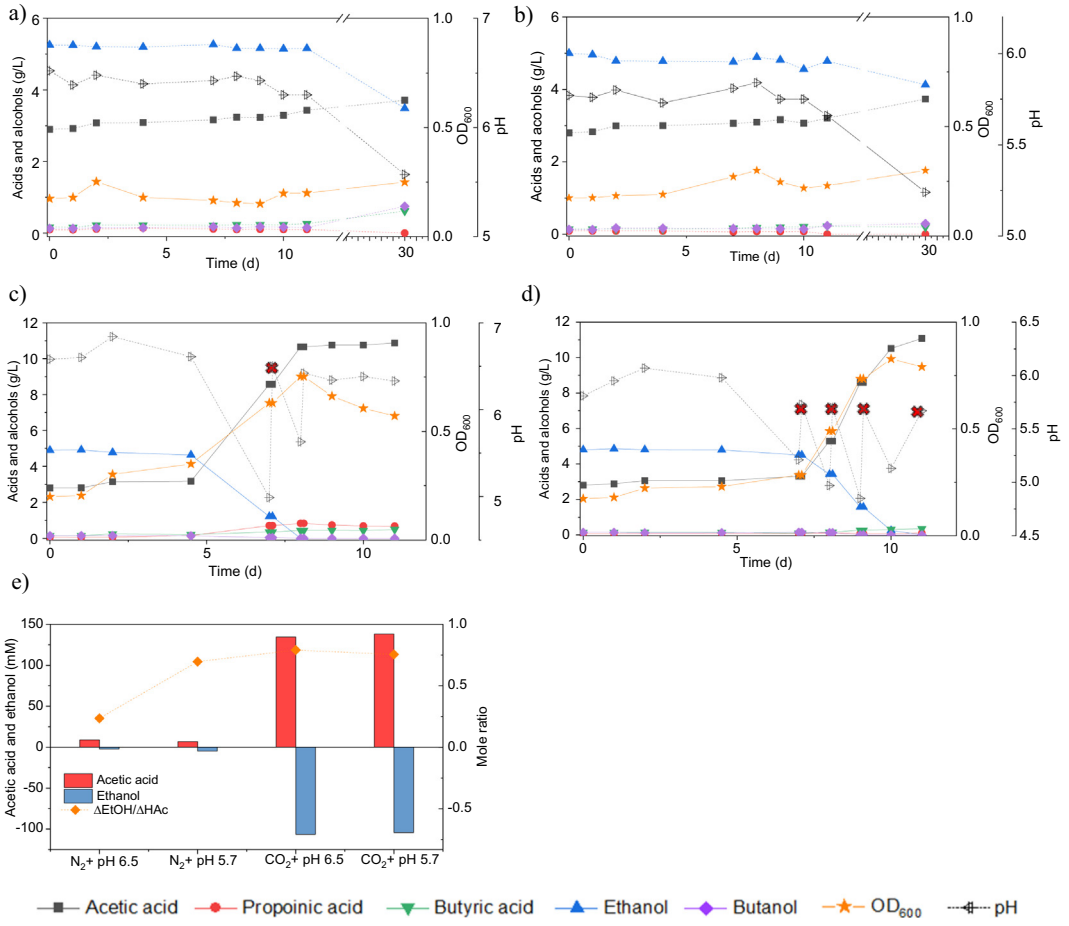

Fig. 4. Effect of exogenous acetic acid and ethanol addition on the production of acetic acid, propionic acid, butyric acid, ethanol and butanol, change of pH and OD_{600} by enriched sludge (sampled day 127 from FB1) in batch tests. a) N_2 + initial pH 6.5, b) N_2 + initial pH 5.7, c) CO_2 + initial pH 6.5 and d) CO_2 + initial pH 5.7 as well as e) Net production of acetic acid and ethanol and molar ratio of consumed ethanol and produced acetic acid. Red cross marks in panel c) and d) represent manual pH adjustment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4 to 1.3 g/L, while 0.82 and 0.95 g/L of, respectively, ethanol and butanol were produced along with a pH increase from 5.7 to 6.0 (Fig. 2a). The pH increase was due to the consumption of acetic acid with concomitant production of neutrally charged ethanol and butanol. The CO consumption rate and CO_2 production rate reached their highest values of, respectively, 68.60 and 24.42 mmol·L^{-1}·d^{-1} at 109 d (Fig. 2e).

During stage V (107–110 d), acetic acid and butyric acid concentrations increased again, up to 7.21 g/L acetic acid and 2.94 g/L butyric acid till 110 d, which was attributed to the transient pH increase, known to stimulate acetogenesis. During this stage, the accumulation of these acids induced a pH decrease to 4.5–5.

Stage VI (111–127 d), at pH often below 5, was a high butanol production stage. On 111–119 d, acetic acid was rapidly consumed, dropping from 7.21 to 1.0 g/L. Interestingly, the ethanol concentration also decreased from 2.2 to 0.8 g/L (discussed below, see Sections 3.2.2 and 4.2), while the butyric acid concentration increased slowly up to its highest concentration of 3.0 g/L. Simultaneously, the butanol concentration increased rapidly up to 4.0 g/L at 119 d, while the butyric acid concentration remained stable. After day 119, both acetic acid and ethanol concentrations decreased to below 1.0 g/L, and also butyric acid was quickly consumed. This was accompanied by a second fast increase of the butanol concentration, which reached 6.8 g/L at 127 d. It was observed that butanol production occurred when the pH of the medium was 5.7, though it raised to 6.4 due to the consumption of acetic acid and butyric acid (Fig. 2c). To sustain continuous butanol production, the pH was regularly, manually, adjusted to 5.7, each time CO was added (Fig. 2c).

3.1.3. Carbon balance of FB1

In Period I (0–99 d), the carbon balance was almost closed and reached 91.0%, to which the unaccounted carbon used for cell growth should be added (Table 1). The level of CO bioconversion to organic compounds (acids and alcohols) and CO_2 (mmol carbon) in Period II (100–127 d) was 21.4% and 46.8%, respectively, reaching 68.2% in total at the end of the incubation period (Fig. 3).

3.2. Conversion pathways for selective butanol production by enriched sludge

3.2.1. CO and syngas conversion

The enriched sludge from FB1 at 127 d was tested in batch assays using pure CO and syngas ($CO/CO_2/H_2/N_2$, 20/20/10/50, *v*/v) as the substrates. After two weeks incubation with CO as the substrate, 1.30 g/L ethanol and 0.30 g/L butanol were produced, while 2.40 g/L ethanol and 0.33 g/L butanol were obtained from syngas (SI Fig. 3a, b). The partial pressure of CO in the syngas in this study was 0.36 bar, which is much lower than in 100% CO at 1.8 bar. The presence of 1.8 bar CO extended the lag phase of bacterial growth (Fig. SI 1), but the cell concentration reached an OD_{600} of 1.72 after 14 days incubation, which was higher than the OD (1.56) obtained using syngas, at the end of the incubation (SI Fig. 3c, d). These batch tests confirmed that the enriched sludge from CO fed FB1 enabled ethanol and butanol production from both CO and syngas.

3.2.2. Ethanol oxidation in the presence of CO_2 by enriched sludge

At high or low initial pH (6.5 or 5.7) with N_2 in the headspace, neither exogenous acetic acid nor ethanol were significantly consumed after 11 days of incubation (Fig. 4a, b). However, after 30 days incubation, 1.5 g/L ethanol was consumed with the concomitant production of 0.4 g/L acetic acid, 0.6 g/L butyric acid and 0.8 g/L butanol along with the pH dropping to 5.67 from the initial pH 6.5 (Fig. 4a). The production of acetic acid could be from ethanol oxidation, and the production of butyric acid suggested that a C_2 to C_4 acid conversion process occurred during the long time (30 d) incubation. At initial pH 5.7, 0.9 g/L ethanol was consumed, while 0.5 g/L acetic acid was produced, and both the butyric acid and butanol concentrations showed a slight increase (<0.1 g/L), while the pH decreased to 5.24 at day 30 (Fig. 4b). The highest cell concentration at initial pH 6.5 and 5.7 reached, respectively, an OD of 0.25 and 0.30 (Fig. 4a, b).

With N_2 in the headspace, at 30 d, the produced carbon was in total 83.8 mmol·L^{-1} C distributed over 13.3 mmol·L^{-1} C acetic acid, 27.2

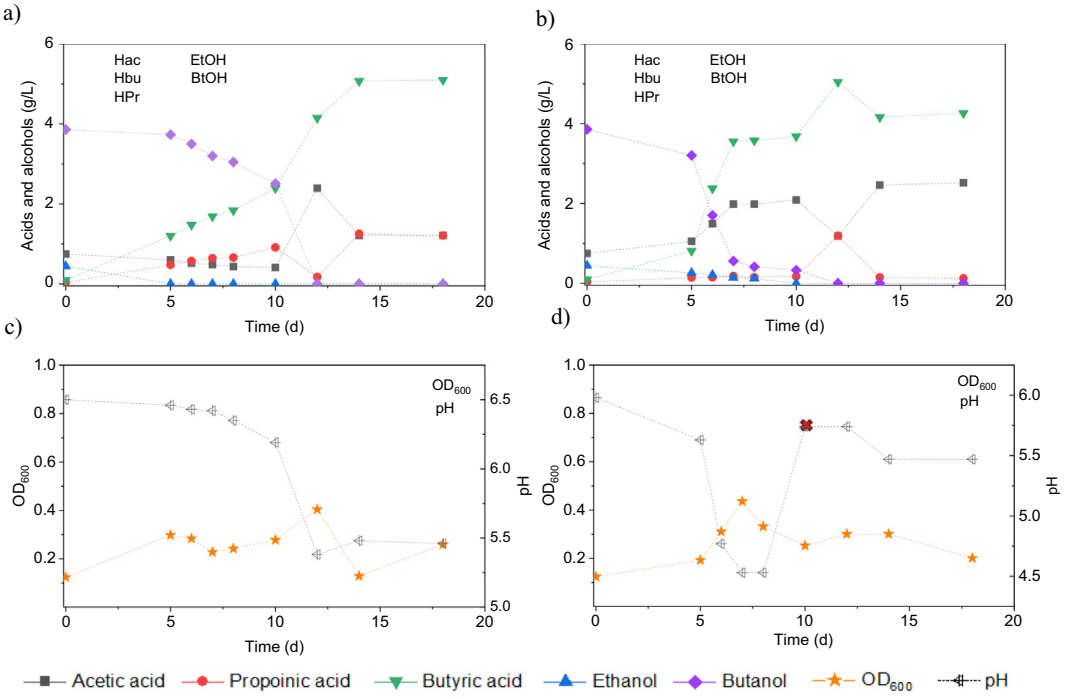

Fig. 5. Production of acetic acid, propionic acid, butyric acid, ethanol, and butanol from exogenous butanol + CO_2 by the enriched sludge sampled at day 127 from FB1. Initial pH of a) 6.5 and b) 5.7 and corresponding changes in pH and OD_{600} of c) and d). Red cross mark represents manual pH adjustment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

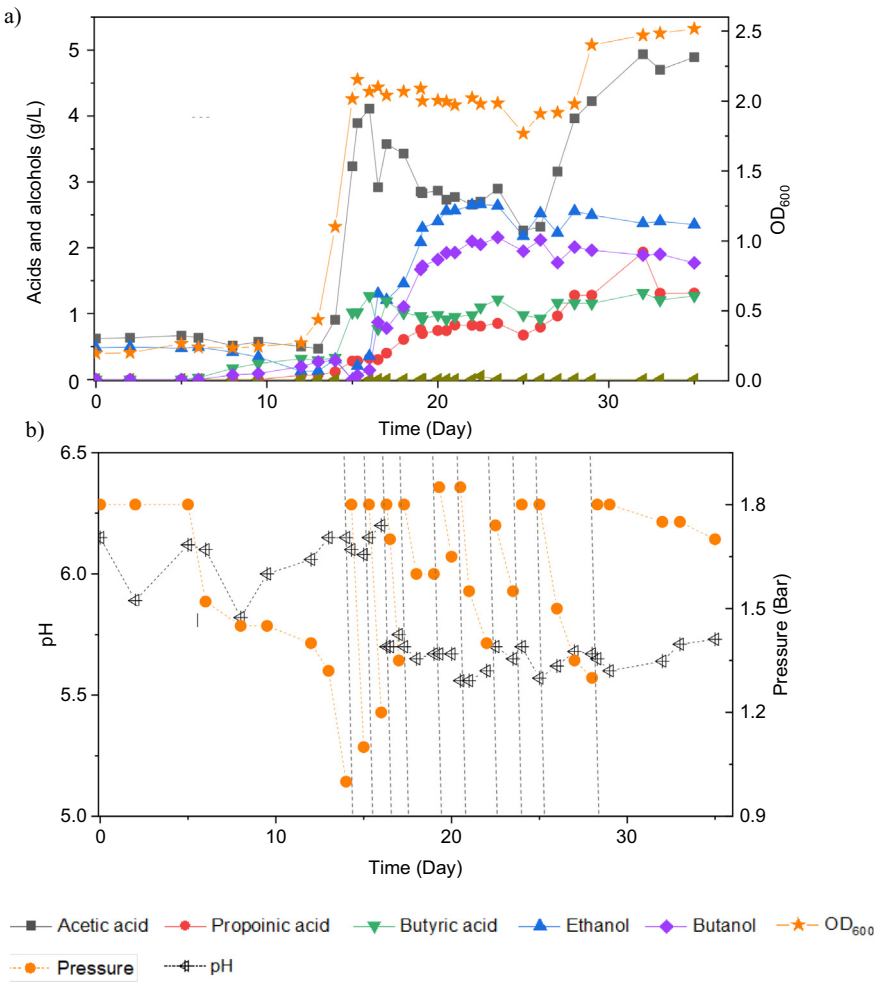

Fig. 6. a) Acetic acid, propionic acid, ethanol, butyric acid, butanol and cell concentrations (OD_{600}) and b) change of pH and gas pressure using CO as the sole substrate by the enriched sludge (sampled day 127) with intermittent CO gas feeding (FB2) on days 0, 14, 16, 17, 18, 19, 21, 22, 24, 26 and 28 d. Dash line in panel b represent CO feeding.

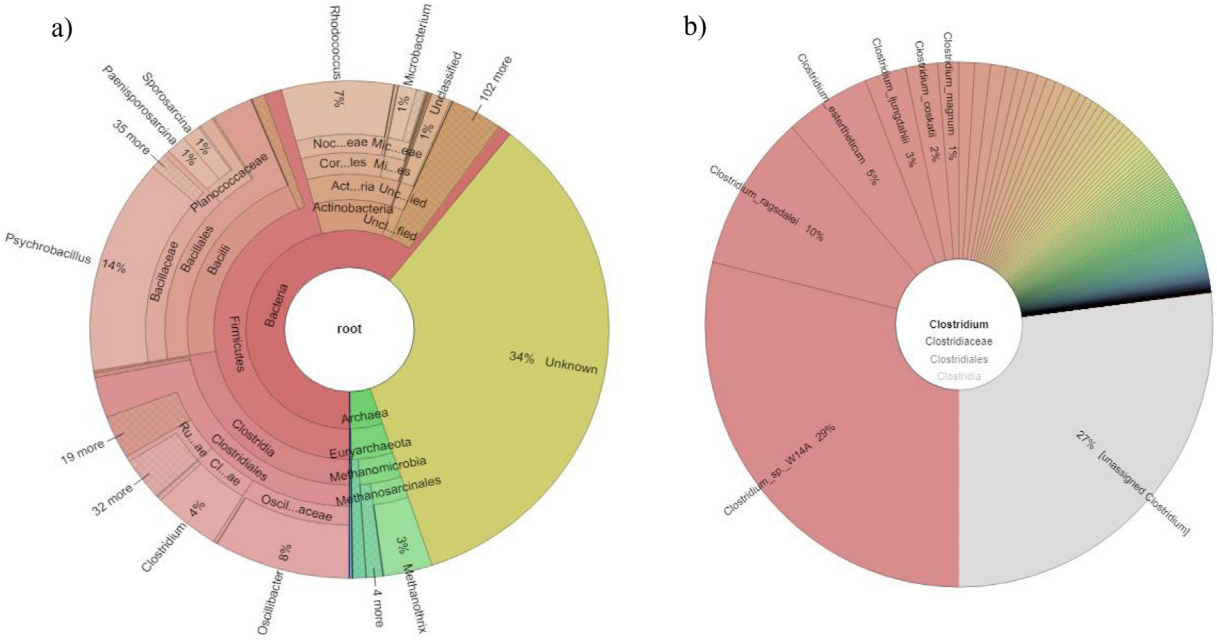

Fig. 7. Relative taxonomic abundance at a) genus and b) *Clostridium* species level of the enriched sludge from FB1 at day 127.

mmoL·L^{-1} C butyric acid and 43.2 mmoL·L^{-1} C butanol, while the total carbon consumed was 82.0 mmoL·L^{-1} C with 65.2 mmoL·L^{-1} C being ethanol and 16.7 mmoL·L^{-1} C likely being 0.5 g/L YE. Thus, the carbon balance of this batch test from consumed ethanol and YE to acetic acid, butyric acid and butanol was almost closed. Hence, part of the ethanol was converted to C_4 compounds.

In the presence of CO_2, ethanol started to be converted to acetic acid after 5 d at initial high pH 6.5 (Fig. 4c) and 7 d at initial low pH 5.7 (Fig. 4d), eventually reaching a total conversion of ethanol to acetic acid. The mole ratio of consumed ethanol to acetic acid, in the presence of CO_2, was 0.80 and 0.76, respectively, at initial pH 6.5 or pH 5.7 (Fig. 4e). This is close to the theoretical ratio of acetic acid production from ethanol and CO_2 (Eq. (7)) (Bao et al., 2019):

$$2CH_3CH_2OH + 2CO_2 \rightarrow 3CH_3COOH\ \Delta G^{\theta} = -32.2\ \text{kJ/mol} \quad (7)$$

3.2.3. Butanol oxidation in the presence of CO_2 by the enriched sludge

With initial pH 6.5, the butanol concentration decreased while the butyric acid concentration increased along with the pH slightly dropping after 5 days incubation (Fig. 5a, c). Thereafter, the butanol concentration further decreased to 2.5 g/L while the pH decreased to 6.2 at day 10. Butanol was subsequently completely consumed within 1 day (Fig. 5a, c). At initial pH 5.7, butanol decreased to 3.1 g/L at day 5 and was then quickly consumed, reaching 0.6 g/L (85% consumption) within 2 days (Fig. 2b, d). Acetic acid and butyric acid accumulated to, respectively, 2.0 and 3.6 g/L along with the pH decreasing to 4.5 (Fig. 5b, d). The pH was then adjusted to 5.7 at day 7 (Fig. 5d). Butanol was completely consumed after 12 days and 2.5 g/L acetic acid and 4.5 g/L butyric acid were obtained at the end of the incubation (Fig. 5b). The cell concentration reached its highest OD_{600} of 0.4 at both initial pH 6.5 and 5.7 (Fig. 5c, d). The mole ratio of butanol consumption to butyric acid production was 0.92 and 1.08, respectively, at initial pH 6.5 and 5.7, which is close to the theoretical ratio of 1 (Eq. (8)) (Schaefer et al., 2010; Fernández-Naveira et al., 2017a).

$$2\ CH_3CH_2CH_2CH_2OH + 2\ CO_2 \rightarrow 2\ CH_3CH_2CH_2COOH + CH_3COOH \quad \Delta G^{\theta} = -498.3\ \text{kJ/mol} \quad (8)$$

3.3. Ethanol and butanol production by the enriched culture at pH 6.2 and 5.7 in FB2

Fig. 6b shows that the gas pressure decreased in incubations of the enriched sludge (sampled on day 127 from FB1), when the pH was controlled at 6.2, from 1.8 bar to 1 bar during 14 days of incubation, during which 0.9 g/L acetic acid and 0.3 g/L butyric acid were produced. After a 2nd CO feeding (pressurization to 1.8 bar) on day 14 at the same pH value, the gas pressure decreased again to 1.1 bar within 24 h and 3.2 g/L acetic acid and 1.0 g/L butyric acid accumulated. It is noteworthy to observe that the small amount ethanol (0.48 g/L) originating from the inoculum was also completely consumed by day 15 (Fig. 6a). With the high CO consumption, the cell concentration doubled between the 1st and 2nd CO supply, i.e., the OD_{600} increased from 1.0 to 2.0 between days 14–16 (Fig. 6a).

Considering the dominant production of acetic and butyric acids, but without any significant ethanol and butanol accumulation at pH 6.0–6.2, the pH was decreased to 5.7 on day 16 to observe its possible effect on solventogenesis (Fig. 6b). 1.8 bar CO decreased to 1.2 bar after 24 h and the concentrations of acetic acid and butyric acid reached, respectively, 4.0 and 1.3 g/L, whereas the ethanol concentration increased to 0.4 g/L at 17 d (Fig. 6a). Subsequently, both ethanol and butanol concentrations increased to, respectively, 2.7 and 2.1 g/L at day 24 after the 8th CO addition (Fig. 6a). Thereafter, the production of both acids and alcohols stabilized (Fig. 6a). Therefore, 50 mL fresh medium was added to re-supply nutrients. From then onwards, the cell concentration increased to an OD_{600} of 2.5 and the acetic acid concentration increased to 4.9 g/L at day 35, while the ethanol and butanol concentrations did not further increase (Fig. 6a).

When operating at a pH of 5.7, alcohols were produced, though it was noted that ethanol and butanol production was inhibited when the gas pressure decreased from 1.8 bar to 1.35 bar (days 16.5–17). This was attributed to the accumulation of CO_2 and the possible reverse reaction of conversion of alcohols back to acids (Eqs. (7), (8)), as observed in previous tests (Figs. 4 and 5). Therefore, 1.8 bar CO was subsequently added every 24 h to avoid CO_2 accumulation since the accumulation of CO_2 may cause ethanol and butanol oxidation (Figs. 4 and 5).

3.4. Microbial community analysis

The relative taxonomic abundance of the enriched sludge sampled from the bioreactor (day 127) when reaching 6.8 g/L butanol is shown in a Krona figure (simplified as Fig. 7). The relative abundance was 61% bacteria, 5% archaea and 34% unknown. The *Firmicutes* phylum occupied 75% of the bacteria, mainly represented by the *Clostridia* (47%) and *Bacilli* (49%) classes (Fig. 7a). The *Clostridiales* order occupied 98% in the *Clostridia* class, which was mainly distributed over the *Ruminococcaceae* 14%, *Clostridiaceae* 21% and 40% *Oscillospiraceae* families (Fig. 7a). The *Clostridium* genus occupied 91% of the *Clostridiaceae* family, distributed as *Clostridium* strain W14A (29%), *C. ragsdalei* (10%), *C. estertheticum* (5%) and *C. ljungdahlii* (3%) (Fig. 7b). Some well-studied solventogenic species such as *C. autoethanogenum*, *C. carboxidivorans* and *C. kluyveri* occupied, respectively, 1%, 0.6% and 0.6% of the *Clostridium* genus (Fig. 7b). The relative abundance in the *Clostridiaceae* family (8%) and *Clostridium* genus (7%) of bacteria in the enriched sludge is much higher than, respectively, 1.7% and 0.3% of the bacteria in the granular sludge inoculum (SI Fig. 3) (He et al., 2020).

4. Discussion

4.1. Selective butanol production by CO fed anaerobic sludge

4.1.1. Selective butanol production

This study showed that a CO gas-fed enrichment can produce a butanol concentration as high as 6.8 g/L (Fig. 2) with a butanol/butyric acid ratio of 12.6 (Table 1). This is, to the best of our knowledge, thus far the highest reported butanol concentration (Table 2). The much higher selective production of butanol with the microbial culture enriched in this study can be explained by the broader metabolic potential of the enriched mixed populations compared to pure cultures, in combination with the pH value and the stressful environmental conditions such as the increased CO partial pressure (Fig. 2a, b). Indeed, the microbial community analysis showed that various *Clostridium* species were enriched, including *C. carboxidivorans* in the class *Clostridia* and other CO converting acetogens in the class *Bacilli*. The positive effect of increased CO partial pressure on cell growth and ethanol production has been reported for some pure strains, e.g., *C. carboxidivorans* (Lanzillo et al., 2020). Hurst and Lewis (2010) studied the effect of the CO partial pressure (P_{CO}) on ethanol production in *C. carboxidivorans* and found that cell growth increased by 440% when increasing the P_{CO} from 0.35 to 2 bar. Ethanol production was not observed when the P_{CO} was 0.35 bar (Hurst and Lewis, 2010). When the P_{CO} was 0.5, 0.7 and 1.05 bar, ethanol was produced in the non-growth phase. Conversely, if P_{CO} was increased to 2 bar, ethanol production was growth-associated. Lanzillo et al. (2020) investigated the cell growth and ethanol/butanol production by *C. carboxidivorans* at a CO pressure varying between 0.5 and 2.5 bar. The best condition for alcohol production was an initial 1.7 bar CO, yielding 0.4 g/L ethanol and 0.13 g/L butanol (Lanzillo et al., 2020). Similarly, this study obtained a selective butanol production in FB1 as well as 2.7 g/L ethanol and 2.1 g/L butanol in FB2 using 1.8 bar CO as the substrate, of which the gas pressure (1.8 bar)

was consistent with the reported optimum CO partial pressure (1.7–2.0 bar) (Hurst and Lewis, 2010; Lanzillo et al., 2020).

Fig. 2a shows that the increase in butanol concentration in the bioreactor with intermittent CO gas feeding occurred in two steps: the first butanol increase occurred at 111–119 d, when the acetic acid and ethanol concentrations decreased, while the butyric acid concentration remained relatively stable. The stable butyric acid concentration was assumed to be due to its simultaneous production from the C_1 gas and conversion to butanol via the WL pathway (Fernández-Naveira et al., 2017a). The second butanol increase was observed at pH 5.7 along with the decrease in butyric acid concentration and reached 6.8 g/L at the end of the incubation (Fig. 2a). The optimal pH for solventogenesis is generally slightly acidic in most acetogenic bacteria, though some strains, e.g., *C. aceticum*, have recently been shown to produce alcohols at near neutral pH (Arslan et al., 2019).

It should be noted that 0.5 g/L yeast extract addition in Period II might also have played a positive role in biomass growth, which can be seen from the shorter adaptation time than in Period I (Fig. 2). Yeast extract is an important, partly undefined, source of nutrients and micronutrients required for microorganisms (Abubackar et al., 2015), especially for syngas fermenting microorganisms, e.g., strain *C. carboxidivorans* P7 (Wan et al., 2017) and *C. autoethanogenum* DSM 10061 (Abubackar et al., 2012). Abubackar et al. (2012) investigated the effect of the yeast extract concentration (0.6–1.6 g/L) on biological solvent production by *C. autoethanogenum* DSM 10061 and used the Minitab analysis with a two level four factor (2^4). Lowering the YE concentration resulted in the production of more reduced compounds such as ethanol. Diender et al. (2016) investigated the production of fatty acids and solvents by a synthetic co-culture of *C. autoethanogenum* and *C. kluyveri* grown on CO. The co-culture was only capable of growing efficiently with 0.5 g/L yeast extract. Yeast extract concentrations lower than 0.5 g/L resulted in strong negative effects on the acid and alcohol production rates, and significantly increased the lag phase. Yeast extract can somewhat favor biomass growth at unfavorable pH values. For instance, in a bioreactor with continuous CO supply and 1 g/L yeast extract, at low pH 5.75, the maximum biomass concentration obtained was comparable to the maximum biomass concentration at pH 6.0 (Abubackar et al., 2015). Therefore, 0.5 g/L yeast extract addition from day 100 onwards (start of Period II) might have played a positive role on cell growth and alcohol production in this study.

4.1.2. Ethanol and butanol production by pH shift from 6.2 to 5.7

The pH control of the CO fed FB2 demonstrated that the shift of pH from 6.2 to 5.7 stimulated both ethanol and butanol production by the enriched sludge (Fig. 6). This agrees with the intermittent gas-fed FB1 experiment, suggesting enhanced butanol production (Period II) when the pH was manually adjusted around 5.7 (Fig. 2a, c). This shift at pH 5.7 in this study is slightly higher than previously reported pH values, between 4.5 and 5.5, that induce solventogenesis in syngas fermentation. Low pH values stimulate ethanol production from CO and syngas as shown by Chakraborty et al. (2019), who achieved ethanol production by anaerobic granular sludge after decreasing the pH from 6.2 to 4.9. The highest butanol concentration of 1.18 g/L was reached after 41 days of incubation at pH 4.9 (Chakraborty et al., 2019).

4.1.3. Carbon balance

The CO conversion efficiency was higher in Period I (91%) compared to Period II (68%) (Table 1). The frequent pH decreases in Period II might have damaged or killed the cells, resulting in the low carbon utilization. Mohammadi et al. (2012) reported a CO conversion efficiency of 93% with *C. ljungdahlii* in a reactor with continuous syngas (55% CO) feeding. In another experiment with *C. ljungdahlii* in a bubble column bioreactor, the CO bioconversion was only 60% from syngas (25% CO) (Morinaga and Kawada, 1990).

4.1.4. Microbial community analysis

C. carboxidivorans is the only reported *Clostridium* species that can produce butanol from C_1 gases (Fernández-Naveira et al., 2016a), although its relative abundance is very low in the enriched sludge (0.6%) (Fig. 7b). It should be noted that the relative abundance of unassigned *Clostridium* spp. is 27% of the *Clostridium* genus and thus some unassigned *Clostridium* spp. might have contributed to the butanol production (Fig. 7b). Considering the diversity of the *Clostridium* genus in the enriched sludge, a broad range of acetogenic organisms can be involved in CO bioconversion to metabolites such as butanol through species interactions, that are not possible in pure cultures. For instance, *Clostridium* species such as *C. autoethanogenum* and *C. kluyveri* have never been observed to produce butanol or hexanol individually in pure monocultures. However, a co-culture of both organisms was found to accumulate both butanol and hexanol (Diender et al., 2016). Considering the mixed culture in the enriched sludge, the positive role of mixed *Clostridium* strains might have contributed to enhanced alcohol production in FB1.

Concerning the other detected bacteria, *Ruminococcus* species of the *Ruminococcaceae* family have been shown to produce H_2 (Kotay and Das, 2008). In the *Oscillospiraceae* family, the relative abundance of the *Oscillibacter* genus reached 97% (Fig. 7a), which is known to be involved in acidogenesis during dark fermentation (Goud et al., 2017) and butyric acid production by microbial electrosynthesis using CO_2 as the substrate (Dessì et al., 2021). Besides the class *Clostridia*, the *Psychrobacillus psychrotolerans* species occupied as high as 57% of the class *Bacilli* and 20% of bacteria (Fig. 7a). The relative abundance of the *Rhodococcus* genus reached 12% of the bacterial population and some species of *Rhodococcus*, such as *Rhodococcus erythropolis* N9T-4 can convert CO to CO_2 under oligotrophic conditions (Fig. 7a) (Ohhata et al., 2007).

4.2. Ethanol and butanol oxidation in the presence of CO_2

Attempts of metabolic pathway elucidation in the enrichment experiments (Fig. 3) suggest that ethanol consumption during butanol production was due to its conversion back to acetic acid in the presence of CO_2. However, ethanol was completely oxidized to acetic acid with CO_2 only after 11 days (Fig. 3c, d), while part of the ethanol was used for C_4 compound production in a N_2 atmosphere (without CO_2) after 30 days of incubation (Fig. 3a, b). Hence, ethanol oxidation to acetic acid was dominant compared to its utilization for C_4 acid conversion with the accumulation of CO_2. The same ethanol oxidation process to acetic acid has been demonstrated in solventogenic acetogens such as *C. aceticum* (Arslan et al., 2019). ^{13}C-labelled ethanol and acetate experiments with another strain, *C. ljungdahlii*, revealed that ethanol production occurred during the exponential phase and that ethanol could then be oxidized to acetate via the aldehyde ferredoxin oxidoreductase pathway in the presence of 1 bar CO and at controlled pH 6.0 (Liu et al., 2020). Though ethanol oxidation to acetic acid had been reported in pure cultures of solventogenic *Clostridium* spp. (Arslan et al., 2019; Liu et al., 2020), butanol oxidation to butyric acid has to the best of our knowledge not been reported before.

Based on the observed compounds consumed and produced during butanol production (Fig. 2), different scenarios of conversion pathways for selective butanol production were considered. Firstly, it was checked (see Section 3.2) if some bacterial populations could have converted acetic acid and ethanol to butyric acid via the reversed β-oxidation pathway, since those two C_2 compounds were sometimes consumed while the concentration of butyric acid increased (Fig. 2a). As a result, butyric acid could then have been converted to butanol via the acetyl-CoA pathway by solventogenic acetogens in the intermittent gas-fed reactor. The reverse β-oxidation pathway using acetate (C_2) as carbon backbone and ethanol (C_2) as an electron donor can lead to *n*-butyrate (C_4) production, which has been described in species such as *C. kluyveri* (Agler et al., 2012; Richter et al., 2016; San-Valero et al.,

2019). However, our experiments with exogenous acetate, ethanol and either CO_2 or N_2 showed that butyric acid production from acetic acid and ethanol was not a relevant mechanism (Fig. 4), while ethanol oxidation to acetic acid in the presence of CO_2 was feasible instead (Fig. 4). Further research on the carbon flow and the biochemical mechanisms of C_2 and C_4 compound formation from CO and CO_2 is thus required, e.g., using nuclear magnetic resonance (NMR) spectroscopy with ^{13}C labelled CO (Gurudata, 2011).

4.3. H_2 production during start-up period

H_2 production was observed during the initial 15–33 days, but then shifted to acetic acid production (Fig. 2d). At each CO feeding, the molar ratio of H_2 production to CO consumption was close to 1 (SI Fig. 2), which is in accordance with the theoretical ratio of H_2 production from CO (Eq. (7)). One explanation is that hydrogen-utilizing acetogens were slowly enriched to become dominant after 30 days of operation along with the accumulation of H_2 and the presence of CO and CO_2 (Fig. 4). Hydrogenic acetogens can indeed be enriched from anaerobic sludge in the presence of CO (Liu et al., 2016).

Considering the accumulation of CO_2 during the fermentation process, it is not possible to discriminate between a direct conversion of CO to acetate (Eq. (1)) and an indirect conversion via H_2 and CO_2 as intermediates (Eqs. (9) and (10)):

$$CO + H_2O \rightarrow H_2 + CO_2 \Delta G^{\theta} = -20.1\ \text{kJ/mol} \quad (9)$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \Delta G^{\theta} = -75.4\ \text{kJ/mol} \quad (10)$$

This could be elucidated by NMR spectroscopy with ^{13}C labelled substrates in future studies.

H_2 production from CO by both pure or mixed cultures has been reported under mesophilic, thermophilic and hyper-thermophilic conditions in pure cultures of *Carboxydothermus hydrogenoformans*, *Carboxydocella thermoautotrophica* and *Thermincola carboxydiphila* (SI Table 1). CO is oxidized by carbon monoxide dehydrogenase (CODH retaining a Ni—Fe active site) to produce CO_2, and electrons are transferred by ferredoxin to an energy-converting hydrogenase that reduces protons to molecular H_2 (Simon et al., 2015). Generally, the growth rates of mesophilic hydrogenogenic bacteria on CO are low and enough biomass needs to develop before the phase of anaerobic CO conversion to H_2 can start (Parshina et al., 2005). Thermophilic conditions are generally more favorable, leading to a higher biohydrogen yield (SI Table 1).

From an energetic point of view, the Gibbs free energy of hydrogen production from CO (−20.1 kJ/mol, Eq. (9)) and water is much higher than for both acetic acid production from CO and water (−154.6 kJ/mol, Eq. (1)) or CO_2 and H_2 (−75.4 kJ/mol, Eq. (10)). The optimal growth pH of the known CO converting hydrogenic strains (SI Table 1) is near neutral under both mesophilic and thermophilic conditions. None of these strains were observed in the enriched sludge from the bioreactor at day 127 (SI Table 1). The *Rhodospirillales* order with relative abundance lower than 0.1% was identified in the enriched sludge. However, H_2 production was observed at pH 5.3–5.5 in this study (Fig. 2c). One possible explanation could be the presence of specific CO-utilizing/H_2-producing strains considering the diverse microbial populations present in anaerobic sludge (Wan et al., 2016). It should be noted that the molar ratio of CO consumption to H_2 production agreed with the theoretical ratio of 1 (Eq. (9)) (Fig. 1d), which confirms that H_2 production originates from CO and not from acetic acid bioconversion.

5. Conclusions

CO and syngas metabolizing solventogenic bacteria were enriched from heat-treated anaerobic granular sludge treating dairy wastewater at pH 5.7–6.5 and they produced up to 6.8 g/L butanol from an initial CO pressure of 1.8 bar in an intermittent gas-fed bioreactor. The high selective production of butanol with the enriched culture could be explained by the broader metabolic potential of the mixed bacterial inoculum compared to pure cultures. Upon the accumulation of CO_2 in the bioreactor, the enriched mixed culture also occasionally reoxidized ethanol and butanol to acetic acid and butyric acid, respectively. Additional tests under controlled pH demonstrated that a low pH (5.7) stimulated ethanol and butanol production by the enriched culture. Although the original sludge hardly contained acetogenic/solventogenic *Clostridia*, the microbial analysis of the enriched ethanol/butanol producing community showed that the applied enrichment procedure efficiently selected for a range of *Clostridium* species, including several known alcohol producers, such as *C. ljungdhalii*, *C. ragsdalei* and *C. coskatii*, in addition to other unidentified species which could include new solventogenic strains.

CRediT authorship contribution statement

Yaxue He: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. **Piet N.L. Lens:** Writing – review & editing, Supervision, Funding acquisition, Project administration. **María C. Veiga:** Resources, Writing – review & editing, Supervision, Funding acquisition. **Christian Kennes:** Conceptualization, Validation, Writing – review & editing, Supervision, Funding acquisition.

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

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References

Abubackar, H.N., Veiga, M.C., Kennes, C., 2012. Biological conversion of carbon monoxide to ethanol: effect of pH, gas pressure, reducing agent and yeast extract. Bioresour. Technol. 114, 518–522.

Abubackar, H.N., Veiga, M.C., Kennes, C., 2015. Ethanol and acetic acid production from carbon monoxide in a Clostridium strain in batch and continuous gas-fed bioreactors. Int. J. Environ. Res. Public Health 12 (1), 1029–1043.

Agler, M.T., Spirito, C.M., Usack, J.G., Werner, J.J., Angenent, L.T., 2012. Chain elongation with reactor microbiomes: upgrading dilute ethanol to medium-chain carboxylates. Energy Environ. Sci. 5 (8), 8189–8192.

Allen, Toby D., Caldwell, Matthew E., Lawson, Paul A., Huhnke, Raymond L., Tanner, Ralph S., 2010. Alkalibaculum bacchi gen. nov., sp. nov., a CO-oxidizing, ethanol-producing acetogen isolated from livestock-impacted soil. Int. J. Syst. Evol. Microbiol. 60 (10), 2483–2489.

Arantes Ana, L., Moreira João, P.C., Martijn, Diender, Parshina Sofiya, N., Stams Alfons, J.M., Madalena, Alves M., Alves Joana, I., Sousa Diana, Z., 2020. Enrichment of anaerobic syngas-converting communities and isolation of a novel carboxydotrophic Acetobacterium wieringae strain JM. Front. Microbiol. 11, 58.

Arslan, K., Bayar, B., Abubackar, H.N., Veiga, M.C., Kennes, C., 2019. Solventogenesis in clostridium aceticum producing high concentrations of ethanol from syngas. Bioresour. Technol. 292, 121941.

Bao, S., Wang, Q., Zhang, P., Zhang, Q., Wu, Y., Li, F., Tao, X., Wang, S., Nabi, M., Zhou, Y., 2019. Effect of acid/ethanol ratio on medium chain carboxylate production with different VFAs as the electron acceptor: insight into carbon balance and microbial community. Energies 12 (19), 3720.

Benomar, S., Ranava, D., Cárdenas, M.L., Trably, E., Rafrafi, Y., Ducret, A., Hamelin, J., Lojou, E., Steyer, J.P., Giudici-Orticoni, M.T., 2015. Nutritional stress induces exchange of cell material and energetic coupling between bacterial species. Nat. Commun. 6, 6283.

Buchfink, B., Xie, C., Huson, D.H., 2015. Fast and sensitive protein alignment using DIAMOND. Nat. Methods 12, 59–60.

Cai, M., Liu, J., Wei, Y., 2004. Enhanced biohydrogen production from sewage sludge with alkaline pretreatment. Environ. Sci. Technol. 38 (11), 3195–3202.

Chakraborty, S., Rene, E.R., Lens, P.N.L., Veiga, M.C., Kennes, C., 2019. Enrichment of a solventogenic anaerobic sludge converting carbon monoxide and syngas into acids and alcohols. Bioresour. Technol. 272, 130–136.

Charubin, K., Papoutsakis, E.T., 2019. Direct cell-to-cell exchange of matter in a synthetic *Clostridium* syntrophy enables CO_2 fixation, superior metabolite yields, and an expanded metabolic space. Metab. Eng. 52, 9–19.

Dessì, P., Lakaniemi, A.M., Lens, P.N., 2017. Biohydrogen production from xylose by fresh and digested activated sludge at 37, 55 and 70°C. Water Res. 115, 120–129.

Dessì, P., Sánchez, C., Mills, S., Francesco, G.C., Isipato, M., Umer, Z.I., Collins, G., Piet, N.L., Lens., 2021. Carboxylic acids production and electrosynthetic microbial community evolution under different CO2 feeding regimens. Bioelectrochemistry 137, 107686.

Devarapalli, M., Atiyeh, H.K., 2015. A review of conversion processes for bioethanol production with a focus on syngas fermentation. Biofuel Res. J. 2 (3), 268–280.

Devarapalli, M., Atiyeh, H.K., Phillips, J.R., Lewis, R.S., Huhnke, R.L., 2016. Ethanol production during semi-continuous syngas fermentation in a trickle bed reactor using Clostridium ragsdalei. Bioresour. Technol. 209, 56–65.

Diender, M., Stams, A., Sousa, D.Z., 2016. Production of medium-chain fatty acids and higher alcohols by a synthetic co-culture grown on carbon monoxide or syngas. Biotechnol. Biofuels 9 (1), 82.

Fast, A.G., Schmidt, E.D., Jones, S.W., Tracy, B.P., 2015. Acetogenic mixotrophy: novel options for yield improvement in biofuels and biochemicals production. Curr. Opin. Biotechnol. 33, 60–72.

Fernández-Naveira, Á., Abubackar, H.N., Veiga, M.C., Kennes, C., 2016a. Efficient butanol-ethanol (BE) production from carbon monoxide fermentation by *Clostridium carboxidivorans*. Appl. Microbiol. Biotechnol. 100 (7), 3361–3370.

Fernández-Naveira, Á., Abubackar, H.N., Veiga, M.C., Kennes, C., 2016b. Carbon monoxide bioconversion to butanol-ethanol by Clostridium carboxidivorans: kinetics and toxicity of alcohols. Appl. Microbiol. Biotechnol. 100 (9), 4231–4240.

Fernández-Naveira, Á., Veiga, M.C., Kennes, C., 2017a. H-B-E (hexanol-butanol-ethanol) fermentation for the production of higher alcohols from syngas/waste gas. J. Chem. Technol. Biotechnol. 92 (4), 712–731.

Fernández-Naveira, Á., Abubackar, H.N., Veiga, M.C., Kennes, C., 2017b. Production of chemicals from C1 gases (CO, CO_2) by Clostridium carboxidivorans. World J. Microbiol. Biotechnol. 33 (3), 43.

Fernández-Naveira, Á., Veiga, M.C., Kennes, C., 2019. Selective anaerobic fermentation of syngas into either C2–C6 organic acids or ethanol and higher alcohols. Bioresour. Technol. 280, 387–395.

Ganigué, R., Sánchez-Paredes, P., Bañeras, L., Colprim, J., 2016. Low fermentation pH is a trigger to alcohol production, but a killer to chain elongation. Front. Microbiol. 7, 702.

Goud, R.K., Arunasri, K., Yeruva, D.K., Krishna, K.V., Dahiya, S., Mohan, S.V., 2017. Impact of selectively enriched microbial communities on long-term fermentative biohydrogen production. Bioresour. Technol. 242, 253–264.

Guo, Y., Xu, J., Zhang, Y., Xu, H., Yuan, Z., Li, D., 2010. Medium optimization for ethanol production with Clostridium autoethanogenum with carbon monoxide as sole carbon source. Bioresour. Technol. 101 (22), 8784–8789.

Gurudata, N., 2011. 13C nuclear magnetic resonance of oximes. i. Solvent and isotope effects on the 13C chemical shifts of acetoxime. Can. J. Chem. 50 (12), 1956–1958.

He, Y., Chiara, C., Flora, M., Lens, Piet N.L., 2020. Homoacetogenesis and solventogenesis from H2/CO2 by granular sludge at 25, 37 and 55°C. Chemosphere 128649.

Humphreys, C.M., Minton, N.P., 2018. Advances in metabolic engineering in the microbial production of fuels and chemicals from C1 gas. Curr. Opin. Biotechnol. 50, 174–181.

Hurst, Kendall M., Lewis, Randy S., 2010. Carbon monoxide partial pressure effects on the metabolic process of syngas fermentation. Biochem. Eng. J. 48 (2), 159–165.

Huson, D.H., Mitra, S., Ruscheweyh, H.J., Weber, N., Schuster, S.C., 2011. Integrative analysis of environmental sequences using MEGAN4. Genome Res. 21 (9), 1552–1560.

Kennes, D., Abubackar, H.N., Diaz, M., Veiga, M.C., Kennes, C., 2016. Bioethanol production from biomass: carbohydrate vs syngas fermentation. J. Chem. Technol. Biotechnol. 91, 304–317.

Kotay, S.M., Das, D., 2008. Biohydrogen as a renewable energy resource—prospects and potentials. Int. J. Hydrog. Energy 33 (1), 258–263.

Lanzillo, F., Giacomo, R., Francesca, R., Russo, E.M., Marzocchella, A., 2020. Batch syngas fermentation by clostridium carboxidivorans for production of acids and alcohols. Processes 8 (9), 1075.

Li, S., Huang, L., Ke, C., Pang, Z., Liu, L., 2020. Pathway dissection, regulation, engineering and application: lessons learned from biobutanol production by solventogenic clostridia. Biotechnol. Biofuels 13, 39.

Liu, K., Atiyeh, Hasan K., Stevenson, Bradley S., Tanner, Ralph S., Wilkins, Mark R., Huhnke, Raymond L., 2014. Mixed culture syngas fermentation and conversion of carboxylic acids into alcohols. Bioresour. Technol. 152, 337–346.

Liu, Y., Wan, J., Han, S., Zhang, S., Luo, G., 2016. Selective conversion of carbon monoxide to hydrogen by anaerobic mixed culture. Bioresour. Technol. 202, 1–7.

Liu, Z., Jia, D., Zhang, K., Zhu, H., Zhang, Q., Jiang, W., Gu, Y., Li, F., 2020. Ethanol metabolism dynamics in Clostridium ljungdahlii grown on carbon monoxide. Appl. Environ. Microbiol. 86 (14), e00730-20.

Maddipati, P., Atiyeh, H.K., Bellmer, D.D., Huhnke, R.L., 2011. Ethanol production from syngas by *Clostridium* strain P11 using corn steep liquor as a nutrient replacement to yeast extract. Bioresour. Technol. 102 (11), 6494–6501.

Mende, D.R., Waller, A.S., Sunagawa, S., Järvelin, A.I., Chan, M.M., Arumugam, M., Raes, J., Bork, P., 2012. Assessment of metagenomic assembly using simulated next generation sequencing data. PLoS One 7 (2), e31386.

Mohammadi, M., Habibollah, Y., Ghasem, N., Abdul, R.M., 2012. Sustainable ethanol fermentation from synthesis gas by Clostridium ljungdahlii in a continuous stirred tank bioreactor. J. Chem. Technol. Biotechnol. 87 (6), 837–843.

Monlau, F., Trably, E., Barakat, A., Hamelin, J., Steyer, J.P., Carrere, H., 2013. Two-stage alkaline–enzymatic pretreatments to enhance biohydrogen production from sunflower stalks. Environ. Sci. Technol. 47 (21), 12591–12599.

Morinaga, T., Kawada, Naoki, 1990. The production of acetic acid from carbon dioxide and hydrogen by an anaerobic bacterium. J. Biotechnol. 14 (2), 187–194.

Munasinghe, P.C., Khanal, S.K., 2010. Biomass-derived syngas fermentation into biofuels: opportunities and challenges. Bioresour. Technol. 101 (13), 5013–5022.

Ohhata, N., Yoshida, N., Egami, H., Katsuragi, T., Tani, Y., Takagi, H., 2007. An extremely oligotrophic bacterium, Rhodococcus erythropolis N9T–4, isolated from crude oil. J. Bacteriol. 189 (19), 6824–6831.

Ondov, B.D., Bergman, N.H., Phillippy, A.M., 2011. Interactive metagenomic visualization in a web browser. BMC Bioinformatics 12 (1), 385.

Parshina, S.N., Sipma, J., Nakashimada, Y., Henstra, A.M., Smidt, H., Lysenko, A.M., Lens, P.N., Lettinga, G., Stams, A.J., 2005. Desulfotomaculum carboxydivorans sp. nov., a novel sulfate-reducing bacterium capable of growth at 100% CO. Int. J. Syst. Evol. Microbiol. 55 (5), 2159–2165.

Phillips, J.R., Atiyeh, H.K., Tanner, R.S., Torres, J.R., Saxena, J., Wilkins, M.R., Huhnke, R.L., 2015. Butanol and hexanol production in Clostridium carboxidivorans syngas fermentation: medium development and culture techniques. Bioresour. Technol. 190, 114–121.

Richter, H., Molitor, B., Diender, M., Sousa, D.Z., Angenent, L.T., 2016. A narrow pH range supports butanol, hexanol, and octanol production from syngas in a continuous co-culture of Clostridium ljungdahlii and Clostridium kluyveri with in-line product extraction. Front. Microbiol. 7, 1773.

San-Valero, P., Fernández-Naveira, Á., Veiga, M.C., Kennes, C., 2019. Influence of electron acceptors on hexanoic acid production by Clostridium kluyveri. J. Environ. Manag. 242, 515–521.

Schaefer, C.E., Yang, X., Pelz, O., Tsao, D.T., Streger, S.H., Steffan, R.J., 2010. Anaerobic biodegradation of iso-butanol and ethanol and their relative effects on BTEX biodegradation in aquifer materials. Chemosphere 81 (9), 1111–1117.

Shen, Y., Brown, R., Wen, Z., 2014. Syngas fermentation of *Clostridium carboxidivoran* P7 in a hollow fiber membrane biofilm reactor: evaluating the mass transfer coefficient and ethanol production performance. Biochem. Eng. J. 85, 21–29.

Shen, S., Wang, G., Zhang, M., Tang, Y., Gu, Y., Jiang, W., Wang, Y., Zhuang, Y., 2020. Effect of temperature and surfactant on biomass growth and higher-alcohol production during syngas fermentation by *Clostridium carboxidivorans* P7. Bioresour. Bioprocess. 7 (1), 1–13.

Simon, K.M.R., Lee, H.S., Lim, J.K., Kim, T.W., Lee, J.H., Kang, S.G., 2015. One-carbon substrate-based biohydrogen production: microbes, mechanism, and productivity. Biotechnol. Adv. 33 (1), 165–177.

Stams, A.J., Van Dijk, J.B., Dijkema, C., Plugge, C.M., 1993. Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Appl. Environ. Microbiol. 59 (4), 1114–1119.

Stoll, I.K., Herbig, S., Zwick, M., Boukis, N., Sauer, J., Neumann, A., Oswald, F., 2018. Fermentation of H2 and CO2 with Clostridium ljungdahlii at elevated process pressure–first experimental results. Chem. Eng. Trans. 64, 151–156.

Sun, X., Atiyeh, H.K., Kumar, A., Zhang, H., Tanner, R.S., 2018. Biochar enhanced ethanol and butanol production by clostridium carboxidivorans from syngas. Bioresour. Technol. 265, 128–138.

Ukpong, M.N., Atiyeh, H.K., Lorme, M.J.M.D., Kan, L., Zhu, X., Tanner, R.S., Wilkins, M.S., Stevenson, B.S., 2012. Physiological response of *Clostridium carboxidivorans* during conversion of synthesis gas to solvents in a gas-fed bioreactor. Biotechnol. Bioeng. 109 (11), 2720–2728.

Wan, J., Jing, Y., Zhang, S., Angelidaki, I., Luo, G., 2016. Mesophilic and thermophilic alkaline fermentation of waste activated sludge for hydrogen production: focusing on homoacetogenesis. Water Res. 102, 524–532.

Wan, N., Sathish, A., You, L., Tang, Y.J., Wen, Z., 2017. Deciphering Clostridium metabolism and its responses to bioreactor mass transfer during syngas mass transfer during syngas fermentation. Sci. Rep. 7, 10090–10100.

Wang, J., Yang, X., Chen, C.C., Yang, S.T., 2014. Engineering clostridia for butanol production from biorenewable resources: from cells to process integration. Curr. Opin. Chem. Eng. 6, 43–54.

Yu, J., Liu, J., Jiang, W., Yang, Y., Sheng, Y., 2015. Current status and prospects of industrial bio-production of n-butanol in China. Biotechnol. Adv. 33 (7) 1943-1501.