STEERING CO2 BIO-ELECTROCYCLING INTO VALUABLE COMPOUNDS THROUGH INLINE MONITORING OF KEY OPERATIONAL PARAMETERS

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DOCTORAL THESIS

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Doctoral Programme in Water Science and Technology

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PhD thesis submitted to aim for PhD degree for the University of Girona

Universitat de Girona

CERTIFICADO DE DIRECCIÓN DE TESIS

El Dr. Jesús Colprim Galcerán, la Dra. María Dolors Balaguer Condom y el Dr. Sebastià Puig Broch del "Laboratori d'Enginyeria Química i Ambiental" (LEQUIA) de la Universitat de Girona,

DECLARAMOS:

Que el trabajo titulado "*Steering CO_2 bio-electrorecycling into valuable compounds through inline monitoring of key operational parameters*", que presenta Ramiro Blasco Gómez para la obtención del título de doctor, se ha realizado bajo nuestra dirección y que cumple los requisitos para poder optar a Mención Internacional.

Y para que así conste y tenga los efectos oportunos, firmamos el presente documento.

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Asimismo, firma este certificado el candidato a doctor

Sr. Ramiro Blasco Gómez

Girona, 20 de diciembre de 2019.

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Sinceramente, nunca pensé en embarcarme en hacer una tesis doctoral, ni mucho menos llegar hasta aquí, pero la realidad es caprichosa y ahora me veo reflexionando sobre cuánto le debo a tantísimas personas.

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"Cultivemos la Ciencia por sí misma, sin considerar por el momento las aplicaciones. Éstas llegan siempre, a veces tardan años; a veces, siglos."

"For the present, let us cultivate science for its own sake, without considering its applications. They will always come, whether in years or perhaps even in centuries."

– Santiago Ramón y Cajal

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List of Acronyms

BES	Bioelectrochemical system
CCS	Carbon capture and storage
CCU	Carbon capture and utilization
CO_2	Carbon dioxide
CO	Carbon monoxide
CODH	Carbon monoxide dehydrogenase
CA	Chronoamperometry
CE	Coulombic efficiency
Ce	Counter electrode
CEM	Cation exchange membrane
CV	Cyclic voltammetry
DPV	Differential pulse voltammetry
DET	Direct electron transfer
DIET	Direct interspecies electron transfer
EET	Extracellular electron transfer
Fd	Ferrodoxine
FID	Flame ionization detector
GC	Gas chromatograph
GHG	Greenhouse gas
HRT	Hydraulic retention time
H_2	Hydrogen
H2ase	Hydrogenase
LCA	Life cycle analysis
LSV	Linear sweep voltammetry

MedET	Mediated electron transfer
MIET	Mediated interspecies electron transfer
MDCs	Microbial desalination cell
MET	Microbial electrochemical technology
MEC	Microbial electrolysis cell
MES	Microbial electrosynthesis
MFC	Microbial fuel cells
mMC	Milimolar of C
NADH	Nicotinamide adenine dinucleotide
S_{obs}	Observed diversity
OTU	Operational taxonomic unit
OD	Optical density
OM	Organic matter
pH_2	Partial pressure of hydrogen
pCO_2	Partial pressure of hydrogen
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RE	Reference electrode
SCCA	Short-chain carboxylic acid
SHE	Standard hydrogen electrode
TRL	Technology readiness level
TCD	Thermal conductivity detector
VFA	Volatile fatty acid
WLP	Wood-Ljungdahl pathway
WE	Working electrode

Publications and Communications

Peer reviewed publications that are presented as chapters of this Ph.D. thesis are listed below:

- **Blasco-Gómez, R.**, Ramió-Pujol, S., Bañeras, L., Colprim, J., Balaguer, M. D., and Puig, S. (2019). Unravelling the factors that influence the bio-electrorecycling of carbon dioxide towards biofuels. Green Chemistry, 21, 684–691. Q1 (IF: 9.405). Citations: 2 (Dec. 2019).

- **Blasco-Gómez, R.**, Romans, M., Bolognesi, S., Perona-Vico, E., Colprim, J., Balaguer, M. D., Bañeras, L., Puig, S. (2019). Steering the bio-electrorecycling of carbon dioxide by monitoring key operational parameters and implementing new feeding and inoculation strategies. (*Prepared for submission*)

Summary

Global warming is caused in a large extend by CO_2 emitted by human activities based on burning fossil fuels. Once reached this critical point, the largest emitter countries agreed on decreasing emissions. Therefore, to develop efficient technologies to sustainably produce carbon-neutral (bio)fuels is highly necessary in order to mitigate CO_2 effect in the environment.

CO_2 emissions are nowadays decreasing by the use of novel carbon capture and storage technologies (e.g. pumping it into geological storages). However, they are not fully developed and, at the same time, different CO_2 transformation technologies, such as chemical, photochemical, electrochemical, biological or inorganic, are being investigated. In this sense, microbial electrochemical technologies (METs) represents a novel promising approach to uptake and reduce the CO_2 in-situ using renewable electricity, in a process known as microbial electrosynthesis (MES). Here, microorganisms grown on electrodes under autotrophic conditions use the CO_2 as electron acceptor and electrons provided by an electrode in the form of electricity. During the process, different compounds are produced depending on the metabolic possibilities of the microorganisms present in the system. This work aims to use an enriched culture of selected electroactive microorganisms to evaluate how to steer the CO_2 transformation into high added-value compounds. Thereby, current knowledge gaps existing regarding the steering of this technology are filled.

This Ph.D. Thesis investigated reliable operational procedures for the monitoring of the performances of METs to produce suitable substrates for economically viable downstream applications. The cathodes of two different designs of bioelectrochemical systems (BESs) were inoculated with an enriched culture of a

carboxydotrophic strain and were operated until stable conversion of CO_2 into acetate, ethanol and small amounts of butyrate.

Tubular BES achieved a concomitant production of ethanol and acetate, which were considered crucial for triggering the production of longer carbon chain carboxylates and alcohols in, for example, a coupled chain elongation bioreactor. On the other hand, flat-plate BES showed constant acetate production and showed high resilience and robustness to unexpected operational episodes. In addition, coulombic efficiencies and overall production rates were higher in the flat-plate design, which suggests the need to improve the manoeuvrability by setting threshold values of key parameters that switch between target metabolic pathways. Moreover, improving the reactor design, mass transport limitation, together with reaching a high maturity of the electroactive community must be considered crucial to obtain more reduced compounds from CO_2 and electricity.

Continuous *in-line* monitoring of key parameters (pH, CO_2 dissolved and partial pressure of hydrogen) revealed variations in the current signal and pH values that were correlated with CO_2 depletion and the transition from acetogenesis to solventogenesis in the enriched culture. In addition, new inoculation and feeding strategies, based on previous electrode enrichment with an electroactive biofilm and avoiding periods with low availability of reducing power, showed promising results that should be addressed in future research on CO_2 bio-electrorecycling.

Finally, this Ph.D. Thesis shows that *in-line* monitoring of pH and electron consumption are meaningful operational variables to differentiate between the carboxylate and alcohol production, which opens the door to develop new approaches to control the bio-electrorecycling of CO_2 into biofuels by METs.

Resumen

El CO_2 emitido por las actividades humanas basadas en la quema de combustibles fósiles es el causante, en gran medida, del calentamiento global del planeta. Las consecuencias de este calentamiento son imprevisibles, pero llegados a este punto crítico, y junto con los compromisos acordados por los países que más emiten, es acuciante desarrollar tecnologías eficientes para mitigar el efecto del CO_2 sobre el calentamiento global y producir combustibles renovables de forma sostenible.

Actualmente, existen nuevas tecnologías de captura y almacenamiento de carbono (p.ej. inyección en depósitos geológicos). Sin embargo, esta tecnología todavía no está completamente desarrollada y, paralelamente, se investigan diferentes tecnologías de transformación de CO_2 basadas en procesos químicos, fotoquímicos, electroquímicos, biológicos o inorgánicos. En este sentido, las tecnologías electroquímicas microbianas (MET; siglas en inglés) representan un enfoque novedoso y prometedor en la absorción y reducción del CO_2 *in-situ* utilizando energía eléctrica renovable mediante un proceso conocido como electrosíntesis microbiana (MES; siglas en inglés). Esta tecnología se basa en la utilización de microorganismos cultivados en electrodos en condiciones autotróficas en las cuales utilizan el CO_2 como aceptor de electrones y un electrodo que les proporciona los electrones en forma de electricidad. Durante este proceso, los microorganismos producen diferentes compuestos dependiendo de las posibilidades metabólicas de los mismos. Esta Tesis Doctoral tiene como objetivo utilizar un cultivo enriquecido de microorganismos electroactivos para evaluar e identificar los parámetros clave que permitan dirigir la transformación de CO_2 en compuestos diana de alto valor agregado. De esta

manera, se pretende avanzar en el conocimiento sobre cómo mejorar la maniobrabilidad de esta tecnología para dirigir los procesos biológicos implicados.

En esta tesis se han estudiado diferentes parámetros de operación que a través de un monitoreo exhaustivo durante la transformación del CO_2 mediante reactores MET. Se utilizaron dos diseños de reactor diferentes (tubular y plano) de sistemas bioelectroquímico (BES; siglas en inglés) que fueron inocularon con un cultivo enriquecido con una cepa carboxidotrófica electroactiva y se operaron hasta logar una electro-conversión estable del CO_2 en ácido acético, etanol y pequeñas cantidades de ácido butírico.

El BES tubular logró una producción concomitante de etanol y acetato, que es considerada clave para desencadenar la producción de carboxilatos y alcoholes de cadena de carbono más largas y complejas en fermentadores acoplados. Por otro lado, el BES de placa plana mostró una producción constante de acetato, así como una alta resiliencia ante episodios operativos inesperados. Además, tanto las eficiencias culómbicas como las tasas de producción generales fueron más altas en el diseño de placa plana. Esto sugiere la necesidad de mejorar su maniobrabilidad identificando los valores umbral de parámetros clave que actúan de interruptores para activar/inactivar las rutas metabólicas de interés. Además, un diseño de reactor más óptimo y una alta madurez de la comunidad electroactiva deben considerarse como aspectos cruciales para obtener compuestos más reducidos y con más valor de mercado a partir de CO_2 y electricidad.

El monitoreo en línea de los parámetros clave (pH, CO_2 disuelto y presión parcial de hidrógeno) reveló variaciones en la señal de demanda de corriente y valores de pH correlacionados con el agotamiento de CO_2 y la transición entre rutas

productoras de ácido acético a otras que resultan en la producción de etanol. Además, se desarrollaron nuevas estrategias de inoculación y alimentación del reactor, basadas en el enriquecimiento previo de electrodos con un biofilm electroactivo y evitando períodos con baja disponibilidad de poder reductor, que mostraron resultados prometedores y que deberían abordarse en futuras investigaciones sobre la conversión bioelectroquímica de CO_2.

Esta tesis destaca la importancia del monitoreo en línea del pH y consumo electrónico como variables operativas significativas para seleccionar entre la producción de carboxilatos y alcoholes, lo que abre la puerta para desarrollar nuevos enfoques que faciliten el control de las tecnologías electroquímicas microbianas durante la conversión bioelectroquímica de CO_2 en biocombustibles.

Resum

El CO_2 emès per les activitats humanes basades en la crema de combustibles fòssils és el causant, en gran mesura, de l'escalfament global del planeta. Les conseqüències d'aquest escalfament són imprevisibles, però arribats a aquest punt crític, i juntament amb els compromisos acordats pels països que més emeten, és urgent desenvolupar tecnologies eficients per mitigar l'efecte de el CO_2 sobre l'escalfament global i produir combustibles renovables de forma sostenible.

Actualment, hi ha noves tecnologies de captura i emmagatzematge de carboni (per exemple injecció en dipòsits geològics). No obstant això, aquesta tecnologia encara no està completament desenvolupada i, paral·lelament, s'investiguen diferents tecnologies de transformació de CO_2 basades en processos químics, fotoquímics, electroquímics, biològics o inorgànics. En aquest sentit, les tecnologies electroquímiques microbianes (MET; sigles en anglès) representen un enfocament nou i prometedor en l'absorció i reducció de el CO_2 *in-situ*, utilitzant energia elèctrica renovable mitjançant un procés conegut com electrosíntesi microbiana (MES; sigles en anglès). Aquesta tecnologia es basa en la utilització de microorganismes cultivats en elèctrodes en condicions autotròfiques a les quals utilitzen el CO_2 com a acceptor d'electrons i un elèctrode que els proporciona els electrons en forma d'electricitat. Durant aquest procés, els microorganismes produeixen diferents compostos depenent de les possibilitats metabòliques dels mateixos. Aquesta tesi doctoral té com a objectiu utilitzar un cultiu enriquit de microorganismes electroactius per avaluar i identificar els paràmetres clau que permetin dirigir la transformació de CO_2 en compostos diana d'alt valor afegit. D'aquesta manera, es pretén avançar en el coneixement sobre com millorar la

maniobrabilitat d'aquesta tecnologia per a dirigir els processos biològics implicats.

En aquesta tesi s'han estudiat diferents paràmetres d'operació que a través d'un monitoratge exhaustiu durant la transformació de l'CO_2 mitjançant reactors MET. Es van utilitzar dos dissenys de reactor diferents (tubular i pla) de sistemes bioelectroquímics (BES; sigles en anglès) que van ser inoculats amb un cultiu enriquit amb una soca carboxidotrófica electroactiva i es van operar fins aconseguir l'electro-conversió estable de el CO_2 en àcid acètic, etanol i petites quantitats d'àcid butíric.

El BES tubular va aconseguir una producció concomitant d'etanol i acetat, que es considerada clau per desencadenar la producció de carboxilats i alcohols de cadena de carboni més llargues i complexes en fermentadors acoplats. D'altra banda, el BES de placa plana va mostrar una producció constant d'acetat, així com una alta resiliència davant d'episodis operatius inesperats. A més, tant les eficiències culómbicas com les taxes de producció generals van ser més altes en el disseny de placa plana. Això suggereix la necessitat de millorar la seva maniobrabilitat identificant els valors llindar de paràmetres clau que actuen d'interruptors per activar / inactivar les rutes metabòliques d'interès. A més, un disseny de reactor més òptim i una alta maduresa de la comunitat electroactiva, han de ser considerats com aspectes crucials per obtenir compostos més reduïts i amb més valor de mercat a partir de CO_2 i electricitat.

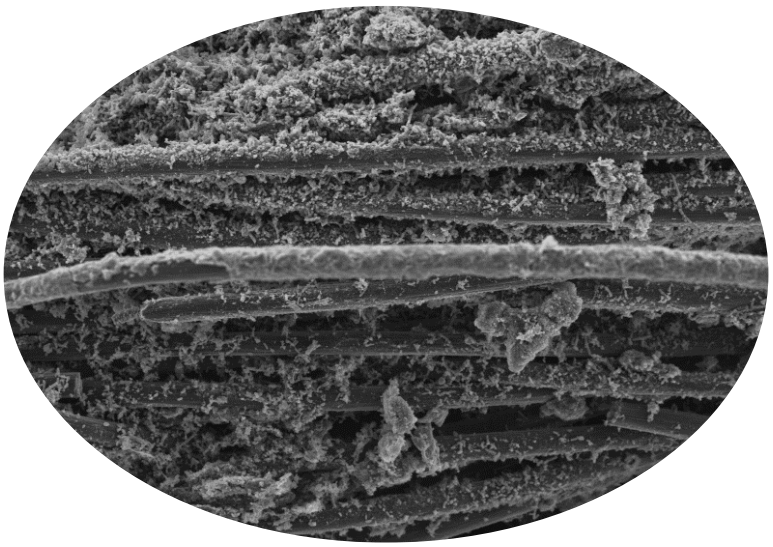
El monitoratge en línia dels paràmetres clau (pH, CO_2 dissolt i pressió parcial d'hidrogen) va revelar variacions en el senyal de demanda de corrent i valors de pH correlacionats amb l'esgotament de CO_2 i la transició entre rutes productores d'àcid acètic a d'altres que resulten en la producció d'etanol. A més, es van desenvolupar noves estratègies d'inoculació i alimentació del reactor, basades en

l'enriquiment previ d'elèctrodes amb un biofilm electroactiu i evitant períodes amb baixa disponibilitat de poder reductor, que van mostrar resultats prometedors i que s'haurien d'abordar en futures investigacions sobre la conversió bioelectroquímica de CO_2.

Aquesta tesi destaca la importància de la monitorització en línia de l'pH i consum electrònic com a variables operatives significatives per seleccionar de la producció de carboxilats i alcohols, el que obre la porta per desenvolupar nous enfocaments que facilitin el control de les tecnologies electroquímiques microbianes durant la conversió bioelectroquímica de CO_2 en biocombustibles.

Chapter 1

INTRODUCTION



1.1 Background

The rise of atmospheric concentration of carbon dioxide (CO_2) from 280 to over 400 ppm caused by the emission of anthropogenic CO_2 is considered a real threat to ecosystems worldwide (IPCC 2019; Sala et al. 2000). CO_2 is considered a greenhouse gas (GHG) responsible of many environmental issues such as the increase of earth temperature and ocean acidification (Doney et al. 2009). Among the strategies proposed to mitigate this GHG's emissions are (i) reduce global energy based on fossil fuels, (ii) capture and storage of CO_2 emissions and (iii) develop carbon-neutral fuels (Lal 2008).

Despite of the huge efforts to cut off the emissions by establishing a global regulatory framework, the results demonstrate that these policies are not being effective and an alternative is urgent. One approach might be given by new technologies that are yet to be commercialized that would help to mitigate global change (Williams et al. 2012).

Up to date, those technical solutions have been mainly focused on carbon capture and storage (CCS) (Figure 1), an alternative based on the collection, compression, transport and storage of CO_2 in geological deposits (e.g. depleted oil and gas fields, deep coal seams and saline formations). Unfortunately, the process is not yet economically feasible and current research efforts are targeting the reduction of the cost. Besides, the significant public opposition and concern about the risk for the environment and human health of short-term and long-term leakages might represent a major drawback (van Alphen et al. 2007).

On the other hand, carbon capture and utilization (CCU) aims to convert the CO_2 through several chemical processes (e.g. photochemical, electrochemical,

biochemical and microwave-assisted) which include the use of solid adsorbents (e.g. membranes) and biological methods (Thakur et al. 2018; Choi, Drese, and Jones 2009).

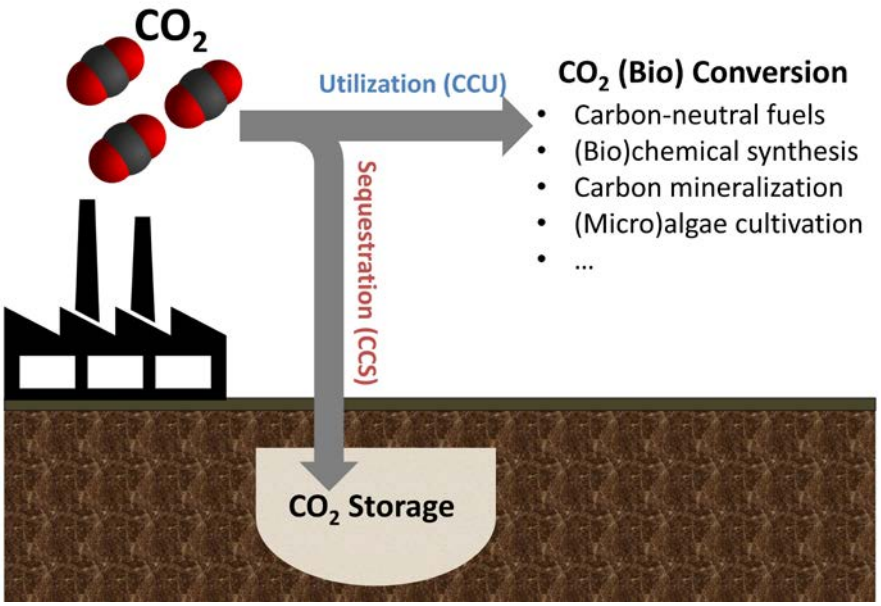


Figure 1. Scheme of the current strategies to reduce the atmospheric CO_2 concentration.

In the last few decades, many CCU technologies (e.g. chemical, photochemical, electrochemical, biological, reforming, and inorganic transformations) which use CO_2 for the generation of so-called carbon-neutral fuels are at the research and development stage (Mikkelsen, Jørgensen, and Krebs 2010). They range from those nearing commercialization, such as electrocatalytic reduction, to technologies being explored in a lab-scale, such as catalytic, photocatalytic, CO_2 polymerization, and biohybrids, to those only now being imagined, such as molecular machine technologies (Bushuyev et al. 2018). Among those technologies, the ones based on the chemical and biological CO_2 transformation into a wide range of chemicals compounds are considered very promising

(Jajesniak, Ali, and Wong 2014; Jouny, Hutchings, and Jiao 2019). Notwithstanding they require an energy source that could come from renewable energy sources (e.g. wind or solar) and therefore meeting the carbon neutrality of its compounds, since they would not imply GHG emissions. In fact, from the applicability point of view, this approach has the potential to (i) mitigate CO_2 emissions, (ii) transform it into valuable compounds, and (iii) serve as storage of renewable electricity in liquid or gas compounds. The nature of the products made by CO_2 and renewable electricity make it easy to store, transport or use when the availability of energy is limited. However, there are still limiting factors such as extremely large surface and volumes, energy intense processing steps and/or chemicals and expensive catalysts, which compromise their economical and the technological feasibility (Haszeldine 2009).

Biological CO_2 mitigation is based on the capability of some CO_2 fixing microorganism (e.g. *algae*, *cyanobacteria*, *β-proteobacteria*, *Clostridia* and *Archaea*) to convert CO_2 into organic compounds through different metabolic pathways (Jajesniak, Ali, and Wong 2014). In this sense, photosynthesis and the *Wood-Ljungdahl* (WLP) are considered the most ancient carbon fixation pathways. The second one is used by homoacetogenic bacteria or acetogens, also referred to as the reductive acetyl-CoA pathway (Martin 2012). The main and most interesting advantage of biological CO_2 utilization is that CO_2 is converted into biomass and marketable compounds such as bio-diesel, biofuels or their precursors and other commodity chemicals of high added-value.

1.1.1 Energy concerns during biological CO_2 conversion

The biological production of carbon-neutral chemical compounds from CO_2 requires the use of energy, which could potentially come from renewable sources. On the one hand, plants carry out photoautotrophic growth to convert CO_2 and water into biomass by means of sunlight as power source. This process is known as photosynthesis and its conversion efficiency reaches the 4.5% as maximum (Barber and Tran 2013). The biomass generated can be used for different purposes such as the production of the first generation biofuels, which are limited by the competition for land and water used for food production (Sims et al. 2010). Notwithstanding, photosynthetic microalgae growth for biofuel production is considered a promising bioenergy field with huge potential (Chisti 2007). However, this technology needs large surface areas for growing and high energy consumption for harvesting, which are its major drawbacks so far.

On the other hand, the use of acetogenic bacteria to accomplish CO_2 reduction through the WLP is nowadays considered as an interesting alternative to the abovementioned options. This biological conversion can be driven in bioelectrochemical system (BES) by using suitable microorganisms as biocatalyst and electricity as reducing power to produce carbon-neutral commodities and/or fuels. This term was firstly coined in 2010 as microbial electrosynthesis (MES) and represents a sustainable alternative to similar existing processes (Nevin et al. 2010; Rabaey and Rozendal 2010). In fact, MES could overtake conventional photosynthesis in terms of energy efficiency when renewable energy such as solar power drives the microbial CO_2 conversion (Nichols et al. 2015; Nangle et al. 2017; Liu et al. 2017; H. M. Woo 2017).

1.2 Microbial electrochemical technologies

Microbial electrochemical technologies (METs) are a promising technological platform for different applications in environmental and industrial biotechnology (Schröder, Harnisch, and Angenent 2015). MET are based on extracellular electron transfer (EET) processes carried out by microorganisms capable to interact with electrodes (Berk and Canfield 1964; Hill and Higgins 1981). Even though these species were firstly reported in 1910 (Potter 1910), their potential applications were identified and developed at a laboratory level 100 years later (Arends and Verstraete 2012; Schröder 2011). Overall, the scope of METs is broad, ranging from bioelectricity generation, bioremediation, fermentation and the production of chemicals.

The basis of a MET reactor is an anode and a cathode separated by an ion exchange membrane (Figure 2). On the one hand, oxidation processes occur in the anode where electrons are delivered to the electrode and protons are released to the medium. These protons diffuse through the semipermeable membrane to the cathode compartment and the equivalent negative charge flows towards the cathode through an external electric circuit.

On the other hand, protons transferred from the anode serve as reducing equivalents in the cathode to carry out reduction processes. When living organisms contribute in the catalytic processes, both chambers are called bioanode and biocathode respectively.

Depending on the application, microorganisms performing some redox reaction can be placed in one or both chambers (Rabaey et al. 2009). The broad amount

of processes studied in BES might be classified in three different groups or concepts: energy, product, and sustainability (Arends and Verstraete 2012).

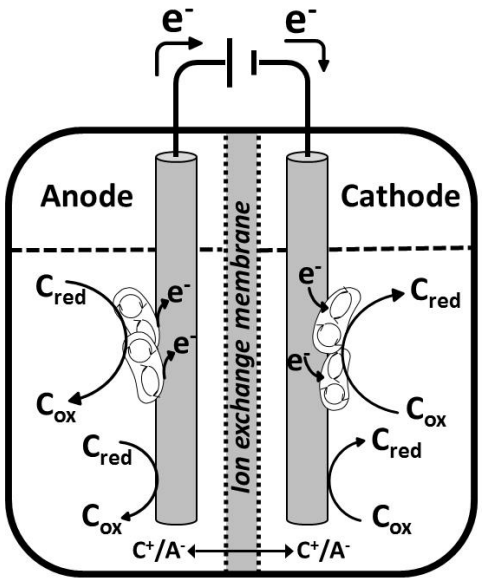


Figure 2. Schematic representation of the reactions taking place in a bioelectrochemical system.

1.2.1 Thermodynamics of METs

Thermodynamics in BES are determined by the redox potential of the reactions taking place in the anode and the cathode. Half-cell potentials of both chambers are reported using the standard hydrogen electrode (SHE) potential as reference, which has a potential of zero at standard conditions. The theoretical half-cell potentials are calculated using equation 1.1 (Logan et al. 2006).

$$\mathrm{E_{An}} = \mathrm{E^{0}_{An}} - \frac{\mathrm{RT}}{\mathrm{nF}} \ln\left(\frac{[\mathrm{products}]^{\mathrm{p}}}{[\mathrm{reactants}]^{\mathrm{r}}}\right) \qquad \text{(Eq. 1.1.)}$$

Where E_{An} is the half-cell potential of the anode (V), E_{an}^{0} is the half-cell potential (V) at standard conditions, R represents the universal gas constant (8.31 J mol^{-1} K^{-1}), T is the temperature in Kelvin, n represents the number of electron taking part in the overall process and F is the Faraday's constant (95485 C mol e^{-1}). The quotient is the ratio of products concentration divided by reactants concentration, raised to their respective stoichiometric coefficients (p, r). The calculation of the cathode potential (E_{Cat}) should be calculated using the same equation.

The overall cell potential (E_{cell}) determines the process spontaneity, which is calculated according to equations 1.2 and 1.3.

$$\mathrm{E_{cell}} = \mathrm{E_{cathode}} - \mathrm{E_{anode}} \qquad \text{(Eq. 1.2)}$$

$$\Delta \mathrm{G} = -\mathrm{n} \cdot \mathrm{F} \cdot \mathrm{E_{cell}} \qquad \text{(Eq. 1.3)}$$

Where E_{cell} corresponds to the cell voltage (V), E_{Cat} and E_{An} are the half-cell potentials (V) for cathode and anode respectively. ΔG corresponds to the Gibbs free energy (J) of the overall process.

If the resulting Gibbs free energy is a negative value, the process is spontaneous; energy will be produced in the form of electricity, as in the well-known microbial fuel cells (MFC). On the contrary, if the ΔG value is positive, the process will not take place spontaneously, which means that the process would require an external energy supply to be triggered. These systems are known as microbial electrolysis cells (MEC) (Logan and Rabaey 2012). Table 1 presents the standard reactions of a typical BES, where the spontaneity of the process relies on the cell potential. Gibbs free energy depends directly on the operation parameters (e.g. pH, temperature, pressure, internal resistances, etc.). Reactions theoretically spontaneous might turn non-spontaneous under different experimental

conditions. This is the main reason of operating BES under either a poised cathode/anode potential (potentiostatic mode), or a fixed current supply (galvanostatic mode) in order to carry out the processes of interest.

Table 1. Thermodynamic spontaneity calculation of different processes in BES, under standard conditions (T= 298 K; P= 1 atm; pH= 7). Data obtained from Rabaey and Rozendal, 2010; Thauer et al., 1977.

Anode reaction	E^0_{an}	Cathode reaction	E^0_{cat}	E_{cell}	electrons involved	ΔG^0 (kJ)
Degradation of acetate	-0.28	Reduction of oxygen	0.82	1.10	2	-417
		Nitrate reduction to nitrite	0.42	0.7	2	-108
		Reduction of H^+ to H_2	-0.41	-0.69	2	533
		Reduction of CO_2 to methane	-0.24	-0.52	8	401
		Reduction of CO_2 to acetate	-0.28	-0.56	8	432
Water oxidation	0.82	Nitrate reduction to nitrite	0.42	-0.40	2	77
		Reduction of H^+ to H_2	-0.41	-1.23	2	237
		Reduction of CO_2 to methane	-0.24	-1.06	8	818
		Reduction of CO_2 to acetate	-0.28	-1.10	8	849

1.2.2 Applications of METS

Up to date, the most known application of METs has been electricity generation from wastewater treatment in a thermodynamically spontaneous process using MFCs (Bond and Lovley 2003; Rabaey et al. 2003; Chaudhuri and Lovley 2003). Here, the biodegradation of organic matter in the bioanode is coupled to oxygen reduction in the cathode, since oxygen is the most suitable electron acceptor in MFCs (Logan et al. 2006). In fact, different types of wastewater such as domestic wastewater (Ahn and Logan 2013), industrial and agriculture wastewater (Vilajeliu-Pons et al. 2016; Rabaey et al. 2005), and landfill leachate (Puig et al. 2011) have been used to produce electricity at bioanodes (Sleutels et al. 2012).

Moreover, the range of potential applications of BES has been recently expanded (Logan and Rabaey 2012). Apart from electricity generation, applications such as the production of chemicals through MECs and MES, water recycling with microbial desalination cells (MDCs), analytical application (e.g. microbial biosensors and Biocomputing) or remediation of contaminated sites (C. Koch et al. 2016) are nowadays under study using METs. In addition, the inclusion of METs in existing wastewater treatment plants to take advantage of the previously mentioned applications looks nowadays promising (Osset-Álvarez et al. 2019; Batlle-Vilanova et al. 2019).

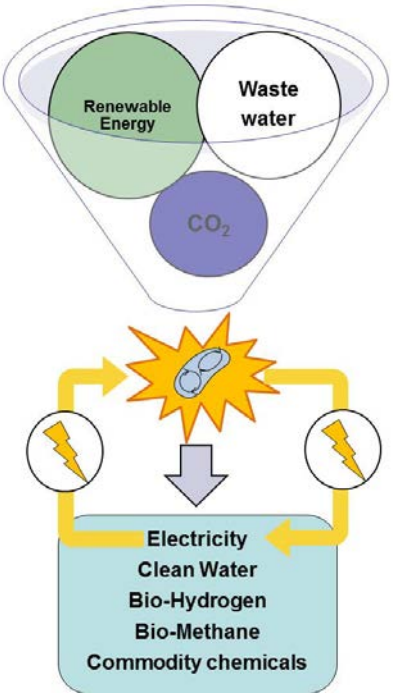


Figure 3. Scheme of substrates used and products obtained with METs.

The reduction of oxidized compounds into chemicals in the cathode chamber might be either biotical or abiotically induced. The overall cell potential may be negative, which thermodynamically is unfavourable and requires the addition of external energy. Anodic acetate oxidation coupled to chemical hydrogen (H_2) evolution in the cathode has been pointed out as a plausible solution to produce fuels (H_2) in the cathode while acetate is oxidized in the anode by electroactive microorganisms (Selembo, Merrill, and Logan 2010; Ambler and Logan 2011; Carmona-Martínez et al. 2015). However, the expensive catalysts required (e.g. nickel, platinum), inefficiency and lack of sustainability leaded to the search of an alternative: electroactive microorganisms. This perfect candidate to alternative cathode catalyst had a low cost, self-regeneration capability and broad metabolism diversity that would allow the removal of harmful and unwanted

compounds or the production of marketable compounds (He and Angenent 2006).

1.3 Biocathodes

Some microorganisms called electrotrophs are capable of up-taking electrons from an electrode that works as energy source. The reducing power provided by the electrode drives interesting metabolic pathways that reduce oxidized compounds into marketable target molecules. Biocathodes are a potential biotechnology platform for industry due to the capability of biocathodic communities to interact with the electrode, grow under cathodic conditions and use the reducing potential to carry out reactions of industrial interest. The interaction microorganism-electrode can be direct or indirect (Figure 4). There is a wide range of possible reductive reactions that allows either removal or production of target compounds (Logan and Rabaey 2012; Jain and He 2018). These reactions are performed by the biocatalysts that interact with the electrode (electron donor) in many ways. Indeed, biocathodes and their potential new applications have attracted an increasing scientific attention in the last ten years (Figure 7). An approach to improve and better understand the interactions between the electrode and the electroactive microorganisms include the manipulation of the electrode in terms of chemical and topographical features (Guo et al. 2015).

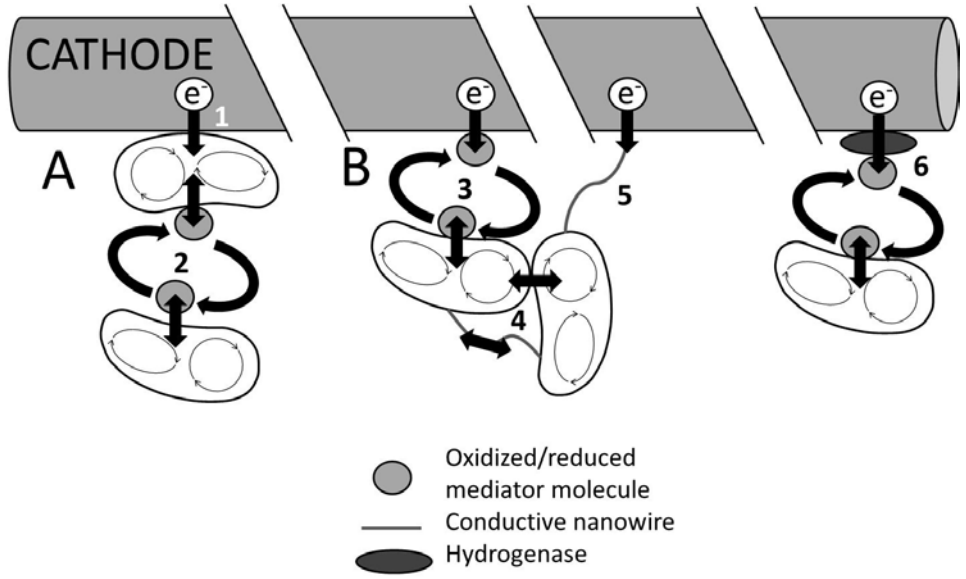


Figure 4. Electron transfer mechanisms between the cathodic electrode and the microorganisms. A. Direct electron uptake. B. Indirect electron uptake. 1. Direct electron transfer (DET). 2. Mediated interspecies electron transfer (MIET). 3. Mediated electron transfer (MedET). 4. Direct interspecies electron transfer (DIET). 5. DET via nanowires. 6. MedET via hydrogenase enzymatic activity.

Biocathodes were firstly used in MFCs to improve oxygen reduction process during electricity production (Clauwaert et al. 2007). Although, its biotechnological potential has been broadened through new applications such as (i) contaminant removal or (ii) production of target chemical compounds. Moreover, biocathodes have been used to decontaminate water (Narcís Pous et al. 2017) with high levels of nitrate (Narcís Pous et al. 2013; Narcis Pous et al. 2015; Virdis et al. 2010), perchlorate (Butler et al. 2010; Mieseler et al. 2013; J. J. Li et al. 2015), sulphate (Coma et al. 2013) or metals (Nancharaiah, Venkata Mohan, and Lens 2015).

Biocathodes have also been used to obtain chemical compounds using protons or CO_2 as electron acceptors to obtain hydrogen or building blocks respectively

(de Vrieze et al. 2020). Recently, photosynthetic biocathodes are considered an interesting alternative for CO_2 bio-electro recycling and biomass production (Sevda et al. 2019). These biocathodes take advantage of the capability of some photosynthetic microorganisms to uptake energy from the sun and either collect it as a capacitor (Wu et al. 2013) or donate the reducing power to other microorganisms through DIET. The sustainable energy production could be coupled to industrial effluent treatment (Luo et al. 2017; Xiao and He 2014). Microalgae placed in the cathode might reduce CO_2, supply oxygen, treat wastewater and lead to biomass production. However, further research must be conducted regarding seasonal variations, rigorous life cycle analysis (LCA), potential microalgae strains and adapt the electrode surface area to the special characteristics of the photosynthetic biocatalysts.

1.3.1 Electrotrophic microorganisms

A vast number of microorganisms can interact with conductive materials that are in their surroundings. These species are called electroactive microorganisms and are used in different types of bioelectrochemical systems. The ones that donate electrons are called exoelectrogens and are typically iron-reducing bacteria (e.g. Geobacter sulfurreducens) that produce high power densities at moderate temperatures. Also, under proper media and growth conditions, common yeasts to extremophiles such as hyperthermophilic archaea can also generate high current densities. On the other hand, microorganisms that can consume electrical current and uptake electrons from cathodes (electrotrophs) are less diverse than exoelectrogens. Electrotrophs can use diverse terminal electron acceptors for cell respiration, including carbon dioxide, enabling a variety of novel cathode-driven reactions (Logan et al. 2019). Unfortunately, monitoring the exchange of electrons is nowadays a challenge, as there is not any available method to measure directly

the current transport between microorganisms. Examples of electrotrophs are nitrate reducers (e.g. *Geobacter metallireducens*) (Gregory, Bond, and Lovley 2004), sulphate reducers (e.g. *Desulfovibrio spp.*), oxygen reducers (e.g. *Klebsiella pneumoniae*) (Rhoads, Beyenal, and Lewandowski 2005), metal reducers (Nancharaiah, Venkata Mohan, and Lens 2015), hydrogen producing bacteria (e.g. *Desulfovibrio spp.*) (Aulenta et al. 2012), carbon dioxide reducers (e.g. *Sporomusa spp*. and *Clostridium spp.*) and archaea (e.g. *Methanobacterium spp.*) that produce methane from carbon dioxide (Perona-Vico et al. 2019; Saheb-Alam et al. 2019).

Electrotrophic microorganisms are known to be less diverse than exoelectrogenic and to have no common or prototypical traits (Logan et al. 2019) Among them there is an interesting group called "weak electricigens" (Doyle and Marsili 2018), which corresponds to microorganisms considered electroactive, either through their own mechanisms or exogenously-added mediators, that produce/consume weak current. They are always present in open cultures communities and might have a role related to syntrophic relationship between electrotrophs (see Figure 4, interaction number 4). These symbiotic relationships are highly beneficial partnerships from the industrial point of view, since they improve yields and therefore bring this technology closer to future real application. In this sense, genetic engineering and synthetic biology are potential platforms to increase the electron uptake efficiencies and improve the competitiveness of future technologies based on that phenomenon.

1.4 CO_2 Bio-electrorecycling

MET has become a promising platform to produce industrially relevant organic compounds from CO_2 by homoacetogenic bacteria (Figure 5). These bio-products (e.g. bio-methane, bio-hydrogen, carboxylates and alcohols) are an alternative to current fossil-fuel-derived commodities (Figure 6).

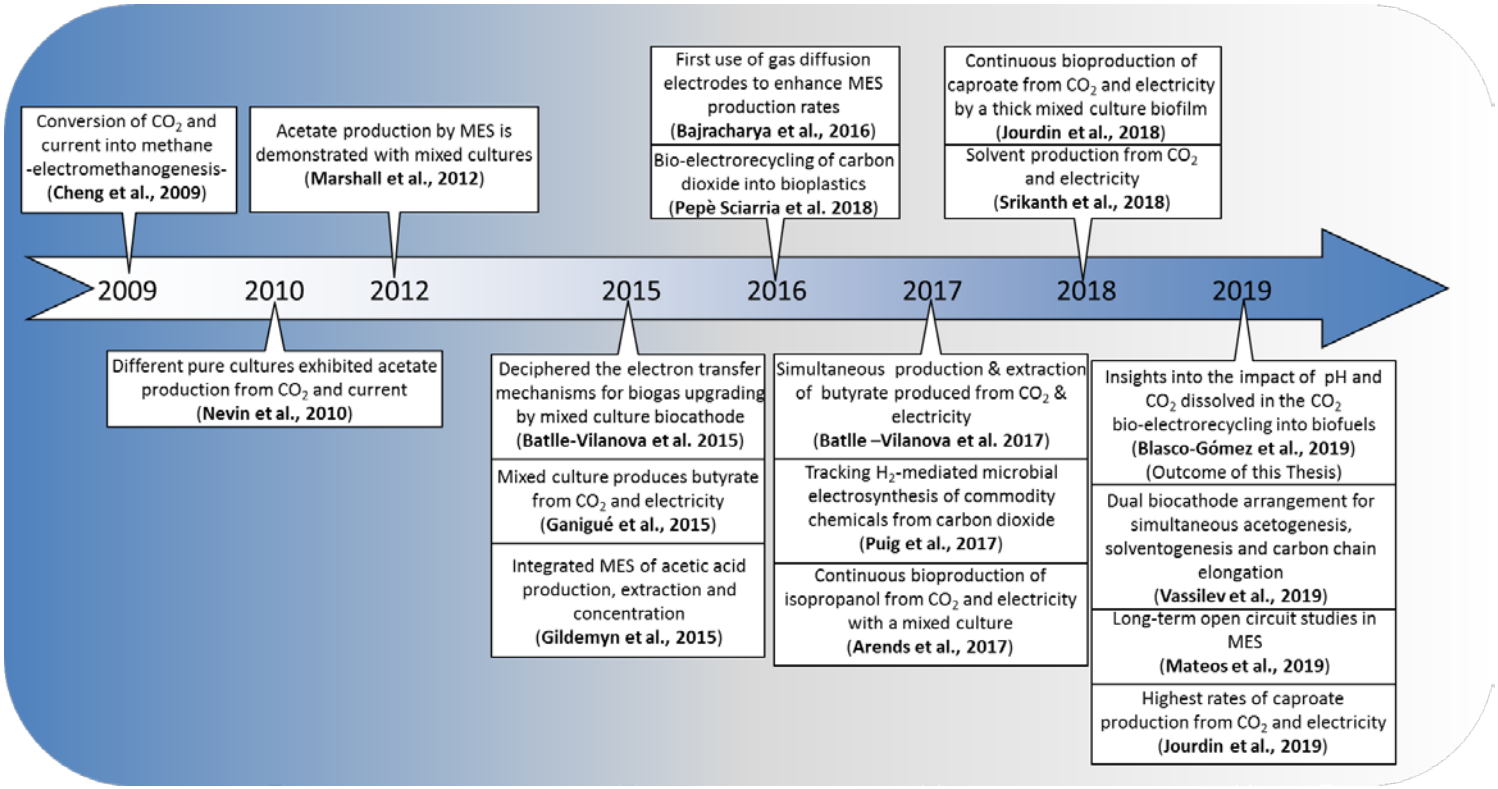


Figure 5. Timeline showing some of the most important achievements in CO_2 bio-electrorecycling throughout the history.

In 2009 scientist proved that an external supply of electron can trigger biochemical reactions or steer them to target products. That year, Cheng and colleagues investigated the bioelectrochemical production of methane from the biotic reduction of CO_2 with H_2 biotically and abiotically provided in the surface of the cathode (Cheng et al. 2009). The effect of the cathode potential (Villano et al. 2010), other operational parameters, production mechanisms and microorganisms involved were consequently studied (Van Eerten-Jansen et al. 2012, 2015; Van Eerten-Jansen, Veldhoen, et al. 2013). This process is commonly known as electro-methanogenesis (Blasco-Gómez et al. 2017).

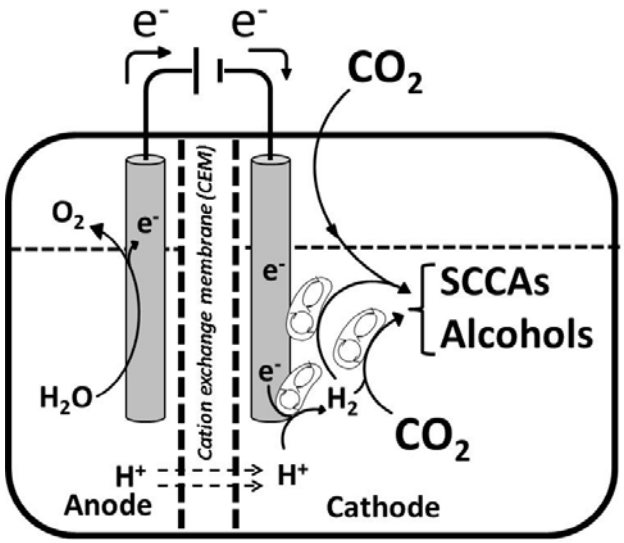


Figure 6. Schematic representation of a bioelectrochemical system that converts CO_2 towards short-chain carboxylic acids (SCCAs) and alcohols.

One year later (2011) Nevin and co-workers provided electrons and CO_2 to the homoacetogen *Sporomusa ovata* grown at the cathode of a BES that was used as biocatalysts for synthesize acetate (Nevin et al. 2010). This was the first attempt to bioelectrochemically produce extracellular multicarbon compounds with METs.

Later on, Nevin et al. showed the ability of other acetogens that were also capable to drive the process (Nevin et al. 2011).

As an alternative, open cultures were also used as biocatalysts for CO_2 electro-conversion into commodities in 2012 (Marshall et al. 2012). In this case, the mixed community showed a concomitant production of acetate and methane at a poised potential of -0.59 vs. SHE. Marshall and co-workers suggested the use of hydrogen as electron shuttle and pointed methanogens as competitors of acetogens for electron consumption. The use of specific inhibitors to methanogenesis helped to overcome such hurdle and place acetate as the main compound produced in the reactor (Marshall et al. 2012). Afterwards, the same authors improved the adaptation of the community and assessed its performance in a long-term operation. They demonstrated the importance of working with a mature community to improve product yields in a MET reactor (Marshall et al. 2013).

Many following studies were focused on the bio-electrorecycling of CO_2 into acetate (Suman Bajracharya et al. 2015; Jiang et al. 2013; Jourdin, Freguia, et al. 2015; Patil, Arends, et al. 2015; Xafenias and Mapelli 2014). Operational parameters such as the cathode potential and the reactor configuration were pointed out as crucial in the product selectivity (Jiang et al. 2013; Xafenias and Mapelli 2014). In addition, the performance of modified cathodic electrodes was assessed in order to improve product yields (Zhang et al. 2013). For instance, acetate production was enhanced by using reticulated vitreous carbon modified with carbon nanotubes (Jourdin et al. 2014), nickel nanowires (Nie et al. 2013), metal oxide–carbon hybrid material (Cui et al. 2017), gas diffusion electrodes (Suman Bajracharya et al. 2016), polarized stainless steel (Soussan et al. 2013) or graphene (N. Aryal et al. 2017; Song et al. 2018). The use of biocompatible metal-based

electrodes have shown promising results (Siegert et al. 2014; Kracke et al. 2019), especially the use of Zinc for the bio-electrorecycling of CO_2 into acetate (Jiang et al. 2019). Other parameters such as the biocatalyst strain using either pure cultures (Suman Bajracharya et al. 2015; Nabin Aryal et al. 2017) or previously adapted open cultures were studied (Patil, Arends, et al. 2015).

At the same time, Batlle-Vilanova and co-workers aimed to decipher electron transfer mechanisms between the electrode and a mixed community during the bio-electroconversion of CO_2 into methane (Batlle-Vilanova et al. 2015). They performed fundamental studies and concluded that H_2 is the main electron donor in the process. Protons evolve biotically or abiotically into hydrogen gas in the electrode surface and the hydrogenotrophic methanogens are the main actors that drive bio-electrorecycling of CO_2 into methane. This electron shuttle was found crucial for electron delivery to the microorganisms during MES (Puig et al. 2017).

The operational parameters needed to expand the product portfolio from CO_2 and electricity were identified. Products such as butyrate (Ganigué et al. 2015) and isopropanol (Arends et al. 2017), ethanol (Srikanth, Singh, et al. 2018; Blasco-Gómez et al. 2019) and caproate (Jourdin et al. 2018, 2019) were bioelectrochemically produced at BES working in batch or continuous mode or even in open circuit conditions (Mateos, Escapa, et al. 2019). Novel reactor designs allowed to carry out simultaneous bioprocesses that require different conditions (Vassilev et al. 2019) in the same reactor or coupling other fermentative process to produce bioplastics (e.g. polyhydroxyalkanoates, PHAs) from just CO_2 and electricity using acetate and butyrate as direct metabolic precursors (Pepè Sciarria et al. 2018). In this sense, CO_2-rich gaseous waste stream resulted from the

separation of biohydrogen by membrane technologies are considered potential inlets for bioelectrorecycling systems (Bakonyi et al. 2020).

Not only the production, but also several extraction methods were evaluated for in-situ separation of carboxylates using three-chamber reactor systems (Gildemyn et al. 2015), ion-exchange resins (S. Bajracharya et al. 2017) and liquid membranes (Batlle-Vilanova et al. 2017).

Lately, scientific interest on CO_2 bio-electro recycling seems to keep increasing (Figure 7). This specific application of METs has been deeply studied in recent doctoral thesis defended since 2015 in many different Universities worldwide (Jourdin 2015; Batlle-Vilanova 2016; Suman Bajracharya 2016; Mateos 2018; Vassilev 2019). However, the decrease in the number of publications in 2019 seems anecdotal based on the trend in 2020 and might show the shift towards a more practical rather than fundamental research. Ongoing projects funded by the European Union under the Horizon 2020 programme dealing with CO_2 bioelectrorecycling (Table 2) are focused on expanding the product portfolio, applying genetic engineering tools and coupling METs with current fermentation-related technologies and renewable power sources (Table 2). In addition, other initiatives are undertaken globally to take advantage of the capability of some electroactive microorganisms to convert gaseous feedstocks into valuable chemicals (Schievano, Pant, and Puig 2019).

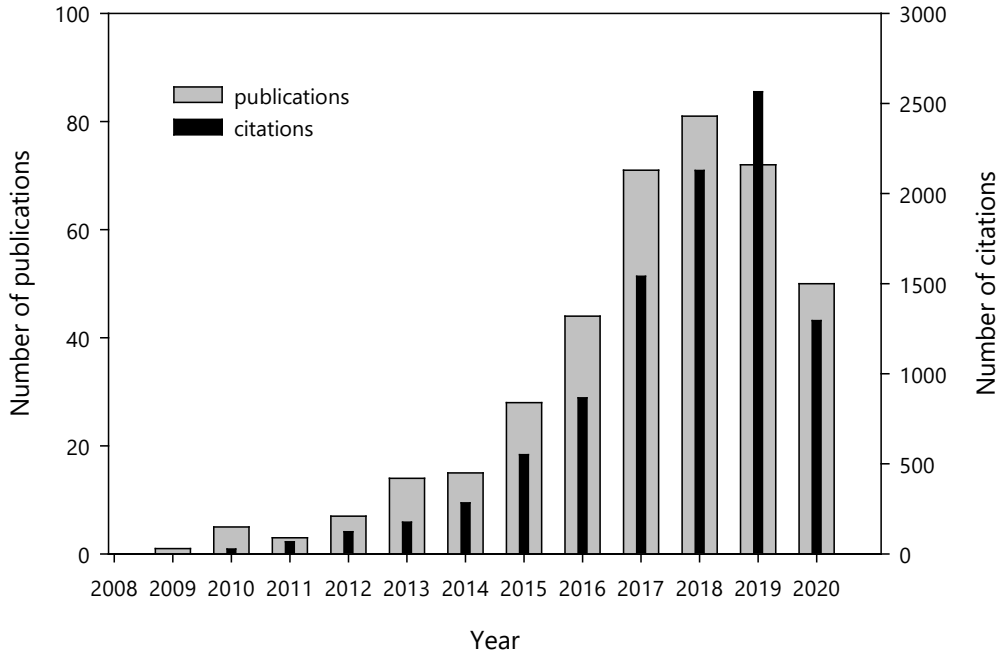


Figure 7. Appearance in the literature of the bioelectrochemical CO_2 conversion in biocathodes. Results found by searching the word "CO_2 Biocathode" in "All fields" in Scopus database (last access: 06/040/2020).

Table 2. Main objectives of ongoing European research projects dealing with CO_2 bio-electrorecycling.

Acronym (years)	Logo	Objective	Website
BIORECO$_2$VER (2018-2021)		CO_2 + renewable energy into lactate and iso-butene	http://bioreco2ver.eu
BAC-TO-FUEL (2018-2021)		CO_2 + solar energy into biofuels (short chain alcohols: methanol, ethanol, butanol)	http://bactofuel.eu
CELBICON (2016-2019)		Atmospheric CO_2 (air) and renewable energy into bioplastics (Poly-Hydroxy-Alkanoates; PHA), isoprene, lactic acid, methane, etc. and development of recovery technologies	http://www.celbicon.org
BIOCON-CO_2 (2018-2021)		CO_2 from the iron, steel, cement, and electric power industries into commodities for chemicals and bioplastics	https://biocon-co2.eu

1.4.1 Electron transfer mechanisms in CO_2 bioelectrorecycling

Different electron transfer routes have been reported between cathodes and electroactive microbes. The main two of them are direct electron transfer (DET) and mediated electron transfer (MET) (Figure 4). In case of DET, membrane proteins such as *c*-type cytochromes and hydrogenases have been identified as

electron bridges (M. Rosenbaum et al. 2011), although cellular conductive structures known as nanowires are also likely to play an important role in the electron exchange mechanisms (Reguera et al. 2006; D. Lovley and Walker 2019). Moreover, electron exchange between two different species can be stablished through syntrophic interactions via direct interspecies electron transfer (DIET) and/or mediated interspecies electron transfer (MIET) (Rotaru et al. 2014). In case of DIET, this mechanism is more energetically conservative due to the direct transfer of electrons between the two individuals, avoiding the production of any intermediate (D. R. Lovley 2011; A. J. M. Stams and Plugge 2009).

Mediated electron transfer (MedET) mechanisms involve intermediate soluble redox compounds that are used by electroactive microorganisms to exchange electrons with the electrode surface (Schröder 2007). Biologically secreted compounds (e.g. flavins, riboflavins, quinones and phenazines) and other non-biologically excreted molecules (e.g. humic acids, thionine, viologens, methylene blue, and sulfur species) act as external mediators for electron exchange (Coursolle et al. 2010; Reguera et al. 2006; Freguia et al. 2009; Pham et al. 2008; Angenent et al. 2004a; a J. M. Stams et al. 2006). Moreover, hydrogen can be produced biotically or abiotically by bio or non-biocatalyst located on the electrode surface or in the bulk. This gas acts as electron carrier in MedET and MIET mediating electron transfer between the electrode and the microorganisms.

In case of CO_2 bio-electrorecycling several abovementioned mechanisms occur (Figure 8). Specifically, microorganisms use: (i) protons and electrons directly when attached to the electrode, (ii) H_2 abiotically produced on the electrode surface, (iii) H_2 biotically produced by H_2 producing bacteria, (iv) intermediate products that serve as electron donors (e.g. formate, acetate or ethanol) or (v) conductive particles, shuttle molecules and nanowires that provide reducing

power via DIET and MIET. These mechanisms have been previously studied in the literature (Batlle-Vilanova et al. 2015; Puig et al. 2017; Walker et al. 2018; Malvankar and Lovley 2014).

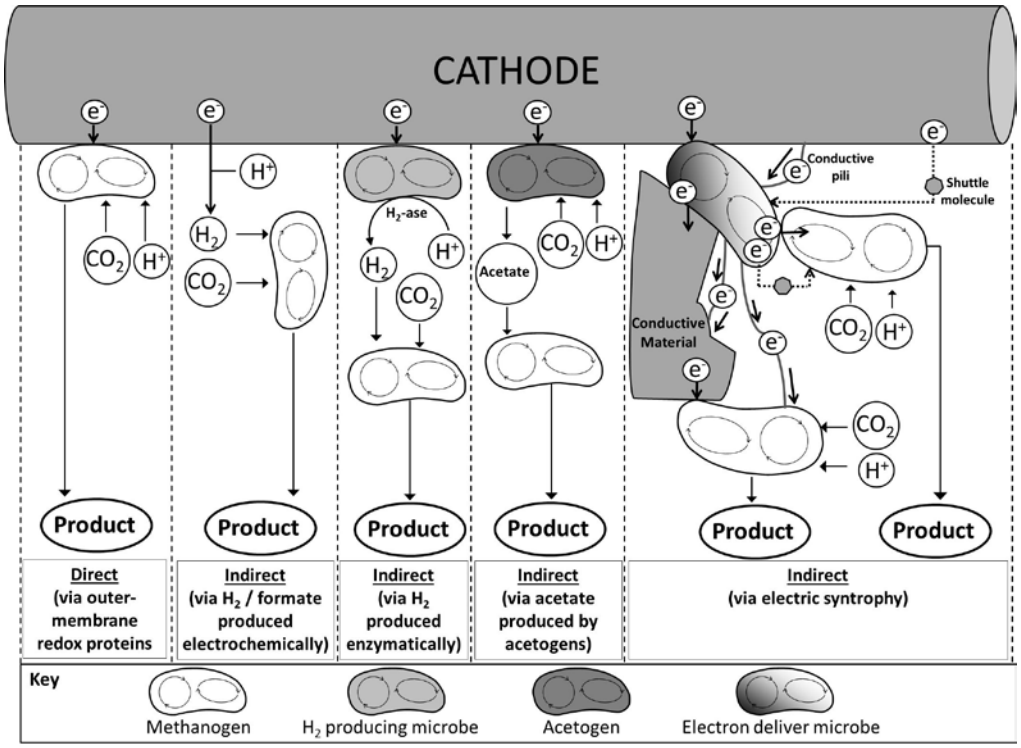
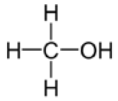
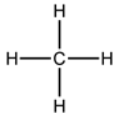
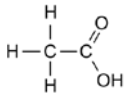
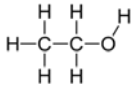
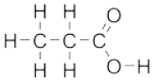
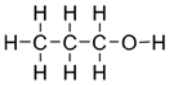
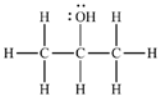
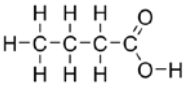
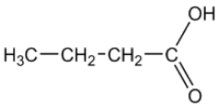
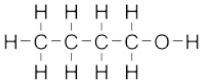


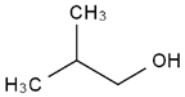
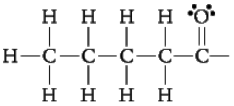
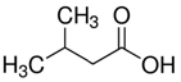
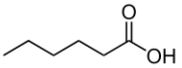
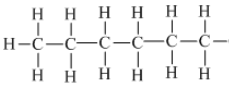
Figure 8. Proposed electron transfer mechanisms within the biocathode compartment. Even though H_2 is the only intermediate molecule (bio)electrochemically produced on the surface of the electrode shown in the figure, formate or other molecules must be also considered to play the same role as electron carrier. Sizes of the circles do not correspond to any proportions. Adapted from (Blasco-Gómez et al. 2017).

1.4.2 Product portfolio of CO_2 Bio-electrorecycling

Several compounds have been produced from only CO_2 and (renewable) energy as carbon source and reducing power respectively. Methane, a gaseous compound, was firstly targeted (Cheng et al. 2009; Blasco-Gómez et al. 2017), although other products such as carboxylate and solvents were also detected and subsequent studies were conducted in order to promote their specific production (ter Heijne et al. 2017). These compounds vary from C1 to C6 (Table 3), which refers to the number of carbon-atoms in their molecular chain. Carboxylates (e.g. acetic acid, butyric acid, lactic acid, caproic acid) and alcohols (e.g. ethanol, butanol, 2,3-butanediol, hexanol) comprise nowadays the product portfolio of MES using CO_2 as the sole carbon source. Also, new reactor configurations and the possibility to couple other fermenting processes to MET expanded the product portfolio towards more complex molecules such as bio-polyesters (polyhydroxyalkanoates, -PHAs-) (Pepè Sciarria et al. 2018), wax esters (Lehtinen et al. 2017), terpenes (Krieg, Sydow, et al. 2018), or proteins (Molitor, Mishra, and Angenent 2019). However, the major compound produced in a single stage BES performing CO_2 bio-electrorecycling is commonly acetate (May, Evans, and LaBelle 2016), which can be produced electrochemically under biologically relevant conditions at $\mathbf{E_o'}$=-0.28V vs. SHE (Rabaey and Rozendal 2010).

Table 3. Products that can be theoretically obtained from CO_2 and electricity by MES.

Final product	Reaction	Nº e⁻ required	Molecule	Molecular weight (g/mol)
Methanol	$CO_2 + 6H^+ + 6e^- \leftrightarrow CH_4O + H_2O$	6		32.04
Methane	$CO_2 + 8H^+ + 8e^- \leftrightarrow CH_4 + 2H_2O$	8		16.04
Acetic acid	$2CO_2 + 8H^+ + 8e^- \leftrightarrow C_2H_4O_2 + 2H_2O$	8		60.05
Ethanol	$2CO_2 + 12H^+ + 12e^- \leftrightarrow C_2H_6O + 3H_2O$	12		46.068
Propionic acid	$3CO_2 + 14H^+ + 14e^- \leftrightarrow C_3H_6O_2 + 4H_2O$	14		74.0785
Propanol	$3CO_2 + 18H^+ + 18e^- \leftrightarrow C_3H_8O + 5H_2O$	18		60.095
Isopropanol	$3CO_2 + 18H^+ + 18e^- \leftrightarrow C_3H_8O + 5H_2O$	18		60.095
Butyric acid	$4CO_2 + 20H^+ + 20e^- \leftrightarrow C_4H_8O_2 + 6H_2O$	20		88.11
Isobutyric acid	$4CO_2 + 20H^+ + 20e^- \leftrightarrow C_4H_8O_2 + 6H_2O$	20		88.11
Butanol	$4CO_2 + 24H^+ + 24e^- \leftrightarrow C_4H_{10}O + 7H_2O$	24		74.12

Iso-butanol	$4CO_2 + 24H^+ + 24e^- \leftrightarrow C_4H_{10}O + 7H_2O$	24		74.12
Valeric acid	$3CO_2 + 26H^+ + 26e^- \leftrightarrow C_5H_{10}O_2 + 8H_2O$	26		102.13
Isovaleric acid	$3CO_2 + 26H^+ + 26e^- \leftrightarrow C_5H_{10}O_2 + 8H_2O$	26		102.13
Hexanoic acid	$6CO_2 + 32H^+ + 32e^- \leftrightarrow C_6H_{12}O_2 + 10H_2O$	32		116.158
Hexanol	$6CO_2 + 36H^+ + 36e^- \leftrightarrow C_6H_{14}O + 11H_2O$	36		102.16

The *Wood-Ljungdahl* pathway is considered the most energetically efficient among the metabolic pathways to reduce CO_2 into organic compounds (Figure 9). In this pathway, acetogenic microorganisms reduce CO_2 using H_2 as electron donor (Fast and Papoutsakis 2012) into Acetyl-CoA, which is a central intermediate of the metabolic route. Different microorganism are then capable to use Acetyl-CoA as building block for the production of a wide range of commodities (D. R. Lovley and Nevin 2013). In addition, other minor metabolic pathways such as the Calvin cycle or the Arnonn-Buchannan cycle have been described during the CO_2 bioelectro-recycling (Wenzel et al. 2018; Vassilev et al. 2018).

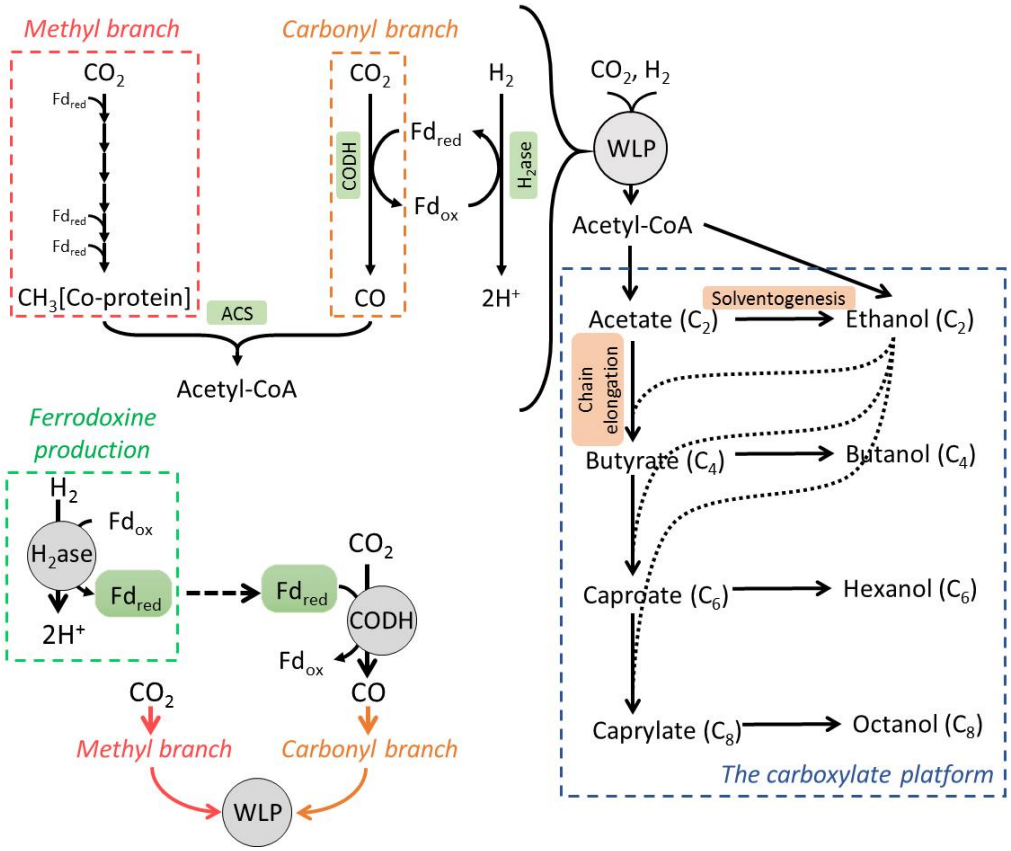


Figure 9. Schematic representation of the *Wood-Ljungdahl* pathway (WLP) and its link to the carboxylate platform. Extra reducing power is obtained by the activation of hydrogenases (H_2ase) that reduce ferrodoxine (Fd) that is subsequently used by carbon monoxide dehydrogenase (CODH) which catalyses the reduction of CO_2 into carbon monoxide (CO) within the carbonyl branch of the WLP.

The use of pure cultures demonstrated that they are useful tools to do fundamental research focused on the elucidation of the different metabolic pathways involved in CO_2 bio-electro recycling. Additionally, they can be genetically modified to enhance the production of a target compound. However, mixed cultures showed higher yields and potential as biocatalyst inoculum for a foreseeable technological solution.

A review of the different pathways for microbial production of commodities with mixed cultures showed the importance of acetate as substrate for its reduction into butyrate, ethanol or larger compounds through secondary fermentation processes (Agler et al. 2011). Furthermore, acetate can be added as feedstock in conventional fermentation (Steinbusch et al. 2011) or BES (Van Eerten-Jansen, Ter Heijne, et al. 2013), known as electro-fermentation (Schievano et al. 2016), to produce longer chain carboxylates such as butyrate or caproate. However, ethanol is the key player in the CO_2 bio-electro recycling into C4-C6 carboxylates and their correspondent solvents. It serves together with the electrode (i.e. hydrogen) as electron donor to the microbes involved in the chain elongation (Jiang et al. 2020). This pathway, where ethanol is oxidised to gain carbon, energy and reducing equivalents to elongate C2 into C4-C6 is called reverse ***β***-oxidation. It is considered the most important pathway in such biotechnological process (Angenent et al. 2016). Ethanol is biologically produced via solventogenesis (Figure 10), which consists in the reduction of acetate under growth limiting conditions (i.e. substrate limitation, product toxicity, and acidification) (Ramió-Pujol et al. 2015a). These bacteria produce alcohols from acetyl-CoA during the transition from exponential growth to stationary phase. For this purpose, they require additional reducing equivalents in the form of reduced nicotinamide adenine dinucleotide (NADH). In fact, two moles of NADH are needed stoichiometrically to produce one mole of ethanol. The same energy input is required for the production of butanol from butyryl-CoA. CO and H_2 are the only reducing equivalents sources under autotrophic growth and H_2 is the only one present in biocathodes, which makes a high partial pressure of H_2 as crucial parameter for triggering the solventogenic route.

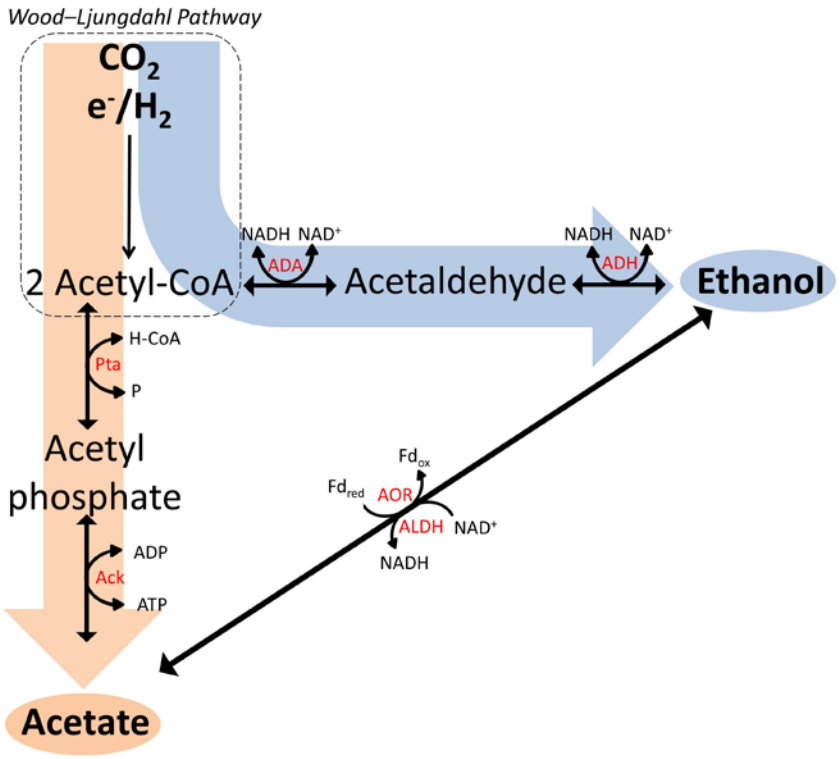


Figure 10. Schematic representation of the acetate and ethanol production from CO_2 and electricity. The WLP reduces inorganic carbon to acetyl-CoA. Noteworthy, the enzymes involved in each reaction are labelled in red. Acronyms: AOR, aldehyde:ferredoxin oxidoreductase; Ack, acetate kinase; Pta, phosphate acetyltransferase; ADA, acetaldehyde dehydrogenase (acetylating); ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase.

1.4.3 Challenges of CO_2 bio-electro recycling

Up to date, many barriers have been pointed out to limit CO_2 bio-electro recycling into commodities (Figure 11). The role of operational parameters such as pH, CO_2 availability and pH_2 are still not well defined, although are suggested to critically affect the performance and selectivity during MES (Blasco-Gómez et al. 2019). Moreover, there are also many biological constraints related to the operation mode of the system (e.g. batch, fed-batch or continuous). For instance, the

limitation of nutrients (e.g. cofactors that work as trace elements) and presence of inhibitors secreted to the extracellular space compromises the biocathodic activity (Nimbalkar et al. 2018; Ammam et al. 2016; Czerwińska-Główka and Krukiewicz 2020). A higher biofilm complexity provides tolerance in case of some perturbations that lead to stress conditions (Borole et al. 2011). The type of inoculum and its growth stage directly affect to the resilience, robustness and selectivity of the system. From the electrochemical point of view, parameters such as the potential applied, the presence of electron sinks and parasitic metabolisms, type of cathodic/anodic electrode, membrane used and the overpotential influence the CO_2 bio-electro recycling process and its efficiency. In fact, the potential applied determines the current density and the availability of reducing equivalents in the medium that is necessary for a better steering of the whole process. The more negative the potential is poised, the more reduced compounds are obtained, since there is more current density and reducing equivalents available for the microorganisms. In addition, the inherent limitation of the gas-liquid mass transfer affects directly on the availability of gaseous substrates in the medium for their further usage by the electroactive microorganisms. In addition, applying new biophysical and computational methods to understand the structural aspects, dynamics, and energetics of CO_2 bio-electrorecycling seem

crucial. This would help to balance the large mismatch in abiotic charge generation versus biotic charge consumption (Su and Ajo-Franklin 2019).

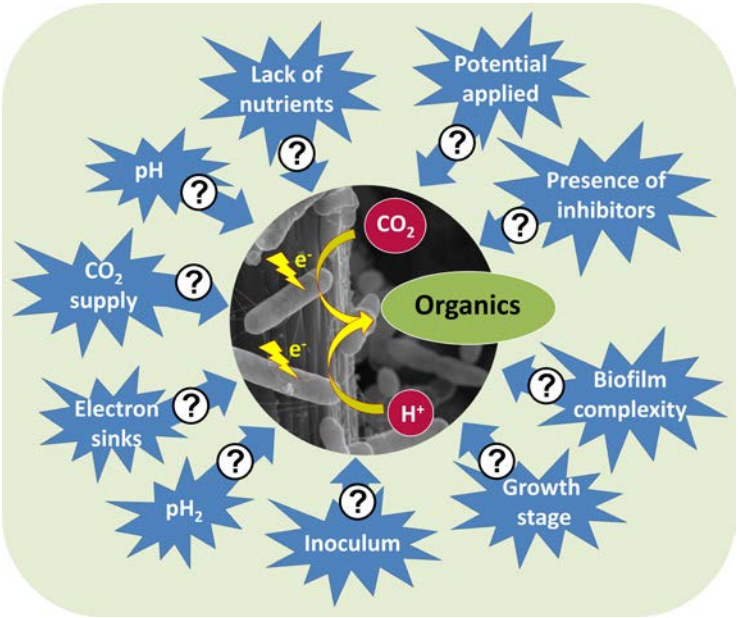


Figure 11. Main hurdles limiting CO_2 bio-electrorecycling.

The lack of bioavailable energy in the system might also be a hurdle when at low partial pressure of hydrogen (pH_2) is present in the biocathode to carry out the reactions of interest. H_2 is required to reduce Fd which will be subsequently used in the carbonyl branch to reduce CO_2 into Acetyl-CoA via the WLP. Moreover, this H_2 actives the energy-converting hydrogenases that will create a ion gradient utilized by an ATP-synthase and provide ATP to the electroactive bacteria present in the biocathode (Schoelmerich and Müller 2019). This lack of energy could be solved by either poising a more negative potential or circumventing H_2 present in the headspace continuously to the bacteria. This would besides improve the overall efficiency of the process by increasing the CO_2 conversion rate (Mateos, Sotres, et al. 2019). In addition, some studies showed that after an electric supply interruption (open circuit) event (≤64h), the biocathodic mixed community shows

a quick response and acetate is immediately produced (Anzola Rojas, Zaiat, et al. 2018), which suggests resilience in the technology. However, if the power interruption remains for longer time (6 weeks), acetoclastic and hydrogenotrophic methanogenesis becomes dominant over acetogenesis and methane is produced in the system (Mateos, Escapa, et al. 2019).

1.4.4 Recent advances in CO_2 bio-electrorecycling: towards a better product selectivity

Up to date, many efforts have aimed at lifting METs towards a more feasible stage from the economic and technical point of view. In this sense, achieving better product selectivity must be crucial, since the market continuously changes and demands different compounds over time based on their profitability. In fact, compounds such as formic acid and ethanol produced from CO_2 and electricity showed higher returns than acetic acid, propionic acid or methanol, in a study of the economic feasibility of CO_2 bio-electrorecycling (Christodoulou et al. 2017).

There are two main approaches to achieve better product selectivity in MES: (i) set the conditions to drive the metabolic pathways towards a specific compound production or (ii) couple an effective extraction process downstream or in-situ by modifying the system design in order to concentrate the target product.

The first approach is based on the existing knowledge on the affection of each operation parameter in the prevalence of a certain metabolic pathway, whose end-product is the target compound, over the rest metabolic activity. Parameters such as pH, CO_2 loading rate, pH_2 ,applied potential, hydraulic retention time (HRT) and biofilm thickness have been pointed out to have a direct role in the

product selectivity (LaBelle et al. 2014; Mohanakrishna, Vanbroekhoven, and Pant 2016; Jourdin et al. 2016, 2019; Blasco-Gómez et al. 2019).

In case of the second approach, many studies have shown the possibility to recover acetic acid or butyric acid through the use of membranes (Gildemyn et al. 2015; Verbeeck, Gildemyn, and Rabaey 2018), hollow fibre liquid membranes (Batlle-Vilanova et al. 2017), resins (S. Bajracharya et al. 2017) or novel system designs that compartmentalize different bioelectrosynthetic processes (Vassilev et al. 2019).

Many efforts have focused on increasing the production rates of specific compounds (Chiranjeevi and Patil 2019). The use of new electrodes (e.g. gas diffusion electrodes, new biocompatible transition-metal-based cathodes, etc.) (Suman Bajracharya et al. 2016; Alvarez-Gallego et al. 2012; Sakai et al. 2016; Jourdin et al. 2014; Song et al. 2018; Kracke et al. 2019) enhance the CO_2 availability and improve the attachment to the biocathodic communities to the electrode, leading to higher yields and more robust systems. Moreover, modifying the existing electrodes has leaded to a better product selectivity (Nabin Aryal et al. 2018; Rengasamy et al. 2018; Chen et al. 2016; Nie et al. 2013; Zhang et al. 2013). The enrichment of certain homoacetogenic strains within the biocathodic community facilitates the selectivity and enhances acetic acid production (Patil, Arends, et al. 2015; Nabin Aryal et al. 2017; Tremblay and Zhang 2015). In addition, the optimization of the electrolyte solution with trace elements (e.g. cofactors for enzymes involved in the WLP) increases yields of both acetate and ethanol by *Sporomusa ovata* (Ammam et al. 2016). Bipolar membranes were successfully

utilized in MES, in which bioanode was used to supply electrons to the cathode for the CO_2 bio-electrorecyling into acetate (Y. Xiang et al. 2017).

1.4.5 Moving forward to a more sophisticated CO_2 bio-electrorecycling

METs aiming at bio-electrorecycle CO_2 are currently taking advantage of all new advances in bio-based technologies such as fermentation or photobiotechnology. Besides the trail regarding upscaling (discussed in section 1.4.6), advances in the elucidation of metabolic routes and proteins involved unblock a powerful tool: synthetic biology. This platform helps in doing fundamental research in order to improve yields and product range (Kracke et al. 2018; Glaven 2019; Chiranjeevi and Patil 2019), which would lead to make this technology technically and economically feasible. Nowadays, engineering living organisms is a reality in the field of electromicrobiology (Teravest and Ajo-Franklin 2016; M. A. Rosenbaum et al. 2018). The widely known electrotroph *Shewanella oneidensis* has been modified to overexpress extracellular electron transfer activity using transcriptional regulation tools (West, Jain, and Gralnick 2017) and CRISPRi-sRNA (Cao et al. 2017), as well as to reduce acetoin into 2,3-butanediol (Tefft and Teravest 2019). Also, new advances in the utilization of CO_2 as sole carbon source to produce biomass by engineered *Escherichia coli* and *Pichia pastoris* open new perspective in the use of BES to produce food (Gleizer et al. 2019; Gassler et al., n.d.). In this case, the modified organisms uses formate and methanol as reducing power, which can be produced electrochemically from CO_2 (Hegner et al. 2019; Hegner, Rosa, and Harnisch 2018; H. Xiang et al. 2020; Xu et al. 2006; Shen, Ichihashi, and Matsumura 2005). However, these studies are scarce and still a long way to go, since carbon fixation requires a relatively large set of genes, most of which involve complex, largely unexplored regulation (Fast and Papoutsakis 2012).

From the point of view of the product, selectivity towards ethanol production is critical. This product is the key element that triggers the CO_2 bio-electrorecycling towards more complex compounds (e.g. caproate) through the reverse **β**-oxidation pathway (Jiang et al. 2020; Reddy, ElMekawy, and Pant 2018), which are more interesting for the current bio-commodity market. Despite of all research efforts made so far on that issue, a robust system that selectively produces ethanol from CO_2 and electricity in long-term operation and interesting yields has not been developed yet (Table 4). In fact, the current trend is to design compartmentalized BES as flexible platforms to drive only the metabolic pathways of interest by controlling key operational parameters (Vassilev et al. 2019), or couple it with other well-stablished fermentation processes as discussed previously (section 1.4.4). Nevertheless, as mentioned above, the use of advanced engineered microorganisms that produced biofuels from CO_2 (e.g. ethanol, butanol) through improved electron accepting mechanisms are promising approaches in the field of bioelectrochemistry (J. E. Woo and Jang 2019; Kracke et al. 2018).

Table 4. Overview of the main features and performance of ethanol-producing biocathodes fed with CO_2 as the sole carbon source.

Voltage (V vs. SHE)	T (ºC)	pH	Inoculum	Feeding	Products	Maximum [ethanol] (mmol C ethanol)	Max. Ethanol-to-acetate ratio (**)	Ref.
-0.9	30	7.0-4.5	Anaerobic sludge	20:80 (CO_2:N_2)	Acetate, butyrate, ethanol	2.56	<1	(Suman Bajracharya et al. 2016)
-0.8/-0.9	37	7.0	Anaerobic sludge + *C. ljungdahlii*	80:20 (CO_2:N_2)	Acetate, butyrate, ethanol	21.71	<1	(Suman Bajracharya et al. 2017)
-0.69	25	<5.0	*Sporomusa ovata* DSM-2662	80:20 (CO_2:N_2)	Acetate, ethanol	26.3	<1	(Ammam et al. 2016)
-0.40	30	7.3	Anaerobic bog sediment	80:20 (CO_2:N_2)	Butanol, ethanol, acetate, propionate, butyrate	0.7	<1 (0.084)	(Zaybak et al. 2013)
-0.6	30	7.0	Enriched homoacetogenic consortia	20:80 (CO_2:N_2)	Acetate, ethanol	3.52	<1	(Mohanakrishna, Vanbroekhoven, and Pant 2016)
-50 mA / -5 $A{\cdot}m^{-2}$(*)	30	7.6-5.0	Anodic effluent of a MFC and a UASB digesting microalgae (1:1)	90:10 (N_2:CO_2)	Butyrate, isopropanol, ethanol, acetate, formate, propionate, acetone	11.72	<1	(Arends et al. 2017)
-0.8	38	4.2-6.3	Carboxydotrophic mixed culture (dominated by *Clostridium* spp.)	CO_2	Acetate, butyrate, ethanol, butanol	20.4	<1 (0.522)	(Batlle-Vilanova et al. 2017)
-0.8	29	6.0	Mixed culture obtained from corroded metal surface	CO_2	Acetate, butyrate, propionate, ethanol, butanol, methanol	n.r.	<1	(Srikanth, Kumar, et al. 2018)
-0.8	29	8.0–4.36	Mixed culture obtained from corroded metal surface	CO_2	Methanol, ethanol, butanol, acetate, butyrate, formic acid	n.r.	>1	(Srikanth, Singh, et al. 2018)
-0.8	35	4.9-5.2	Mixed culture obtained from a BES performing acetatogenesis from bicarbonate	CO_2	Acetate, butyrate, isobutyrate, caproate, ethanol, butanol, isobutanol, hexanol	56.1	<1	(Vassilev et al. 2018)
-0.8	25	7.0-5.3	Enriched culture of Isolate I-19	CO_2	Acetate, ethanol	35.65	>1 (1.54)	(Blasco-Gómez et al. 2019)

1.4.6 Successful examples in the upscaling of CO_2 Bio-electrorecycling

Unfortunately, successful experiences in up-scaling CO_2 bio-electrorecycling technologies are rare. Only in the field of electromethanogenesis, some companies have launched full-scale prototypes that are currently in the market (Blasco-Gómez et al. 2017), which lifts up the application to a technology readiness level (TRL) of 8-9 (Figure 12). Electrochaea (available online: http://www.electrochaea.com) is currently developing a new solution (BioCat) based on the bio-electrochemical reduction of CO_2 into methane via the Power-to-Gas concept (Geppert et al. 2016).

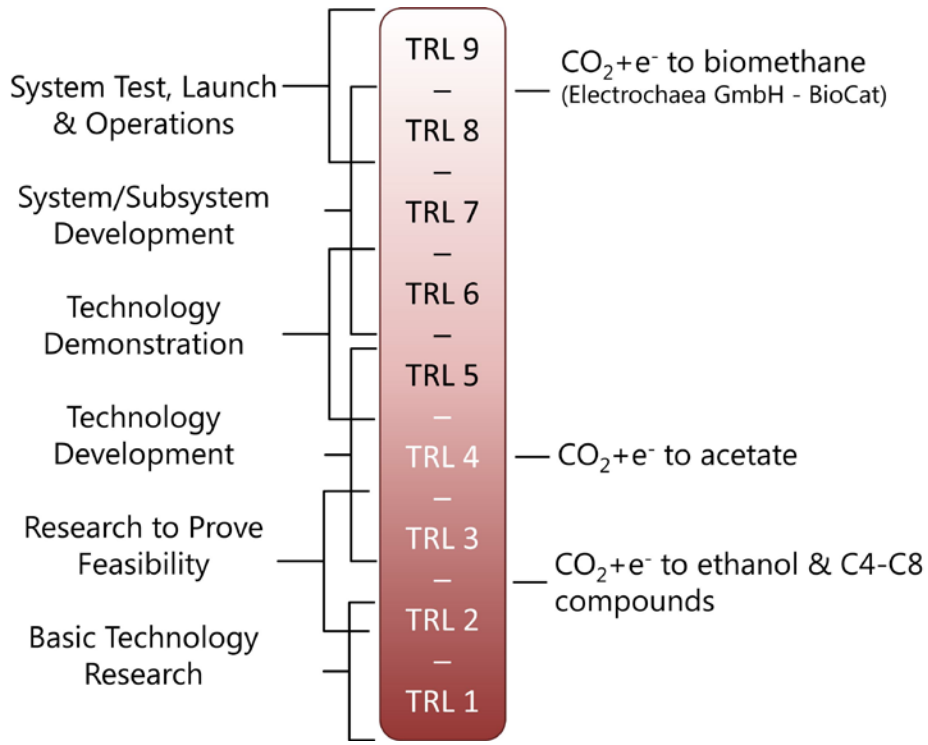


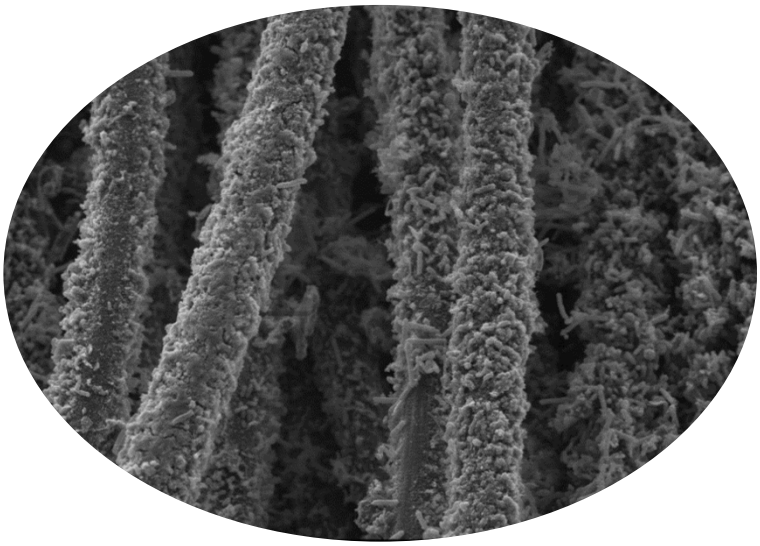
Figure 12. Technology readiness level (TRL) of CO_2 bio-electrorecycling technologies.

MES processes, which still have to undergo optimizations to be ready for scale up in an economical point of view, must transfer knowledge from the upscaling carried out in photobiotechnology to speed up its development (Enzmann et al. 2019). In fact, combining scale up and numbering up may lead to industrially relevant scales in bioelectrochemical processes allowing an industrial application of the technology in near future.

Despite of all the above mentioned needs, some studies with bigger reactors appeared to show that upscaling is an issue that must be firstly solved to continue the progress in CO_2 bio-electrorecycling. Electrotrophic biofilm maintenance, improvable mechanical design, variability in substrates and anode-cathode distance were pointed out by Tian and co-workers as bottle-necks identified when up-scaling this technology (Tian et al. 2019). In addition, a proper distance between electrodes to avoid ohmic losses, electrolyte mixing to decrease mass transport limitations, correct surface-to-volume ratios that affects volumetric current densities and production yields, chemical short circuits and an effective way to deal with solids are the main concerns pointed out by Krieg et al. (Krieg, Madjarov, et al. 2018).

Chapter 2

OBJECTIVES



2.1 Problem definition

CO_2 bio-electrorecycling is an electrochemical process used to drive microbial metabolism for the reduction of CO_2 into industrially relevant organic compounds as an alternative to current fossil-fuel-derived commodities. After a decade of research on this technology, there are still several open questions that need to be addressed. Efforts on reaching competitive production rates and energy conversion efficiencies in microbial-compatible electrolytes must be addressed in order to push up the TRL and make the CO_2 bio-electrorecycling a sustainable technology. From the one hand, to explore the role of different operational parameters (geometry of the reactor, CO_2 dissolved, pH and pH_2) that affect the biological performance of electroactive bacteria. On the other hand, define thresholds of these parameters that induce the activation or deactivation of metabolic routes of interest to bring this technology closer to full-scale.

This Ph. D thesis aims to provide some insights into the CO_2 bio-electrorecycling in METs and contribute to fill the existing knowledge gaps.

2.2 Objectives

When this Ph.D thesis started in 2016, some studies were done as proof of concept of the possibility to use METs for the conversion of CO_2 into methane, acetate and butyrate. However, studies focused on how to steer BES towards the production of other compounds was not yet studied. For this reason, the main objective of the Ph.D thesis was:

- To perform a thorough study of how key operation parameters such as CO_2 dissolved, pH and pH_2 affect to the activation or deactivation of the main metabolic pathways involved in the CO_2 bio-electrorecycling and how to steer electrosynthesis towards a selective production of target compounds.

Once the main objective was achieved, the secondary objectives were in the line of using such knowledge into the manoeuvrability of the BES. Therefore, the identified operational parameters were modified and monitored for the control of the CO_2 bio-recycling process towards the production of target compounds such as ethanol and butyrate through solventogenesis and chain elongation respectively.

- To steer CO_2 bio-electrorecycling process toward ethanol production by adjusting the identified key operational parameters (CO_2 dissolved, pH and pH_2).
- To steer CO_2 bio-electrorecycling process toward butyrate production by modifying the feeding and inoculation strategies.

The chapters of this Ph.D. thesis are outlined (Figure 13) according to the objectives in various chapters:

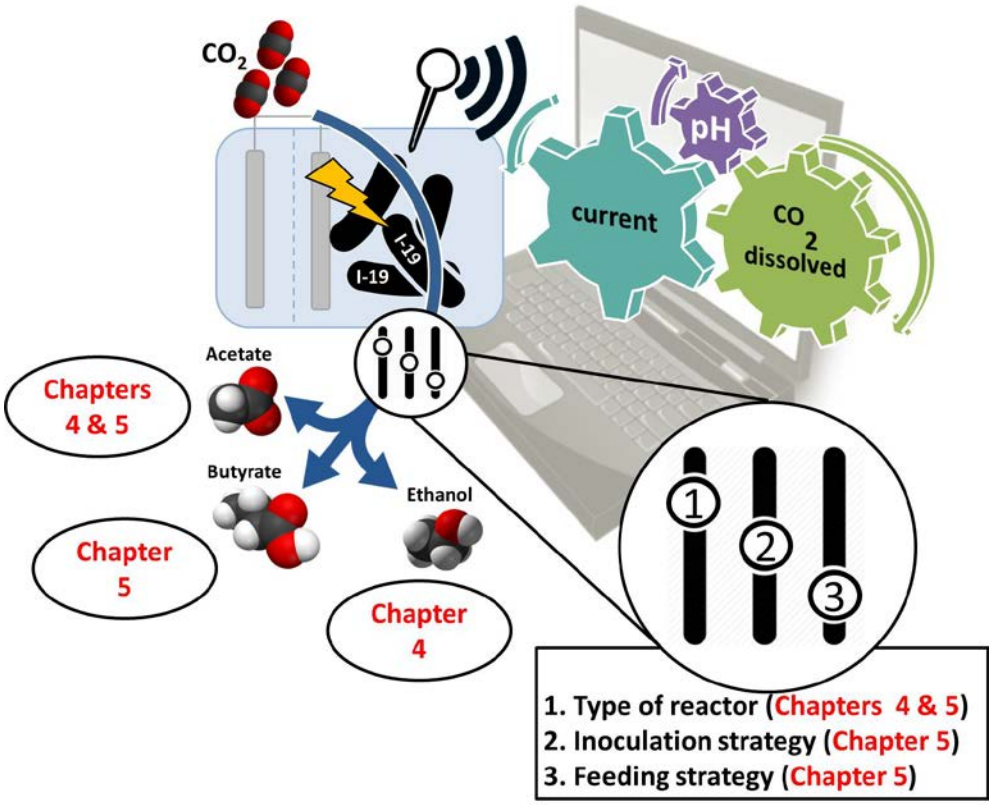
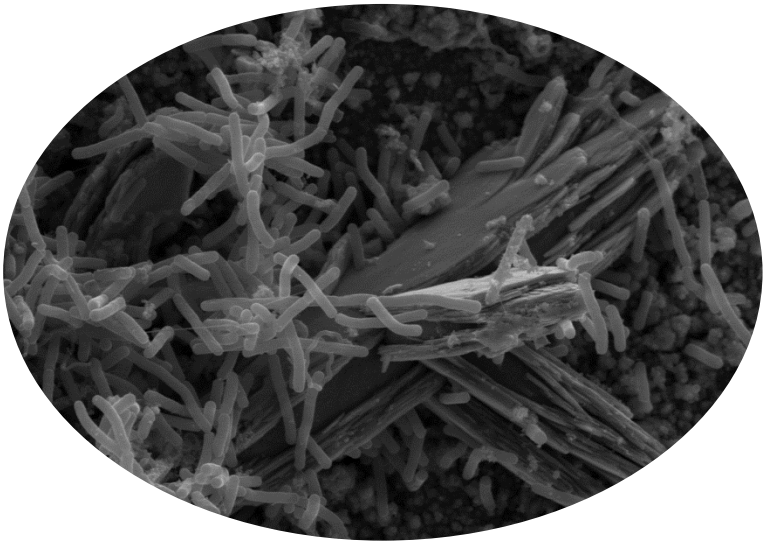


Figure 13. Schematic representation of the outline followed in this Ph.D. thesis. Thereby, here is explored the production of acetate, ethanol and butyrate using electricity and carbon dioxide as reducing power and carbon source respectively, in a biocathode of a BES. An exhaustive monitoring of crucial operational parameters was carried out in chapter 4 and 5. Different reactors types (tubular and flat-plate) were used in chapter 4 and 5 to produce those reduced compounds. Also, novel inoculation strategies and feeding strategies were tested in the CO_2 bio-electrorecycling.

Chapter 3

MATERIALS & METHODS



3.1 Bioelectrochemical systems set-ups

Two different BES configurations were used during the experiments: i) tubular reactor (Figure 14 and Figure 15) and ii) flat-plate reactor (Figure 17).

3.1.1 Tubular BES

Each tubular BES consisted of an inner concentric cathode and an outer anode compartments separated by a 580 cm^2 cation exchange membrane (CEM) (CMI-1875T, Membranes international, USA) (Figure 14; Figure 15 and Figure 21). The anode consisted of 280 cm^2 of commercial carbon cloth as electrode material (Thickness 490 µm; NuVant's ELAT, LT2400W, FuelCellsEtc, USA) connected to the potentiostat with a titanium wire (1mm Ø, Alfa Aesar, USA), while the cathode chamber was filled with granular graphite (model 00514, diameter 1.5–5 mm, EnViro-cell, Germany). Both materials increase electrode surface to volume ratio and sustain biofilm growth. A stainless steel wire (0.44mm Ø, Feval Filtros S.L., Spain) was placed along the graphite granule bed and connected to the power source. The systems were operated in a three-electrode configuration with a potentiostat (BioLogic, Model VSP, France), which controlled the cathode potential at −0.8 V vs. SHE and monitored the current density. The biocathode was used as working electrode, the anode as counter electrode, and an Ag/AgCl electrode 0.197 V vs. SHE (sat. KCl, SE11-S Sensortechnik Meinsberg, Germany) placed in the cathode chamber was used as reference electrode. The system was always operated at fixed voltage.

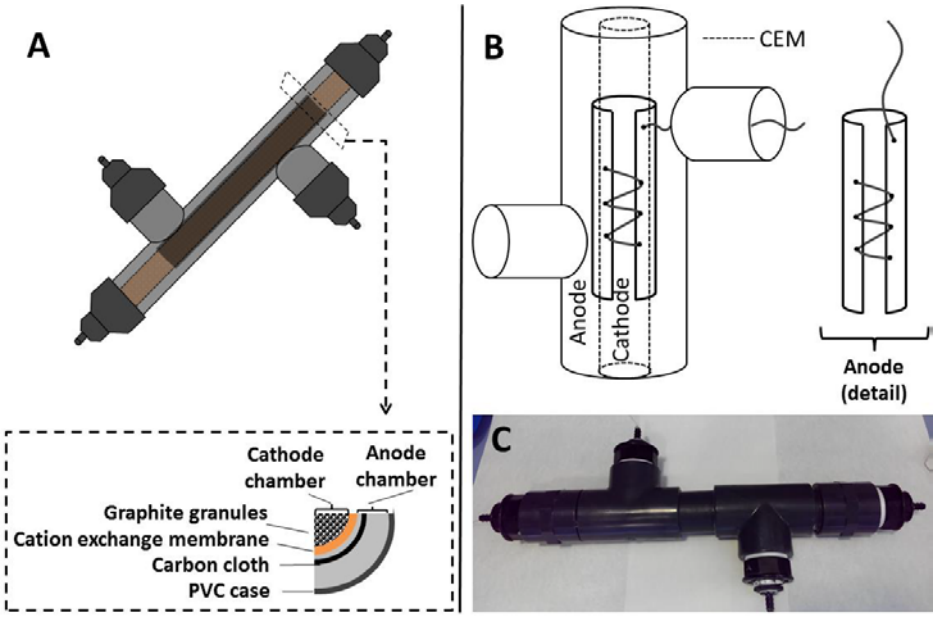


Figure 14. Schematic representation (A, B) and picture (C) of the tubular reactor design.

Both anode and cathode were connected to external buffer tanks allowing for the recirculation of liquid (4.5 L h^{-1}), increasing internal velocity. Moreover, cathodic buffer tank housed ports for CO_2 feeding, and sampling of liquid and gas phases. The total working volume of both chambers was 1 L, whereby 0.3 L corresponded to the cathode chamber and 0.7 L to the buffer tank, while the headspace volume of the cathode chamber was 0.2 L. Each BES was operated at 25 ± 1 °C and was kept in the dark.

The pH was in-line measured using a sensor (model 5303, Crimson, Spain) placed in the cathode recirculation loop (right after the BES outlet) to monitor and control the pH with a transmitter (MultiMeter MM44, Crison, Spain). The pH sensor was then connected to a data acquisition device (RSG40, Memograph M, Endress+Hauser, Switzerland) to register the value at five minutes intervals.

CO_2 dissolved in-line measurements in the liquid phase were carried out using a sensor (InPro® 5000, Mettler-Toledo, USA), which was placed conveniently to measure the CO_2 dissolved content in the outlet of each system. The data was collected in a 2-channel transmitter (M800 Multi-Parameter transmitter, Mettler-Toledo, USA) that acted as datalogger registering data at 5 minutes interval.

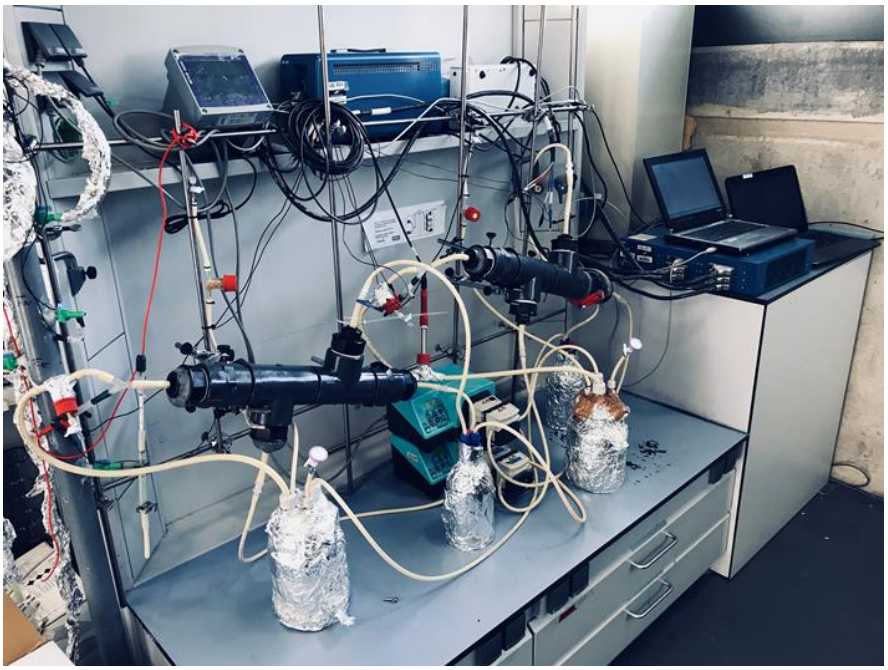


Figure 15. Real overview of the tubular duplicate reactors setup.

3.1.2 Flat-plate BES

The new geometry was chosen to increase the ratio electrode surface versus chamber volume as much as possible and set both electrodes closer to each other. Therefore, the substrate availability was increased, whereas mass transfer limitations and ohmic losses were minimized. Each flat-plate BES

used during the experiments consisted of two methacrylate compartments of 185 mL each (Figure 16; Figure 17 and Figure 32). A cation exchange membrane (CEM) (CMI-1875T, Membranes international, USA) of 82.7 cm^2 (4.4 cm width and 18.8 cm length) was used to separate both compartments.

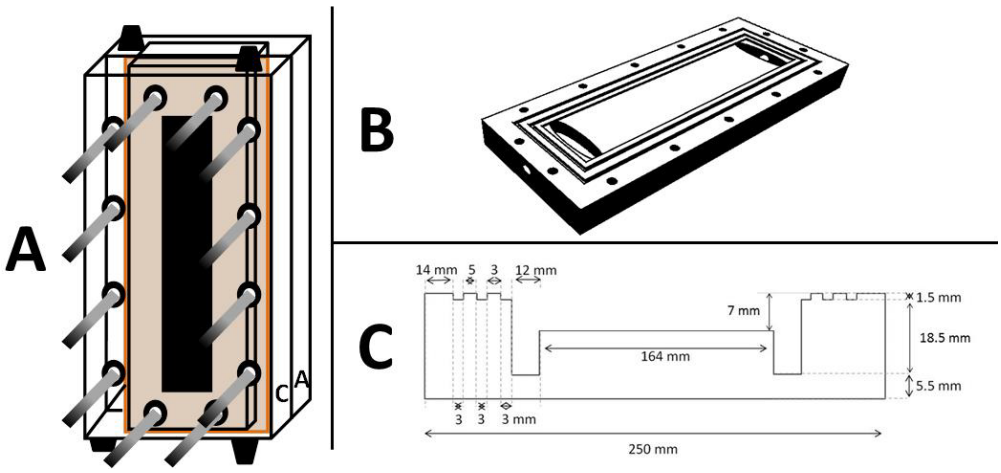


Figure 16. Schematic representation (A), chamber overview (B) and dimensions (C) of the flat-plate reactor design.

Due to space limitations and the necessity to incorporate abovementioned improvements to the BES reactors, electrode materials varied compared to the tubular reactor. The anode consisted of 129 cm^2 (4.3 cm width and 15 cm length) of commercial carbon cloth (Thickness 490 µm; NuVant's ELAT, LT2400W, FuelCellsEtc, USA) connected to a carbon rod (0.45 cm diameter and 4.4 cm of length, Mersen Iberica, Spain). A 129 cm^2 carbon cloth (similar to the anode) was used as cathode electrode connected to the power source through a stainless steel wire (0.44mm Ø, Feval Filtros S.L., Spain). The BES was operated in a three-electrode configuration with a potentiostat (BioLogic, Model VSP, France), which controlled the cathode potential at −0.8 V vs. SHE (Standard Hydrogen Electrode) and monitored the current density. The biocathode was used as working electrode, the anode as

counter electrode, and an Ag/AgCl electrode 0.197 V vs. SHE (sat. KCl, SE11-S Sensortechnik Meinsberg, Germany) placed in the cathode chamber was used as reference electrode.

External buffer tanks (wet volume of 150 ml) were coupled to both anode and cathode chambers respectively and two pumps (Watson-Marlow 323S, Watson-Marlow Fluid Technology Group, USA) recirculated anolythe and catholyte at a constant flow of 4.5 L h^{-1}. Cathodic buffer tank housed ports for CO_2 feeding, and sampling the gas phase. The liquid samples were taken between the outlet of the cathodic chamber and the pH/dissolved CO_2 probes. The total working volume of both chambers was 335 mL, whereby 185 mL corresponded to the cathodic/anodic chamber and 150 mL to each buffer tank, while the headspace volume of the buffer tank was around 165 mL. Both BES were operated at room temperature (25 ± 1 °C) and kept in the dark throughout the entire experiment.

Figure 17. Real overview of the flat-plate duplicate reactors setup.

In contrast to the tubular BES, the continuous pH measurements were carried out here using two pH probes (IH40AT, Ionode, Australia) coupled to a wireless emitter (Bluebox-pH, Instrumentworks, Australia) sent real-time data to an Apple Ipod (Ipod Touch 6th Gen, USA) that gathered the information through the Dataworks data app. These probes were placed, together with the CO_2 dissolved (same as the Tubular BES) in the outlet of the cathodic chamber and registered data at 5 minutes intervals.

3.2 Experimental procedures

All studies presented in this thesis have been conducted using CO_2 as the main carbon source. BESs were operated in batch mode, feeding CO_2 at the beginning of the cycle. Parameters such as current signal, pH and CO_2 dissolved were

measured inline every 5 minutes. In addition, the total pressure (P_T) was also continuously measured in case of the flat-plate systems.

Liquid and gas phase samples were taken in periods from 2 to 3 days, unless otherwise stated. Chemical analyses performed in the experimental work are detailed in section 3.3. These analyses allowed calculating production rates of each compound and CO_2 consumption rates (section 3.4) of the systems operated. The electrochemical techniques used to operate all BES was chronoamperometry (CA), although occasionally other techniques were also performed (see section 3.5).

The feeding strategies varied depending on the purpose of the study. In case of the tubular systems, the CO_2 gas was sparged into the liquid synthetic medium until saturation. On the contrary, in case of the flat-plate BES, the CO_2 was injected to the headspace until the total pressure reached 350 mbar, which means that the amount of CO_2 fed varied between feeding events.

The inoculum, used in the case of the tubular BESs was I-19 (*Eubacterium limosum* isolate), while an electrode-attached mixed culture was pre-grown in the cathode electrode of the flat-plate BESs. An exhaustive characterization was carried out right before the electrode was placed in the cathodic chamber (see section 3.7).

In case of the applied cathode potential (-0.8V vs. SHE), operation mode (batch) and culture medium (modified ATCC1754 PETC) were the same in all experiments described in this Ph.D. thesis.

3.3 Chemical analysis

Conductivity was analysed at the end of each batch with an electric conductivity meter (EC-meter basic 30+, Crison, Spain). Both The pH and CO_2 dissolved in-line measurements were taken with the abovementioned probes at intervals of 5 minutes.

The optical density (OD) of the catholyte was periodically measured to control the growth of the bulk microbial community with a spectrophotometer (CE 1021, 1000 Series, CECIL Instruments Ltd., UK) at a wavelength of 600 nm.

Gas samples of the tubular systems were collected with a glass syringe (500 μL Hamilton Samplelock Syringe, Hamilton, USA) and subsequently analysed in an Agilent 7890A (Agilent Technologies, USA) gas chromatograph (GC) equipped with a HP-Molesieve column and a thermal conductivity detector (TCD). On the other hand, the gas samples of the flat-plate BES were analysed using a glass syringe (500 μL Hamilton Samplelock Syringe, Hamilton, USA) in an Agilent 490 Micro GC (Agilent Technologies, USA) equipped with a Molsieve 5Å and PoraPLOT U columns in parallel coupled to a TCD detector.

The pressure of the gas phase in the headspace of the BES was measured with a differential manometer (Model-Testo-512; Testo, Germany) in case of the experiments carried out with the tubular BES. On the other hand, the total pressure in the flat-plate BES was continuously measured every 5 minutes using a two pressure transducers (CirrusSense TDWLB Gen1, Transducer Direct, USA) that sent real-time information to an Apple Ipod (Ipod Touch 6th Gen, USA) that gathered the information through the Dataworks data app.

Carboxylic acids and alcohols present in the liquid phase were analysed every two-three days with an Agilent 7890A (Agilent Technologies, USA) gas GC equipped with a DB-FFAP column and a flame ionization detector (FID). Before the analyses, each sample was firstly acidified with ortho-phosphoric acid (85%, Scharlau, Spain) and subsequently crotonic acid (Scharlau, Spain) was used as internal standard to ensure the results obtained.

3.4 Calculation of production rates

3.4.1 Gas product distributions

The quantities of each gaseous compound in the headspace were calculated by means of the gas composition analysis, the known volume of the headspace and the total pressure measured. Also, the composition of the headspace and the known total pressure allowed for the calculation of the partial pressure of each compound and therefore, considering the Henry's law, the concentration of gas dissolved in the liquid phase (calculated according to the equation 2.1.).

$$[\mathrm{i}]_{\mathrm{liq}} = \mathrm{K}_{\mathrm{h,i}} \cdot \mathrm{P}_{\mathrm{i}} \qquad \text{(Eq. 2.1)}$$

Here *i* refers to the compound itself; $[i]_{liq}$ is the concentration of the compound in mol L^{-1} contained in the liquid phase; $K_{h,i}$ corresponds to the Henry's constant in mol L^{-1} atm^{-1} of the compound at the experimental temperature. P_i (atm) is the partial pressure of the compound present in the gas phase, while P_i is calculated from the total pressure and the molar fraction of *i* in the gas phase, according to the following equation (eq. 2.2).

$$P_i = P_t \cdot y_i \qquad \text{(Eq. 2.2)}$$

Where P_t corresponds to the total pressure (atm) measured in the reactor and y_i is the molar fraction of each gaseous compound (*i)* present in the gas phase.

$K_{h,i}$ was calculated as function of the temperature according to the experimental conditions using the equation 2.3.

$$K_{h,i} = K_i^{\theta} \cdot \exp\left[\frac{-\Delta H_{sol}}{R} \cdot \left(\frac{1}{T} - \frac{1}{T^{\theta}}\right)\right] \qquad \text{(Eq. 2.3)}$$

Where K_i^{θ} corresponds to the Henry's constant of the compound *i* at standard temperature (T^{θ}=298.15 K); and ΔH_{sol} is the enthalpy of dissolution of the compound *i*. Table 5 shows Henry's constants and enthalpies of dissolution of gaseous compounds measured in the present thesis.

Table 5. $\Delta H_{sol}/R$ and Henry's constant standard values of different gas compounds, used for calculation in Equation 2.3

Compound	$\frac{-\Delta H_{sol}}{R}$ (K)	K_i^{θ} (mol L^{-1} atm^{-1}) (Sander 2015)
CO_2	2400	3.4 x 10^{-2}
H_2	500	7.8 x 10^{-4}

Partial pressure of hydrogen (pH_2) was calculated from the composition of the gas measured in the headspace and the total pressure of the biocathodic chamber. The concentration of dissolved CO_2 and H_2 in mM were calculated through the Henry's law at room temperature ($K_{CO_2}^{\theta}$ = 3.4·10^{-}

^{2} mol L^{-1} atm^{-1}; $K_{H_2}^{\theta}$ = $7.8 \cdot 10^{-4}$ mol L^{-1} atm^{-1}, T=25ºC). In addition, it was assumed that CO_2 saturation point was reached right after feeding with the gas (by bubbling into the medium), and therefore all the H_2 was flushed from the system.

The total pressure of the gas phase in the headspace of the reactor was measured after feeding and before the sampling with a differential manometer (Model-Testo-512; Testo, Germany). A pH sensor (model 5303, Crison, Spain) was placed in the cathode recirculation loop of each reactor to monitor and control the pH with a transmitter (MultiMeter MM44, Crison, Spain) connected to a data acquisition device (RSG40, Memograph M, Endress+Hauser, Switzerland). Moreover, CO_2 dissolved in-line measurements were periodically performed in the liquid phase with a sensor (InPro® 5000, Mettler-Toledo, USA), which was placed conveniently to measure the CO_2 dissolved content of the cathodic outlet. Gas samples from each reactor were collected at the headspace of the buffer tank with a glass syringe (5 mL, Model 1005, Hamilton, USA) before taking liquid samples, and subsequently analysed in a second channel of an Agilent 490 Micro-GC (Agilent Technologies, USA) equipped with an HP-Molesieve column and a thermal conductivity detector (TCD).

3.4.2 Liquid phase composition

The compounds present in the liquid phase were analysed in terms of product concentration in moles per litre. Since CO_2 was used as sole carbon source and organic products were synthetized from it, all the concentrations were expressed as the concentration of carbon (C) in each molecule in the form of moles of C per

litre (mMC). The moles of C were calculated for each compound according to the molecular weight and the number of carbon atoms contained in each molecule, as shown in the following equation (2.4).

$$\mathrm{mMC} = \frac{\mathrm{C_i} \cdot \mathrm{n_{C,i}}}{\mathrm{M_i}} \qquad \text{(Eq. 2.4)}$$

Where C_i corresponds to the concentration of the specific product (i) in the liquid phase (in mg L^{-1}); $n_{C,i}$ is the number of carbon atoms contained in the molecular structure of the compound and M_i corresponds to the molecular weight of i (in mg $mmol^{-1}$).

The concentration (C_i) of short and medium carboxylic acids (SCCAs; MCCAs) and alcohols present in the liquid phase were analysed with an Agilent 7890A (Agilent Technologies, USA) gas chromatograph (GC) equipped with a DB-FFAP column and a flame ionization detector (FID).

These values correspond to the samples taken before feeding the cathode with CO_2.

3.4.3 Organic carbon production rate

The moles produced of each compound were presented as function of time and normalized to liquid volume through the equation 2.5. The production rates were expressed as mM C d^{-1}.

$$\text{Production rate} = \frac{\mathrm{C_f} - \mathrm{C_i}}{\mathrm{t}} \qquad \text{(Eq. 2.5)}$$

Where, C_f is the concentration of a certain compound at the end of the batch in mM C, C_i is the concentration of a certain compound at the

beginning of the batch in mM C and t (d) is the time between the CO_2 feeding point and the measuring point.

3.5 Electrochemical analyses

Potentiostats (SP-50 and VSP models, BioLogic, France) (Figure 18) working in a three-electrode configuration controlled the voltage and fixed the cathode potential. The cathode acted as working electrode (WE), reference electrode (RE) was an Ag/AgCl electrode 0.197 V vs. SHE (sat. (sat. KCl, SE11-S Sensortechnik Meinsberg, Germany) placed in the cathodic chamber for potentiostatic control. Finally, a counter electrode (Ce) was placed in the anodic chamber. The system was operated in CA mode with a fixed potential of -0.8V vs. SHE. Electrochemical-related parameters such as current signal, cell potential and power consumed were continuously monitored and registered once every 5 minutes. All the voltage values that appear throughout this thesis are reported versus SHE.

Electrochemical analyses were performed to understand the electroactivity and electrochemical capacity of each BES (Harnisch and Freguia 2012). These techniques used in this thesis were based on voltage scanning and a thorough analysis of the response given by the current signal. Among them, cyclic voltammetry (CV), linear sweep voltammetry (LSV), or differential pulse voltammetry (DPV) were performed when necessary.

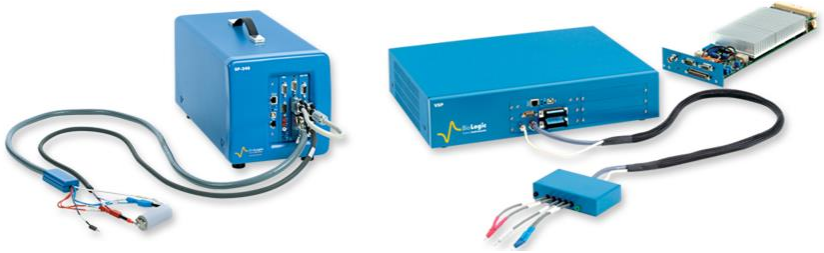


Figure 18. Bio-logic SP-50 (left) and VSP (right) potentiostat models used in this thesis.

3.5.1 Coulombic and energy efficiencies

The calculation of the coulombic efficiency (CE) is based on the comparison of the energy consumed and the energy contained in the final compounds produced, which is the reason why is expressed in percentage (5). This equation is summarized by Patil and colleagues (Patil, Gildemyn, et al. 2015). The CE was calculated using the following equation (Eq. 2.6).

$$\mathrm{CE} = \frac{\mathrm{F} \cdot \Sigma_{\mathrm{i}} \mathrm{M}_{\mathrm{e,i}} \cdot \Delta_{\mathrm{e,i}}}{\int \mathrm{I}\, \mathrm{dt}} \cdot 100 \qquad \text{(Eq. 2.6)}$$

Where F is the Faraday's constant; $M_{p,i}$ correspond to the moles of product (i); $\Delta_{e,i}$ is the difference (in degree) of reduction between substrate and product (i.e. moles of electrons per mol of product); and $\int I\, dt$ is the integration of the current supplied over time.

3.6 Enrichment of electroactive bacteria on the electrode

3.6.1 Inoculum: I-19

A new isolate named I-19 was obtained from sheep manure pellets by Ramió-pujol (Ramió-Pujol 2016). According to the similarity of an almost complete 16S rRNA gene sequence fragment, I-19 could be identified as a *Eubacterium limosum* isolate. It showed the ability to produce an equivalent molar production of acetate and ethanol in batch reactors maintained under strict autotrophic conditions with syngas at 37 ºC and also in BES fed with CO_2 (Blasco-Gómez et al. 2019).

I-19 cultures inoculated in the tubular reactors were incubated at 37ºC under mild agitation in a rotary shaker at 100 rpm. A modified ATCC1754 PETC medium was used for growth and maintenance (Table 13). Medium was prepared anaerobically and pH was adjusted to 6. Cultures were flushed on a two to three day basis with syngas (32% CO, 32% H_2, 28% N_2 and 8% CO_2) for at least five minutes, and kept at an overpressure of 100 kPa at 37ºC (Ramió-Pujol 2016). Serum sealed tubes (Ochs Gläsgeratebau, Germany) or gas-tight bottles (100 mL), were used for routine cultivation of the isolate. Transfers to freshly prepared medium were performed at a 10 to 20 % ratio.

Previously to its inoculation in the flat-plate BESs, the isolate was activated in 200 mL serum bottles and maintained in organic medium at 30ºC under anaerobic conditions fed periodically with CO_2:H_2 (80:20% *v/v*) (Praxair, Spain).

3.6.2 Fermentation cell

The fermentation cell used for the enrichment of I-19 on the electrode was made of methacrylate and had a total volume of 410 mL (15 cm length, 10 cm width and 2.7 cm depth) (Figure 19,1). The cell was connected to a buffer tank of 300 mL of total volume. A pump (Watson-Marlow 505U, Watson-Marlow Fluid Technology Group, USA) was continuously recirculating medium at a constant rate of 7 L h^{-1}. The outlet was used to take samples. Three carbon cloths (Thickness 490µm; NuVant's ELAT, LT2400W, FuelCellsETc, USA) of 129 cm^2 (4.3 cm width and 15 cm length) were placed inside along the fermenter chamber. In addition, three spacers were placed between each carbon cloth to avoid accumulation of gas when feeding the chambers and contact between each other (Figure 19,2). One of these carbon cloths was used to analyse the biological community.

Figure 19. Pictures of the fermentation cell (1) already assembled (2) and during its operation.

Before inoculation, the fermenter cell was sterilized exposing it under UV light for 20 minutes. The chamber was continuously flushed with pure N_2 gas (99.9%, Nippon gases, Spain) in order to avoid the presence of O_2.

The cell was inoculated filled with 500 mL of organic medium (Table 14) and subsequently inoculated with 200 ml of a I19 inoculum that was previously activated with the same organic medium and fed with CO_2:H_2 (80:20% *v/v*) (Praxair, Spain) later on.

3.6.3 Operation of the fermentation cell

The gas and liquid phases in the system were sampled twice a week right before feeding with gas. The gas sample was taken in the headspace of the buffer tank and collected in a 5 mL vacutainer for its further analysis. The liquid sample was taken directly from a sample port located in the outlet of the fermenter cell. Gas samples were analysed with an Agilent 490 Micro GC (see section 3.3). Liquid samples were analysed with an Agilent 7890A (Agilent Technologies, USA) gas GC (see section3.3).

After each sampling event the cell was opened in an anaerobic chamber (90 % N_2, 5% CO_2, 5% H_2; *v/v*) in absence of oxygen (Vynyl Anaerobic glove chamber, CoyLab, USA) and a sample of 0.25 cm^2 from the "sacrificial" carbon cloth was extracted to study the biofilm formation. The samples were taken from three different areas of the carbon cloth (both extremes and the middle). Then the samples were stored in a freezer under -20ºC. In addition, a 10 ml sample from the liquid phase was collected and subsequently centrifuged (Centrifuge 5702R, Eppendorf, Germany) at 4400 rpm under 4ºC for 20 minutes. The supernatant was discarded and the pellet was stored in the freezer at -20ºC for further analysis.

After taking liquid samples, the medium was replaced with modified ATCC1754 ETC medium whose pH was previously adjusted to 6 and flushed with N_2 (Praxair, Spain) to ensure the absence of O_2. The system was then placed in the original position and reconnected to the buffer tank. Afterwards, the system was fed with CO_2:H_2 (80:20% *v/v*) until saturation and the normal operation was re-stablished.

3.6.4 Transfer of electrode from open-circuit to close-circuit.

After 45 days of operation, the operational conditions were comparable to BES (pH and autotrophy) and a thin layer of biofilm was visible with the naked eye. Then, two of the carbon cloths were placed in the cathodic chambers of two flat-plate BES (see section 3.1.2) using anoxic conditions in the anaerobic chamber (Vynyl Anaerobic glove chamber, CoyLab, USA). The system was setup accordingly, both chambers (cathode and anode) were then filled with fresh modified ATCC1754 ETC medium saturated with pure CO_2 (99,95%, Praxair, Spain), and connected to the potentiostat (VSP model, Bio-Logic, France) to start the experiments.

3.7 Microbial characterization analyses

3.7.1 DNA extraction and microbial analyses

In order to characterize the biological community present on the cathode and in the bulk solution, conventional polymerase chain reaction (PCR) were performed. DNA extractions were performed from (i) the pieces of carbon cloth extracted and (ii) the pellets resulted from the centrifugation of the bulk liquid. The cell lysis was

achieved through bead-beating with the addition of sterilized 0.2 g of 0.1 mm diameter glass beads and 0.3 g of 0.1 mm diameter silica beads in sterilized 2 mL Eppendorf tubes. The pellets resulted from the settlement of bulk liquid community were re-suspended in 0.31 mL of lysis buffer (40 mM EDTA, 50 mM Tris-HCl; pH of 8.3) and 0.75 M of sucrose. This solution was then added to the previously mentioned *Bead-Beater* tubes and followed the protocol described elsewhere (Llirós, Casamayor, and Borrego 2008) involving the use of hexadecyltrimethylammonium bromide. In the case of the lysis of the cells forming the biofilm attached to the carbon cloth, each 0.25 cm^2 piece collected during each sampling was introduced in the 2-mL *Bead-beater* Eppendorf tube and 0.31 mL of lysis buffer was added. The extraction was also achieved through a protocol described elsewhere (Llirós, Casamayor, and Borrego 2008).

A specific PCR was performed to both set of samples in order to identify the presence of the isolate I-19 using specific primers (EL1F/EL1R) for the detection of *Eubacterium limosum*, previously designed by the GEMM research group of the University of Girona (data not published). PCRs were performed adding 1 µL of sample in a total volume of 20 µL and a final concentration of nucleotides (dNTPs) of 0.4 mM (Applied Biosystems, USA), 1.5 mM of $MgCl_2$ (Applied Biosystems, USA), 0.25 µM of each primer and AmpliTaq® 360 DNA Polymerase at 0.025U/reaction (Applied Biosystems, USA).

The samples were amplified in a thermocycler GeneAmp PCR system 2700 (Applied Biosystems, Thermo Fisher Scientific Inc., USA) with the following steps: 4 minutes at 94 ºC (denaturation), 35 cycles of 30 seconds at 94 ºC (denaturation), 30 seconds at 60 ºC (annealing) and 90 seconds at 72 ºC (extension). Finally, the samples were exposed at 72 ºC for 10 minutes to carry out a final extension and at 4 ºC until the thermocycler was stopped.

DNA extracts were finally analysed by agarose (1.5 %) gel electrophoresis, and visualized after staining with GelRed™ (Biotium Inc., USA) and a molecular weight marker (GeneRuler™ 100bp DNA Ladder Plus) with a UV lamp.

3.7.2 Quantitative analysis through qPCR

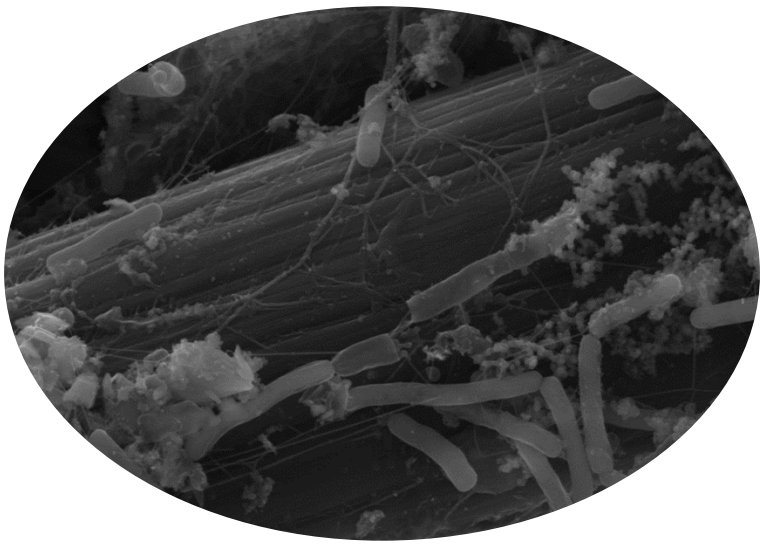
The determination of the amount of biomass attached to the electrode and present in the bulk liquid was achieved through quantitative PCR (qPCR). Notwithstanding, a previous quantification was carried out using Qubit® (Qubit® 2.0 fluorimeter, Invitrogen, USA). The qPCR was based on SYBR green technology and targeted the 16S rRNA gene. 10 µL of each sample analysed contained 6 µL of a LightCycler® 480 SYBR® Green (Roche Molecular Systems, USA) mix and 3 µL of MiliQ water, and 0,5 µL of each primer (341F/534R) whose design is available elsewhere (López-Gutiérrez et al. 2004). The mixture was added to a 96 microplate and was subsequently added 2 µL of sample (or 1 µL of sample plus 1 µL of MiliQ water), therefore each well contained 12 µL of solution. The microplate was introduced in a LightCycler® 96 (Roche Molecular Systems, USA) that performed a pre-incubation cycle, 40 cycles of amplification and one cycle of analysis of the formed amplicons as stated elsewhere (López-Gutiérrez et al. 2004). The fluorescent signal data was processed with LightCycler software (v.1.1.) and a pattern line resulted from the alignment of all the dilutions of the amplified 16S rRNA was used to obtain the number of copies obtained per sample.

3.7.3 Analysis of the biological community

The structure of the biological community in both the electrode (carbon cloth) and the bulk liquid was analysed through sequencing of each sample. The sequencing process followed is detailed by Perona-Vico and co-workers (Perona-Vico et al. 2019).

Chapter 4

Unravelling the factors that influence the bio-electrorecycling of carbon dioxide towards biofuels.



Redrafted from:

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4.1 Background and objectives

The production of chemicals and liquid fuels from wastes has recently attracted global attention. For that reason, new resource recovery technologies are in great demand. Among them, the use of reactors containing mixed microbial populations has emerged as an interesting option to convert wastes into sustainable target products through fermentation (Agler et al. 2011).

In this sense, approaches such as METs are considered promising due to its ability to use widely available CO_2 waste streams as the sole carbon source (Rabaey and Rozendal 2010; Nevin et al. 2010; Pepè Sciarria et al. 2018). In METs, electroactive organisms fix CO_2 into valuable products by means of bioelectrocatalytic processes (Figure 20). To date, acetate is the main product obtained by METs solely fed with CO_2, although many authors have demonstrated that C4 or C6 compounds can also be produced (Ganigué et al. 2015; Jourdin et al. 2018; Batlle-Vilanova et al. 2017; H. Li et al. 2012). In spite of these proof-of-concept experiments, there is a lack of understanding of not only the biological processes that occur in the reactor, but also how environmental conditions hamper product selectivity towards longer C-chain products. In the majority of examples available to date using homoacetogenic METs, hydrogen is concomitantly produced in the biocathode as an intermediate step in acetogenesis, thus diminishing mass transfer effects.

Furthermore, chain elongation has been considered an interesting bioprocess to convert short-chain carboxylates (e.g., acetate [C2] and n-butyrate [C4]) into medium-chain carboxylates (e.g., n-caprylate [C8] and

n-caproate [C6]) with hydrogen gas as a side product in the presence of organic electron donors, e.g. ethanol, lactate and carbohydrates (Angenent et al. 2016). The electron donor requirement for chain elongation to take place makes METs a potential initial process that might provide a suitable substrate to a coupled chain elongating reactor. Even though other authors have stressed the idea that chain elongation could be performed in the same MET reactor (Reddy, ElMekawy, and Pant 2018; Vassilev et al. 2018). Vassilev et al. recently gave some valuable insights on the putative metabolic pathways active during chain elongation in a CO_2-fed biocathode (Vassilev et al. 2018).

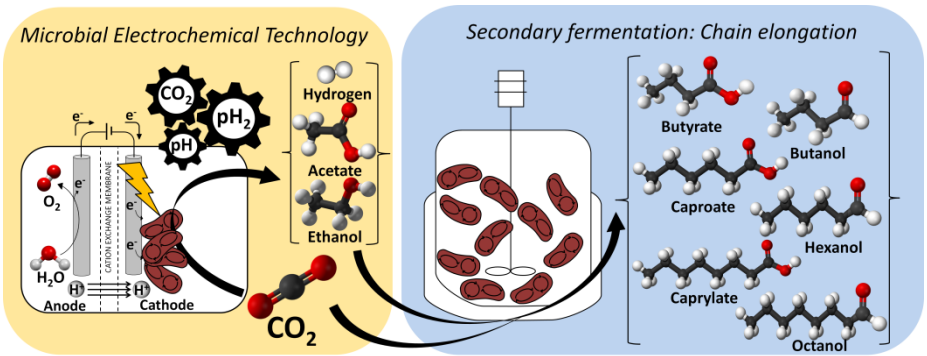


Figure 20. Proposed scheme of the bio-electrorecycling of CO2 into biofuels with a coupled chain elongation reactor.

In this way, ethanol was considered as the most interesting target compounds in CO_2-fed biocathodes from the sustainable and economic point of view (Christodoulou et al. 2017). Unfortunately, ethanol has been rarely detected in the catholyte of CO_2 reducing biocathodes (Suman Bajracharya et al. 2017; Ammam et al. 2016; Zaybak et al. 2013; Mohanakrishna, Vanbroekhoven, and Pant 2016; Arends et al. 2017; Batlle-Vilanova et al. 2017; Srikanth, Kumar, et al. 2018; Jabeen and Farooq 2016; Vassilev et al. 2018). Only

recently, Srikanth and colleagues were successful at producing ethanol over other products by using a gas diffusion electrode (Srikanth, Singh, et al. 2018). However, how different operational conditions lead to the production of ethanol in METs remains poorly understood. Notwithstanding this, pH, carbon and/or reducing equivalents availability have been pointed out as key factors for selective ethanol production (Roghair et al. 2018; Gunda, Vanbroekhoven, and Pant 2018). Indeed, reducing equivalents (mainly in the form of hydrogen) not only control the specific productivity, but also the final product spectrum (Blanchet et al. 2015). Therefore, deciphering the basis of the bioelectrochemical ethanol production in biocathodes only fed with CO_2 should be considered to achieve a maintained solvent production.

In the present work we aimed at providing key elements for sustained maintenance and operation conditions for a tuneable production ethanol-to-acetate ratio in MET reactors inoculated with an enriched homoacetogenic bacterium. Our results provide new insights on how to operate future MET reactors under a tighter control on product selectivity, which will make MET an alternative bio-based technology for bio-ethanol production.

4.2 Methodology

4.2.1 BES Set-up

Two tubular BES were set and named Reactor 1 and Reactor 2. Each BES had the same features as indicated in subsection 3.1.1 and were operated under a specific configuration (Figure 21).

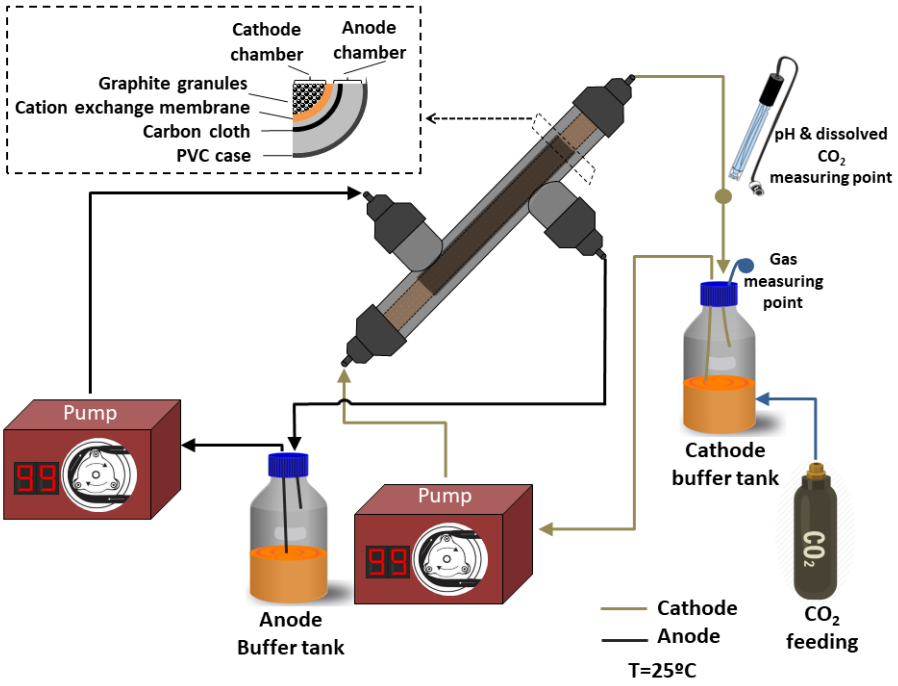


Figure 21. Schematic representation of the tubular BES setup.

4.2.2 Microorganism and growing conditions

The cathodes were initially inoculated with 100 mL (10% v/v) of an exponentially growing culture of a new isolate named I-19, obtained from sheep manure pellets. According to the similarity of an almost complete 16S rRNA gene sequence fragment, I-19 could be identified as a *Eubacterium limosum* isolate. It showed the ability to produce an equivalent molar production of acetate and ethanol in batch reactors maintained under strict autotrophic conditions with syngas at 37 ºC (Ramió-Pujol 2016), making it a good candidate to be tested in this experiment. Moreover, previous studies were performed to prove its electroactivity through CV (Figure 22).

I-19 cultures were incubated at 37ºC under mild agitation in a rotary shaker at 100 rpm. A modified ATCC1754 PETC medium was used for growth and maintenance (Table 13). Medium was prepared anaerobically and pH was adjusted to 6.00. Cultures were flushed on a two to three day basis with syngas (32% CO, 32% H_2, 28% N_2 and 8% CO_2) for at least five minutes, and kept at an overpressure of 100 kPa at 37ºC (Ramió-Pujol 2016). Serum sealed tubes (Ochs Gläsgeratebau, Germany) or gas-tight bottles (100 mL), were used for routine cultivation of the isolate. Transfers to freshly prepared medium were performed at a 10 to 20% ratio.

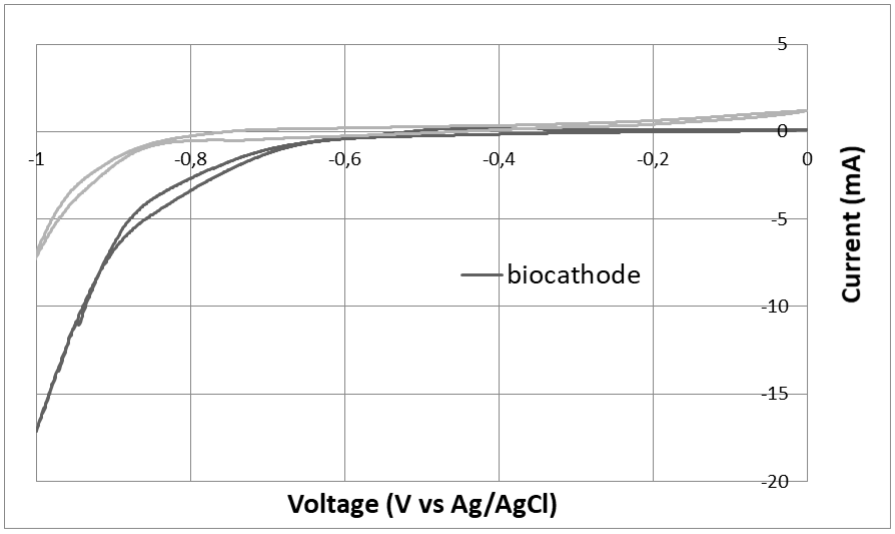


Figure 22. Cyclic voltammetry tests performed under abiotic (grey) and in in the presence of the I-19 (dark grey) to prove its capability to interact with the cathode. This technic shows a higher current demand with the same electrode under the same conditions, which could be directly related to the biocatalyzed hydrogen production.

4.2.3 BES Start-up and operation

Both BES were operated in batch-mode. Anode and cathode were filled with low-buffered mineral medium, which was prepared based on a modified ATCC1754 PETC medium adjusted to pH 5.4 (Batlle-Vilanova et al. 2017). Inorganic carbon in the form of CO_2 was used as the only carbon source, which was supplied every 2 to 3 days by sparging pure CO_2 (99.9%, Praxair, Spain) for over 5 minutes into the buffer tank. The frequency at which CO_2 was bubbled was based on the consumption rate of the system. Thereby, a low range pH (5.3-5.9) was maintained and also enabled to monitor the switch of metabolic pathways in the biological community.

Liquid and gas samples were collected regularly before feeding with CO_2 to analyse the production of volatile fatty acids (VFAs) and alcohols, and to characterize the gas composition, respectively.

4.3 Results & discussion

4.3.1 Performance of the BES reactors

The two reactors were inoculated at a 20% ratio (v/v) with an exponentially growing I-19 cells cultured with syngas (32% CO, 32% H_2, 28% N_2 and 8% CO_2) at 37ºC (Ramió-Pujol 2016). Electrochemical behaviour and product evolution over time for both reactors was similar during the first days after inoculation (Figure 23).

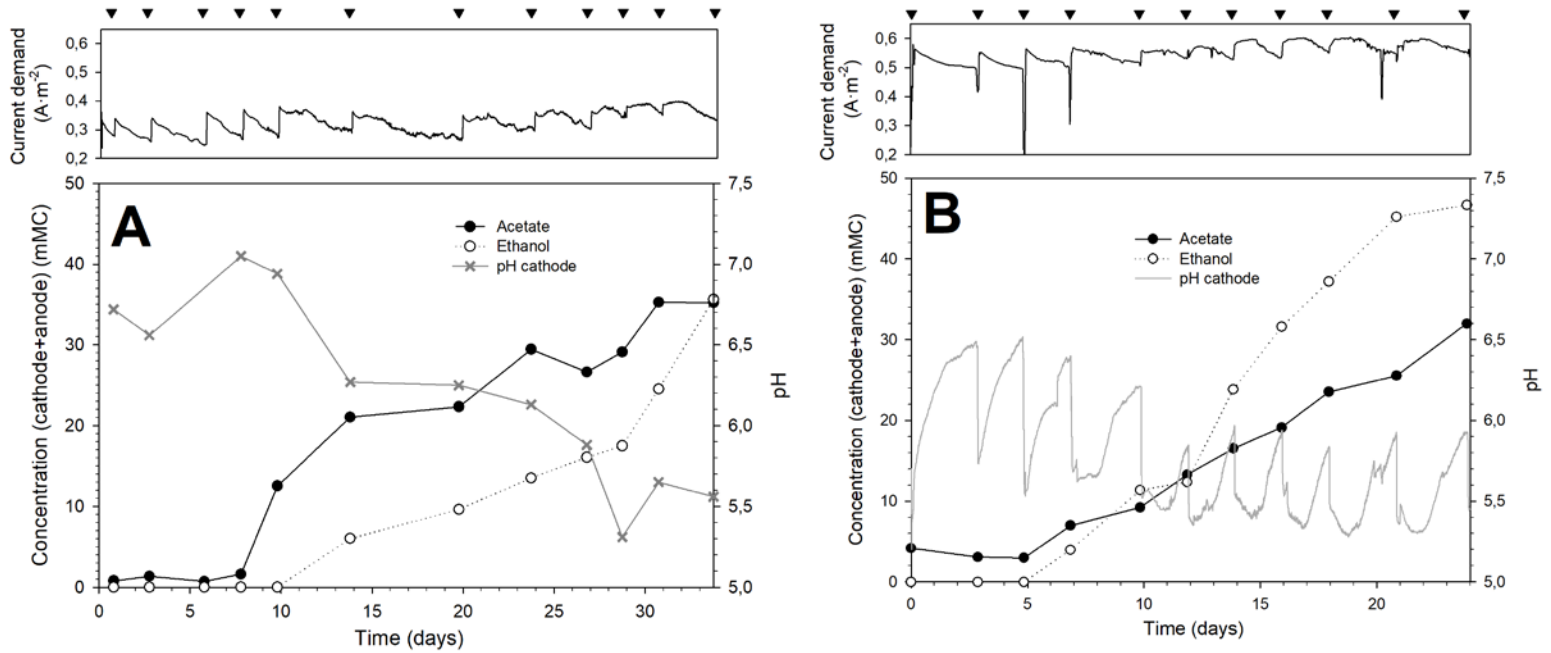


Figure 23. Evolution of current signal, pH and total concentration of acetate and ethanol over time in Reactor 1 (A) and 2 (B). Black inverted triangles on the top part of the figure indicate sampling and subsequent flushing with CO_2.

Neither VFAs nor alcohols were detected before five days of operation while hydrogen production was detected since the beginning of the experiment. Part of this hydrogen might have been biotically produced, as suggested by other authors that worked with CO_2-fed biocathodes performing acetogenesis (Anzola Rojas, Mateos, et al. 2018). Indeed, after a short adaptation period of 5 to 7 days, homoacetogenic activity was observed in both biocathodes by the production of acetic acid. It should be noticed that neither butyrate nor butanol were detected in the catholyte at any time of the experiment. The lack of production could be more likely explained by the inactivation of the β-oxidation pathway at the working conditions or, alternatively, by the lack of key enzymes required for such reactions, described previously by Vassilev and co-workers (Vassilev et al. 2018).

In case of the reactor 1 (Figure 23A), acetate was firstly detected after the 7th day, whereas ethanol production started later. The sequenced production might be explained by the need of a minimum acetate accumulation in the catholyte to trigger ethanol production via the acetate re-assimilation pathway (Bertsch and Müller 2015). Total yields for the whole production period in Reactor 1 (35 days) was 60 ± 59 mg ethanol $m^{-2}_{electrode}$ d^{-1} and 63 ± 67 mg acetate $m^{-2}_{electrode}$ d^{-1}. The maximum concentration of ethanol and acetate reached in the biocathode were 0.82 g ethanol L^{-1} and 1.06 g acetate L^{-1}, which correspond to an ethanol-to-acetate ratio of 0.78 (g ethanol/g acetate). Concomitantly to acetic acid production pH decreased during the overall test from 7 to 5.5. In addition, CE was maintained around 14 ± 7% along the experiment. Related to the H_2 in the gas phase, after each feeding by CO_2 sparge, pH_2 plummeted to zero and increased once H_2 was formed and accumulated in the headspace before subsequent feeding event,

achieving a pH_2 mean value of 1.03 ± 0.02 atm. The fact that H_2 was detected in the headspace suggests that the community was not able to use all produced H_2, leading to an increase of pH_2 in the system.

Performance of Reactor 2 was similar to Reactor 1 in terms of acetate and ethanol production (Figure 23B). Ethanol and acetate were produced with similar yields than reactor 1 (1.5 referred to g ethanol/g acetate). In this case, the system yielded 82 ± 48 mg ethanol $m^{-2}_{electrode}$ d^{-1} and 99 ± 62 mg acetate $m^{-2}_{electrode}$ d^{-1} since day 5. The high deviations shown in the yields (also in reactor 1) can be explained by the lack of pH control, since sampling was carried out in an inconstant basis (2-3 days) and product concentration varied. The maximum concentration of ethanol and acetate achieved in the biocathode was 1.08 and 0.87 g L^{-1} respectively, which in case of ethanol is among the highest reported under the working conditions (Table 4). In addition, the CE was maintained at 12 ± 10 % along the experiment and the maximum pH_2 reached in the system at the end of every cycle was 1.03 ± 0.03 atm.

Interestingly, current signal was able to record significant physiological changes between feeding events (Figure 24, Figure 27). The current profile rapidly increased after feeding with CO_2 in periods when organics were not produced, and subsequently started to slowly decrease until the next feeding event. Inversely, during production of organics, the current signal profile showed a rapid increase after feeding with CO_2 followed by a plateau that ended up in a slow decrease until CO_2 was supplied again.

However, reactor 2 demanded significantly more current than reactor 1 along the whole experiment, which could be explained by side reactions that act as electron sinks and have not been quantified. The higher reducing

power present in the biocathode chamber of reactor 2 might also explain the increase in ethanol concentration found in the catholyte, since this compound requires stoichiometrically 12 moles of electrons to form 1 mole of product, whereas the production of 1 mole of acetate requires 8 moles of electrons.

The continuous measurement of pH in Reactor 2 (Figure 23B) revealed differences in the periods between CO_2 supplies depending on the production of organics. When no organics where produced (0 to 7 days), pH plummeted immediately after feeding with CO_2 and increased continuously afterwards. In contrast, in periods when organic products were formed, the pH remained at low values (between 5.3 and 5.5) outlining a baseline followed by a subsequent increase until the next CO_2 load. Figure 23B shows that the current signal profile in reactor 2 followed the same trend than in reactor 1. This profile outlined a typical plateau when organic products were formed with a subsequent stepwise decrease.

4.3.2 Overall carbon balance

An overall bio-electroconversion of CO_2 to organics was calculated as a carbon balance. Reactor 1 averaged a conversion into acetate and ethanol of 28 ± 12 % of the total C supplied in the form of CO_2 (25.7 ± 0.2 mmol C_{CO2}), whereas reactor 2 converted the 27 ± 9 % of the total C supplied (26 ± 1 mmol C_{CO2}). During the bioelectrochemical process the yield of acetate and ethanol produced from C_{CO2} consumed (mmol $C_{acetate/ethanol}$ $mmol^{-1}$ C_{CO2}) was of 0.67 ± 0.20 mmol $C_{acetate}$ $mmol^{-1}$ C_{CO2} and 0.33 ± 0.20 mmol $C_{ethanol}$ $mmol^{-1}$ C_{CO2} in reactor 1. On the other hand, reactor 2 yielded 0.33 ± 0.32 mmol $C_{acetate}$ $mmol^{-1}$ C_{CO2} and 0.67 ± 0.32 mmol $C_{ethanol}$ $mmol^{-1}$ C_{CO2},

which partially explains why reactor 2 demanded more current that reactor 1 throughout the whole experiment. These values are indeed comparable to other CO_2-reducing biocathodes in the literature (Pepè Sciarria et al. 2018; Mohanakrishna, Vanbroekhoven, and Pant 2016; Batlle-Vilanova et al. 2017), pointing out biomass production, bicarbonate formation or non-analysed compound production as main sinks for the C not used by the system.

4.3.3 Thorough study of acetogenic and solventogenic shifts in CO2-fed BES

In order to understand the dynamics of the system acetate + ethanol producing phases were thoroughly analysed by taking samples between consecutive CO_2 feeding points compared to non-production phases. As expected, pH changes in non-producing periods were most likely linked to the use of protons for the catalysis of H_2 on the electrode surface (Figure 24; replicate shown in Figure 27). Consumption of protons conducted to an increase of pH and consequently, CO_2 was converted to bicarbonate (pK_a = 6.35 at standard conditions). In addition, the current signal started to decrease immediately after feeding, which suggested a high dependence of this parameter on the availability of protons in the catholyte. Current signal decreased once the pH increased due to the protons used in the bioelectrocatalytic process to produce H_2, which resulted in the appearance of this gas in the headspace. However, extra hydrogen was not required due to the lack of acetate production and thus the current signal dropped.

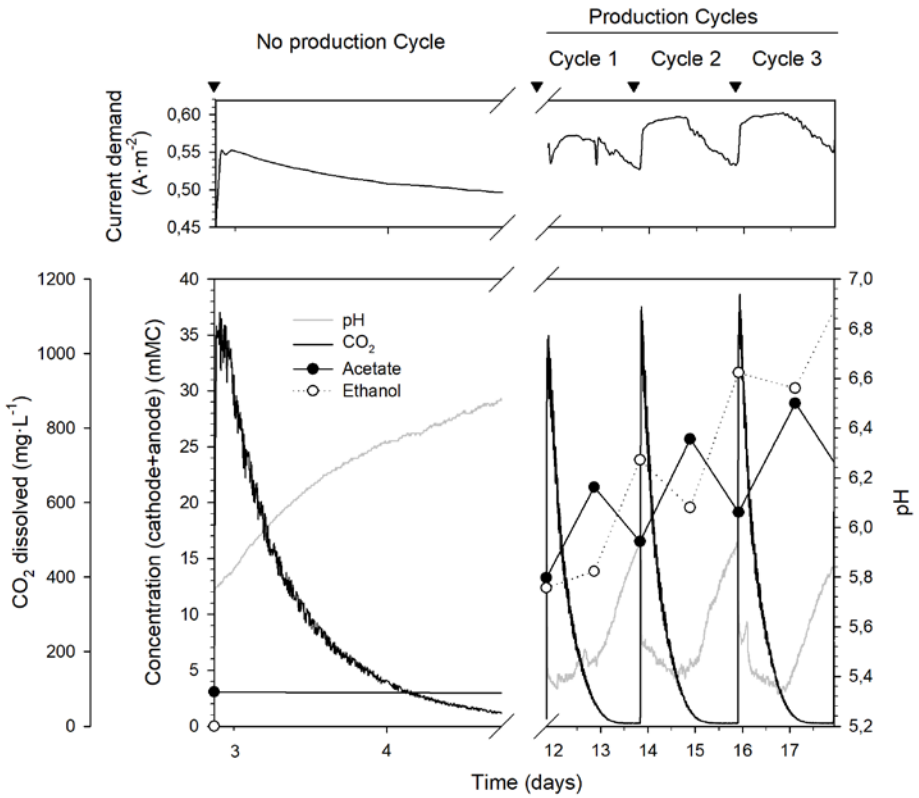


Figure 24. Current demand signal, pH, CO_2 dissolved and total concentration of acetate and ethanol in the reactor 2. Black inverted triangles on the top part of the figure indicate sampling and subsequent flushing with CO_2.

During production cycles (1, 2 and 3) acetate was produced immediately after feeding with CO_2, which corresponded to high CO_2 availability, low pH and low pH_2 in the biocathode. This is related to the acetogenic phase where CO_2 was reduced to acetate in the presence of H_2 previously accumulated in the biocathode (reactions 1 and 2, Table 6) (Figure 25). This statement also supports the maintenance of pH as a result of the neutralization of the acetic acid production through the hydrogen evolution. In addition, the slight increase of the current signal is most likely related to electron feed for H_2 production and electroacetogenesis. Cycles

2 and 3 showed that part of the ethanol was consumed in the first acetogenic phase, which may have served as an additional electron donor, together with H_2 (reaction 4, Table 6).

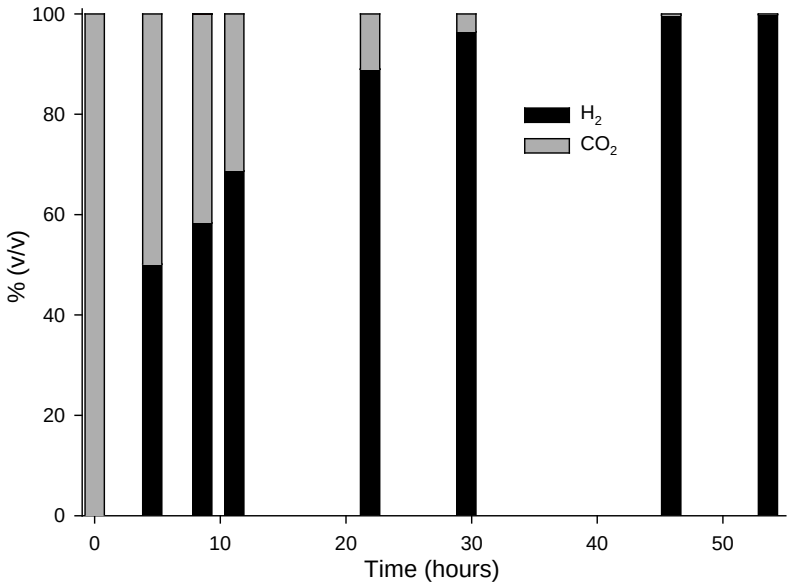


Figure 25. Variation in the gas composition of the headspace of reactor 2 between CO_2 feeding events.

Table 6. Reaction taking place in the biocathodes

Ref. Text	Reaction	Potential required Gibbs free energy	Reference
(1)	$2H^+ + 2e^- \rightarrow H_2$	E°'= -0.41V vs. SHE	(Rabaey and Rozendal 2010)
(2)	$4H_2 + 2CO_2 \rightarrow Acetate^- + H^+ + 2H_2O$	ΔG°'= -94.9 kJ mol^{-1}	(Dolfing, Larter, and Head 2008)
(3)	$Acetate^- + H^+ + 2H_2 \rightarrow Ethanol + H_2O$	ΔG°'= -9.1 kJ mol^{-1}	(Steinbusch, Hamelers, and Buisman 2008)
(4)	$Ethanol + H_2O \rightarrow Acetate^- + H^+ + 2H_2$	ΔG°'= 9.1 kJ mol^{-1}	(Steinbusch, Hamelers, and Buisman 2008)

The samples taken at the end of each cycle suggested the recovering of the solventogenic phase, since ethanol was produced and enough reducing power was reached from the increasing of the pH_2. In contrast, acetate was consumed, which might be explained by the fact that it was reduced to ethanol with molecular hydrogen as electron donor (reaction 3, Table 6) via solventogenesis. This is also supported by the increase of the pH likely linked to hydrogen (reaction 1, Table 6) together with ethanol production (reaction 3, Table 6) (Figure 26).

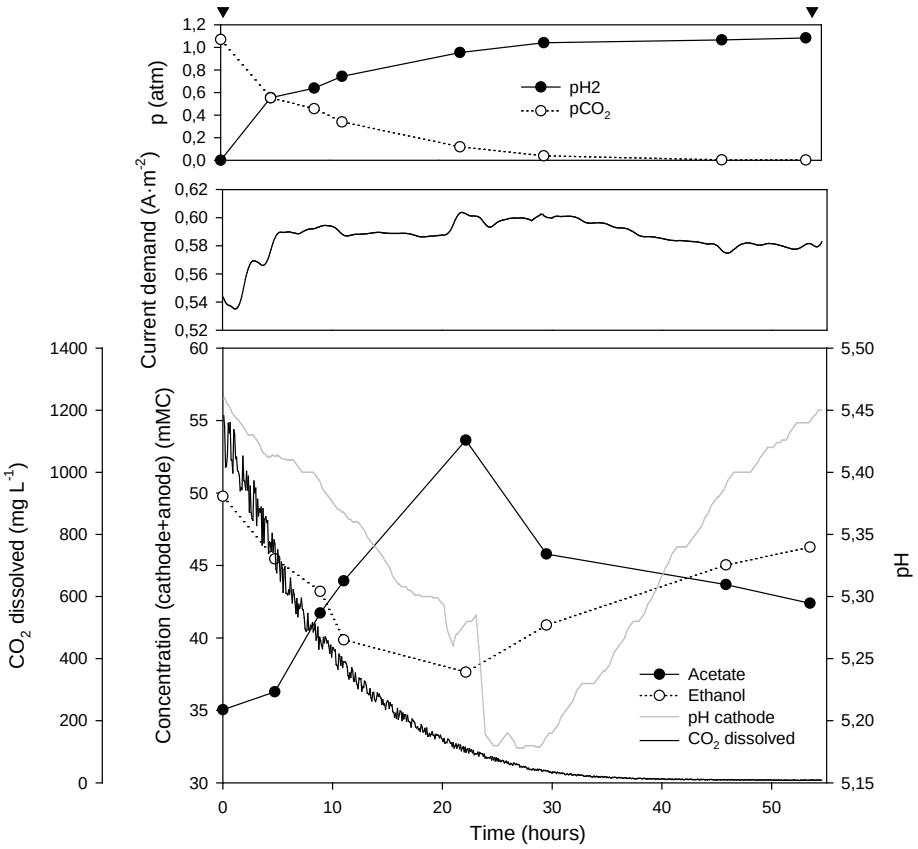


Figure 26. Current demand, pH, pH_2, CO_2 dissolved and total concentration of acetate and ethanol in a production cycle of reactor 2. Black inverted triangles on the top part of the figure indicate sampling and subsequent flushing with CO_2.

Moreover, the increase of pH matches with the current signal decrease. During this phase, CO_2 availability was low (<100 mg $CO_{2dissolved}$ L^{-1}) and the pH_2 was high (1.03 ± 0.02 atm), which was pointed out as a triggering factor for solventogenesis (Agler et al. 2011; Angenent et al. 2004b).

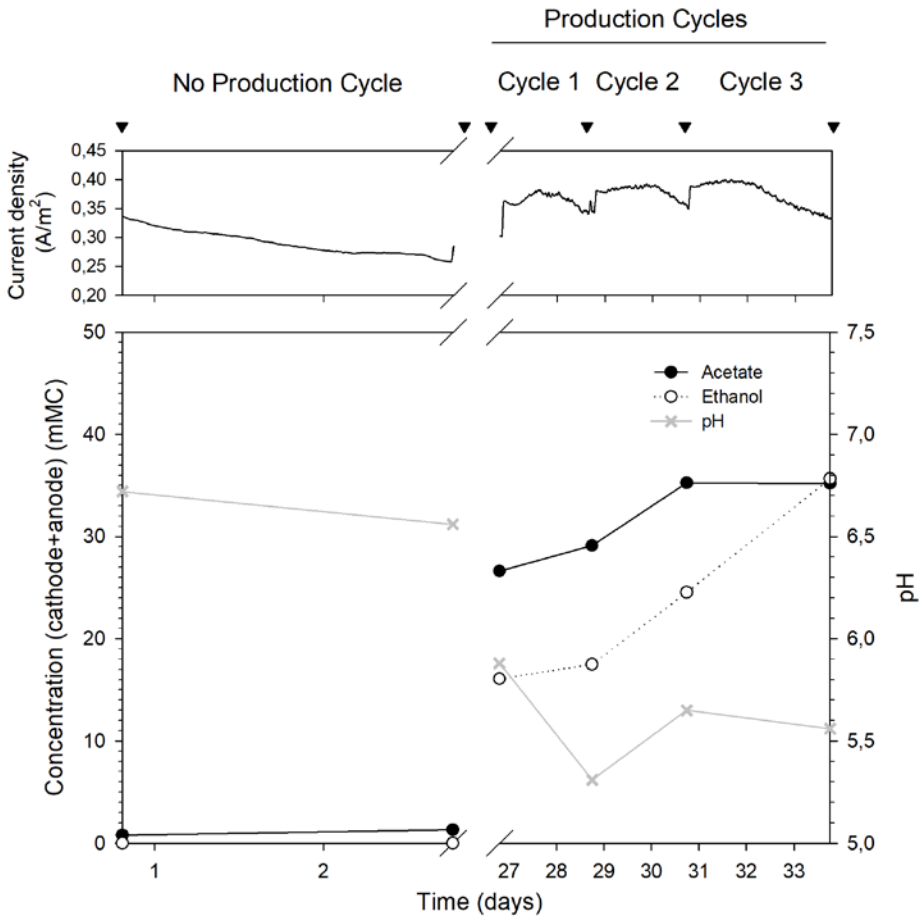


Figure 27. Current signal, pH and total concentration of acetate and ethanol in the reactor 1. Black inverted triangles on the top part of the figure indicate sampling and subsequent flushing with CO_2.

4.3.4 Thermodynamic approach to reveal the role of H_2 and CO_2 in the bioelectrochemical solvent production

The system produced H_2 as main product from the beginning of the experiment and was presumably the main source of reducing power. Moreover, H_2 was produced and accumulated in the biocathode headspace between flushing events, which increased the pH_2 to values over 1 atm. Many authors have stated that solventogenesis is triggered under high pH_2 and a low pH not only in METs (Steinbusch, Hamelers, and Buisman 2008), but also in fermentative processes with *Clostridium* spp. and others (Ganigué et al. 2016; Ramió-Pujol et al. 2015a, 2015b). Moreover, the accumulation of undissociated organic acids (pH ≈ pK_a; $pK_{acetate}$ = 4.76) may be toxic for bacteria, which could eventually force solventogenesis to occur and decrease the amount of VFAs as a detoxifying process (Jones and Woods 1986).

In addition, the surplus of H_2 in the headspace of the BES reactor, along with the CO_2 not consumed, may feed a hypothetical second stage fermenter where carbon chain elongation would ultimately take place. This should be considered as an advantage since it could raise the production rate and improve the energetic balance, thus increasing the overall potential of the system (Angenent et al. 2016).

A thermodynamic approach was used to assess the Gibbs free energy required in the biocathode to perform reactions 2 (electroacetogenesis) and 3 (solventogenesis) from experimental data gathered in reactor 2. Acetogenesis resulted more spontaneous than solventogenesis under the experimental conditions of this work. This was indeed concluded by Dolfing

et al. and Steinbusch et al. in standard conditions, which set the Gibbs free energy of reaction 2 and 3 as being -94.9 and -9.1 kJ mol^{-1} respectively (Dolfing, Larter, and Head 2008; Steinbusch, Hamelers, and Buisman 2008). Furthermore, the effect of the pH on the critical pH_2 was assessed depending on different values of partial pressure of CO_2 (pCO_2) and ethanol-to-acetate ratio (Figure 28). The model (Figure 28A) showed that an increase of pCO_2 from 0.02 to 1 atm would decrease the critical pH_2 required for bioelectrochemical acetate production from $2.7 \cdot 10^{-6}$ to $3.9 \cdot 10^{-7}$ atm at a pH of 5.5. Also, within the pH range observed in the reactors, the critical pH_2 required by electroacetogenesis increased with lower pH values. For instance, under 0.02 atm of pCO_2, an increasing of the pH_2 from $1.2 \cdot 10^{-6}$ to $4.9 \cdot 10^{-6}$ atm would be necessary in case the pH decreased from 7 to 4.5.

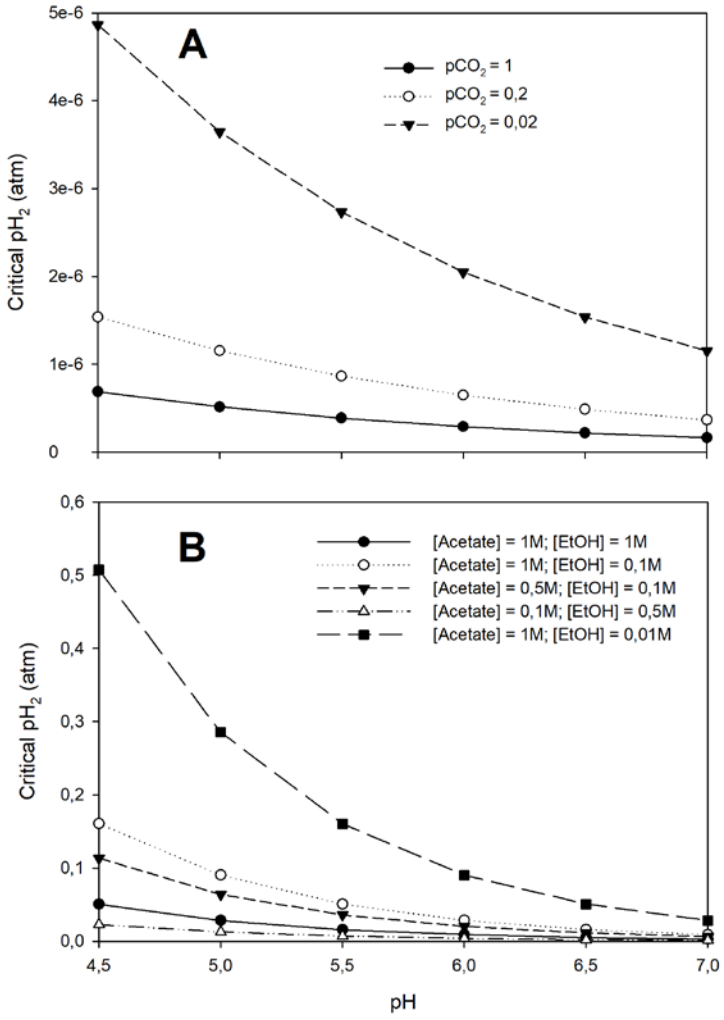


Figure 28. Critical pH_2 required for bioelectrochemical production of 1M of acetate depending on pH, pCO_2 (A). Critical pH_2 required for solventogenesis depending on pH and acetate/ethanol (EtOH) concentrations (B).

On the other hand, the ethanol-to-acetate ratio should be also considered as a key parameter in order to decrease the pH_2 requirements for solventogenesis to occur. Figure 28B showed that the higher the ethanol-to-acetate ratio was, the lower the pH_2 was required to perform ethanol production from acetate and molecular hydrogen. Likewise, critical pH_2 values required for ethanol production increases with lower pH values. The

lower critical pH_2 required to perform solventogenesis under the working pH values (pH ≈ 5.5) would have been $7.2 \cdot 10^{-3}$ atm if the ethanol-to-acetate ratio had been 3.9. However, with an ethanol-to-acetate ratio of 0.78, which is closer to the achieved in this work, the critical pH_2 would be set to $1.6 \cdot 10^{-2}$ atm.

4.3.5 Insights on controlling the ethanol-to-acetate ratio

A maintained equimolar production of ethanol and acetate opens up new opportunities to the use of METs as initial stages for secondary fermentation processes. An ethanol-to-acetate ratio over 8.3 (mMC ethanol per mMC acetate) was proposed by Kucek et al. as the ideal substrate to produce C6-C8 carboxylates (i.e. n-caproate, n-caprylate) (Kucek, Spirito, and Angenent 2016), which is still far from the maximum ratio reached in this work (2.0 ethanol-to-acetate ratio, mMC ethanol per mMC acetate) and other solvent-producer biocathodes found in the literature (Arends et al. 2017; Suman Bajracharya et al. 2017; Ammam et al. 2016).

According to our results, a tight control of pH, pH_2 and CO_2 availability will lead to a tuneable system. The continuous measurement of the above mentioned parameters suggested pH of 5.4 and CO_2 availability of 100 mg $_{CO2}$ L^{-1} as critical values to achieve selectivity. Shift between desired products might be accomplished by modifying those parameters (Figure 29 and Table 7). Moreover, the current consumption profile could be used as indicator to start feeding CO_2 when a turning point is detected. On the other hand, an increase of pH_2 by decreasing CO_2 feeding frequency will lead to trigger solventogenesis. An enhanced control of these parameters will ideally lead to a tuneable system in which different ethanol-to-acetate ratios can be

attained. Also, the simultaneous acetic acid and ethanol production may have taken place due to the increase of hydrogen producers' activity present in the biocathode while CO_2 was fed to the system. Once CO_2 was massively supplied, acetogenesis took place due to the high availability of both CO_2 and the biotically produced H_2. Simultaneously, due to the drop of pH (pH < 5) during the CO_2 feeding, the solventogenic pathway was used by the bacteria to convert VFAs into its correspondent alcohol (acetate into ethanol) as a detoxifying process. This statement is supported by Vassilev and co-workers that observed the same phenomenon under similar conditions and also confirmed the presence in the reactor microbiome of the genes required to perform solventogenesis.

Table 7. Overview of the preferential routes observed depending on the operation parameters.

pH_2	pH	Dissolved CO_2	Preferred route/s	Ref.
Low	Low	High	Acetogenesis & solventogenesis	(Srikanth, Singh, et al. 2018)
High	High	Low	Acetogenesis	(Zaybak et al. 2013)
Low	High	High	Acetogenesis	(Mohanakrishna, Vanbroekhoven, and Pant 2016)
High	Low	Low	Solventogenesis	This study

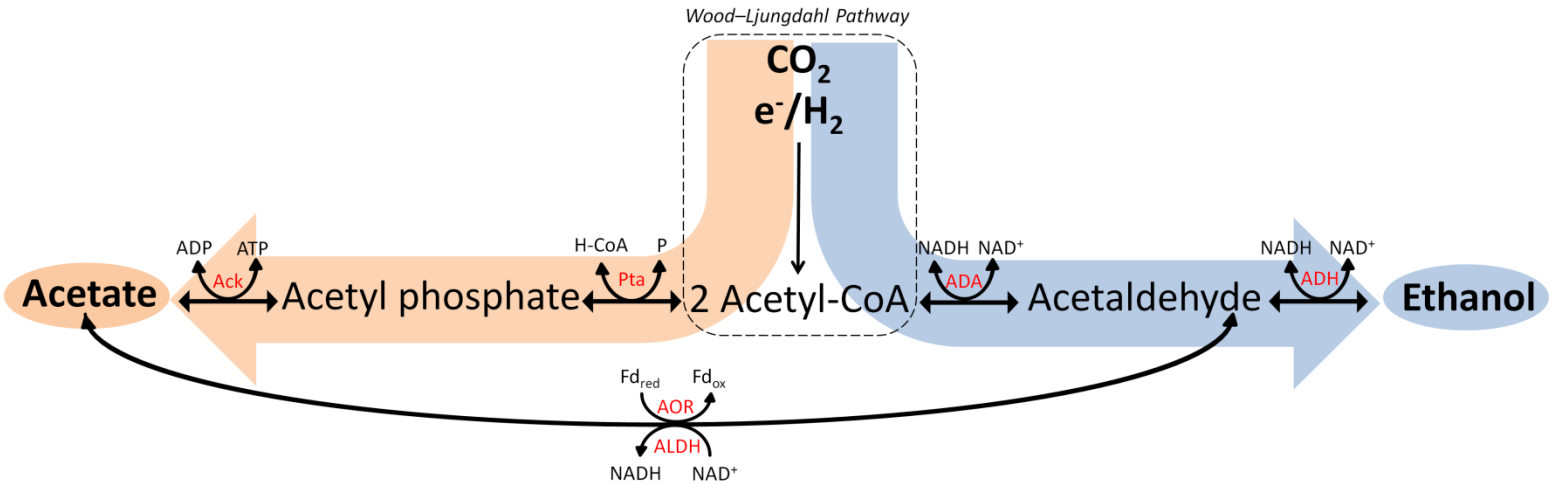


Figure 29. Acetate and ethanol production pathways from CO_2 and electricity. The Wood–Ljungdahl pathway reduces inorganic carbon to acetyl-CoA. Noteworthy, the enzymes involved in each reaction are labelled in red. Acronyms: AOR, aldehyde:ferredoxin oxidoreductase; Ack, acetate kinase; Pta, phosphate acetyltransferase; ADA, acetaldehyde dehydrogenase (acetylating); ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase.

This study provides an approach to accomplish better control of the system based on the current signal profile as an indicator of the main metabolic pathway taking place in the biocathode (Figure 30). The stabilization or slight increase of the current signal after feeding event related to acetate production would allow the operator to drive the reaction towards acetogenesis in case more acetate is needed as substrate. This would be easily accomplished by increasing the amount of CO_2 dissolved over 100 mg L^{-1} by either setting a higher feeding frequency or switching to continuous mode, thus achieving a tighter control. Whereas, under the proper conditions of low pH (<5.4) and CO_2 availability (<100 mg L^{-1}) plus high pH_2 (≥1 atm of total pressure), the longer the current decreases after acetogenesis, the higher the ethanol will be produced, leading to an increased ethanol-to-acetate ratio. In addition, the maintenance of the above described phases would be directly related to the time that the suggested values of pH, pH_2 and CO_2 dissolved are kept in the biocathode. Further research should be focused on studying the resilience of the system and the relationship between the key parameters in order to develop a real in line tool to control the production of organics in a CO_2-fed biocathode.

Also, is worthy to underline that these statements are closely related to the working conditions and the strain used in this work. The fact that CO_2 dissolved had never been directly measured carefully in previous works might be the reason why this parameter has never been pointed out as critical. Therefore, a much more exhaustive look at the role of pH_2, pH and specially CO_2 dissolved in other MET systems must be carried out in forthcoming studies.

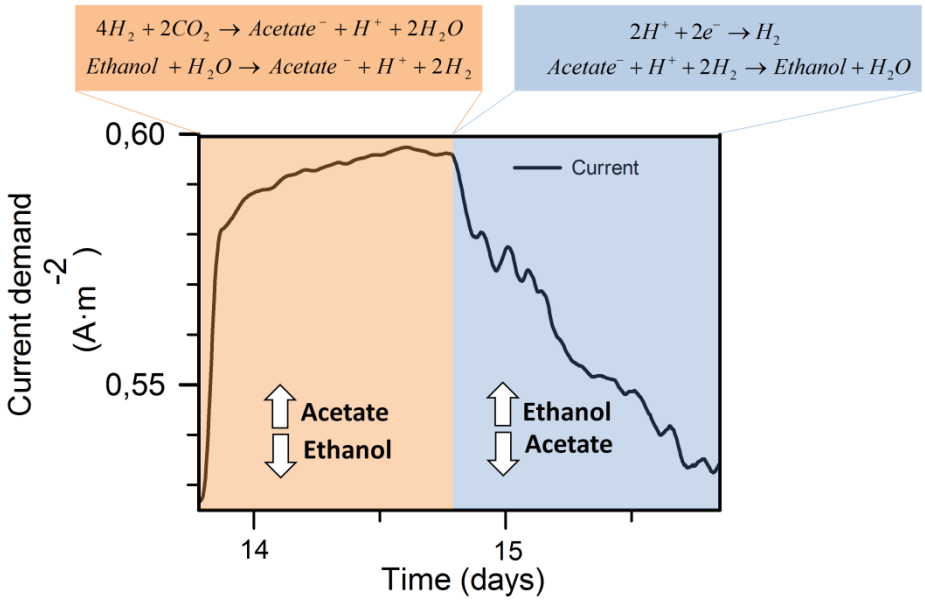


Figure 30. Tentative main reactions taking place within a typical current signal profile observed in a production cycle of the reactor 2.

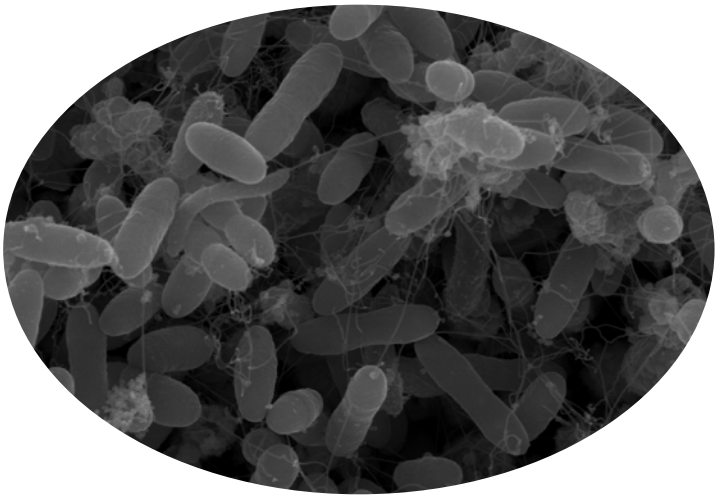
4.4 Final remarks

The present study supports the idea of converting CO_2 into acetate and ethanol in the same BES reactor. pH, CO_2 dissolved and pH_2 were key parameters to reach an equimolar bioelectro-production of both compounds in an optimum ratio as potential substrate for performing chain elongation in a coupled fermenter.

Recurrent patterns observed in current signal and pH profiles were directly linked to CO_2 conversion into either acetate or ethanol. Those parameters, together with the CO_2 availability in the medium, were found as key operational variables to control acetogenesis over solventogenesis or vice versa. Thus, the first approach of a real-time control system for the bioelectro-recycling of CO_2 to valuable building blocks was presented.

Chapter 5

Steering bio-electrorecycling of carbon dioxide towards target compounds through novel inoculation and feeding strategies



Redrafted from the paper in preparation for submission:

Steering bio-electrorecycling of carbon dioxide towards target compounds through novel inoculation and feeding strategies, **Blasco-Gómez, R.**, Bolognesi, S., Romans, M., Bañeras, L., Colprim, J., Balaguer, M. D., Puig, S. (2020)

5.1 Background and objectives

The production of chemicals and liquid fuels from waste gaseous streams using microbial electrochemical technologies (METs) has attracted an increasing attention. The capability of some microorganisms to act as biocatalyst in the reduction of CO_2 into commodity chemicals by means of renewable electricity is the base of a sustainable platform to mitigate global warming (Rabaey and Rozendal 2010; Nevin et al. 2010).

Although acetate has been the main product obtained by most attempts aiming to convert CO_2 into building blocks, recent studies have demonstrated that C3-C6 compounds such as isopropanol, butyrate, butanol, caproate and hexanol may be obtained through the bio-electrorecycling of CO_2 (Ganigué et al. 2015; LaBelle and May 2017; Batlle-Vilanova et al. 2017; Arends et al. 2017; Jourdin et al. 2018; Vassilev et al. 2018; Jourdin et al. 2019). Some authors pointed out the adjustment of operational parameters or novel reactor designs as plausible strategies to improve the selectivity required for the production of larger carbon compounds. For instance, setting high CO_2 loading rates (173 L d^{-1}) and high hydraulic retention times (14 days) during operation leaded to an increased production of butyrate and caproate (Jourdin et al. 2019). Furthermore, the addition of an extra cathodic chamber open the possibility to separate process that required different pH values and improves the selectivity of processes driven by uncoupled metabolic pathways (Vassilev et al. 2019).

Despite all the progress mentioned, the economic feasibility of this resource recovery technology is subjected to a better knowledge of how to steer the process (Prévoteau et al. 2020). It is highly necessary to choose the target

compound based not only on the market but also on the requirements for its recovery (Christodoulou et al. 2017). Such tighter control should be accomplished by defining the operational parameters that would allow driving the reactions towards the production of either carboxylates or alcohols. In this sense, pH, CO_2 dissolved and hydrogen partial pressure (pH_2) were reported as critical operational parameters to switch from acetogenesis to solventogenesis in bioelectrochemical systems (Blasco-Gómez et al. 2019) (Chapter 4).

Putative values of key-parameters that are likely to switch between metabolic pathways involved in CO_2 transformation are required to be precise in order to move towards scaling up the technology. Therefore a continuous monitoring of those parameters during the bio-electrorecycling of CO_2 is essential to improve the selectivity and competitiveness of METs (Figure 31; Chapter 4).

Moreover, an effective biofilm settled on the surface of the electrode that acts as biocatalyst is required to achieve the production of more complex molecules in a long-term operation (Jourdin et al. 2018). Therefore, new methodologies to reinforce the attachment of the electroactive biofilms and achieve higher thicknesses must be also stablished.

The main objective of this study was to give new insights into the CO_2 bio-electrorecycling to C2-C4 compounds. First, a new inoculation protocol to favour the attachment of an electroactive community on the electrode surface was stablished. Second, a novel CO_2 feeding strategy to increase the reducing power at favourable thermodynamic conditions was tested to reach more reduced compounds. Third, a closer look at key operational

parameters such as pH, CO_2 dissolved and pH_2 permitted better product selectivity.

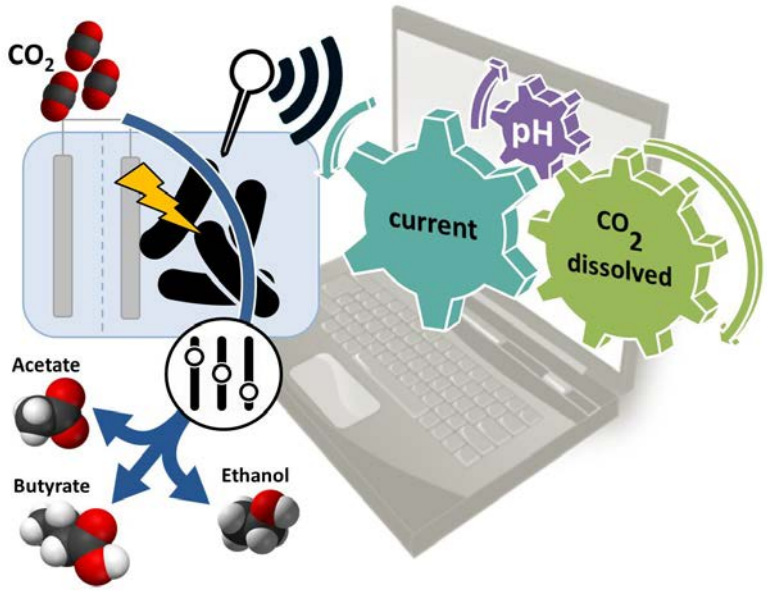


Figure 31. Graphical scheme of the real-time monitorization of the CO2 bio-electrorecycling into acetate, ethanol and/or butyrate.

5.2 Methodology

5.2.1 BES Set-up

Two flat-plate BESs were set and named Reactor 3 and Reactor 4 (Figure 17; Chapter 4). Each reactor of the duplicated BESs had the same features as indicated in subsection 3.1.2 (Figure 32).

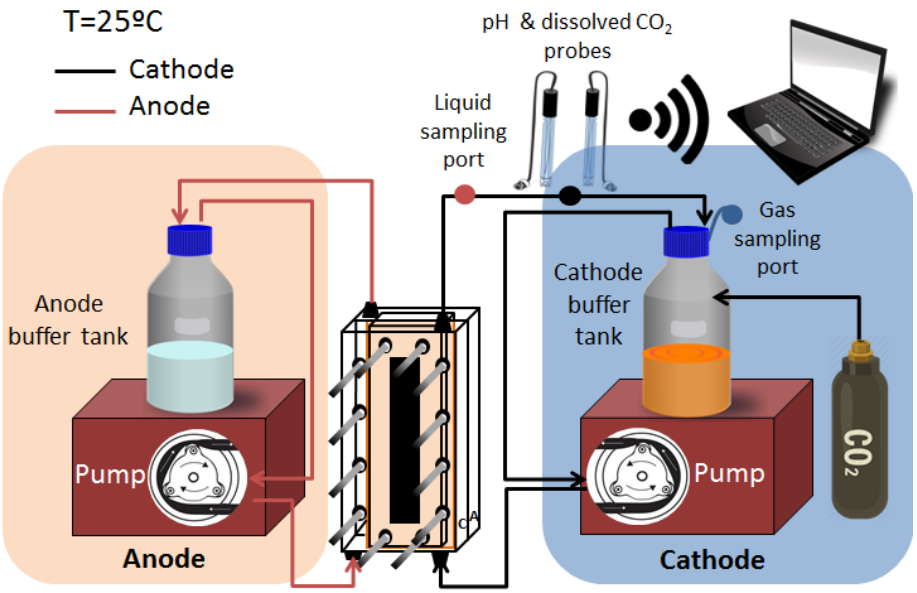


Figure 32. Schematic representation of the flat-plate BES setup (reactors 3 and 4).

5.2.2 Microorganism, inoculation and growing conditions

Both cathodic electrodes (carbon cloth) were initially enriched with potentially electroactive bacteria in a fermenter and progressively adapted to autotrophic conditions. The fermenter was inoculated with the isolate I-19 whose features were indicated elsewhere (Blasco-Gómez et al. 2019) and fed under autotrophic conditions with CO_2/H_2 (80:20% *v/v*, Praxair, Spain) gas mixture for 45 days as stated previously in section 3.6.3. A characterization of the community in both the carbon cloth and the bulk liquid was carried out (section 3.7.3), as well as a quantification through qPCR (3.7.2). After 45 days of operation, the electrodes were placed in the cathodes of BES reactor 3 and reactor 4 under the anaerobic chamber, filled with anaerobic fresh medium and subsequently connected to the

potentiostat. Thereby, the electron donor (H_2) was substituted by an electrode.

5.2.3 Fermentation cell and transfer to the BES

A fermentation cell (410 mL) was used for the enrichment of electroactive microorganisms on the surface of the electrode. The fermenter and its operation are described previously in section 3.6.2 and 3.6.3 respectively. Both the biofilm and the planktonic community were monitored with qPCR, as indicated in section 3.7.2., in order to assess their growth and suitability to perform the bio-electrorecycling of CO_2. In addition, the protocol followed to transfer the electrodes into the BESs is detailed in section 3.6.4

5.2.4 BES Start-up and operation

Both BES reactors were operated in batch-mode as stated in section 3.2. In this case, inorganic carbon in the form of CO_2 was used as the only carbon source, which was supplied every 2-3 days by feeding pure CO_2 (99.9 %, Praxair, Spain). Two feeding strategies were followed to assess their impact in the compounds obtained and its production rates. First strategy followed was to bubble with CO_2 until saturation, whereas the second strategy consisted of feeding with CO_2 until an overpressure of 350-400 mbar was reached in the headspace without flushing remaining H_2 from the previous production stage. The frequency chosen for feeding with CO_2 was higher (3 days) in the first two batches and lowered from the third batch onwards. Thereby, a pH range of 3.8-5.3 was maintained and enabled to monitor the switch of metabolic pathways within the biological community. A slightly higher pH was measured at the beginning of each batch, which corresponded to the initial pH of the fresh medium added (pH ≈ 6).

5.3 Results and discussion

5.3.1 Fermentation phase

An electroactive biofilm capable to bio-electrorecycle CO_2 was previously grown on a carbon cloth by using a fermenter fed with CO_2 and H_2 as carbon and reducing power sources respectively. This cell was inoculated with a mixed culture enriched with the isolate I-19 growth in organic medium (Table 14). The system was operated for 45 days and fed with CO_2:H_2 (80:20% *v/v*) every 2-3 days. As shown in Figure 33, initial concentrations of carboxylates and alcohols indicate that they were introduced with the inoculum. Also the organic matter (OM) plotted correspond to the mix of organic compounds contained in the medium at the beginning of the experiment (0-28 days). Subsequent replacement of the medium during the feeding event diluted the OM from an initial concentration of 15 g C L^{-1} to 2.75 10^{-5} g C L^{-1} at day 28, when is assumed to be finally depleted. The main compounds produced during the first phase of operation were acetate and ethanol, which reached a concentration of 43.3 and 67.7 mM C respectively. Due to the increasing amount of acids present in the medium, the pH decreased to 4.75 and was rapidly adjusted to 5.5 by replacing old with fresh medium.

The fermenting activity decreased between days 25 and 35 due to both the depletion of OM and the low pH values. At this moment, the community was shifting between heterotrophic to autotrophic conditions. This adaptation might have also been reinforced by the exposure to conditions leading to acid crash (Ramió-Pujol et al. 2015b). Under lower values of pH than the acetate pK_a (4.76), it does not dissociate and crosses the cell membrane which would end up in the death of the bacteria due to stress (Jarboe, Royce, and Liu 2013; Maddox et al. 2000). Although the working temperature (25 ºC) helped to reduce the metabolic

activity and the acidic conditions are unlikely (Ramió-Pujol et al. 2015b), pH was adjusted to 5.5 at day 28 to avoid the acid crash and stimulate autotrophic activity.

After the day 35, an ethanol production peak can be observed that was followed by a consumption leading to caproate (C6) production through chain elongation.

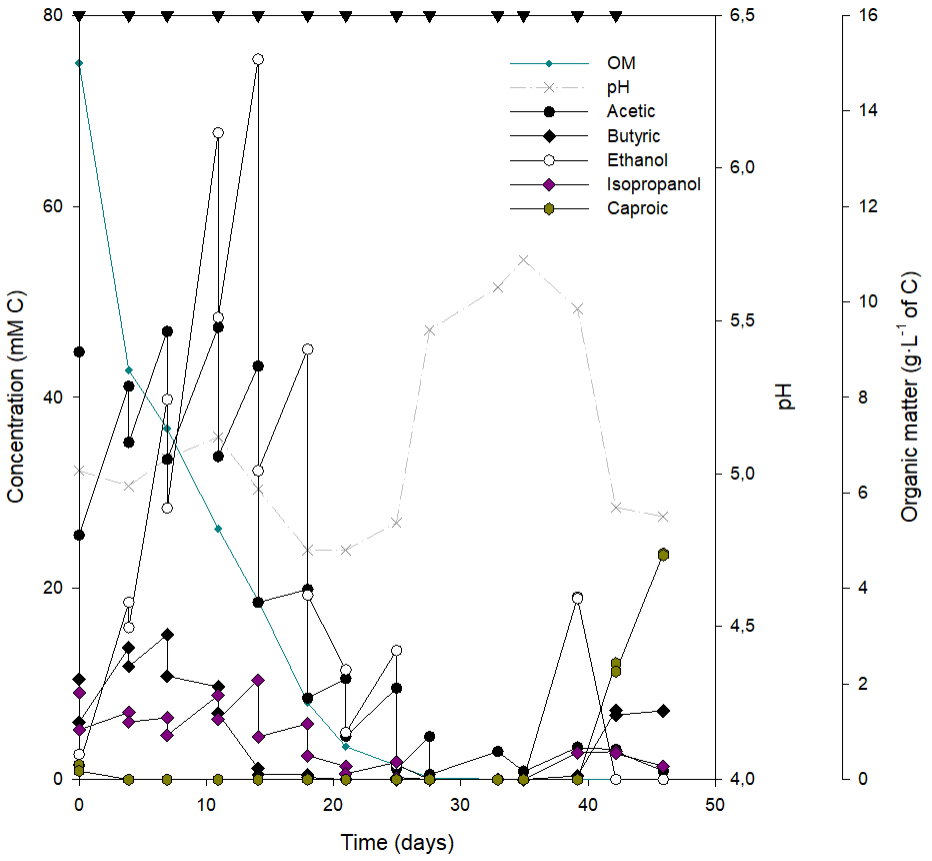


Figure 33. Evolution of the values of organic matter (OM), pH, carboxylates and alcohols in the fermenting medium during the fermentation cell operation. Black inverted triangles indicate the feeding events with CO_2:H_2 (80:20, *v/v*). The shift from heterotrophic to autotrophic was set at day 28.

5.3.2 Microbial enrichment through fermentation

Biofilm formation on the carbon cloth was monitored along the fermentation process in order to assess its suitability to be placed in a BES. Carbon cloth samples were taken during each sampling event to study the formation of biofilm on the surface of the electrode material. In addition, samples from the bulk liquid were also analysed. In this case, I-19 was the targeted bacteria to assess the biological growth, since is the most interesting electroactive bacteria from the point of view of a further CO_2 bio-electrorecycling process. The presence of I-19 was confirmed through the use of specific primers in a PCR, whose specific amplification fragment was expected at 432 bp (Figure 34). This is the first time the isolate is described to form biofilm on carbon cloth.

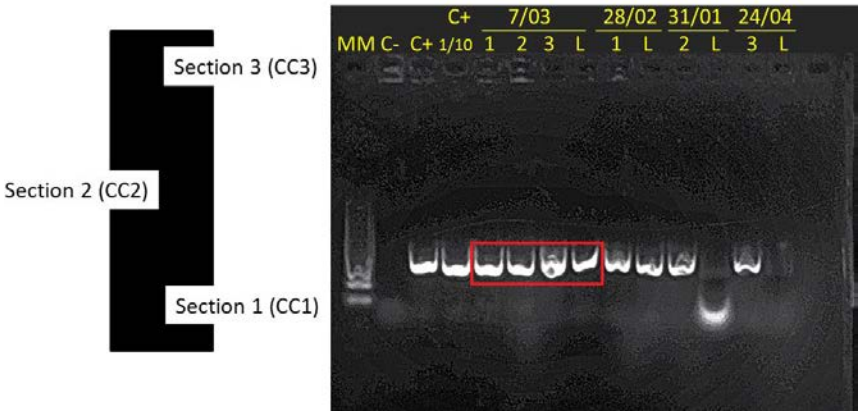


Figure 34. Electrophoresis gel that confirms the presence of I-19 in the biofilm. In the left figure the three sampling areas of the carbon cloth are shown. In the right, the gel is presented with the I19 specific marker (MM), negative (C-) and positive (C+) controls, a dilution of the positive control (C+ 1/10), and the confirmation of the presence of I19 in the three sections along the experiment (4 samples). The nomenclature used for the three sections of carbon cloth (CC1, CC2 and CC3) corresponds to its proximity to the inlet, being CC1 the closest to that point.

In addition, the growth was tracked throughout the experiment through a qPCR (Figure 35). The quantification of I19 in the carbon cloth and the bulk liquid was correlated to the amount of copies of 16S rRNA amplified per cm^2 of carbon cloth and mL of liquid media, respectively. The average efficiency of the qPCR was 1.98 (98%) and the R-squared of the straight line was 0.999.

The observed growth during the first 25 days might be caused by the availability of organic matter. On the other hand, the adaptation to autotrophic conditions and the decrease of the pH affected negatively the biofilm and the planktonic communities by compromising its growth.

The biofilm reached $6.07 \cdot 10^6$ DNA copies per cm^2 of carbon cloth, which means that, assuming homogeneous coverage, each carbon cloth placed as electrode in the flat-plate BESs contained over $3.9 \cdot 10^8$ DNA copies per cm^2.

Additionally, the OD_{600} values of the bulk liquid dropped at the beginning of the experiment which explains the attachment of such biomass on the surface of the carbon cloth and therefore the biofilm growth.

On the other hand, the bulk community reached $4.83 \cdot 10^6$ DNA copies per mL of liquid, which means that there were $3.38 \cdot 10^9$ I-19 cells in the fermenter bulk.

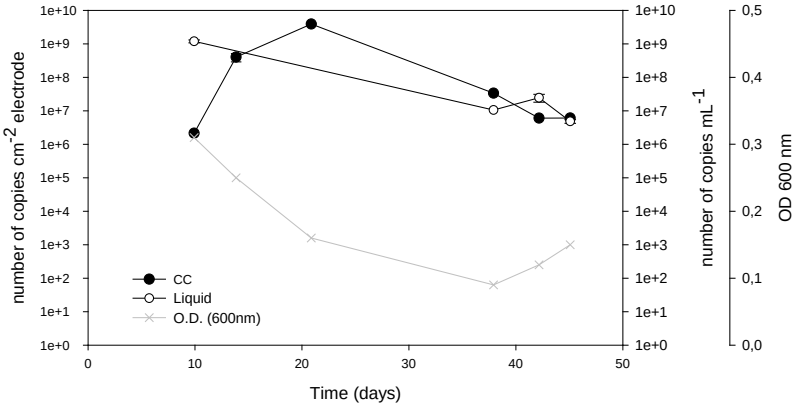


Figure 35. qPCR results showing the dynamics of the biofilm (CC) and bulk liquid communities during the fermentation experiment. Optical density was also measured as an indicator of cell growth in the bulk liquid.

5.3.3 Analysis of the biological community structure

Three samples from the three regions along the carbon cloth (129 cm^2) and one sample of the bulk liquid in the last day of operation (day 45) were characterized in order to study the biological community present. This community is the one subsequently inoculated in the flat-plate BESs. There were a total of 236958 sequences identified with high quality. The most abundant was the bulk liquid (169.631) and the less was the piece of the carbon cloth furthest to the feeding inlet (CC3). The diversity coverage measured was higher than 95%. The number of operational taxonomic units (OTUs) identified, which is considered an index of observed diversity (S_{obs}), were 60 and 45 for the three carbon cloth samples and the bulk liquid, respectively. In addition, the microbial diversity was calculated through the Shannon's index (H') and resulted in 3.203 for the three carbon cloth samples together and 2.944 for the bulk liquid respectively. This shows the activity compartmentalization and the differences between the biofilm and the bulk liquid

communities suggested by other authors (Berlanga and Guerrero 2016), which is also observed in the CO_2 bio-electrorecycling reactors (Puig et al. 2017; Wenzel et al. 2018). Interestingly, phylum relative abundance varied along the carbon cloth (Figure 36), which might be explained by the difference in terms of nutrient availability and physical-chemical conditions inside the fermenter. The taxonomic identification of the sequences determined that the major phylums presented in both the biofilm and the planktonic communities were *Firmicutes*, *Actinobacteria* and *Proteobacteria*.

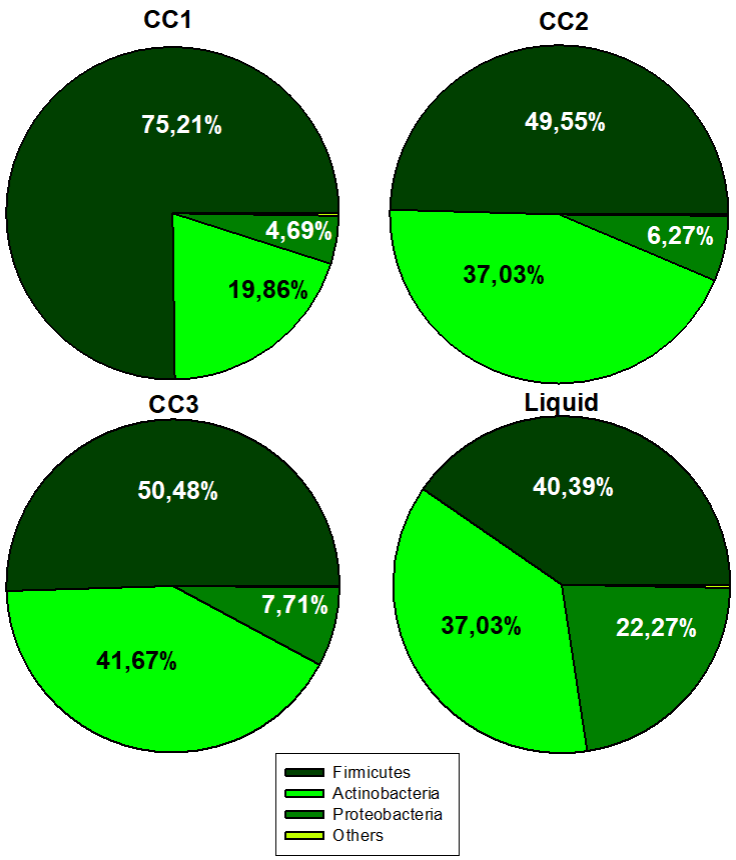


Figure 36. Phylogenetic composition of the microbial community presented in the biofilm and the bulk liquid. CC1 corresponds to the closest region of carbon cloth to the inlet, CC2 to the medium and CC3 to the farthest. Phylums presented in a relative abundance below 1% (e.g. *Cianobacteria, Acidobacteria, Pactescibacteria*) were classified as others.

The main differences observed were not only between biofilm samples and planktonic, but also between different areas along the carbon cloth. The region CC1 of carbon cloth had a higher availability of nutrients due to its proximity to the gas inlet and a higher turbulence. Compared to the rest carbon cloth regions, the predominant phylum (*Firmicutes*) had a higher relative abundance (75.2%

compared to 49.6% and 50.5% of CC2 and CC3 respectively). The samples from CC2 and CC3 showed a closer community structure to the planktonic. Although, *Proteobacteria* was more abundant in the bulk liquid (22.3%) compared to the carbon cloth (4.69%, 6.27% and 7.71%).

From the genus point of view, substantial differences were observed also between sampling points (Figure 37). Even though the fermenter was inoculated with an enriched culture of the isolate I-19 (corresponding to *Eubacterium* in Figure 37), only represents the 18.7% of the total sequences identified in the carbon cloth (CC1). The dominant genera presented in all samples were *Cellulomonas* and *Clostridium* (group 12). Interestingly, the presence of *Burkholderia* ensured the anoxic environment due to its activity as oxygen scavenger (Cournet et al. 2010; Marshall et al. 2016). Moreover, this genera is known to be electroactive and capable of producing short chain carboxylic acids (Yabuuchi et al. 1992).

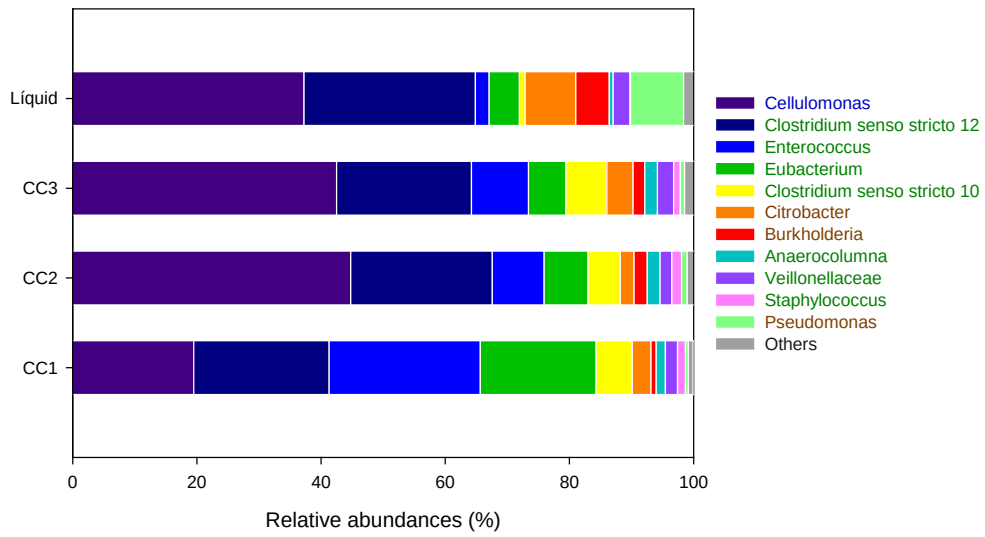


Figure 37. Genera diversity observed in the microbial communities of the fermenter correlated to their relative abundances. Colours of in the font of the legend are referred to the different phylums identified: *Firmicutes* (green), *Actinobacteria* (blue) and

Proteobacteria (brown). All the genera with relative abundances below 1% were classified as others.

The presence of *Eubacterium limosum* (strain JMC 6421), comparable to isolate 1-19, was ensured through BLASTn comparisons with cultured bacterial representatives showing 100% of identity. This specie was found in the biofilm at the closest point of the inlet, where the carbon source (CO_2 dissolved) availability was higher. On the other hand, the presence of I-19 was not massive in the bulk liquid. Here *Cellulomonas* and *Clostridium* were the most abundant, together with *Pseudomonas*, which is a genera with high capability of electron exchange and therefore very active in syntrophic interactions (Bosire and Rosenbaum 2017), which were also linked with CO_2 bio-electrorecycling (Jourdin, Freguia, et al. 2015; Mateos et al. 2018).

Eubacterium spp. is known for its capability to produce fuels and chemicals (Marshall, LaBelle, and May 2013), but the fact that were not observed in the catholyte suggests that its utilization is ubiquitous in the fermenter by other species (e.g. *Clostridium*) to produce more complex molecules such as butyrate and caproate (Zhu et al. 2015; Reddy, ElMekawy, and Pant 2018).

The presence of the genus *Cellulomonas* might be explained by the presence of organic matter at the beginning of the experiment, required for its development. This genus is capable of producing acetate, ethanol and propionate (Wakarchuk et al. 2016). *Enterococcus* has been related to EET and pyruvate production (Keogh et al. 2018). *Citrobacter* is electroactive and able to produce propanediol (Zhou et al. 2015). The genus *Anaerocolumna* is known to produce acetate, ethanol and hydrogen from organic matter (Ueki et al. 2016). *Veillonellaceae* produces propionate and its growth is favoured in BESs reactor (Sasaki et al. 2018). *Sporomusa* is a widely known electroactive genus involved in the CO_2 bio-

electrorecycling into acetate (Nabin Aryal et al. 2017). Finally, *Staphylococcus* has been described as electron acceptor and proved its electroactivity (Christin Koch and Harnisch 2016).

Despite of the fact that relative abundance does not match in many cases with relative activity, the high diversity of genera observed and its proven electroactivity ensures its suitability as inoculum in a BES.

5.3.4 BES operation

The two carbon cloth resulted from the enrichment with microorganisms suitable for CO_2 conversion were placed in the flat-plate BES (reactor 3 and 4) and connected to the potentiostat (poised at -0.8 V vs. SHE) as described in section 3.6.4. Both reactors (3 and 4) were considered duplicates, since the operational conditions were identical. Reactors 3 and 4 were operated during 274 and 176 days respectively using different operation strategies in each batch (Table 8).

Table 8. Operation conditions of each batch during the experiment. In the feeding strategy row, "S" corresponds to CO_2 saturation and "NS" means CO_2 non-saturation of the medium.

	Batch						
	1	2	3	4	5	6	7
Reactor 3 (days)	0-57	57-107	107-141	141-176	176-191	204-239	239-274
Reactor 4 (days)	0-57	57-107	107-141	141-176	-	-	-
Feeding per week	3	3	2	2	2	2	2
Feeding strategy	S	S	NS	NS	NS	NS	NS

The first batches (1 and 2) corresponded with the time of adaptation to the new electrochemical conditions (Figure 38 and Figure 39). In this case, the reducing power was provided directly by the electrode in form of electrons, instead of externally through the bubbling of hydrogen gas. The only carbon source was CO_2, similar to the fermenting phase, although in this case was provided pure (99.9% *v/v*) without the hydrogen. The adaptation period lasted around 40 and 30 days in reactors 3 and 4, respectively. An initial consumption of all the organic compounds was observed. Ethanol peaks (170 and 97 mMC) identified the first days in both reactors could be explained by the fermentation of remaining caproate from the fermenting broth previously added or due to a contamination during the transfer process, since ethanol was used to reach axenic conditions. After such adaptation phase, both reactors started to produce acetate until the end of the first batch, once the pH was stabilized between 5.5 and 4. In this phase, the current demand increased to 1.4 and 1.1 A $m^{-2}_{electrode}$ and more reducing equivalents were available. During the first batch until the day 58 each system produced a maximum concentration of 64.80 and 42.52 mMC of acetate (productivity: 3.85 ± 2.51 and 2.17 ± 1.64 g $m^{-2}_{electrode}$ d^{-1}) in reactor 3 and 4 respectively (Table 9). This decreased the pH to values below 4 in the reactor 4, which might have triggered an episode of acid crash and the production stopped. Coulombic efficiencies averaged 58 ± 41 % in reactor 3 and 57 ± 40 % in reactor 4 (Table 10).

A new batch (batch 2) was then started simultaneously in both systems by replacing 150 mL of catholyte with fresh medium. The anode medium was replaced with 150 mL of new fresh medium adjusted to pH of 2 in order to maintain a high concentration of protons in the anodic chamber since the beginning of the subsequent new batch. In case of batch 3 onwards, both anolyte

and catholyte were entirely replaced. During the second batch, the availability of nutrients, low pH (below 4.5) and the increasing current density (2 and 1.6 $A \cdot m^{-2}$) leaded to a concomitant production of acetate and ethanol in reactor 3.

Once the new feeding strategy was implemented, the third batch showed the highest productions rates of acetate in reactor 3 (7.46 ± 3.87 g $m^{-2}_{electrode}$ d^{-1}). Moreover, a concomitant production of ethanol leaded to chain elongation towards butyrate when ethanol concentrations were 12 and 6 mMC in reactor 3 and 4, respectively. Butyrate maximum concentrations reached 27 and 19 mMC with average production rates of 0.99 ± 0.94 and 0.36 ± 0.38 g $m^{-2}_{electrode}$ d^{-1} in reactors 3 and 4, respectively. Ethanol reached concentrations of 12 and 5 mM C with average production rates of 0.65 ± 0.71 and 0.83 ± 0.50 g $m^{-2}_{electrode}$ d^{-1}. In this batch CEs increased reaching 89 ± 35% in reactor 3 and 21 ± 26% in reactor 4. During this period, small amounts of propionate, valerate and caproate were detected (not shown) in both reactors, although not higher than 5 mMC in any case.

In Batch 4 ethanol was also produced concomitantly with acetate production. However only reactor 4 increased both acetate and butyrate production rates compared to the previous batch. Production rates increased from 5.50 to 5.92 0.43±0.51 g acetate $m^{-2}_{electrode}$ d^{-1} and from 0.36 to 0.43 g butyrate $m^{-2}_{electrode}$ d^{-1}.

Batches 5, 6 and 7 in reactor 3 were not relevant in terms of organic production, where both production rates and coulombic efficiencies decreased. This might be explained by oxygen intrusion due to the opening of the cathodic chamber while feeding when the created overpressure led the thrown of the reference electrode out of its port.

Table 9. Production rates of reactors 3 and 4 along the experiment.

	Production rates ($g\ m^{-2}_{electrode}\ d^{-1}$)					
	Reactor 3			Reactor 4		
Batch	Acetate	Ethanol	Butyrate	Acetate	Ethanol	Butyrate
1	3.85±2.51	0.08±0.17	0.08±0.07	2.17±1.64	0.04±0.04	0.09±0.22
2	7.04±6.11	0.59±0.67	0.21±0.14	2.66±1.88	0.10±0.14	0.31±0.27
3	7.46±3.87	0.65±0.71	0.99±0.94	5.50±2.71	0.83±0.50	0.36±0.38
4	5.15±3.20	0.54±0.31	0.25±0.18	5.92±4.00	0.17±0.12	0.43±0.51
5	3.21±2.79	0.18±0.20	0.20±0.14			
6	5.67±2.49	0.08±0.07	0.35±0.25			
7	5.76±3.31	0.11±0.15	0.24±0.09			

Table 10. Coulombic efficiencies of reactors 3 (R3) and 4 (R4) along the experiment.

		Batch						
		1	2	3	4	5	6	7
R3	CE (%)	58±41	29±22	89±35	59±34	41±39	46±27	48±29
	J ($A\ m^{-2}$)	0.54±0.46	1.00±0.38	1.38±0.23	1.56±0.22	1.29±0.36	1.44±0.32	1.82±0.26
R4	CE (%)	37±26	27±23	21±26	28±28	-	-	-
	J ($A\ m^{-2}$)	0.56±0.48	0.91±0.31	1.13±0.30	1.86±0.72	-	-	-

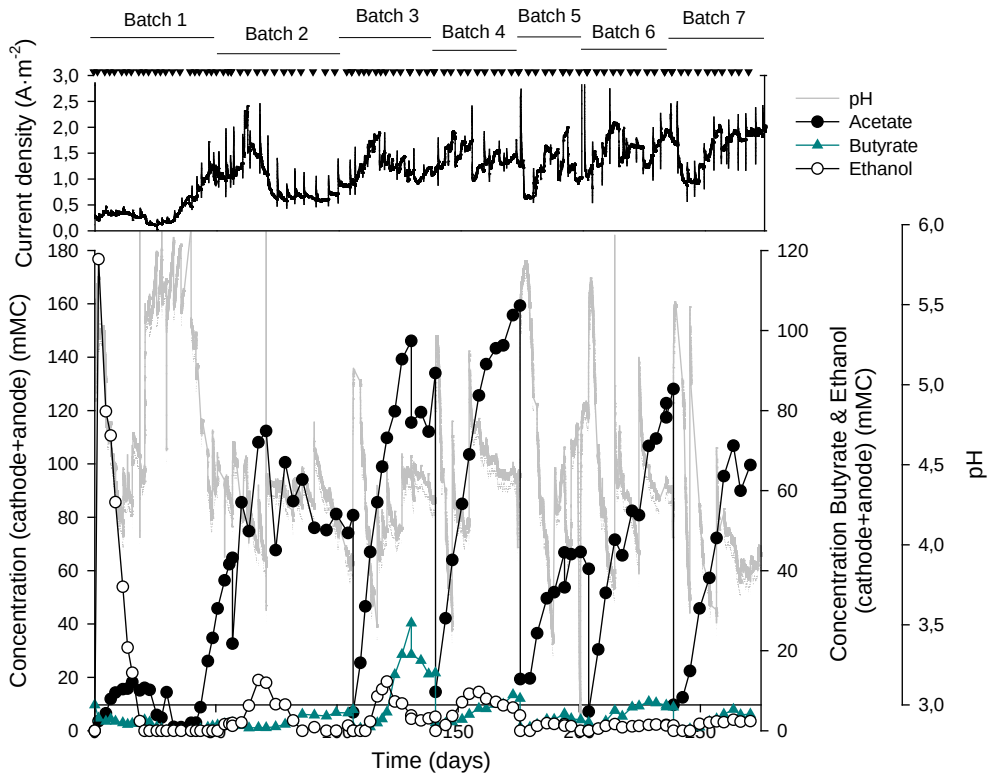


Figure 38. Evolution of current signal, pH and total concentration of acetate, butyrate and ethanol over time in Reactor 3. Black inverted triangles on the top part of the figure indicate sampling and subsequent flushing with CO_2.

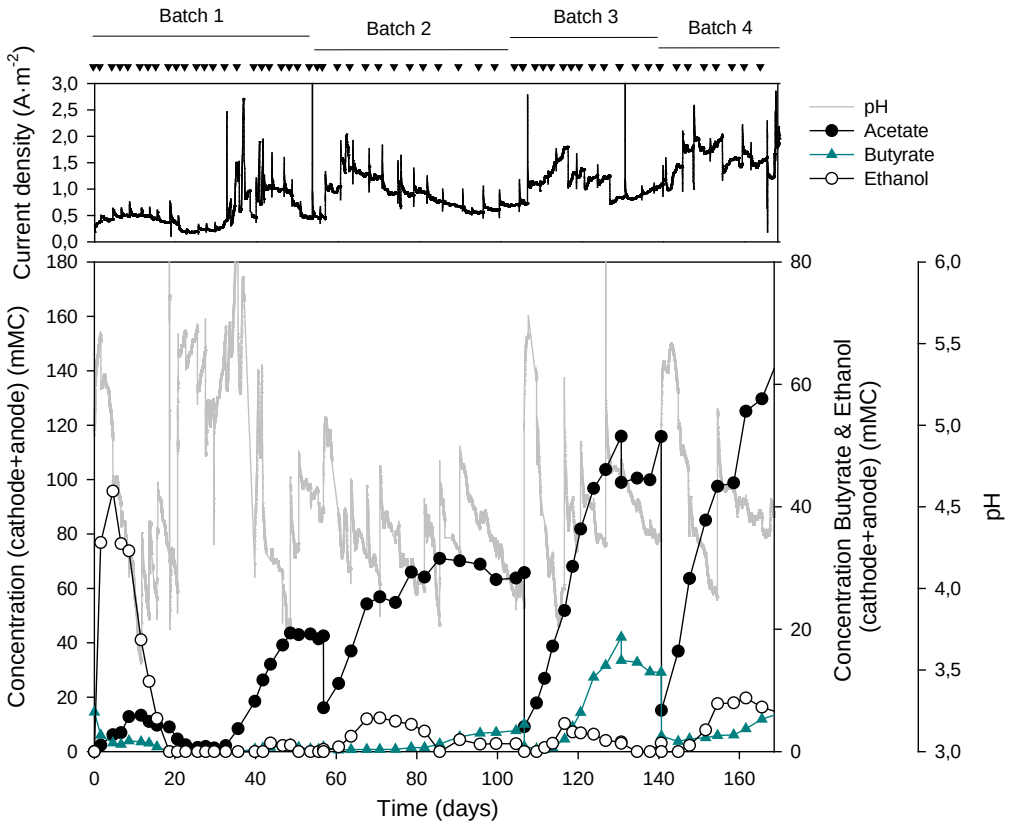


Figure 39. Evolution of current signal, pH and total concentration of acetate, butyrate and ethanol over time in Reactor 4. Black inverted triangles on the top part of the figure indicate sampling and subsequent flushing with CO_2.

As mentioned above, the increase in the current density leaded to the presence of more reducing equivalents in the medium, mainly in the form of H_2, which permitted reactions (e.g. ethanol production) that required more energy to be thermodynamically favourable (Raes et al. 2017; Blasco-Gómez et al. 2019).

In order to favour the production of ethanol and more reduced compounds, a new feeding strategy was followed in both systems based on maintaining the reducing power and not fully saturate the catholyte with CO_2 (explained in section

3.2). This strategy was implemented after batch 2 and resulted in better product selectivity. In addition, feeding rate frequency was lowered and switched from 3 to 2 times per week, which decreased the partial pressure of pCO_2 between feeding points, which has been pointed out as an strategy to trigger chain elongation (Jourdin et al. 2019).

The continuous monitoring of the pH and the CO_2 dissolved in the liquid allowed to make decision accordingly to favour ethanol production, which would lead to an increase of the ethanol-to-acetate ratio triggering chain elongation-based reactions. However, the lack of continuous ethanol production obstructed this objective and therefore, butyrate productions were detected for short periods of time along the experiment.

The fact that the current signal was slightly different in each system explained the differences in production rates. Reactor 3 showed higher current signal than reactor 4 throughout the experiment.

Nevertheless, both systems showed high resilience and robustness to operational changes and unexpected situations (e.g. power cuts, medium emptying, oxygen intrusion, run out of carbon source, etc.). In fact, the expected microbial lag phases at the beginning of each batch were non-existent. This is surprising due to the lack of nutrients and high concentration of inhibitory compounds accumulated at the end of the previous batch. In this case, organics production started right after fresh medium was added and reached maximum production rates of 18 and 12 g $m^{-2}_{electrode}$ d^{-1} of products at different points of the experiment.

5.3.5 Effects of the new feeding strategy on the product spectrum

The new strategy followed in this experiment allowed to avoid the lack of reducing power present (*in-situ* bioH_2) in the reactor after being fed during batch experiments. To date, all experiences reported in the literature were based on the direct addition of CO_2 by bubbling it in the catholyte. This flushed all the H_2 produced biotically or abiotically in the cathode during previous batches and therefore the initial stage of the subsequent batch lacked enough reducing power. In this case, the headspace was not flushed and CO_2 was injected until the total pressure reached 350 mbar. Moreover, the CO_2 not converted remained in the system and the conversion efficiency increased, as an alternative to continuous recirculation (Mateos, Sotres, et al. 2019).

The new feeding strategy was implemented from batch 3 onwards in both systems and favoured the production of butyrate (Figure 40 and Figure 41). This most likely happened because the conditions for ethanol production (low pH and high pH_2) were reached since the beginning and ethanol was concomitantly produced and oxidized to trigger chain elongation through reverse ***β***-oxidation to produce butyrate (Figure 42). Since the pH is not controlled in these experiments due to the low values observed during the whole operation time, the parameter most influenced by this change was the initial pH_2 and therefore reducing equivalents available since the beginning of the feeding event.

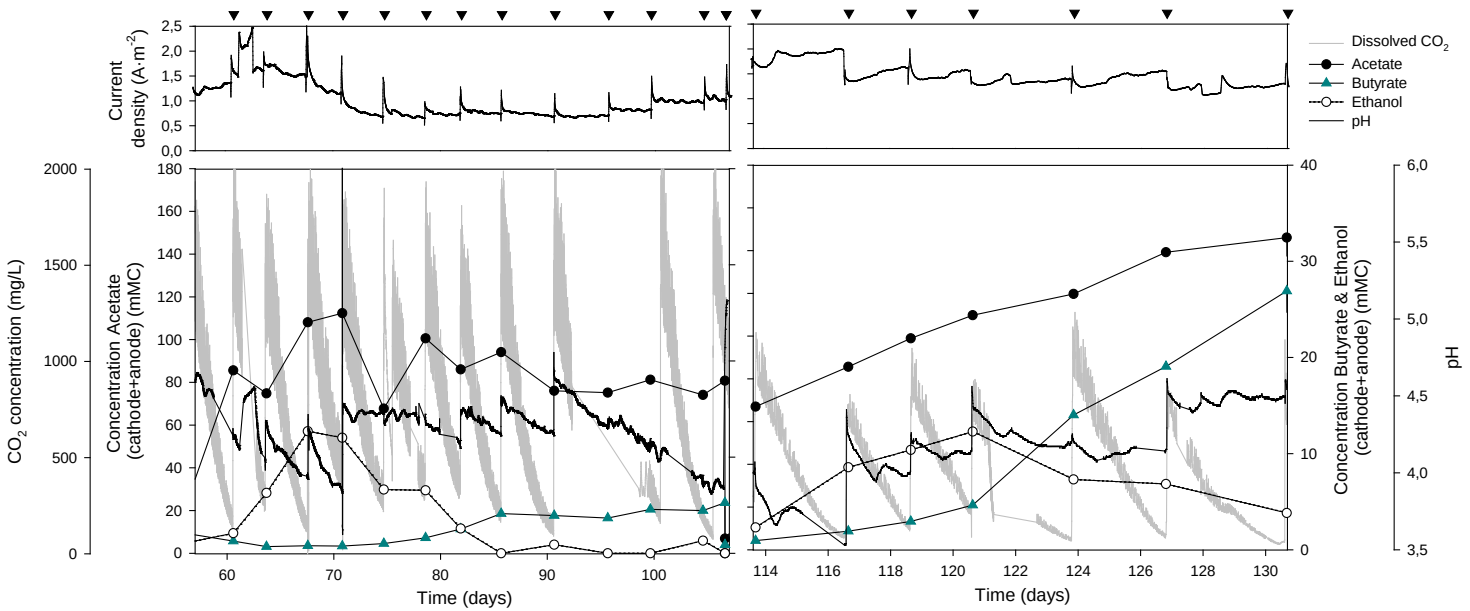


Figure 40. Comparison between batch 2 (left) and 3 (right) of Reactor 3 with different feeding strategies. The new feeding strategy was implemented at the beginning of batch 3.

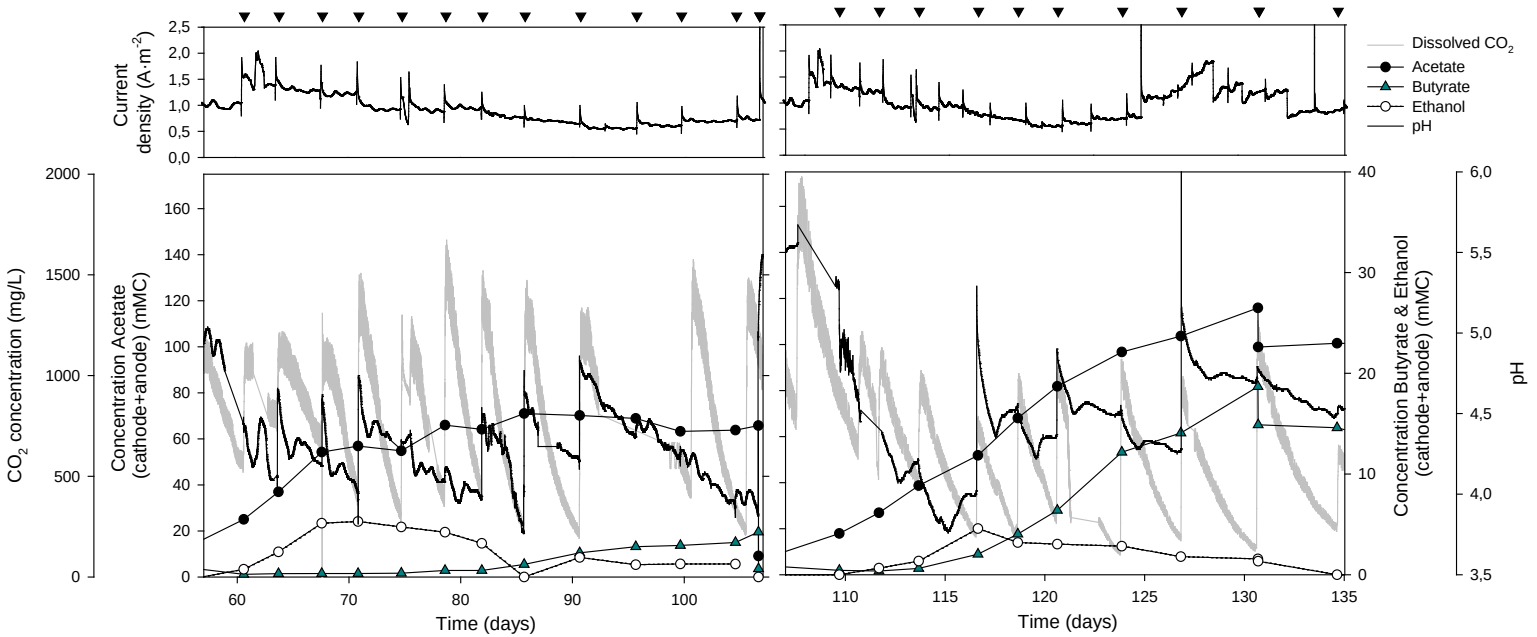


Figure 41. Comparison between batch 2 (left) and 3 (right) of Reactor 4 with different feeding strategies. The new feeding strategy was implemented at the beginning of batch 3.

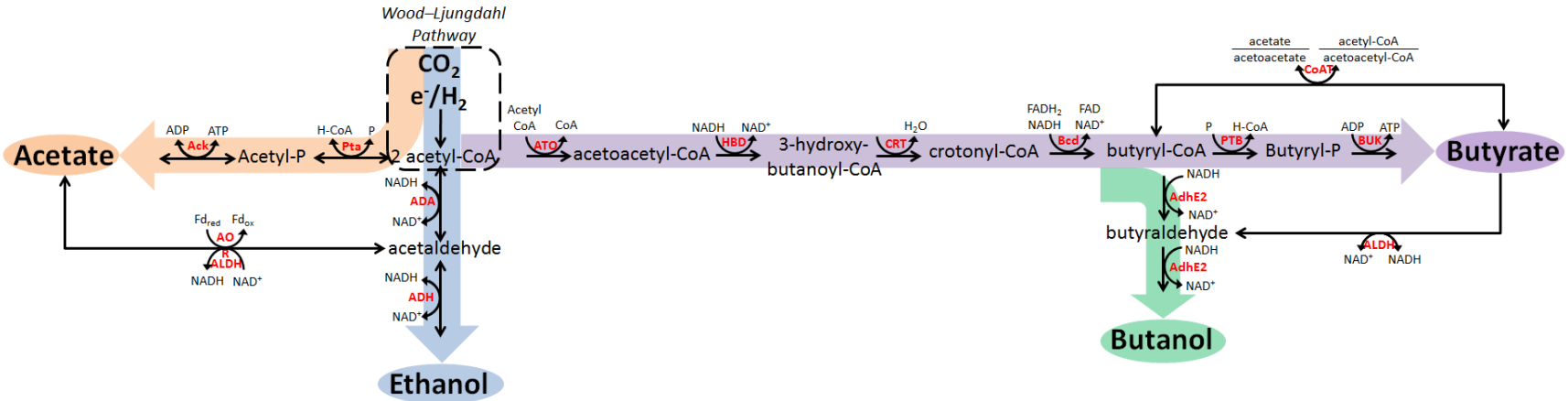


Figure 42. C2-C4 carboxylates and solvents production pathways from CO_2 and electricity. The Wood–Ljungdahl pathway reduces inorganic carbon to acetyl-CoA. Noteworthy, the enzymes involved in each reaction are labelled in red. Acronyms: AOR, aldehyde:ferredoxin oxidoreductase; Ack, acetate kinase; Pta, phosphate acetyltransferase; ADA, acetaldehyde dehydrogenase (acetylating); ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; ATO, acetyl-CoA acetyltransferase; HBD, 3-hydroxy butyryl-CoA dehydrogenase; CRT, crotonase; Bcd, butyryl-CoA dehydrogenase; PTB, phosphate butyryltransferase; BUK, butyrate kinase; CoAT, acetate CoA/acetoacetate CoA; AdhE2, aldehyde alcohol dehydrogenase.

5.3.6 Product selectivity

The lack of a continuous ethanol production, despite of the favourable operational parameters, might be explained by the low current density reached in both systems (<2 A m^{-2}). This parameter has been already pointed out as crucial to trigger chain elongation by other authors (Jourdin et al. 2019). In fact, a clear increase of this parameter was shown in both BESs (Figure 40 and Figure 41) due to (i) the biocathodic biofilm growth and the (ii) selection pressure towards electroactive species. This suggests that more reduced compounds are likely to be obtained with this reactor in a long-term operation.

The low pH values required to trigger ethanol productions might have not been reached due to pH gradients present in the biocathode. This low pH conditions might also have compromised the acetate production rate, since it dramatically decreases when pH is below 5 (LaBelle et al. 2014). Kang and co-workers measured a persistent alkalinity in the proximity of the electrode during CO_2 electroreduction (Yang, Kas, and Smith 2019), which is where the electroactive biofilm habits and therefore the most affected by this phenomenon. However, ethanol production prevailed over butyrate production in reactor 3 when pH was lower than 4.3. When pH value increased, chain elongation was clearly triggered (Figure 43, Figure 44 and Figure 45).

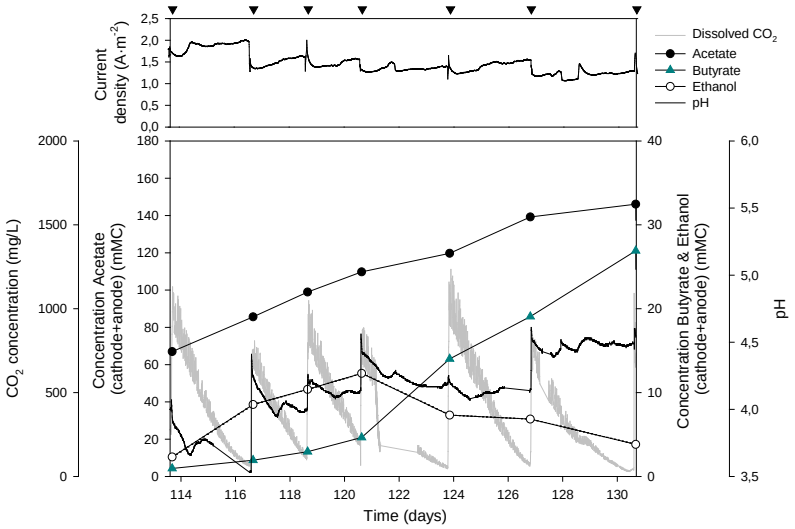


Figure 43. Evolution of current signal, pH, CO_2 dissolved and total concentration of acetate, butyrate and ethanol in Reactor 3 (batch 3). Black inverted triangles on the top part of the figure indicate sampling and subsequent flushing with CO_2.

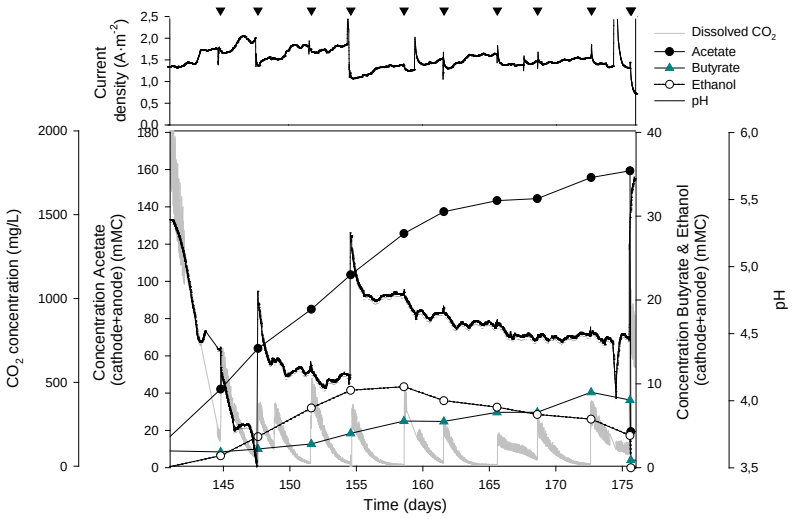


Figure 44. Evolution of current signal, pH, CO_2 dissolved and total concentration of acetate, butyrate and ethanol in Reactor 3 (batch 4). Black inverted triangles on the top part of the figure indicate sampling and subsequent flushing with CO_2.

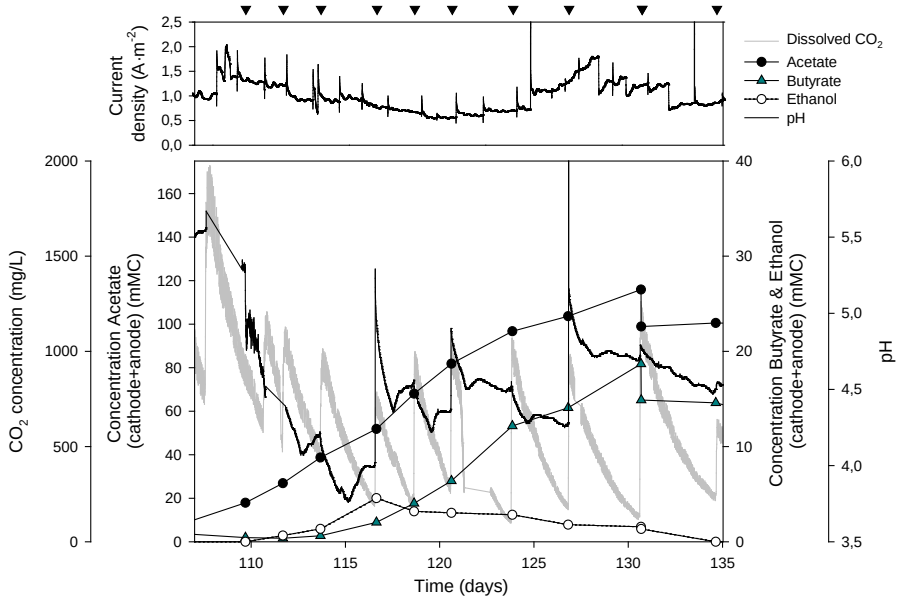


Figure 45. Evolution of current signal, pH, CO_2 dissolved and total concentration of acetate, butyrate and ethanol in Reactor 4 (batch 3). Black inverted triangles on the top part of the figure indicate sampling and subsequent flushing with CO_2.

5.4 Final remarks

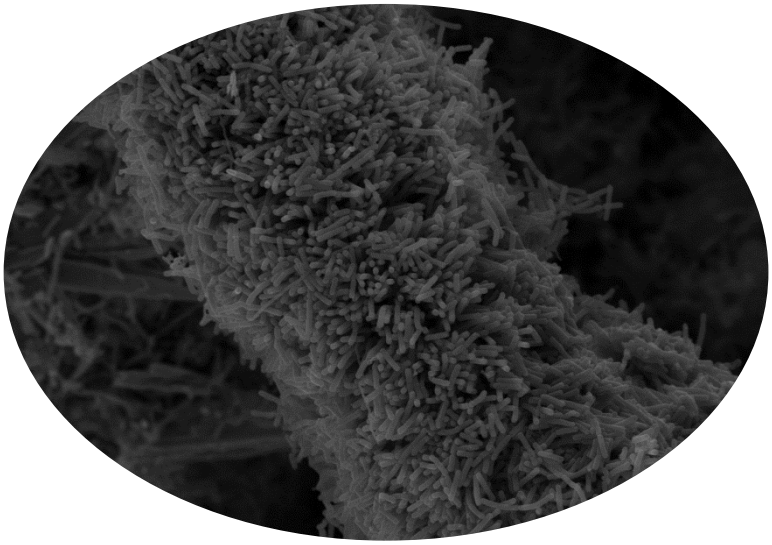
This study establishes the basis of a new methodology to promote electroactive biofilm formation on the surface of an electrode prior to its connection to a power source. This methodology is based on a previous electrode-enrichment through fermentation prior to its operation in a BES.

The presence in the biofilm formed of the target electroactive microorganism (I-19) after the fermentation and subsequent autotrophic conditions was demonstrated. In addition, the community analysis revealed a compartmentalization of the system. The biofilm community not only differs from the planktonic one, but also between different points along the growth surface. This suggests the existence of a substrate gradient that underlies the concomitant occurrence of different metabolic pathways during the fermentation process.

This pre-enriched biofilm was able to produce acetate, butyrate and ethanol during the CO_2 bio-electrorecycling for over 270 (reactor 3) and 170 days (reactor 4). Acetate was the main compound produced, whereas butyrate and ethanol were produced intermittently when the new feeding strategy based on the maintenance of reducing power since the beginning of the CO_2 feeding was implemented. Nevertheless, both systems showed high resilience and robustness to operational changes and disturbances.

Chapter 6

General discussion



General discussion

High rates of bioelectrochemical acetate production from CO_2 have been observed in previous studies (Jourdin, Grieger, et al. 2015; May, Evans, and LaBelle 2016). However, the operational conditions required to accomplish the steering of the bio-electrorecycling of CO_2 into more reduced compounds remain unclear. In this Ph.D. thesis, operational parameters such as pH, CO_2 dissolved and pH_2 were continuously monitored and showed high impact in the product selectivity. In addition, different reactor architectures and configurations were also assessed to accomplish a better control over the production of target compounds.

Performance of the tubular and flat-plate BES

The geometry of BES is considered one of the key points to improve rates, productivities and, in general, sustainable performances. In this PhD Thesis, two designs (tubular and flat-plate) were tested under comparable conditions of potential applied and biocatalyst used. The tubular BES showed an equimolar production of acetate and ethanol from the bio-electrorecycling of CO_2. On the other hand, flat-plate BES reactors showed higher production rates of acetate as major product and accomplished chain elongation into butyrate (Table 11). Different product spectrum obtained (alcohols or acids) underlies the importance of the reactor design. Tubular reactors had better manoeuvrability in terms of achieving ethanol production through solventogenesis over acetogenesis. However, the production rates and coulombic efficiencies were not as higher as the ones obtained in the flat-plate reactors. Current density demands were different between reactor types, fact that discards the idea of being a driving parameter that governs the production of more reduced compounds in the case

of the enriched culture of I-19. This highlights the importance of reactor design depending on the target compound. In addition, improve the biofilm thickness and mass transport was crucial to enhance the growth of more electroactive species that will help to increase both current density and product selectivity.

Table 11. Comparison of the two sets of reactors used in this Ph.D. Thesis.

	Productivity ($g_{product}$ m^{-2} d^{-1})				
Reactor	Acetate	Butyrate	Ethanol	CE (%)	J (A m^{-2})
1 & 2 (Tubular)	0.12 ± 0.29	-	0.08 ± 0.009	13.00 ± 8.5	0.47 ± 0.03
3 & 4 (Flat-plate)	4.76 ± 3.01	4.76 ± 3.01	0.31 ± 0.34	40.00 ± 29.00	1.29 ± 0.31

These outcomes suggest that a higher substrate availability (flat-plate) resulted in the enhancement of acetate production. Moreover, the minimized mass transfer limitations and ohmic losses achieved with the flat geometry due to closer distance between electrodes, dramatically increased the yields of the system, which led to an increasing of the CE from 13 to 40%. This number, rather low, is among the CE achieved for the same bioelectrochemical process in the literature (May, Evans, and LaBelle 2016). This might be explained by the leak of hydrogen that was not quantified in the calculations, together with the presence of parasitic reactions that act as electron sink. For instance, reactions of reduced compounds present in the buffer solution of the cathodic culture medium (e.g., sulfides, Fe^{2+}) with oxidants generated in the anode chamber (e.g., O_2, H_2, or Cl_2).

Economic comparison and scalability

The geometry and the cost of each type of reactor must be taken into account to foreseen the future scaling up towards a full-scale prototype. The cost of tubular reactor and flat plate were 362 and 522 €/unit, respectively (Table 12). Despite cost increase might not correlate with size increase during scale-up, these results show that tubular shaped are around 30% cheaper than flat-plate reactors at a lab-scale. Therefore, tubular reactors might be a good candidate from the economic point of view for its further planification towards full scale.

Table 12. Cost of building each type of reactor.

Reactor type	Cost (€)*				TOTAL (€/unit)
	Chassis	Cathodic electrode	Anodic electrode	Membrane	
Tubular	61.93	5.13	172.71	122.64	**362,41**
Flat-plate	359.37	105.13	55.70	2.00	**522,20**

***Pumps, analytical probes, reference electrode and potentiostats are not included because were equally used in both reactor types.**

The reactor design must incorporate elements that serve to overcome the existing limitations of the technology. By addressing those hurdles, the odds of moving forward a possible implementation of the technology will rise considerably.

The scaling up must be carried out maintaining a close distance between the electrodes. This means that the tubular reactor will be scaled up by increasing the height, whereas the flat-plate should be stacked and several units must be arranged in series. Moreover, flat-plates must keep the rectangular shape, since squared shapes contain high amount of dead spaces that would drastically decrease their overall performance (Vilà-Rovira et al. 2015).

In addition, the switch between synthetic and real streams must be taken into account when foreseeing the potential scalability of MET systems. For that reason, the avoidance of potential damages of the reactor elements must be considered. Calcification and/or affections due to corrosive reagents have to be addressed by placing a pre-treatment unit for the inlet or designing the reactor in accordance to those aspects.

Thermodynamic approach to reveal the role of H_2 and CO_2 in the bioelectrochemical solvent production

A thermodynamic approach was used to assess the Gibbs free energy required in the biocathode to perform electro-acetogenesis and solventogenesis from experimental data gathered with the tubular BES reactors. Acetogenesis resulted more spontaneous than solventogenesis under the experimental conditions of operation. At standard conditions, the Gibbs free energy of acetogenesis and solventogenesis were set at -94.9 and -9.1 kJ mol^{-1} respectively. Furthermore, the effect of the pH on the critical pH_2 was assessed depending on different values of pCO_2 and ethanol-to-acetate ratio. The model developed in section 4.3.4 showed that an increase of pCO_2 from 0.02 to 1 atm would decrease the critical pH_2 required for bioelectrochemical acetate production from $2.7 \cdot 10^{-6}$ to $3.9 \cdot 10^{-7}$ atm at a pH of 5.5. Also, within the pH range observed in the reactors, the critical pH_2 required by electroacetogenesis increased with lower pH values.

Insights on controlling the ethanol-to-acetate ratio

Parameters such as pH, CO_2 dissolved and pH_2 were found critical to reach an equimolar bioelectro-production of acetate and ethanol in an optimum ratio as potential substrate for performing chain elongation in a coupled fermenter. Recurrent patterns observed in current signal and pH profiles were directly linked to CO_2 conversion into either acetate or ethanol. Those parameters, together with the CO_2 availability in the medium, were found as key operational variables to control acetogenesis over solventogenesis or vice versa. The continuous measurement of these parameters suggested pH of 5.4 and CO_2 availability of 100 mg_{CO2} L^{-1} as critical values to achieve selectivity. Lowering those values would lead to a transition towards solventogenesis, whereas maintaining the values above the mentioned thresholds would trigger acetogenesis as the prevalent pathway. In addition, current consumption profile could be used as indicator to start feeding CO_2 when a turning point is detected.

Thus, the first approach of a real-time control system for the bioelectro-recycling of CO_2 to valuable building blocks was presented in this Ph.D. Thesis.

Microbial pre-enrichment of a biocathode through fermentation: a new inoculation strategy

A pre-enrichment of the biofilm on the surface of the carbon cloth that acted afterwards as electrode was carried out feeding with CO_2 (carbon source) and H_2 (reducing power). The formed biofilm was subsequently used as biocatalyst in a BES (reactor 3 and 4). This strategy showed a short period of activation of the BES and a new protocol of electroactive bacteria growth was successfully developed.

The starting inoculum consisted of an enriched culture of the isolate I-19 and after the fermentation resulted in a developed community in both the biofilm (carbon cloth) and the bulk liquid of the fermenter.

The phyla *Firmicutes*, *Actinobacteria*, and *Proteobacteria* showed most abundance in both the planktonic community and the biofilm. However, *Firmicutes* relative abundance was higher in the biofilm than in the bulk liquid, where *Proteobacteria* was more abundant. In addition, the high diversity of genera observed and its proven electroactivity ensured its suitability as inoculum in a BES (Figure 46).

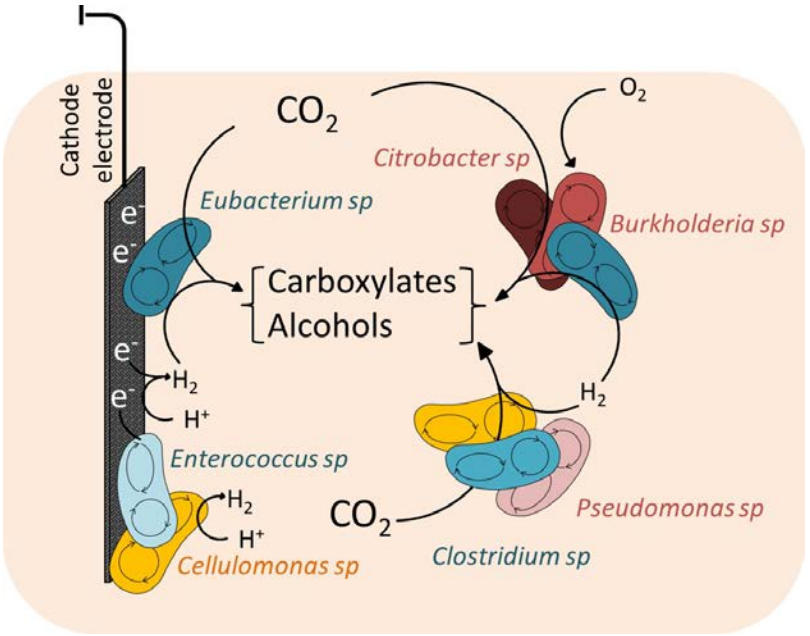


Figure 46. Putative abiotic and abiotic reactions from CO_2 and electricity taking place in the biocathode from the communities found in the biofilm and the bulk liquid of the enrichment process through fermentation.

Effects of the new feeding strategy on the product spectrum

This pre-enriched biofilm was able to produce acetate, butyrate and ethanol during the CO_2 bio-electrorecycling for over 270 days. Acetate was the main compound produced, whereas butyrate and ethanol were produced intermittently when the new feeding strategy based on the maintenance of the reducing power in the reactor, in the form of H_2, at the beginning of the CO_2 feeding event was implemented. This new strategy enables the microbial community to have access to a surplus of reducing power right after each feeding event. This favours reduction reactions from the thermodynamics point of view and allows producing more reduced compounds such as butyrate from the beginning. In addition, coulombic efficiencies are higher and the novel feeding strategy improves the resilience and robustness of the system to operational disturbances.

Outlook and future perspectives

Nowadays, the production of commodity chemicals using METs enters into a stage where selectivity and a tighter control of the overall process must be taken as a priority. Further research on this aspect has to be carried out in future Ph.D. Thesis, which must address how to better control the biocatalyst and couple new technologies such as artificial intelligent that will allow a remote and continuous control of the systems. Moreover, new advances in analytic technologies should be implemented in METs to serve as a tool to continuously measure key parameters and drive the process towards the production of target compounds.

Proofs of concept presented recently at lab scale showed the potential of METs and truly place this technology among the most promising sustainable

alternatives in the field of chemical production. These advancements should boost new initiatives at fundamental research level to further explore new biocatalyst and electrode materials. Also, a deeper understanding of electron transfer mechanisms between the electroactive microorganisms and electrodes will have a tremendous impact in the field. This, together with the recent development of synthetic biology and genetic engineering will lead to develop and better control metabolic routes related to the production of more interesting compounds.

Thermodynamics and hydrodynamics will be fundamental to achieve more efficient reactors and bring the technology closer to economic feasibility.

Scaling up this technology and the use of real streams are nowadays crucial to advance towards the development of the first full-scale prototypes. This will permit METs to compete with other existing bio-based technologies (i.e. fermentation, enzymatic, photochemical, etc.) and chemical-based technologies such as petrochemical with higher TRLs.

Chapter 7

General conclusions

General conclusions

This Ph.D. Thesis investigated reliable operational procedures for the monitoring of the performances of METs to produce suitable substrates for economically viable downstream applications. This work aimed at studying and levelling key operational conditions in model electrosynthesis reactors in order to obtain carbon-neutral compounds from CO_2. The cathodes of two different designs of BESs were inoculated with an enriched culture of a carboxydotrophic strain and were operated until stable conversion of CO_2 into acetate, ethanol and small amounts of butyrate

Tubular BES achieved a concomitant production of ethanol and acetate, which were considered crucial for triggering the production of longer carbon chain carboxylates and alcohols in, for example, a coupled chain elongation bioreactor. On the other hand, flat-plate BES showed constant acetate production and showed high resilience and robustness to unexpected operational episodes. In addition, coulombic efficiencies and overall production rates were higher in the flat-plate design, which suggests the need to improve the manoeuvrability by setting threshold values of key parameters that switch between target metabolic pathways. Moreover, improving the reactor design, mass transport limitation, together with reaching a high maturity of the electroactive community must be considered crucial to obtain more reduced compounds from CO_2 and electricity.

Continuous *in-line* monitoring of key parameters (pH, CO_2 dissolved and pH_2) revealed variations in the current signal and pH values that were correlated with

CO_2 depletion and the transition from acetogenesis to solventogenesis in the enriched culture. In addition, new inoculation and feeding strategies, based on previous electrode enrichment with an electroactive biofilm and avoiding periods with low availability of reducing power, showed promising results that should be addressed in future research on CO_2 bio-electrorecycling.

Finally, this Ph.D. Thesis shows that *in-line* monitoring of pH and electron consumption are meaningful operational variables to differentiate between the carboxylate and alcohol production, which opens the door to develop new approaches to control the bio-electrorecycling of CO_2 into biofuels by METs.

Chapter 8

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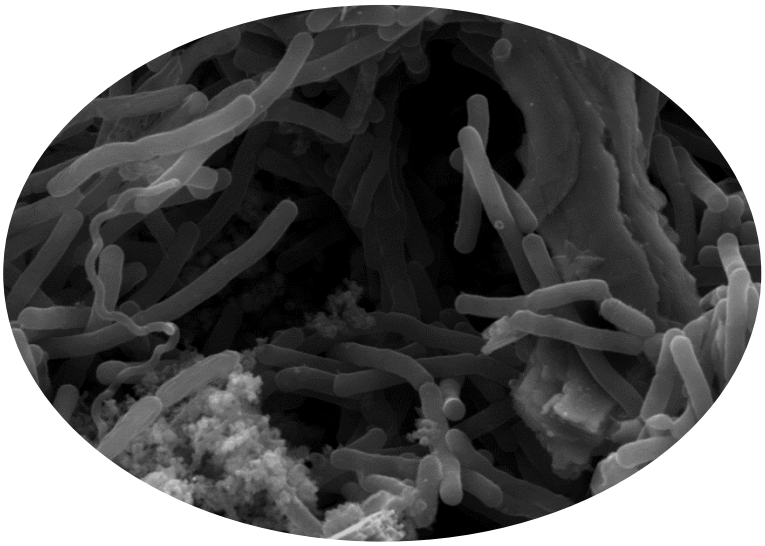

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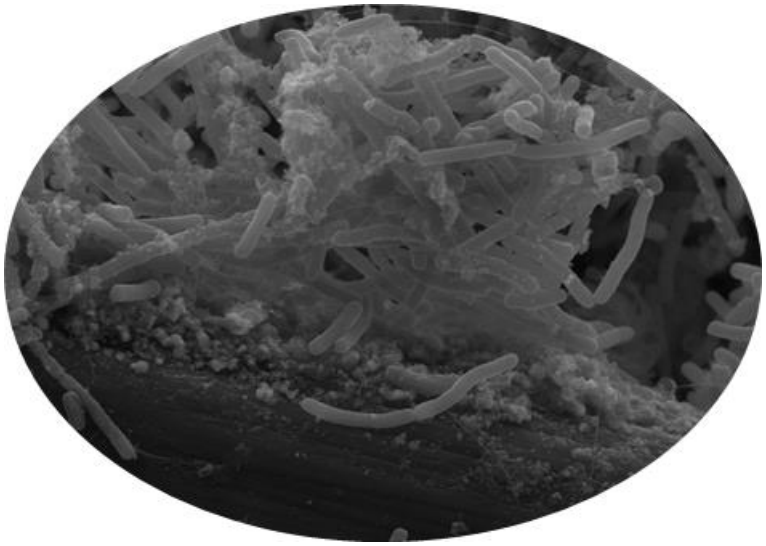
Chapter 9

Appendix



Media composition

Table 13. Composition of the modified ATCC1754 ETC medium used in the BES reactors of this Ph.D. thesis.

Medium component	($g\ L^{-1}$)	Trace metal solution component	($mg\ L^{-1}$)	Vitamin solution component	($\mu g\ L^{-1}$)
KH_2PO_4	0.1	Nitrilotriacetic acid	20.0	Biotin	20.0
NaCl	0.8	$MnSO_4 \cdot H_2O$	10.0	Folic acid	20.0
NH_4Cl	1.0	$Fe(SO_4)_2(NH_4)_2 \cdot 6H_2O$	8.0	Pyridoxine hydrochloride	100.0
$MgCl_2 \cdot 6H_2O$	0.2	$CoCl_2.6H_2O$	2.0	Thiamine hydrochloride	50.0
KCl	0.1	$ZnSO_4 \cdot 7H_2O$	0.002	Riboflavin	50.0
$CaCl_2 \cdot 2H_2O$	0.02	$CuCl_2 \cdot 2H_2O$	0.2	Nicotinic acid	50.0
MES	1.95	$NiCl_2 \cdot 2H_2O$	0.2	DL- calcium pantothenate	50.0
Cysteine HCl	0.4	$Na_2MoO_4 \cdot 2H_2O$	0.2	Vitamin B12	1.0
		Na_2SeO_4	0.2	p-aminobenzoic acid	50.0
		Na_2WO_4	0.2	Lipoic acid (Thioctic acid)	50.0

Table 14. Composition of the organic medium used to grow I-19 in the fermenter of the chapter 5.

Medium component	(g L^{-1})
Tryptone	5
Meat extract	5
Fructose	2.5
NaCl	5
Soluble starch	0.5
Cys-HCl	0.25
Sodium acetate	1.5
Yeast extract	1.5