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Clostridium ljungdahlii as a biocatalyst in microbial electrosynthesis – Effect of culture conditions on product formation

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ABSTRACT

Microbial electrosynthesis enables the production of value-added chemicals from CO_2 and electrons provided by an electrode. *Clostridium ljungdahlii* is an electroactive acetogen that potentially could be used in microbial electrosynthesis systems. However, the optimal operational parameters for microbial electrosynthesis using *C. ljungdahlii* are not known. Here, we explored the effects of yeast extract, pH, and cathode potential. A low initial pH increased the rate of acetate production from CO_2 and H_2 in serum bottle cultures. When cultivated in bioelectrochemical systems, the optimal coulombic efficiency (i.e. close to 100 %) was observed at a cathode potential between −0.8 V and −1.0 V, while the highest productivity was reached at −1.0 V. Addition of yeast extract to the medium was needed to ensure reproducible results. Using cyclic voltammetry, we detected hydrogen-mediated extracellular electron transfer of *C. ljungdahlii* during growth on CO_2 in a bioelectrochemical system. These results show that operational parameters should be chosen carefully to maximise the efficiency of microbial electrosynthesis.

1. Introduction

Bioelectrochemical systems (BESs) rely on the interaction between microorganisms and insoluble solid electrodes (Logan et al., 2019). Extracellular electron transfer (EET) by anodophilic bacteria, which donate electrons to electrodes, drives the generation of electricity in microbial fuel cells and could be used for sustainable energy production (Santoro et al., 2017). Microbial electrosynthesis (MES) is attracting increasing attention as a means to produce multi-carbon compounds from CO_2, by supplying microorganisms with electrons derived from an electrode using an external power source (Das et al., 2020). A crucial advantage of the low operational voltages required for MES is that the electrical energy may come from renewable sources, such as solar, wind, tidal, and geothermal, or even surplus electricity from a power plant (Nevin et al., 2010). In this way, electroactive bacteria can store renewable electrical energy in the form of chemical bonds. Additionally, altering the redox balance of electroactive bacteria may induce production of value-added chemicals (Choi et al., 2014; Logan et al., 2019), as exemplified by the valorisation of residual carbon sources, such as wastewater, CO_2, and glycerol (Kondaveeti and Min, 2015; Kong et al., 2020; Roume et al., 2016).

Pure-culture MES using CO_2 has been tested with several different acetogens, including *Clostridium ljungdahlii*, *Clostridium scatologenes*, *Acetobacterium woodii*, and *Moorella thermoacetica* (Nevin et al., 2010, 2011). *C. ljungdahlii* is a model homoacetogen capable of CO_2 and CO fixation, which has been employed for both MES and microbial fuel cells (Bajracharya et al., 2015; Han et al., 2016; Nevin et al., 2011). While *A. woodii* monocultures have been shown incapable of utilising electrons from an electrode; *Acetobacterium* spp. have emerged as dominant in several MES studies employing mixed cultures (Marshall et al., 2012, 2013; Saheb-Alam et al., 2018). Recently, MES with mixed or pure cultures has been shown to convert CO_2 into acetate, as well as C_4–C_6 fatty acids and the corresponding alcohols (Liu et al., 2018; Vassilev et al., 2018). Several factors, including pH, acid concentration in the culture broth, and degree of reduction of the substrate, are known to affect the product profile of mixed cultures (Das et al., 2020; Vees et al., 2020). Low pH has been reported to favor solventogenesis by solventogenic acetogens (Grimalt-Alemany et al., 2018). Vassilev et al.

Abbreviations: AOR, aldehyde:ferredoxin oxidoreductase; BES, bioelectrochemical system; CP, cathode potential; CE, coulombic efficiency; CV, cyclic voltammetry; EET, extracellular electron transfer; MES, microbial electrosynthesis.

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(2018) maintained the culture pH at around 5 by periodically sparging 100 % CO_2 and successfully demonstrated production of acetate, butyrate, isobutyrate, caproate, and the corresponding alcohols by a mixed culture. The mildly acidic pH allowed *Clostridium* spp. to become dominant, enabling reverse-beta oxidation and production of butyrate and caproate (Vassilev et al., 2018). Reverse-beta oxidation for chain elongation in a BES has been demonstrated also for pure-culture MES with *C. scatologenes*, whereby potentials below −0.8 V vs. Ag/AgCl resulted in the production of acetate, as well as low titres of butyrate and ethanol (Liu et al., 2018).

Recently, acetogens have garnered increasing attention as biocatalysts for the fermentation of syngas, or synthesis gas, a mixture consisting primarily of H_2, CO, and CO_2. Syngas is produced during pyrolysis or gasification of carbonaceous materials, or from CO-rich off-gases generated by steel mills (Sun et al., 2019). Acetogens can convert syngas components to alcohols (mainly ethanol) and other chemicals through the Wood-Ljungdahl pathway and its downstream ramifications. *C. ljungdahlii* has been reported to produce ethanol, acetate, formate, lactate, and 2,3-butanediol during syngas fermentation (Kopke et al., 2011; Tanner et al., 1993). Ethanol, acetate, as well as longer-chain alcohols, such as *n*-butanol, *n*-hexanol, and *n*-octanol, were detected in a co-culture of *C. ljungdahlii* and *Clostridium kluyveri* fermenting syngas (Richter et al., 2016a).

Ethanol is produced by solventogenic homoacetogens through the aldehyde:ferredoxin oxidoreductase (AOR) pathway or the aldehyde/alcohol dehydrogenase pathway (Liu et al., 2020). The latter converts acetyl-CoA into acetaldehyde using NADH; whereas the former converts undissociated acetic acid, which is reassimilated into cells at low pH, to acetaldehyde using reduced ferredoxin as a cofactor (Liu et al., 2020; Richter et al., 2016b). Therefore, when ethanol production from CO or CO_2 is desired, it is important to maintain a low pH of the culture, to boost diffusion of the generated undissociated acetic acid into the cell. The source of nitrogen has also been reported to affect ethanol production (Abubackar et al., 2012; Cotter et al., 2009; Phillips et al., 2015). Nitrogen-limiting conditions, elicited by starving the cultures for a carbon and nitrogen source such as yeast extract, promote solventogenesis in *Clostridia* (Engel et al., 2019b; Klask et al., 2020; Phillips et al., 2015). Yeast extract boosts cell growth at the beginning of cultivation, but its impact on ethanol production in MES remains unknown.

In this study, we investigated the operational parameters for the production of value-added commodity chemicals from CO_2 using *C. ljungdahlii*-based MES. We systematically tested various parameters, including cathodic potential (CP), addition of yeast extract, and pH, in two different set-ups: a BES with a cathode as electron donor, and in serum bottles with H_2 as electron donor. The resulting information will facilitate the development of more efficient bioprocesses using *C. ljungdahlii* as a biocatalyst.

2. Materials and methods

2.1. Bacteria, pre-cultures, and media

C. ljungdahlii (DSM-13528) was obtained from DSMZ (Braunschweig, Germany). The bacterial stock was stored as 1.5-mL aliquots in anoxic 20 % glycerol at −80 °C. The cryostock was streaked onto YTF (yeast extract-tryptone-fructose) agar plates in an anaerobic Whitley M95 Workstation (Don Whitley Scientific Ltd., Bingley, UK). Once the colonies were visible, a single colony was inoculated in 40 mL anaerobically prepared modified DSMZ879 medium with or without 1 g/L yeast extract, under 2 bars of over-pressurised gas (80:20 = H_2:CO_2) in 200-mL rubber stopper-sealed serum bottles. The pre-cultures were placed horizontally in a shaking incubator and incubated at 160 rpm and 37 °C until they reached an optical density at 660 nm (OD_{660}) of 0.3 (without yeast extract) or 0.4–0.7 (with yeast extract).

The modified DSMZ879 medium contained the following: 20 g/L 2-ethanesulfonic acid monohydrate, 1 g/L NH_4Cl, 0.1 g/L KCl, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.8 g/L NaCl, 0.01 g/L KH_2PO_4, 0.02 g/L $CaCl_2{\cdot}2H_2O$, 0.25 g/L sodium acetate, 50 mg/L $FeSO_4{\cdot}7H_2O$, and 0.6 g/L L-Cysteine·HCl. The medium was supplemented with 1 mL 0.1 % w/v Na-resazurin solution, 10 mL modified Wolin's mineral solution (containing 1.5 g/L nitrilotriacetic acid, 3 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L $MnSO_4{\cdot}H_2O$, 1 g/L NaCl, 0.1 g/L $FeSO_4{\cdot}7H_2O$, 0.18 g/L $CoSO_4{\cdot}7H_2O$, 0.1 g/L $CaCl_2{\cdot}2H_2O$, 0.18 g/L $ZnSO_4{\cdot}7H_2O$, 0.01 g/L $CuSO_4{\cdot}5H_2O$, 0.02 g/L $KAl(SO_4)_2{\cdot}12H_2O$, 0.01 g/L H_3BO_3, 0.01 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.03 g/L $NiCl_2{\cdot}6H_2O$, 0.3 mg/L $Na_2SeO_3{\cdot}5H_2O$, and 0.4 mg/L $Na_2WO_4{\cdot}2H_2O$), and 10 mL Wolin's vitamin solution (containing 2 mg/L biotin, 2 mg/L folic acid, 10 mg/L pyridoxine·HCl, 5 mg/L thiamine·HCl, 5 mg/L riboflavin, 5 mg/L nicotinic acid, 5 mg/L Ca·D-pantothenate, 0.1 mg/L vitamin B12, 5 mg/L *p*-aminobenzoic acid, and 5 mg/L a-lipoic acid). The pH of the medium was set to either 5.7 or 5.0 before autoclaving. When testing the effect of yeast extract on *C. ljungdahlii* performance, 1 g/L of yeast extract was added to the medium before pH adjustment and autoclaving.

2.2. Serum bottle experiments

Serum bottles were inoculated with the pre-culture to an initial OD_{660} of 0.05, irrespective of yeast extract content. The headspace of the bottles was filled with 2 gauge bars of either 100 % CO_2 gas or 80:20 H_2:CO_2. During cultivation, the gas in the headspace was replaced with fresh gas when the pressure fell below 1 gauge bar. Samples of 1 mL were taken every 24 h to determine OD_{660}, pH, and metabolite composition. Each serum bottle experiment was performed in triplicate.

2.3. BES reactor operation

H-type BES reactors (Adams & Chittenden Scientific Glass Coop, Berkeley, CA, US) were used for MES. The cathode and anode chambers of the BES were connected by an NW40 high-temperature chain clamp (Evac AG, Grabs, Switzerland) and separated by a Nafion 117 membrane (d = 4 cm; Alfa Aesar, Heysham, UK) pre-treated as described previously (Flimban et al., 2020). Each chamber contained 250 mL modified DSMZ879 medium without resazurin and supplemented with 1.0 g/L yeast extract when appropriate. A graphite block (2.5 cm × 1.0 cm × 2.5 cm; Alfa Aesar) connected with a titanium wire (Alfa Aesar) was used as cathode, while a platinised titanium wire (MAGNETO special anodes B. V., Schiedam, The Netherlands) was used as anode. Cells from the pre-culture were inoculated to an OD_{660} of 0.01 or 0.06 for cultures containing or lacking yeast extract, respectively. The cultures were sparged continuously with 100 % CO_2 at 10 mL/min. The cathodic potential (CP) was maintained using a MultiEmStat3+ potentiostat (PalmSens, Houten, The Netherlands) controlled by the MultiTrace software (PalmSens). All CPs mentioned in this study were controlled based on the Ag/AgCl (3 M NaCl) reference electrode (BASi, West Lafayette, IN, USA). Briefly, 1 mL of sample was taken from both the cathode and anode chambers every 24 h to determine OD_{660}, pH, and metabolite composition. Each BES experiment was performed in triplicate.

2.4. Analytical methods

OD_{660} of the culture was measured in a WPA S1200+ visible spectrophotometer (Biochrom, Cambridge, UK). The pH was measured with a pH meter (Mettler Toledo, Columbus, OH, USA). Metabolite analysis was performed using a high-performance liquid chromatography system equipped with a refractive index detector (at 40 °C) and a UV detector (210 nm) (Jasco, Tokyo, Japan), as well as a ROA-Organic acid H^+ (8 %) column (Rezex, Torrance, CA, USA). The oven temperature was maintained at 60 °C and the column was eluted with 5 mM H_2SO_4 at 0.6 mL/min. The collected liquid samples were centrifuged at 12,000 rpm for 5 min, followed by filtration through a 0.2-μm nylon syringe filter before sample injection in the chromatographer.

2.5. Stoichiometry and coulombic efficiency (CE) calculations

The stoichiometries for the reduction of H^+ to H_2 and CO_2 into various organic compounds are summarised in Eqs. (1)–(6). Based on the stoichiometries, as well as observed current generation and product yields, the CE of each experiment was calculated using Eq. (7), where n is the number of electrons required (mol e^-/mol product), F corresponds to Faraday's constant (96,485 C/mol), m is the moles of product (mol), I is the current (A), and t is time (s).

$$2e^- + 2H^+ \rightarrow H_2 \quad (1)$$

$$CO_2 + 2e^- + 2H^+ \rightarrow HCOOH \quad (2)$$

$$2CO_2 + 8e^- + 8H^+ \rightarrow CH_3COOH \quad (3)$$

$$2CO_2 + 12e^- + 12H^+ \rightarrow CH_3CH_2OH \quad (4)$$

$$3CO_2 + 12e^- + 12H^+ \rightarrow CH_3CH(OH)COOH \quad (5)$$

$$4CO_2 + 22e^- + 22H^+ \rightarrow CH_3(CHOH)_2CH_3 \quad (6)$$

$$CE\ (\%) = \frac{nFm}{\int_0^t I\,dt} \times 100\% \quad (7)$$

2.6. Cyclic voltammetry (CV)

CV measurements were performed at the end of the experiment carried out with a CP of −1.0 V, and independently for a blank containing only nutrient medium and no bacteria. CV was measured from

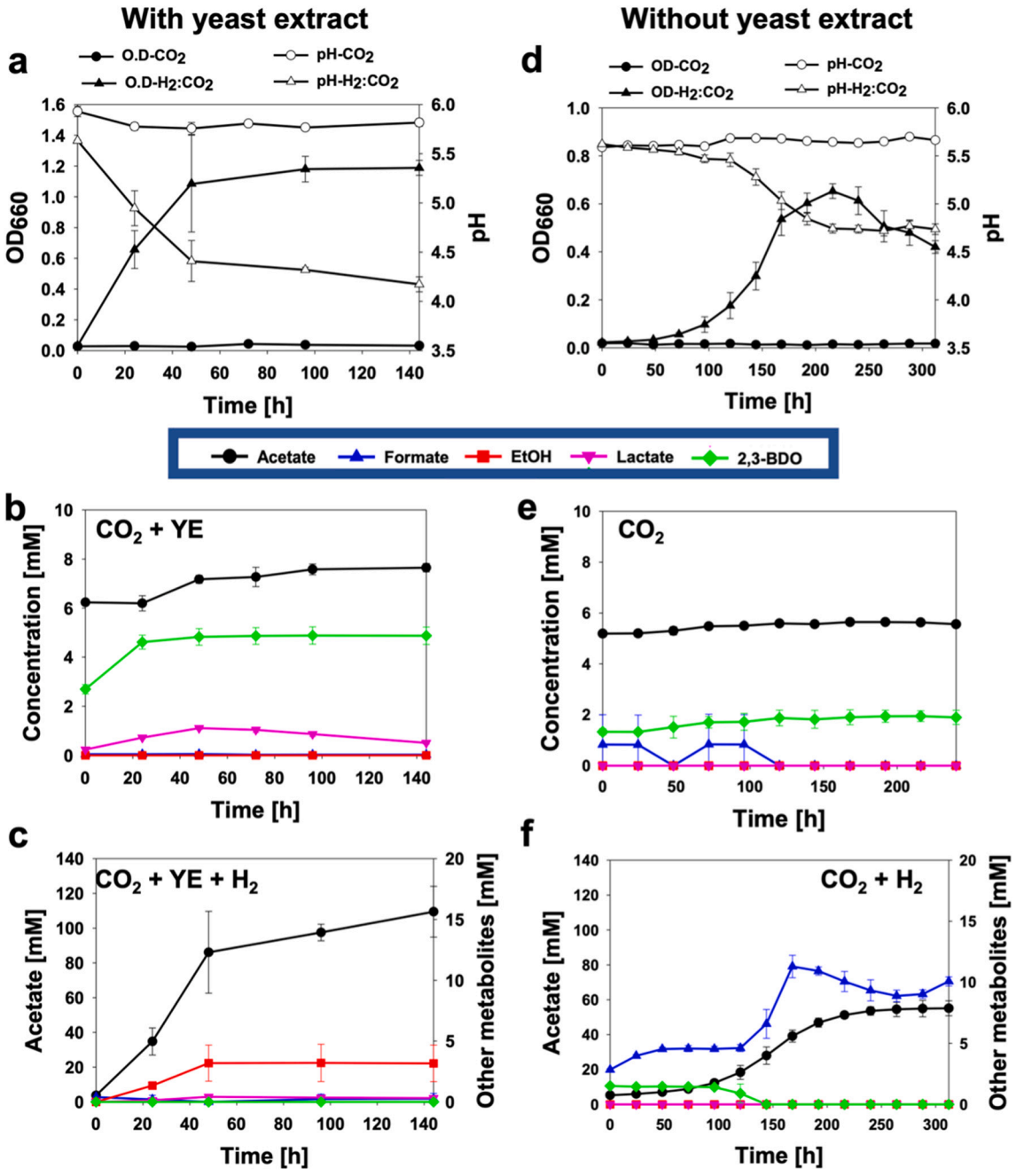

Fig. 1. Growth parameters of *C. ljungdahlii* cultured in serum bottles supplemented (a, b, c) or not (d, e, f) with 1 g/L yeast extract (YE). (a, d) Growth curves and medium pH. (b, c, e, f) Metabolite production under different conditions. Note the different length of experiments (x-axis) for the various set-ups.

−1.4 V to 0.0 V vs. an Ag/AgCl (3 M) reference electrode at a scan rate of 1 mV/s.

3. Results and discussion

3.1. Addition of yeast extract improves the conversion of CO_2 and H_2 in serum bottle cultures

Yeast extract contains different carbon and nitrogen sources, as well as vitamins and trace elements. Owing to its rich nutrient composition, it is commonly used to support bacterial growth. However, yeast extract is omitted when the products of MES are to be generated solely from CO_2 and the electrons originate from the cathode (Marshall et al., 2013; Vassilev et al., 2018). *C. ljungdahlii* has been suggested to have electroactive properties as it consumes current and produces acetate from CO_2 in a BES reactor (Bajracharya et al., 2015; Nevin et al., 2011). Because the medium contained yeast extract in these experiments, we first wanted to verify that *C. ljungdahlii* could produce acetate from CO_2 and yeast extract as a carbon source, without any additional electron donor (i.e. H_2). The impact of yeast extract in culture medium on the metabolic profile of *C ljungdahlii* grown on CO_2 or a gaseous H_2:CO_2 mixture was determined using serum bottle cultures (Fig. 1).

Cultures supplemented with yeast extract but without added H_2 displayed no change in OD_{660} over time and produced only 1.41 ± 0.17 mM acetate, 0.27 ± 0.05 mM lactate, and 2.18 ± 0.35 mM 2,3-butanediol (Fig. 1a and b). Without yeast extract, no growth or metabolite production was observed unless H_2 was supplemented, in which case maximal OD_{660} was ~0.58 ± 0.07, while 55.07 ± 4.28 mM acetate and 10.07 ± 0.37 mM formate were generated (Fig. 1d, e, and f). Cultures supplemented with both yeast extract and H_2 attained a maximal OD_{660} of 1.19 ± 0.05, together with 105.72 ± 14.61 mM acetate, 3.17 ± 1.51 mM ethanol, and 0.31 ± 0.12 mM lactate (Fig. 1a and c). These values were higher compared to when no yeast extract was supplied. As expected, acetate was the main product under all tested conditions. Notably, growth and metabolite production started immediately in cultures supplemented with yeast extract and H_2, while being delayed by around 50 h in cultures devoid of yeast extract (Fig. 1a and d). Growth and productivity of *Clostridium carboxidivorans* were similarly decreased when yeast extract was removed from the culture medium during syngas fermentation (Phillips et al., 2015). A study on an ethanol-producing bacterial community enrichment of syngas-fermenting mixed microbial communities demonstrated that addition of yeast extract at low initial pH yielded a diverse set of metabolites and more ethanol than other conditions (Grimalt-Alemany et al., 2018). Taken together, these findings indicate that the addition of yeast extract to *C. ljungdahlii* cultures clearly improved growth and production of metabolites from CO_2 and H_2, while only negligible amounts of metabolites were produced from yeast extract alone without H_2.

3.2. A low initial pH increases production of acetate from CO_2 and H_2 in serum bottle cultures

While the mechanism for EET by *C. ljungdahlii* in a BES has not been determined, hydrogen-mediated electron transfer is likely involved (Deutzmann et al., 2015; Philips, 2019). Because the operation of BES reactors is much more time-demanding and tedious compared to that of serum bottle cultures, the latter were employed to mimic the conversion of CO_2 to ethanol in a BES consisting of *C. ljungdahlii* supplemented with a gaseous H_2:CO_2 mixture. Ethanol production by *C. ljungdahlii* fermenting CO-rich syngas in a mildly acidic medium (pH 5.5) has been reported previously (Richter et al., 2016b). Varying amounts of ethanol and ethanol: acetate ratios were reported in *C. ljungdahlii* chemostats maintained at different pH values and supplemented with H_2 and CO_2 (Klask et al., 2020). At low pH, undissociated acetic acid is expected to diffuse back into the cells, where it is first converted to acetaldehyde by the AOR pathway and then into ethanol (Richter et al., 2016b). Therefore, it is essential to choose the right operational pH when exploiting *C. ljungdahlii* as a biocatalyst in MES. Several studies on solventogenic homoacetogens have demonstrated an increased production of alcohol in cultures devoid of yeast extract or containing a low amount of it (Abubackar et al., 2012; Cotter et al., 2009; Phillips et al., 2015). Here, *C. ljungdahlii* cultures were grown in serum bottles at a low initial pH (pH 5.0), with or without yeast extract supplementation (Fig. 2a).

Lowering the initial pH from 5.7 to 5.0 increased acetate yield and productivity (Figs. 1c and f, and 2b). At the end of the experiment, 251.02 ± 5.29 mM acetate and 5.44 ± 1.75 mM ethanol were detected when yeast extract was added to the medium, whereas only 176.94 ± 5.09 mM acetate and 2.78 ± 0.09 mM ethanol were recorded in the absence of yeast extract (Fig. 2b). In cultures supplemented with yeast extract, cell growth was the same irrespective of starting pH (Figs. 1a and 2a). In contrast, in cultures lacking yeast extract, the maximal OD_{660} was barely 0.30 ± 0.07 at pH 5.0 but doubled to 0.58 ± 0.07 in those at pH 5.7 (Figs. 1d and 2a). Expectedly, a lower initial pH led also to a lower final pH compared to cultures with a higher initial pH, both in the presence of yeast extract (pH 3.75 vs. 4.17 ± 0.08) and not (pH 3.88 ± 0.14 vs. 4.74 ± 0.06) (Figs. 1a and d, and 2a). *C. ljungdahlii* has been reported to thrive at pH 4.0–7.0 (Tanner et al., 1993).

3.3. The CP determines CE and productivity in a BES

Based on the obtained results from serum bottle experiments, BES reactors were operated with yeast extract and initial pH of 5.0. The choice of CP is perhaps the most important operational parameters of a MES as it influences the abiotic H_2 evolution rate and pH of the cathode chamber. Specifically, abiotic H_2 evolution is higher at a lower CP, which triggers a rise in pH in the cathode chamber. To identify the optimal CP, we ran BES reactors containing *C. ljungdahlii* at different CPs, including −0.6 V, −0.8 V, −1.0 V, and −1.2 V, resulting in CEs of 2788 %, 365 %, 18 %, and 5 % (Table 1).

Notably, BES reactors at CPs of −0.6 V and −0.8 V yielded only 0.65 ± 0.01 mM and 0.91 ± 0.01 mM acetate, respectively (Fig. 3a and b, Table 1). The highest product titre was reached at a CP of −1.0 V, whereby 5.56 ± 0.80 mM acetate, 6.01 ± 0.60 mM formate, and 0.77 mM lactate were detected (Fig. 3c, Table 1). At a CP of −1.2 V, 2.02 ± 1.5 mM acetate, 7.54 ± 0.49 mM formate, and 0.66 ± 0.0 mM lactate were produced (Fig. 3d, Table 1). A CE above 100 % at CPs of −0.6 and −0.8 V can be explained by low current generation and the contribution of yeast extract to product formation (see Supplementary material). Liu et al. (2018) tested different CPs for MES, demonstrating the successful conversion of CO_2 to ethanol and butyrate by a pure culture of *C. scatologenes*. In that experiment, CE decreased as lower CPs were applied. This is in line with our observation using *C. ljungdahlii*, although CEs for *C. scatologenes* remained below 100 % even when yeast extract was added to the medium (Liu et al., 2018). Overall, less lactate and no ethanol were produced in BES reactors compared to serum bottle cultures (Figs. 1 and 3).

Cell growth of *C. ljungdahlii* was not as strong in BES reactors as in serum bottle experiments (Figs. 1, 2, and 3). Most of the growth occurred during the first 24 h. The maximum OD_{660} was 0.04 at −0.6 V, 0.05 at −0.8 V, 0.08 at −1.0 V, and 0.05 at −1.2 V. A similarly minor increase in biomass was observed in a BES with *C. scatologenes* (Liu et al., 2018), although it remains unclear why growth of autotrophic acetogens is so poor in a BES reactor. One problem may be accessibility to the source of electrons at the cathode. If the surface area of the electrode is small and only cells around the cathode have access to the electron source, cell growth will be limited.

Maintaining the operational pH in a conventional BES reactor lacking pH control is hindered by a continuous increase in pH as protons are used for the cathodic reactions during MES (Eqs. (1)–(6)). The Nafion membrane is supposed to counteract this pH increase by allowing the passage of protons from the anode to the cathode chamber. Protons are produced by electrolysis on the anode, but proton diffusion through the

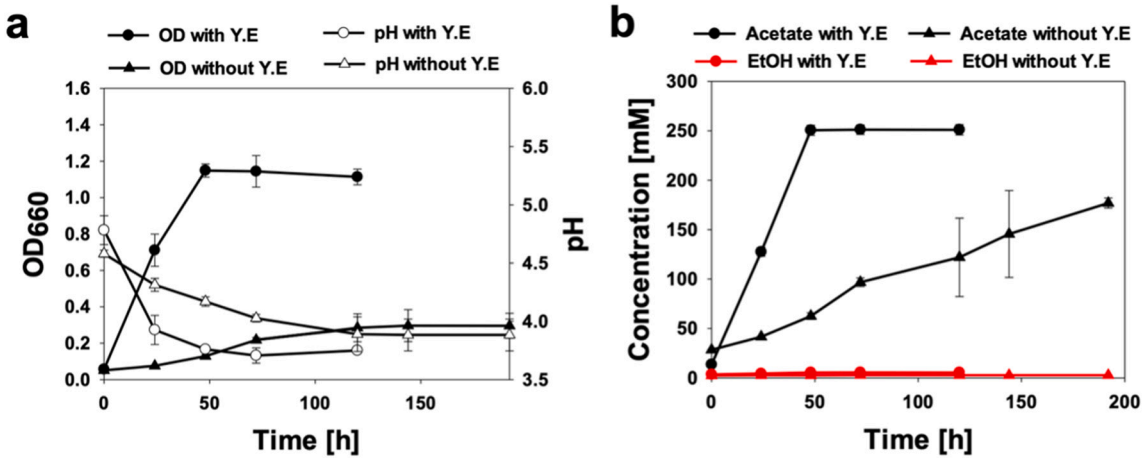

Fig. 2. Growth parameters of *C. ljungdahlii* cultivated in serum bottles with a gaseous mixture of H_2 and CO_2 and supplemented or not with yeast extract (YE). The initial pH was set to 5.0. (a) Growth curves and pH. (b) Metabolite production.

Table 1
Summary of data from the BES experiment at different cathode potentials, with and without addition of yeast extract. Data are presented as the average of three replicate cultures ± standard deviation. For experiments without yeast extract, actual values of the three replicate experiments are reported.

Cathodic potential (V)	Produced metabolites (mM)					Charge equivalents in metabolites (C)	Electrical charge (C)	Coulombic efficiency	Yeast extract (g/L)	pH
	Acetate	Formate	Ethanol	Lactate	2,3-Butanediol					
−0.6	−1.89 ± 0.30	–	–	–	–	−731	108 34 25	–	–	4.9–5.1
−0.8	1.07 0.35 −0.06	–	–	–	–	414 134 −22	766 303 397	~50 %	–	5.0–5.4
−1.0	6.08 2.68 2.14	38.70 13.09 9.28	–	–	–	6081 2298 1721	100,672 26,373 19,903	<1 %	–	4.3–5.8
−0.6	0.65 ± 0.01	–	–	–	–	251 ± 3	9 ± 1	2788 %	1	5.0–5.1
−0.8	0.91 ± 0.01	–	–	–	–	354 ± 3	97 ± 20	365 %	1	5.0–5.2
−1.0	5.56 ± 0.80	6.01 ± 0.60	–	0.77	–	3174 ± 366	17,910 ± 412	18 %	1	4.7–5.5
−1.2	2.02 ± 1.5	7.54 ± 0.49	–	0.66 ± 0.01	–	2274 ± 99	46,279 ± 2528	5 %	1	4.9–5.8

Nafion 117 membrane is still not fast enough to balance the pH difference between the chambers (Chae et al., 2007). The pH of the cathode chamber remained around 5.0 at a CP of −0.6 V, and increased slightly to less than pH 5.2 at −0.8 V (Fig. 3e and f). In contrast, the pH of the cathode chamber rose dramatically during the first day but then decreased gradually over time at a CP of −1.0 V and −1.2 V (Fig. 3g and h). When a low CP (e.g. −1.0 V and −1.2 V) is applied, the pH of the anode chamber decreases rapidly due to protons being generated via electrolysis. The small pH difference between cathode and anode chambers at the beginning cannot compensate for the subsequent rapid increase in pH in the cathode chamber. However, when the difference in pH between the two chambers is sufficiently large, the Nafion membrane can successfully transfer protons from the anode to the cathode chamber.

To better estimate how yeast extract contributed to metabolite production and CE, we ran BES experiments with medium devoid of yeast extract (see Supplementary material). In spite of great variability among triplicate runs (Table 1), production was generally slower in cultures lacking yeast extract, thus confirming the results of serum bottle experiments. At a CP of −0.6 V, the concentration of acetate was found to decrease in the absence of yeast extract. While undissociated acetate is expected to diffuse back into cells at low pH and be further metabolized via the AOR pathway (Richter et al., 2016b), we hypothesize that the available reducing equivalents were insufficient for ethanol production. When *C. ljungdahlii* was grown on a CO:CO_2 mixture, ethanol oxidation in stationary phase was assumed to provide energy and reducing equivalents to support cell viability (Liu et al., 2020). Moreover, inconsistent current consumption in such cultures may explain the poor reproducibility of chemical production (Table 1, see Supplementary material).

3.4. The EET of *C. ljungdahlii* is likely mediated by hydrogen

A couple of mechanisms have been proposed to describe EET of *Clostridia* acetogens, namely extracellular hydrogenase-mediated EET and thermodynamically-shifted hydrogen evolution (Deutzmann et al., 2015; Engel et al., 2019a; Philips, 2019). CV measures the redox potential of an electrochemical reaction and is commonly used to study EET mechanisms (Patil et al., 2012). Here, we used CV to determine the dominant EET mechanism in BES reactors containing *C. ljungdahlii*.

Riboflavin and other flavin-type compounds in yeast extract have been reported to act as exogenous electron mediators in *Lactococcus lactis* (Masuda et al., 2010). Thus, we speculated that exogenous electron mediators found in yeast extract could stabilize the MES. However, no

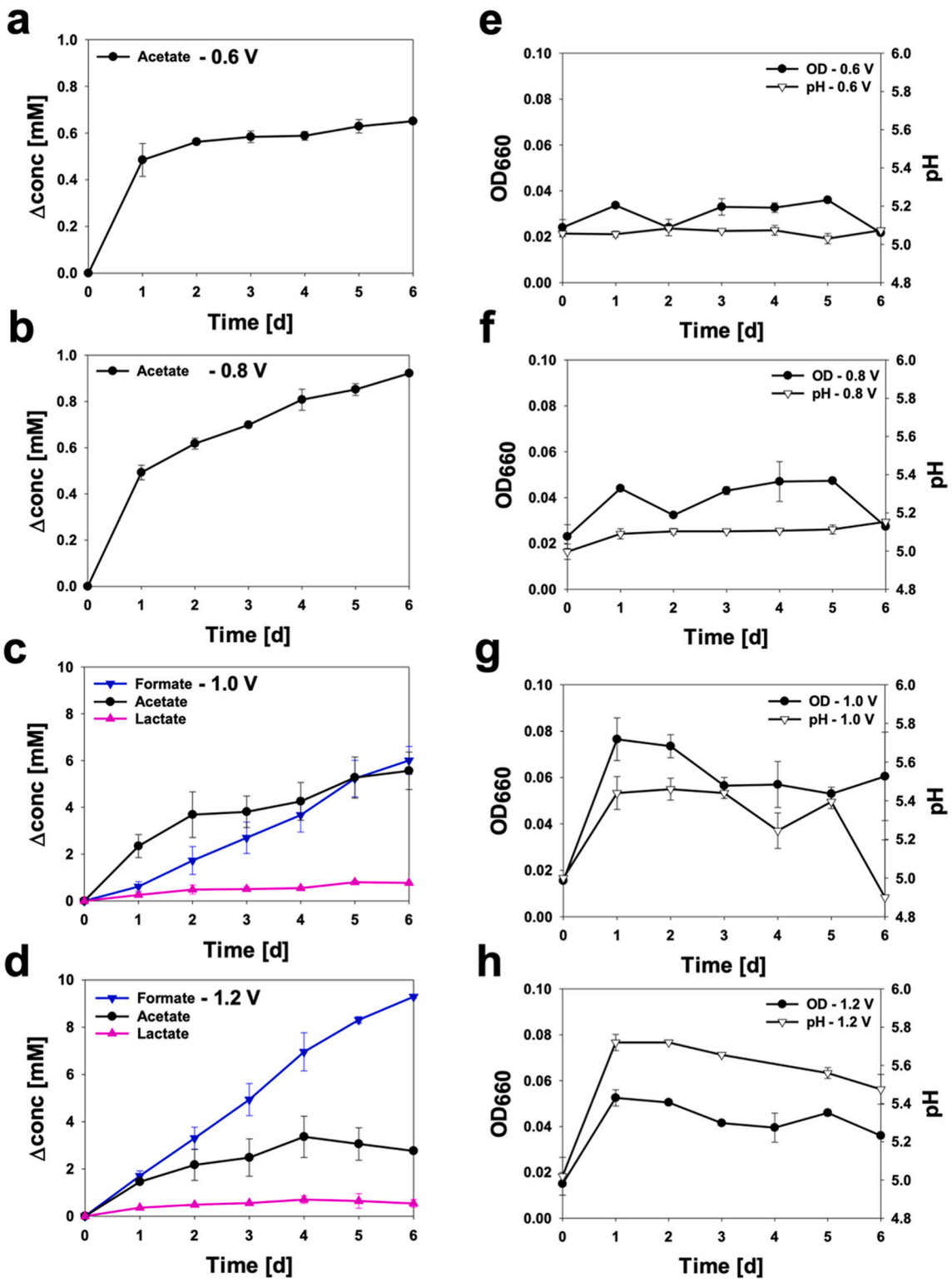

Fig. 3. Time-dependent accumulation of metabolites, pH, and OD_{660} in BES reactors operated at different CPs: (a, e) −0.6 V, (b, f) −0.8 V, (c, g) −1.0 V, and (d, h) −1.2 V. The medium was supplemented with 1 g/L yeast extract.

visible redox peak such as those noted by Masuda et al. (2010) were identified on the CV graphs of our BES cultures supplemented with yeast extract (Fig. 4). Except for the onset of CP being shifted upon addition of yeast extract, no significant difference was observed between CV values of cultures supplemented or not with yeast extract. Therefore, we concluded that yeast extract was not a source of exogenous electron mediators in BES reactors. Compared to abiotic cathodes, the onset of CP was similar in biotic cathodes and fresh medium supplemented with yeast extract, although a steeper current consumption was noted in the latter (Fig. 4). The maximum current at CPs ranging from −1.4 V to −1.2 V was greater for biotic cathodes than fresh medium alone, irrespective of yeast extract supplementation. This phenomenon could be explained by the beneficial effect of hydrogen-consuming *C. ljungdahlii* on H_2 evolution through a lower H_2 partial pressure on the cathode. As a

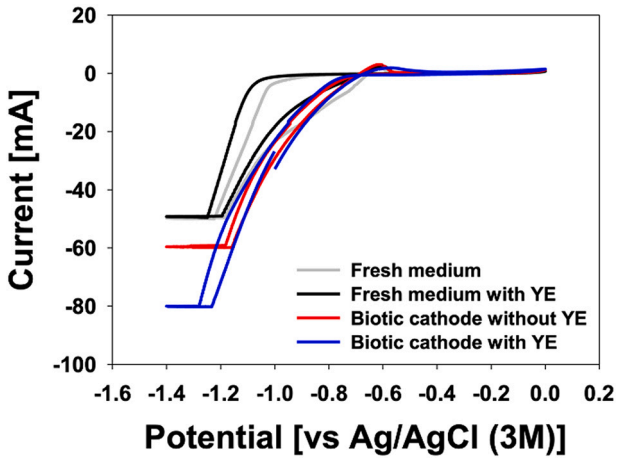

Fig. 4. Cyclic voltammogram of fresh medium and *C. ljungdahlii* cultures in a BES reactor supplemented or not with yeast extract (YE).

result, we suggest that *C. ljungdahlii* employs a hydrogen-mediated EET mechanism on the cathode.

Identifying the EET mechanism of a host strain in MES is important for further process design and operation, because EET determines the CE and productivity of MES (Jourdin et al., 2016). Previously, the involvement of EET in MES has been studied for *Sporomusa ovata* cultures and their cell-free spent medium by testing various CPs in BES reactors (Tremblay et al., 2019). Even though the study did not show all CEs at different CPs, H_2 was detected only in the proximity of the cathode surface at −0.9 V, leading to a CE of 75 % ± 3 % *S. ovata* is a model organism for MES using CO_2, and has displayed the best performance in among homoacetogens tested so far (Tremblay et al., 2019). This may explain why, unlike *C. scatologenes* in a BES reactor and *C. ljungdahlii* in this study, *S. ovata* showed high CE even at low CP (Aryal et al., 2017; Liu et al., 2018; Nevin et al., 2011).

If it is assumed that EET in a BES reactor containing *C. ljungdahlii* is mediated by H_2, it appears clear that H_2 availability determines the efficiency and productivity of such a system. Here, a more negative CP promoted current consumption (Fig. 4) and H_2 evolution, thus resulting in greater H_2 availability. H_2 evolution should be controlled tightly to optimise its availability and utilisation rate in a BES reactor. CE represents the ratio of H_2 utilisation vs. H_2 evolution on the cathode (technically, the ratio of electrons recovered in products to electrons supplied from an electrode) in a system. In this study, we noted a decreasing CE as CP decreased from −0.6 V to −1.2 V (Table 1). In contrast, productivity increased as the CP decreased to −1.0 V CP. At CPs of −1.0 V and −1.2 V, CE was below 100 %. This means that part of the produced H_2 was not fully utilised by *C. ljungdahlii* but was flushed away by continuous gas sparging. At a CP of −0.8 V or −1.0 V, the CE was close to 100 % which is the optimal scenario. Accordingly, the optimal CP would range between −0.8 V and −1.0 V.

Recently, attempts of MES using *C. ljungdahlii* mainly focused on testing new electrode materials. These studies highlight the importance of intermediates (H_2 and CO) for microbial electrosynthesis with *C. ljungdahlii*. Wang et al., modified carbon felt with nickel phosphide (Ni—P) to increase hydrogen evolution reaction rate for metabolically engineered *C. ljungdahlii*, which can produce butyrate (Wang et al., 2020). Acetate and butyrate production was almost doubled, corresponding to increased current flow with slightly increased CE (from 72 % to 81.7 %). Syngas mediated microbial electrosynthesis was also tested using syngas fermenting *C. ljungdahlii* (Zhu et al., 2022). The authors incorporated cobalt phthalocyanine into planar 2D and porous 3D MES cathodes in order to convert CO_2 into CO. The 3D MES cathode achieved the most stable CO production and the acetate production rate from syngas mediated microbial electrosynthesis outperformed similar MES studies using *C. ljungdahlii* (Table 2) (Zhu et al., 2022).

3.5. Future application of *C. ljungdahlii* as a host strain in a BES

While the utilisation of *C. ljungdahlii* as a biocatalyst in MES still needs optimisation, this host organism provides several advantages, including the possibility for more diverse end products from autotrophic growth, the capability to use CO both as a carbon and energy source, as well as flexible substrate utilisation. Furthermore, several recently developed synthetic biology tools for engineering *Clostridium* spp., including CRISPR/Cas9, transposons, and phage serine integrase systems, could aid in the generation of new target strains (Canadas et al., 2019; Huang et al., 2016, 2019; Jin et al., 2020; Joseph et al., 2018; Philipps et al., 2019). Metabolic engineering could improve the performance of *C. ljungdahlii* in MES, and alter the range of products (Liu et al., 2020; Lo et al., 2020; Wang et al., 2020). For example, the first attempt of using metabolic engineering of *C. ljungdahlii* for a BES was to show its exoelectrogenic property (Han et al., 2016). Formate dehydrogenase was overexpressed in *C. ljungdahlii* for NADH regeneration from formate, and the voltage of a sodium formate fed microbial fuel cell with engineered *C. ljungdahlii* was increased 3.8 times compared to the parent strain (Han et al., 2016). The other MES study using engineered *C. ljungdahlii* introduced the butyrate production pathway from *C. acetobutylicum* into *C. ljungdahlii* (Huang et al., 2019; Wang et al., 2020). Acetate production of butyrate-producing engineered *C. ljungdahlii* was increased approximately 3 and 2-fold at −0.6 V and −1.05 V, respectively, compared to the wild type (Wang et al., 2020).

C. ljungdahlii is a type-strain for syngas fermentation and its autotrophic growth and metabolite production have been extensively studied (Whitham et al., 2016; Nagarajan et al., 2013). Utilisation of syngas as a raw material can be expected to improve the economic feasibility of MES if coupled to the production of value-added chemicals (Table 2) (Zhu et al., 2022). CO oxidation by carbon monoxide dehydrogenase gives significant advantages to autotrophic acetogens. CO oxidation produces reduced ferredoxin, which is required for cascade reactions generating ATP, resulting in higher cell growth and ethanol yields from acetate via the AOR pathway (Richter et al., 2016b). Even though MES with CO-containing gas mixtures has been demonstrated successfully using mixed cultures and electron mediators, it is not yet economically feasible or sustainable (Im et al., 2018; Song et al., 2021). Addition of electron mediators is required to circumvent the inhibitory effect of CO on hydrogenases and/or extracellular electron transfer mechanisms (Im et al., 2018; Song et al., 2020, 2021). In the future, strain engineering and optimisation of the MES process is expected to pave the way for industrial application of *C. ljungdahlii* in MES.

In this study, the effect of yeast extract in the medium was tested for *C. ljungdahlii* in serum bottle cultures and a BES. *C. ljungdahlii* produced varying amounts of metabolites depending on the set-up, the main product being acetate. No ethanol production from CO_2 was detected during MES using *C. ljungdahlii*. However, previous study suggests that syngas as a substrate in a BES can improve carbon recovery in products, induce ethanol production and faster production rate during MES (Im et al., 2018; Zhu et al., 2022). Further research is required to integrate syngas fermentation and optimise microbial electrosynthesis for value-added chemical production. In order to achieve that, a strategy for how to perform stable MES long-term should be preceded.

4. Conclusions

Different MES set-ups for *C. ljungdahlii* were tested and the addition of yeast extract resulted in a robust performance. A starting pH of 5.0 resulted in higher acetate productivity than a pH of 5.7. Assessment of various CPs and the ensuing CEs identified the optimal CP as being between −0.8 V and −1.0 V. The highest acetate production was observed at −1.0 V. Based on CV measurements, we propose that *C. ljungdahlii* utilises a hydrogen-mediated EET mechanism when grown in a BES

Table 2
Comparison of performances of previous MES studies using *C. ljungdahlii* and this study.

Cathode potential (versus 3 M Ag/AgCl)	Strain used	Cathode material	Dimension of the electrode	Production rate (mM/d)	Coulombic efficiency	Temp	Set-up	Reference
−0.6 V	*C. ljungdahlii*	Graphite stick	1 × 3 × 0.5 in.	Acetate: ~0.013	88 ± 10 % (*n* = 3)	37 °C	H-cell	(Nevin et al., 2011)
−0.9 V	*C. ljungdahlii*	Graphite felts with stainless steel	5 × 3 cm^2	Acetate: 1st batch: 1, Acetate: 2nd batch: 0.2, Acetate: 3rd batch: -	~40 %	30 °C	H-cell	(Bajracharya et al., 2015)
−0.6 V	*C. ljungdahlii*	Ni-P_{15} coated carbon felt	50 × 50 × 5 mm	Acetate: 0.067	–	25 °C	H-cell	(Wang et al., 2020)
−1.05 V				Acetate: 0.749	–			
−0.6 V	$CLJU_{BAPP}$			Acetate: 0.200	–			
−1.05 V				Acetate 2.83, Butyrate: 1.13	81.7 % (*n* = 2)			
−1.05 V		Carbon felt		Acetate: 1.67, Ethanol: 0.30, Butyrate: 0.45	–			
−1.8 V	*C. ljungdahlii*	Cobalt phthalocyanine	D = 47 mm	Acetate: 33.30, Ethanol: 3.65	10.3 ± 8.7 H2 72.0 ± 3.3 CO 16.9 ± 5.2 acetate 3.2 ± 1.6 ethanol	37 °C	One-pot microbial electrosynthesis system (Continuous recirculation of each electrolyte), YTF medium	(Zhu et al., 2022)
−1.2 V			D = 3.8 cm, *t* = 0.3 cm	Acetate: 27.75, Ethanol: 12.21	42.9 ± 5.3 H2 27.9 ± 6.2 CO 30.4 ± 10.2 acetate 8.6 ± 7.6 ethanol			
−0.6 V	*C. ljungdahlii*	Graphite block	2.5 × 1 × 2.5 cm	0.11	2788 % (n = 3)	37 °C	H-cell	This study
−0.8 V				0.15	365 % (n = 3)			
−1.0 V				Acetate: 0.93, Formate: 1, Lactate: 0.13	18 % (n = 3)			
−1.2 V				Acetate: 0.34, Formate: 1.26, Lactate: 0.11	5 % (n = 3)			

reactor. The present results are expected to facilitate industrial production of value-added chemicals from gaseous substrates using a microbial BES.

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.biteb.2022.101156.

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