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Y BIODIVERSIDAD**

**Grupo de Ingeniería Química, Ambiental y Bioprocesos**

**BIOPROCESSES FOR WASTEWATER  
TREATMENT: INTEGRATION OF  
BIOELECTROCHEMICAL SYSTEMS AND  
OTHER TECHNOLOGIES**

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## ABSTRACT

Wastewater streams (such as cheese whey or domestic wastewater) usually require a biological treatment prior to their disposal to the environment. These treatments have traditionally been performed by conventional technologies, particularly anaerobic digestion (AD). Dark fermentation (DF) would be another example, although not so well-established in the real scale. Bioelectrochemical systems (BESs) have recently developed as a promising technology for biological treatments, as the use of BESs for wastewater treatment allows for a reduction in the energy consumption for these processes. Moreover, integration of BESs with conventional bioprocessing technologies (AD or DF), represents an innovative approach that could optimize the amount of energy recovered from the waste streams, making the process more profitable.

In this context, the main objective of this thesis was to evaluate the integration of BESs and more conventional biological processes (such as AD or DF) as a technology for industrial (cheese whey) and domestic wastewater treatment.

The first step was the determination of the operating conditions that optimize the performance of these bioprocesses. In order to do so, the effect of nitrogen and lactose concentration for the batch-mode dark fermentative processing of cheese whey was studied by means of the response surface methodology (RSM), finding that the best operability area for fermentative  $H_2$  production was at lower values of nitrogen and lactose. Besides, higher hydrogen yields were found when gradually decreasing N content when working in semi-continuous mode. DF of cheese whey resulted in a maximum  $H_2$  production rate of  $0.18 \text{ L } H_2 \text{ L}_r^{-1} \text{ d}^{-1}$ .

The integration, in a two-stage configuration, of biocatalyzed electrolysis as a post-treatment for dark fermentative  $H_2$  production from cheese whey was studied afterwards. In the first stage, batch-mode dark fermentation of cheese whey resulted in a  $H_2$  production of  $0.7 \text{ L-}H_2 \text{ L}_r^{-1}$ , obtaining an acidified effluent as well. After being diluted and amended, the effluent was fed into a microbial electrolysis cell (MEC) in continuous-mode. The  $H_2$  production rate in the second stage was  $0.5 \text{ L-}H_2 \text{ L}_r^{-1} \text{ d}^{-1}$ . As a whole, the combined process resulted in a maximum  $H_2$  yield of  $94.2 \text{ L-}H_2 \text{ kg}_{VS}^{-1}$ , which represents a 20% improvement when compared to cheese whey fermentations yields reported in bibliography.

The integration of electrogens-enriched electrodes and AD in the same reactor was also evaluated. This approach (AD + BES) was used to analyze operation of the system both at overloaded and underloaded (fed with high-strength and low-strength substrates, respectively) conditions, in which AD usually presents inefficiencies.

Given that an excessive acidification by volatile fatty acids (VFA) accumulation is one of the main causes for the collapse of anaerobic digesters, the study was aimed to characterize the behavior of AD + BES integration under acidic conditions. For the case of electrode-containing digesters, it was observed a delay in VFA build-up, as well as an enhancement in CH<sub>4</sub> production during the first 100 h of the process. Although this effect relies on biomass proliferation rather than electrochemical activity, these results allow for an optimization of AD process when working in continuous-mode at short hydraulic retention times (HRTs).

The benefits of using MECs to assist methanogenesis while simultaneously treating low-strength domestic wastewater were also investigated. When a MEC electrode was inserted in what could be considered an underloaded anaerobic digester of wastewater in batch-mode, organic matter degradation and CH<sub>4</sub> production were increased as a result of an enhancement of hydrogenotrophic methanogenesis, preventing inefficiencies associated to homoacetogens proliferation. In continuous mode, longer HRTs were found to be more favorable for optimizing energy recovery.

Finally, two different strategies for the improvement of exoelectrogenic performance in BESs were tested. One of the approaches consisted in the selective enrichment of exoelectrogenic species from a natural soil substrate by testing different voltages applied. A voltage value of +0.2 V seemed to optimize bioelectrocatalytic response. In the other strategy, a non-targeted mutagenesis induction tool based on the overexpression of DNA polymerase IV could be developed. However, for a proper confirmation of these results, further investigations are required.

## RESUMEN

Las aguas residuales (ya sean de origen industrial, como el lactosuero, o doméstico) suelen requerir un tratamiento biológico antes de ser desechadas al medio ambiente. Este tipo de tratamientos ha estado tradicionalmente ligado a tecnologías convencionales, sobre todo la digestión anaerobia (AD). Otro ejemplo sería la fermentación oscura (DF), aunque no está tan implementada a escala real. Recientemente, los sistemas bioelectroquímicos (BESs) han aparecido como una alternativa prometedora a estos tratamientos, dado que el uso de BESs como tratamiento de aguas residuales posibilita una reducción del consumo de energía del proceso. Además, la integración de los BESs con las tecnologías de tratamiento convencionales (AD o DF) representa un enfoque innovador con potencial para optimizar la cantidad de energía recuperada de las aguas residuales, lo que aumentaría la rentabilidad del proceso.

En este contexto, el objetivo principal de esta tesis es evaluar la integración de los BESs y procesos biológicos convencionales (como la AD o DF) como alternativa tecnológica para el tratamiento de aguas residuales tanto de origen industrial (lactosuero) como de origen doméstico.

El primer paso consistió en la determinación de las condiciones de operación que optimizan el rendimiento de estos procesos biológicos. Para ello, el efecto de la concentración de nitrógeno y lactosa para el procesamiento de lactosuero mediante fermentación oscura fue estudiado por medio de la metodología de superficie de respuesta (RSM) bajo condiciones discontinuas, resultando que el área de operación óptima para la producción de  $H_2$  por fermentación se encontraría en los rangos más bajos de concentración de ambos componentes. Adicionalmente, al trabajar en modo semicontinuo, los máximos rendimientos de producción de  $H_2$  se dieron al reducir gradualmente la concentración de N. La fermentación oscura de lactosuero resultó en una tasa máxima de producción de  $H_2$  de  $0.18 \text{ L-H}_2 \text{ L}_r^{-1} \text{ d}^{-1}$ .

A continuación se llevó a cabo la integración, en una configuración de dos etapas, de la electrólisis biocatalítica como postratamiento a la DF, para la producción de  $H_2$  a partir de lactosuero. En la primera etapa, la fermentación oscura de lactosuero en batch resultó en una producción de  $H_2$  de  $0.7 \text{ L-H}_2 \text{ L}_r^{-1}$ , obteniéndose además un efluente de naturaleza ácida. Tras ser procesado, este efluente fue usado como alimentación, operando en modo continuo, para una celda de electrólisis microbiana (MEC). La tasa de producción de  $H_2$  en esta segunda etapa fue de 0.5

$L\text{-H}_2 L_r^{-1}d^{-1}$ . A nivel global, el rendimiento máximo de producción de  $H_2$  del proceso combinado fue de  $94.2 L\text{-H}_2 kg_{VS}^{-1}$ , lo que representa una mejora del 20% en comparación con estudios de fermentación de lactosuero descritos en bibliografía.

La integración de electrodos (previamente enriquecidos en biomasa electrógena) y AD en el mismo reactor también fue estudiada. Esta configuración (AD + BES) se utilizó para analizar la capacidad del sistema tanto en condiciones de sobrecarga como de baja carga orgánica, en las que el proceso de AD suele presentar ineficiencias.

Dado que una acidificación excesiva producida por la acumulación de ácidos grasos volátiles (VFA), es una de las causas principales del colapso de los digestores anaerobios, el estudio fue realizado para evaluar el comportamiento de la integración AD + BES bajo condiciones ácidas. En el caso de los reactores con electrodos, se observa un retraso en la acumulación de AGV, así como un incremento en la producción de  $CH_4$  durante las primeras 100 h de operación. Aunque este efecto está basado más en la proliferación de biomasa que en la actividad electroquímica, los resultados permiten una optimización del proceso de AD al trabajar en modo continuo a bajos tiempos de retención hidráulica (HRTs).

También se investigaron las ventajas de usar MECs como soporte a la producción de metano durante el tratamiento de agua residual urbana de baja carga orgánica. Al insertar un electrodo MEC en lo que podría ser considerado un digestor anaerobio operando en modo discontinuo a baja carga orgánica, se incrementó la tasa de degradación de materia orgánica, así como la producción de  $CH_4$ , debido a un aumento de los procesos de metanogénesis hidrogenotrófica, lo que a su vez permitió evitar las ineficiencias asociadas a la proliferación de homoacetógenas. Al trabajar en modo continuo, los HRTs más largos resultaron ser más favorables para optimizar la recuperación de energía.

Finalmente, se evaluaron dos estrategias diferentes para la mejora de la respuesta electrógena en BESs. Una de ellas consistió en el enriquecimiento selectivo de bacterias exoelectrógenas (a partir de suelo como sustrato) a diferentes valores de voltaje aplicado. Un valor de +0.2 V parece optimizar la respuesta bioelectrocatalítica. En la otra estrategia, se pudo desarrollar una herramienta de inducción de mutagénesis no dirigida a partir de la sobreexpresión de la DNA polimerasa IV. Sin embargo, serán necesarios estudios posteriores para confirmar estos resultados.

## **LIST OF SYMBOLS AND ABBREVIATIONS**

|                        |   |
|------------------------|---|
| <b>Ac<sup>-</sup></b>  | Acetate                                 |
| <b>AD</b>              | Anaerobic digestion                     |
| <b>ADP</b>             | Adenosine diphosphate                   |
| <b>ARB</b>             | Anode-respiring bacteria                |
| <b>ASBR</b>            | Anaerobic sequencing batch reactor      |
| <b>ATP</b>             | Adenosine triphosphate                  |
| <b>BES</b>             | Bioelectrochemical systems              |
| <b>BOD<sub>5</sub></b> | Biological oxygen demand                |
| <b>BP</b>              | Biosolid pellets                        |
| <b>bp</b>              | Base pairs                              |
| <b>But</b>             | Butyrate                                |
| <b>CA</b>              | Chronoamperometry                       |
| <b>CC</b>              | Close-circuit                           |
| <b>CC<sub>e</sub></b>  | Cathode conversion efficiency           |
| <b>CE</b>              | Counter electrode                       |
| <b>C<sub>e</sub></b>   | Coulombic efficiency                    |
| <b>COD</b>             | Chemical oxygen demand                  |
| <b>CSTR</b>            | Continuously stirred tank reactor       |
| <b>CV</b>              | Cyclic voltammetry                      |
| <b>CW</b>              | Cheese whey                             |
| <b>DGGE</b>            | Denaturing gradient gel electrophoresis |
| <b>DNA</b>             | Deoxyribonucleic acid                   |
| <b>DNA pol. IV</b>     | DNA polymerase IV                       |
| <b>DS</b>              | Digested sludge                         |
| <b>E</b>               | Potential                               |
| <b>E<sub>an</sub></b>  | Potential of the anodic half-reaction   |
| <b>E<sub>cat</sub></b> | Potential of the cathodic half-reaction |
| <b>E<sub>emf</sub></b> | Electromotive force                     |

## LIST OF SYMBOLS AND ABBREVIATIONS

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|   |   |
|---|---|
| <b><math>E_{we}</math></b>              | Potential of the working electrode                      |
| <b>EDTA</b>                             | Ethylenediaminetetraacetic acid                         |
| <b>EET</b>                              | Extracellular electron transfer                         |
| <b>EFC</b>                              | Enzymatic fuel cell                                     |
| <b>F</b>                                | Faraday's constant                                      |
| <b>FAD<sup>+</sup>/FADH<sub>2</sub></b> | Flavine adenine dinucleotide                            |
| <b>Fd<sub>red</sub>/Fd<sub>ox</sub></b> | Ferredoxin in its reduced/oxidized form                 |
| <b>FHL</b>                              | Formate hydrogen lyase                                  |
| <b>FHP</b>                              | Fermentative H <sub>2</sub> production                  |
| <b>FID</b>                              | Flame ionization detector                               |
| <b>GC</b>                               | Gas chromatography                                      |
| <b>GI</b>                               | Glucose   |
| <b>HPLC</b>                             | High-pressure liquid chromatography                     |
| <b>HPR</b>                              | H <sub>2</sub> production rate                          |
| <b>HRT</b>                              | Hydraulic retention time                                |
| <b>I</b>                                | Inoculum  |
| <b>i</b>                                | Current density   |
| <b>ICP-AES</b>                          | Inductively-coupled plasma atomic emission spectrometer |
| <b>L<sub>a</sub></b>                    | Liter of anodic chamber                                 |
| <b>Lac<sup>-</sup></b>                  | Lactate   |
| <b>L<sub>r</sub></b>                    | Liter of reactor  |
| <b>MDC</b>                              | Microbial desalination cell                             |
| <b>MEC</b>                              | Microbial electrolysis cell                             |
| <b>MES</b>                              | Microbial electrosynthesis                              |
| <b>MFC</b>                              | Microbial fuel cell                                     |
| <b>N<sub>A</sub></b>                    | Avogadro's constant                                     |
| <b>NADH-FOR</b>                         | NADH ferredoxin oxidoreductase                          |
| <b>NAD(P)<sup>+</sup>/NAD(P)H</b>       | Nicotinamide adenine dinucleotide (phosphate)           |
| <b>OC</b>                               | Open-circuit  |
| <b>OD (600 nm)</b>                      | Optical density at a wavelength of 600 nm               |
| <b>OLR</b>                              | Organic loading rate                                    |



|                                       |   |
|---------------------------------------|---|
| <b>OMC</b>                            | Outer-membrane c-type cytochromes         |
| <b>PAH</b>                            | Polyhydroxyalkanoates                     |
| <b>PAMFC</b>                          | Photosynthetic algal microbial fuel cell  |
| <b>PC</b>                             | Positive control                          |
| <b>PCR</b>                            | Polymerase chain reaction                 |
| <b>PEM</b>                            | Proton exchange membrane                  |
| <b>PFL</b>                            | Pyruvate formate lyase                    |
| <b>PFOR</b>                           | Pyruvate ferredoxin oxidoreductase        |
| <b>P<sub>H2</sub></b>                 | Partial pressure of H <sub>2</sub>        |
| <b>PMFC</b>                           | Plant microbial fuel cell                 |
| <b>Prop</b>                           | Propionate                                |
| <b>PVDF</b>                           | Polyvinylidene fluoride                   |
| <b>Q<sub>H2</sub>/Q<sub>CH4</sub></b> | H <sub>2</sub> /CH <sub>4</sub> flow rate |
| <b>qPCR</b>                           | Quantitative polymerase chain reaction    |
| <b>R</b>                              | Universal gas constant                    |
| <b>RE</b>                             | Reference electrode                       |
| <b>R<sub>int</sub></b>                | Internal resistance                       |
| <b>rRNA</b>                           | Ribosomal RNA                             |
| <b>RSM</b>                            | Response surface methodology              |
| <b>SHE</b>                            | Standard hydrogen electrode               |
| <b>SM</b>                             | Synthetic medium                          |
| <b>TAE</b>                            | Tris-Acetate EDTA                         |
| <b>TCD</b>                            | Thermal conductivity detector             |
| <b>TOC</b>                            | Total organic carbon                      |
| <b>TS</b>                             | Total solids                              |
| <b>TSS</b>                            | Total suspended solids                    |
| <b>V<sub>app</sub></b>                | Voltage applied                           |
| <b>VFA</b>                            | Volatile fatty acids                      |
| <b>VS</b>                             | Volatile solids                           |
| <b>VSS</b>                            | Volatile suspended solids                 |
| <b>WE</b>                             | Working electrode                         |

*LIST OF SYMBOLS AND ABBREVIATIONS*

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|             |                            |
|-------------|----------------------------|
| <b>wt</b>   | Wild-type                  |
| <b>WW</b>   | Wastewater                 |
| <b>WWTP</b> | Wastewater treatment plant |

# *Chapter 1*

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## *General Introduction*



## 1.1. BIOPROCESSING TECHNOLOGIES FOR WASTE MANAGEMENT: ANAEROBIC DIGESTION AND DARK FERMENTATION

Anaerobic digestion has consolidated as one of the most important technologies for waste management and CH<sub>4</sub> production. Dark fermentation has also developed recently (in this case for bio-H<sub>2</sub> production), although it is not so well established yet. Because of the need of reducing the high dependence of fossil fuels present in our society, research in alternative fuels such as CH<sub>4</sub> or H<sub>2</sub> obtained from renewable sources has developed in recent years.

### 1.1.1. Anaerobic digestion

Anaerobic digestion (AD) is a biological process which consists on the biodegradation of organic matter in an oxygen-free environment by means of an anaerobic microorganism consortia. The main product of the AD process is biogas, a mixture of gases which generally comprises CH<sub>4</sub> (48-65 %), CO<sub>2</sub> (36-41 %), N<sub>2</sub> (around 17%), and small proportions of other gases, such as CO, H<sub>2</sub>, H<sub>2</sub>S, etc. (Ward et al., 2008). Anaerobic degradation technologies present some advantages over aerobic waste treatments, as they are less energy-intensive processes and biomass formation rates are lower (allowing for more cost-effective sludge management processes). As a result, anaerobic digestion is a more favorable alternative for waste treatment than aerobic treatments (Abbasi et al., 2012).

AD is a complex process, based on different stages carried out by a wide variety of microorganism species working sequentially or simultaneously (Figure 1.1) (Massé and Droste, 2000). The first stage (**hydrolysis**) consists on the solubilization of insoluble organic matter, as a result of the action of extracellular enzymes secreted by hydrolytic bacteria. In this stage, organic polymers are degraded to more simple compounds (the polymer subunits, e.g. sugars, amino acids, alcohols or long-chain fatty acids). The hydrolytic stage is highly dependent on the complexity of the organic matter fed into the digester, as well as the ability of microorganisms to degrade it. As a result, it is considered the limiting step of the global process.

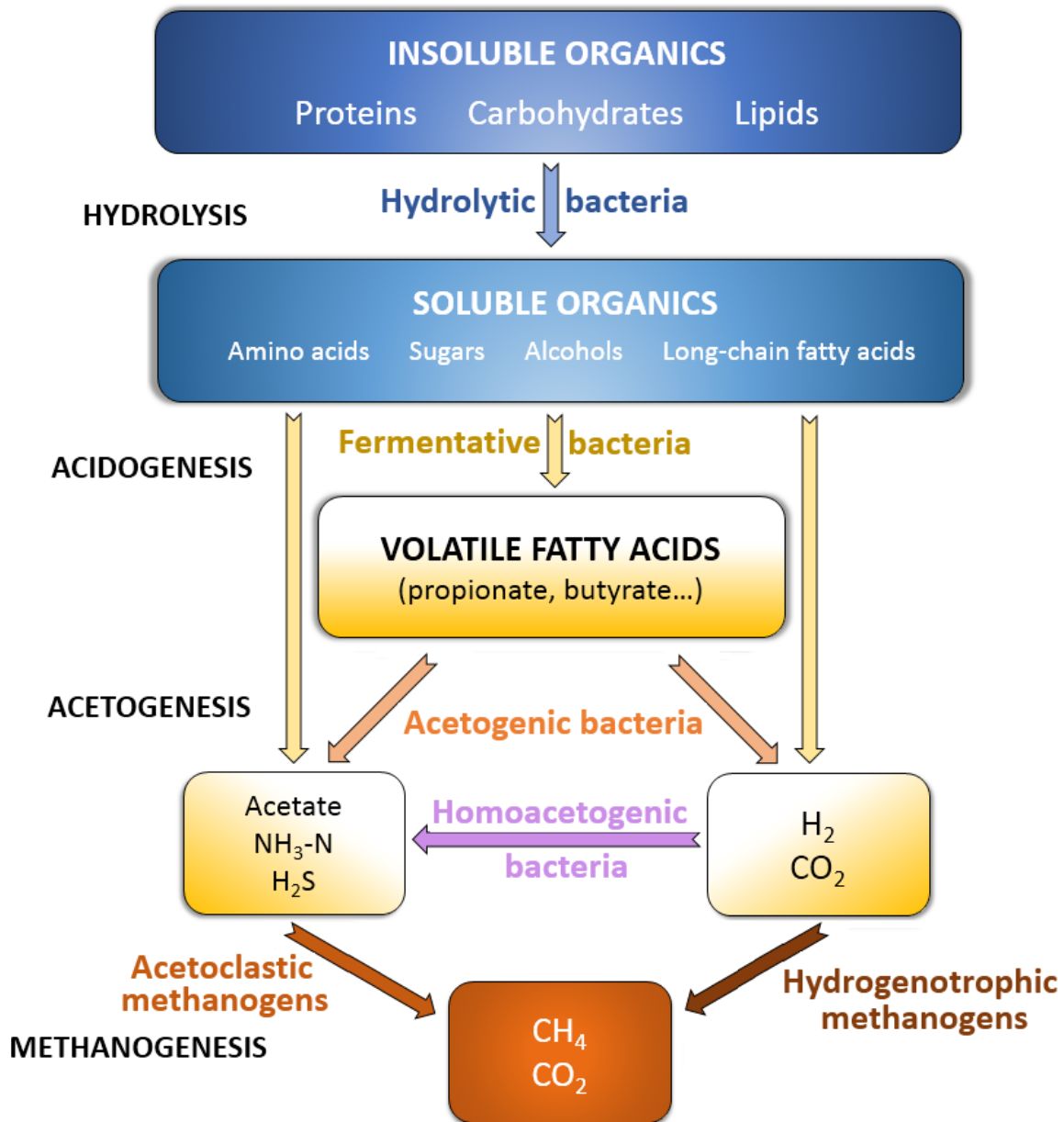
The second phase is known as **acidogenesis**. In this stage, fermentative bacteria metabolize the soluble organic matter produced in the previous phase, generating volatile fatty acids (VFA, mainly acetate, propionate and butyrate) and other organic acids (e.g. lactate or pyruvate). The next step of the AD process (**acetogenesis**) consists on the transformation of VFAs into acetate,

H<sub>2</sub> and CO<sub>2</sub>, which will be the substrates for methanogens in the subsequent stage. During the acetogenic stage, homoacetogenic bacteria might proliferate and produce acetate from H<sub>2</sub> and CO<sub>2</sub>, establishing a competition with hydrogenotrophic methanogens.

Finally, in the **methanogenesis** phase, CH<sub>4</sub> is produced from acetate by means of acetoclastic methanogens (CO<sub>2</sub> is formed as a byproduct). An alternative methanogenic pathway is carried out by hydrogenotrophic methanogens, which produce CH<sub>4</sub> via the reduction of CO<sub>2</sub> with H<sub>2</sub>.

Cheese whey (CW) is regarded as a potential substrate for AD processes. CW is the main byproduct of cheese production in dairy industries, and represents approximately 90% of the total volume of milk and about 55% of its nutrients (González, 1996). It can be used for livestock feeding, as well as for elaborating some foods (e.g. Italian “Ricotta” or Spanish “Requesón”). However, a production of 9 L<sub>CW</sub> kg<sup>-1</sup> cheese has been estimated (González, 1996): given that CW production is higher than its use for these purposes, CW might be considered both as a resource and a waste stream that needs an appropriate management. Although CW composition may vary depending on the type of milk and the type of cheese produced, it usually presents a solids content of about 6.4-6.5% (Gèosta-Bylund and López-Gómez, 2003). As a result of these characteristics, CW poses serious hazard for the environment if not properly treated.

Nevertheless, because of its high organic content, CW presents some difficulties when undergoing the AD process. Since the degradation rate of VFA by methanogens is lower than their production rate by acidogenic bacteria, VFA accumulation occurs in the digester (Prazeres et al., 2012). Therefore, maintaining stable operating conditions is a complex issue (Janczukowicz et al., 2008). In order to solve this problem, several strategies have been studied: for instance, alkalinity supplementation (Gannoun et al., 2008) in order to increase the buffering capacity of the system. A two-stage configuration (separating the acidogenic and methanogenic stages in different reactors) has also been studied, enabling the optimization of operating conditions for both reactors (Saddoud et al., 2007; Fernández et al., 2015). Recently, the integration of AD and bioelectrochemical processes in the same reactor has also been reported in bibliography (see Section 1.2.8.).



*Figure 1.1. Schematic view of the four stages of the anaerobic digestion process (Massé and Droste, 2000).*

### 1.1.2. Dark fermentation

Aside from anaerobic digestion, dark fermentation (DF) has also been proposed as a feasible technology for waste management. In this process, the final product obtained from the

degradation of organic matter is  $H_2$ .  $H_2$  production from biological wastes might be achieved by many processes, two of them being based on biological reactions carried out by microorganisms: photofermentation and dark fermentation (Prazeres et al., 2012).

In contrast to photofermentation, dark fermentation does not require a source of light to occur. Dark fermentation is an anaerobic process for the degradation of organic matter into  $H_2$ , which can be carried out both by mixed or pure cultures. Among the  $H_2$ -producing microorganism species, the most well-known are *Clostridium* and *Enterobacter*. The mechanism for  $H_2$  production starts with the metabolism of organic matter through the Embden-Meyerhof pathway (also known as glycolysis), obtaining pyruvate at the end (Davila-Vázquez and Razo-Flores, 2007). The next step is the transformation of pyruvate into acetyl-CoA. This reaction is catalyzed by a different enzyme depending on the microorganism (Figure 1.2): in *Clostridium*, the enzyme is known as the pyruvate ferredoxin oxidoreductase (Charon et al., 1999); in enteric bacteria, two enzymes are involved (pyruvate formate lyase and formate hydrogen lyase), with formic acid as an intermediate (Axley et al., 1990; Bagramyan and Trchounian, 2003). In both cases, reducing power is generated as a result of these metabolic reactions. This reducing power is used by an enzymatic complex to form  $H_2$  (Axley et al., 1990; Peters, 1999). These enzymatic complexes are generically referred to as hydrogenases, although there are some differences between hydrogenases in *Clostridium* and in enteric bacteria, both structurally and functionally (Figure 1.2). In enteric bacteria, the hydrogenase is a subunit of the formate hydrogen lyase (Axley et al., 1990). In *Clostridium*, the hydrogenase takes the electrons via an intermediate (ferredoxin) (Charon et al., 1999).

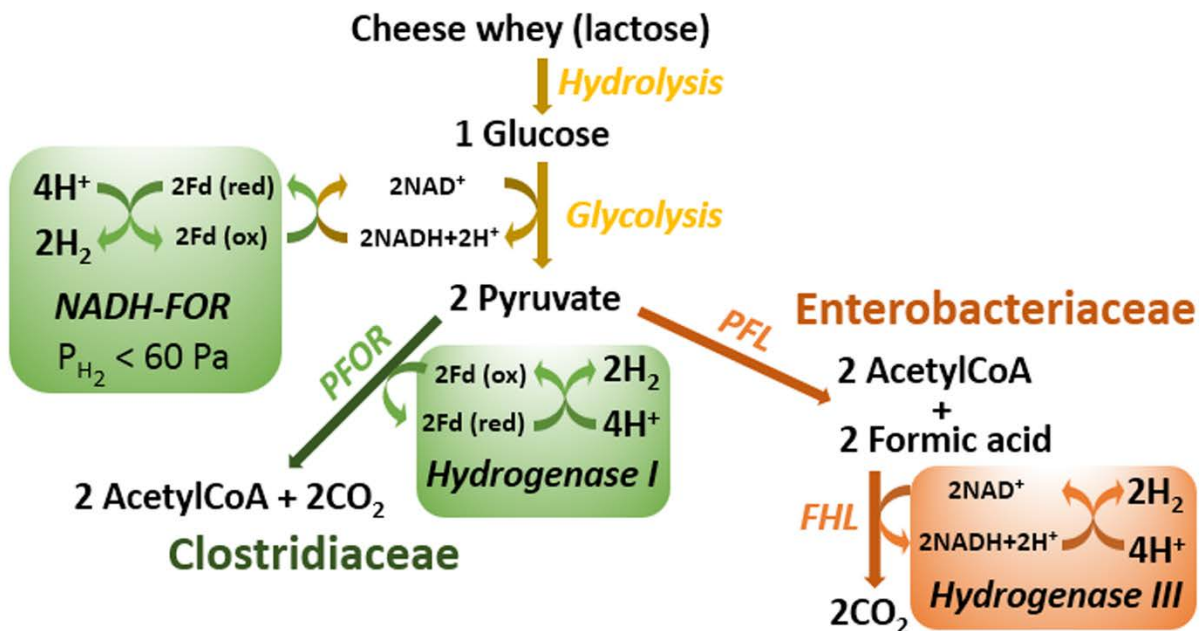
It is important to highlight, however, that the genus *Clostridium* possesses another enzyme, known as NADH ferredoxin oxidoreductase, which allows hydrogenase to use the reducing power produced in the Embden-Meyerhof pathway (Sinha and Pandey, 2011), thereby enhancing the total amount of  $H_2$  produced by *Clostridium* in comparison to *Enterobacter*. Nevertheless, *Enterobacter* are facultative anaerobes while *Clostridium* are strict anaerobes. Because of the capacity of *Enterobacter* to survive an aerobic environment, as well as its ability to turn an initially aerobic environment into an anaerobic one, the use of mixed cultures for DF is advisable.

Dark fermentation of CW has been evaluated in several studies. Theoretically, this process should lead to a theoretical yield of 8 mol of  $H_2$  per mol of lactose (Prazeres et al., 2012). However,



H<sub>2</sub> production by dark fermentation can undergo product inhibition: if partial pressure of H<sub>2</sub> in the fermenter is higher than 60 Pa (Figure 1.2), part of the reducing power is directed towards butyric fermentation instead of acetate production, and the H<sub>2</sub> yield is significantly decreased (Davila-Vázquez and Razo-Flores, 2007).

Because of its high organics content, chemical oxygen demand (COD) removal is one of the main parameters used to assess in dark fermentative processes. COD reductions and sugar consumption around 90% have been reported (Venetsaneas et al., 2009; Azbar and Dokgoz, 2010; Rosales-Colunga et al., 2010). However, the effluent obtained after H<sub>2</sub> production cannot be discharged and a post-treatment is required (Prazeres et al., 2012). As previously stated, using the effluent of dark fermentation as substrate for the anaerobic digestion process has been tested (Venetsaneas et al., 2009; Fernández et al., 2015). On the other hand, Azbar and Dokgoz (2010) integrated dark fermentation and photofermentation. This approach presented the disadvantage of being energy-intensive. A promising alternative (see Section 1.2.8.) consists on the use of the VFA-rich effluent from dark fermentation as a substrate for bioelectrochemical systems.



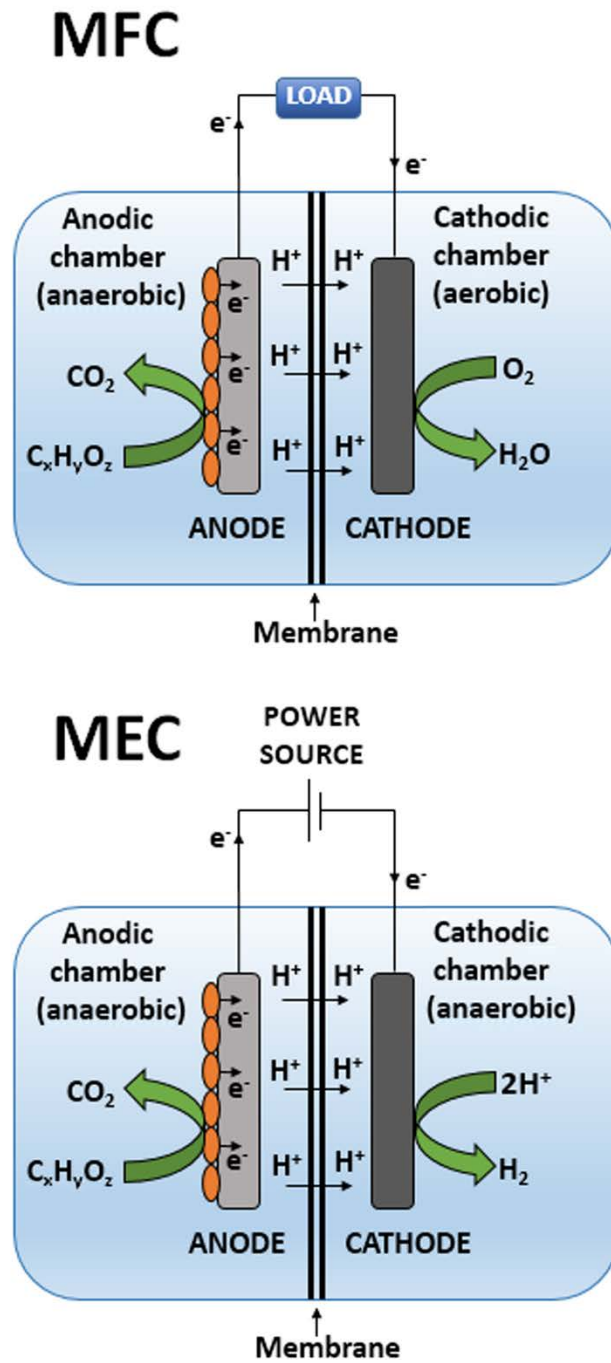
**Figure 1.2.** Different pathways for H<sub>2</sub> production through dark fermentation (NADH-FOR: NADH ferredoxin oxidoreductase; PFOR: pyruvate ferredoxin oxidoreductase; PFL: pyruvate formate lyase; FHL: formate hydrogen lyase).

### 1.2. BIOELECTROCHEMICAL SYSTEMS

In general terms, bioelectrochemical systems (BESs) are bioreactors in which microorganisms attached to the surface of an electrode are used for the catalysis of redox reactions (Rabaey et al., 2007). Since the discovery of electrochemically active microorganisms by Potter (1911), BESs have developed as an emerging technology that combine both biological and electrochemical processes for energy generation (in terms of electricity, H<sub>2</sub>, CH<sub>4</sub> or other useful chemicals), as well as wastewater treatment (Bajracharya et al., 2016). Depending on the process required, different kinds of BESs have flourished recently: microbial fuel cells (MFCs) for electricity production (Liu et al., 2005a; Logan et al., 2006); microbial electrolysis cells (MECs) for the synthesis of chemical valuables (Rozendal et al., 2006; Escapa et al., 2016); enzymatic fuel cells (EFCs) (Leech et al., 2012); microbial desalination cells (MDCs) (Kim and Logan, 2013); plant microbial fuel cells (PMFCs) (Lu et al., 2015); or photosynthetic algal microbial fuel cells (PAMFCs) (Rosenbaum et al., 2010a), among others.

A standard MFC consists of two different compartments, known as the anodic and cathodic chambers, each of them containing an electrode and separated by means of a proton exchange membrane (PEM) (Figure 1.3). In the anodic chamber, organic matter is degraded by electrogenic bacteria, producing CO<sub>2</sub>, electrons and protons. The electrons are transferred to the anode by different mechanisms, and then transported to the cathode through an external circuit, whereas protons reach the cathodic chamber by permeating through the PEM. In the cathodic chamber, electrons react with a terminal electron acceptor (typically O<sub>2</sub>), to form H<sub>2</sub>O (Logan, 2008). Although the maximum electromotive force ( $E_{emf}$ ) in an MFC is theoretically 1.1 V (Rozendal et al., 2007), in practice the  $E_{emf}$  usually falls below 0.6 as a result of potential losses and irreversibilities (Logan et al., 2006).

On the other hand, MECs are aimed to the production of chemical values (initially H<sub>2</sub> or CH<sub>4</sub>) rather than electricity generation. As a result, although not very different to MFCs in general reactor architecture, two main distinctions between MFCs and MECs can be established (Figure 1.3): first, the cathodic chamber must be anaerobic, in order to avoid the oxygen reduction reaction. Second, considering that the hydrogen evolution reaction is not spontaneous, it is necessary to apply a voltage to force the reaction between electrons and protons to occur.



**Figure 1.3.** Structure comparison and different operating characteristics of a MFC and a MEC. The cathodic chamber is aerobic in MFCs and anaerobic in MECs, and a load resistance in MFCs is replaced by a power supply in MECs. As a result, MECs are aimed to  $H_2/CH_4$  and other chemicals production, while electricity is being produced in MFCs.

Although initially designed for H<sub>2</sub> production (Liu et al., 2005b; Rozendal et al., 2006), MEC have also focused on the generation of other value added products, such as CH<sub>4</sub> (Cheng et al., 2009; Fu et al., 2015) or H<sub>2</sub>O<sub>2</sub> (Fu et al., 2010), as well as new bioremediation processes: removal of nitrogen (Arredondo et al., 2015), sulfate (Coma et al., 2013) or nickel (Luo et al., 2015), among others. Indeed, the development of microbial electrosynthesis processes (MESs) for the production of organic compounds from CO<sub>2</sub> (Nevin et al., 2010; Rabaey and Rozendal, 2010; Sadhukhan et al., 2016) has highly increased in the past years.

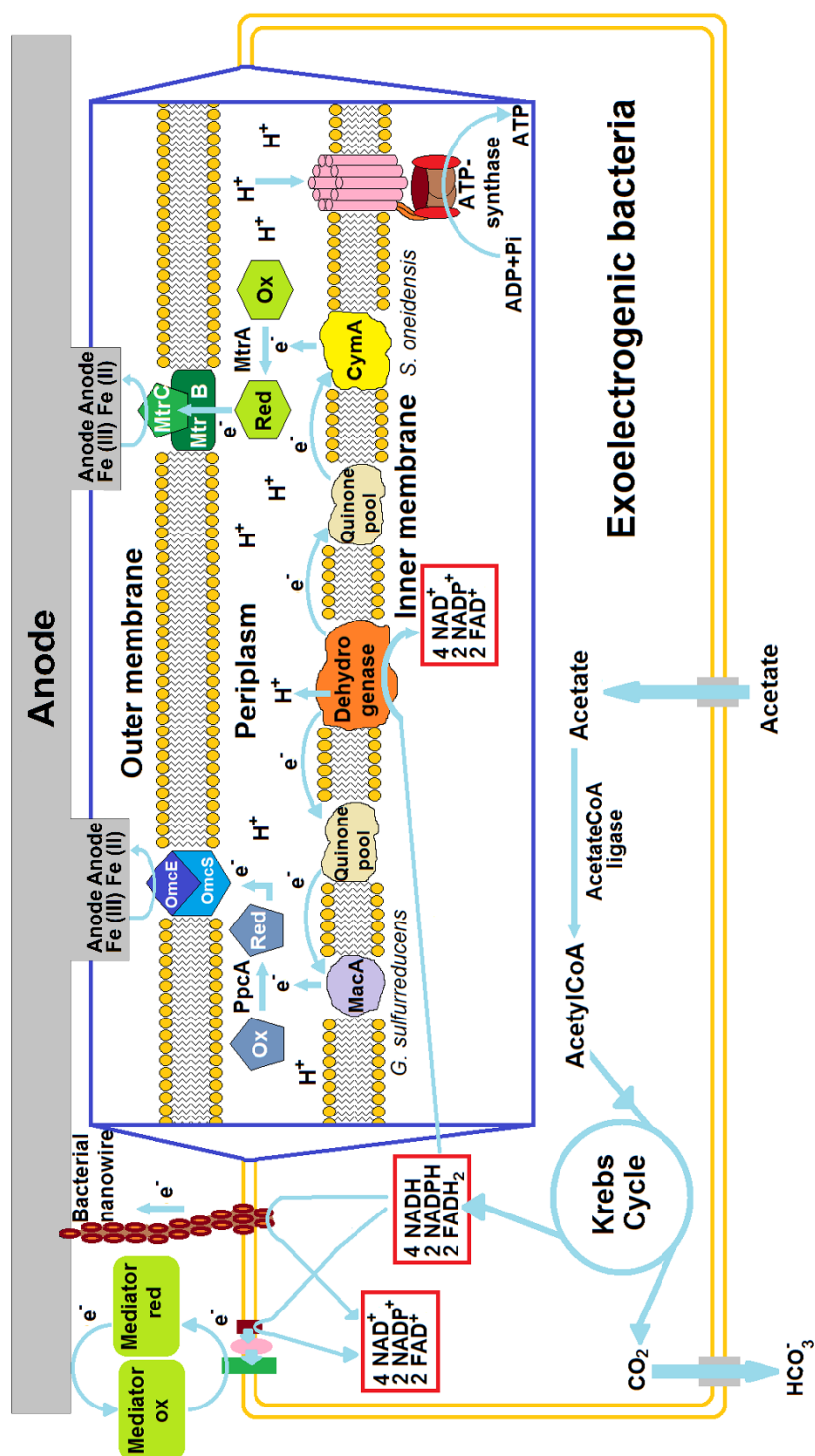
### 1.2.1. Extracellular electron transfer mechanisms

The electron transfer between the microorganisms and the anode is one of the key steps of the whole bioelectrochemical process. Microorganisms able to transfer electrons from the metabolism of organic matter (substrate) to the anode are known as exoelectrogens (Kumar et al., 2015). The most common and well-studied exoelectrogens in MFC and MEC are *Geobacter* and *Shewanella*, whose genomes have already been sequenced (Heidelberg et al., 2002; Methe et al., 2003). However, the diversity of exoelectrogenic species is much greater (Zhang et al., 2006; Mathuriya and Sharma, 2009; Lee et al., 2012). Electron transfer from exoelectrogens to the anode can follow different mechanisms: by nanowires, by using redox shuttles as electron carriers, or by direct transfer between the bacterial cell wall and the anode surface (Figure 1.4) (Yang et al., 2012). Additional models such as inter-bacterial electron transfer by means of pili have also been proposed (Pham et al., 2009).

Bacterial nanowires, with approximately 20 μm length and 8 nm thick, might be the most thermodynamically favorable electron pathway for extracellular electron transfer (EET) (Yang et al., 2012), as their presence significantly increases the electron transfer rate. This is due to the high electronic conductivity they present (6 mS/cm in *Geobacter sulfurreducens*) (Malvankar et al., 2011). Nanowires seem to be composed of pilin monomers with cytochromes attached (Reguera et al., 2005). They emerge from the inner membrane and go through the periplasm towards the extracellular space. In *Shewanella*, it has been speculated that outer membrane c-type cytochromes (OMC) may participate in the electron transfer along the nanowires (Gorby et al., 2006), whereas pili in *Geobacter* seem to be partially covered of OMCs (Leang et al., 2010).

Another electron transfer mechanism is the use of redox shuttles as mediators. This may allow redox processes between the anode and bacteria which originally cannot interact with it due to the long distance between both of them (Lojou et al., 2005). The ideal electron shuttle ought to be water soluble, of low molecular weight, reusable and highly reactive. It should also be non-specific, and have a redox potential between that of the substrate and that of the electrode (Rabaey, 2010). Redox shuttles can be exogenous (either externally added, such as resazurin, or present naturally in the medium, as humic acids) or endogenous (produced by the bacteria; for instance, phenazines, flavins, or quinones). In order to obtain the maximum power output, a continuous MFC based on mediated electron transfer needs to be fed with concentrated substrates and operated at long HRT, so that the endogenous redox mediators can accumulate in the anodic compartment until it becomes non-limiting (Yang et al., 2012).

Finally, the third electron transfer mechanism is by direct contact between c-type cytochromes, these being heme-containing proteins across both the inner and the outer membrane of many Gram negative genera. Cytochromes of *Shewanella oneidensis* and *Geobacter sulfurreducens* have been widely investigated, and appear to transport out of the cell the electrons taken from NAD(P)H by inner-membrane-anchored dehydrogenases during the process of oxidative phosphorylation (Rabaey, 2010). These electrons are initially stored on the quinone pool. In *S. oneidensis*, a tetraheme cytochrome bound to the inner membrane (CymA), takes up electrons from the quinone pool and conducts them to the periplasmic space, so that they can reduce the periplasmic decaheme protein MtrA (Borloo et al., 2011). After that, MtrA transfers the electrons to the outer-membrane protein MtrC. Besides, MtrA seems to be essential for the stability of MtrB, which, on its part, is necessary for the proper localization of MtrC on the outer membrane (Hartshorne et al., 2009). In the end, MtrC uses the electrons to reduce external electron acceptors, such as the solid-phase iron anode. As for *G. sulfurreducens*, MacA, an inner-membrane-bound diheme cytochrome, receives electrons from the quinone pool, and delivers them to the periplasmic triheme cytochrome PpcA. This protein reduces c-type cytochromes which are present on the outer membrane (or on the nanowires, if present), and, as a result, the EET is produced (Lovley, 2008). It has been shown that PpcA may potentially contribute to the proton motive force of the cells, so that ATP can be generated by transferring electrons coupled to trans-membrane proton transport (Pessanha et al., 2006). This will, in the end, result on a higher bacteria growth rate.



**Figure 1.4.** Different extracellular electron transfer mechanisms, from left to right: by means of redox mediators; by means of nanowires; and direct electron transfer by c-type cytochromes in *Geobacter sulfurreducens* and *Shewanella oneidensis*.

Mixed cultures enriched from domestic wastewater or anaerobic sludge have been used as the inoculum for most MEC studies (Logan and Regan, 2006; Call et al., 2009; Kiely et al., 2011). While some isolates from mixed cultures demonstrate electrochemically active properties, most of them exhibit lower current densities when grown as pure cultures compared to the mixed cultures (Logan, 2009). Given that interspecific interactions in mixed cultures are still not clear, further research is to be done in order to elucidate population dynamics in the mixed microbiota, as well as the establishment of syntrophic or symbiotic synergies between different species, and optimize bioelectrodes performance as a consequence.

### 1.2.2. Thermodynamics of bioelectrochemical reactions

In an electrochemical reactor, electrical power production will occur if the overall reaction is thermodynamically favorable. This can be stated from the Gibbs free energy of the reaction.

$$\Delta G_r = \Delta G_r^0 + RT \ln(\Pi)$$

where  $\Delta G_r$  (J mol<sup>-1</sup>) is the Gibbs free energy for the reaction conditions,  $\Delta G_r^0$  (J mol<sup>-1</sup>) is the Gibbs free energy under standard conditions (T = 298.15 K, concentration = 1M and P = 1 atm), R is the universal gas constant (8.314 J mol<sup>-1</sup> K<sup>-1</sup>), T (K) is the temperature and  $\Pi$  is the reaction quotient (the activities of the products divided by those of the reactants, each raised to the power of its stoichiometric coefficient). If the  $\Delta G_r > 0$ , the process does not occur spontaneously, so an external energy input is required to drive the reactions. If the  $\Delta G_r < 0$ , the process is spontaneous and there is no need of external energy input.

Electromotive force ( $E_{emf}$ ) is an indicator of the theoretical maximum attainable potential of a spontaneous process ( $E_{emf} > 0$ ), or the voltage necessary for a non-spontaneous process to occur ( $E_{emf} < 0$ ).  $E_{emf}$  is calculated as:

$$E_{emf} = -\frac{\Delta G_r}{nF}$$

where  $E_{emf}$  (V) is the electromotive force, n is the mol of electrons transferred within the reaction and F is the Faraday's constant (96485 C mol<sup>-1</sup> e<sup>-</sup>).

Electromotive force may also be expressed as the difference between the theoretical cathodic ( $E_{cat}$ ) and anodic ( $E_{an}$ ) potentials (i.e., the potentials of the half-reactions). In Table 1.1,

the half-reactions of a MFC/MEC producing electricity/hydrogen with acetate as electron donor (substrate) are presented, as well as their corresponding reaction potentials under standard conditions (pH=7).

$$E_{emf} = E_{cat} - E_{an}$$

**Table 1.1.** Electrode half-reactions in MFC/MEC.

| Type of BES | Electrode | Reaction  | Potential (V) |
|-------------|-----------|---|---------------|
| <b>MFC</b>  | Anode     | $\text{CH}_3\text{COO}^- + 4\text{H}_2\text{O} \rightarrow 2\text{HCO}_3^- + 9\text{H}^+ + 8\text{e}^-$ | -0.279        |
|             | Cathode   | $\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O}$                                | +0.806        |
| <b>MEC</b>  | Anode     | $\text{CH}_3\text{COO}^- + 4\text{H}_2\text{O} \rightarrow 2\text{HCO}_3^- + 9\text{H}^+ + 8\text{e}^-$ | -0.279        |
|             | Cathode   | $2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2$  | -0.414        |

From data in Table 1.1 it can be calculated that  $E_{emf}$  in a MFC and a MEC is:

$$\text{MFC} \longrightarrow E_{emf} = 0.806 - (-0.279) = 1.085 \text{ V}$$

$$\text{MEC} \longrightarrow E_{emf} = -0.414 - (-0.279) = -0.135 \text{ V}$$

The positive equilibrium voltage for the MFC indicates that acetate consumption and electricity production as a result is a spontaneous process, whereas the negative value for the MEC points out that  $\text{H}_2$  evolution reaction is non-spontaneous and that the application of an external additional voltage of at least 0.135 V is necessary for the reaction to occur. However, due to the internal losses in the system, empirical data have shown that  $V_{app}$ s of 0.25 V must be applied in order to reach a good performance of the MEC system (Cheng and Logan, 2007; Rozendal et al., 2007).

### 1.2.2.1. Internal voltage losses

As stated above, internal voltage losses (also known as overpotentials) are responsible for the differences between theoretical and empirical voltage values in BESs, as they decrease the available voltage in MFC reactors and increase the required voltage application in MEC. The most important kinds of voltage losses are:



- Ohmic losses ( $\eta_{ohm}$ ) are derived from the resistance to ion transfer through the electrolyte in the BES and to electron transfer through the electrodes and interconnections. These losses can be calculated by means of Ohm's law:

$$\eta_{ohm} = IR_{int}$$

where  $I$  is the current flow (A) and  $R_{int}$  is the internal resistance ( $\Omega$ ) of the BES. Ohmic overpotentials can be decreased by using electrolytes with high ionic strength, by reducing the inter-electrode separation and by the use of current collectors for a better distribution of the current throughout the electrode surface.

- Concentration losses ( $\eta_c$ ) are caused by limited mass transfer between the substrate solution and the electrode surface. It can be expressed as (Bagotsky, 2005):

$$\eta_c = \frac{RT}{nF} \cdot \ln \left( \frac{1 + i/i_{l,red}}{1 - i/i_{l,ox}} \right)$$

where  $i$  ( $A\ m^{-2}$ ) is the current density, and  $i_{l,red}$  and  $i_{l,ox}$  ( $A\ m^{-2}$ ) are the limiting currents of reduction and oxidation of the electrochemically active species. A possible manner of reducing concentration overpotentials would be by achieving a better reactor mixing.

- Bacterial metabolic losses are generated during the process of extracellular electron transfer. They are due to the difference between the anode potential and the redox potential of the substrate, which will determine the metabolic energy gain for the bacteria (Logan, 2009).
- Activation losses are associated to the overpotential required to overcome the activation energy of an electrochemical reaction on a catalytic surface. Activation overpotentials can be minimized by using more effective catalysts, by increasing the real surface electrode or by using higher reaction temperatures.

### 1.2.3. Parameters describing the BES performance

During recent years, the number of research studies concerning BESs has strongly increased, evaluating different aspects such as BESs design or operational conditions. As a result of this high variability, several parameters have been described in order to normalize performance of BESs. The most important parameters for systems evaluation are detailed below.

- Treatment efficiency ( $COD_r$ , %). BESs have been proposed as a technology for wastewater treatment, and as such it is important to compute its ability to remove organic matter from a waste effluent. Even though its performance can be evaluated in terms of biochemical oxygen demand (BOD) removal, total organic carbon (TOC) removal and several other parameters (Metcalf, 2003), in this work chemical oxygen demand removal ( $COD_r$ ) has been selected as the preferred parameter to compute the efficiency of the treatment:

$$COD_r = \frac{(COD_{in} - COD_{out})}{COD_{in}}$$

where  $COD_{in}$  and  $COD_{out}$  are the COD concentration of MEC influent and effluent respectively.

- Coulombic efficiency ( $C_e$ , %): has been defined as the ratio of total electronic charges transferred to the anode from the substrate, to maximum possible charges if all substrate removal produced current (Logan et al., 2006). It is calculated as:

$$C_e = \frac{\int_0^{86400} I dt}{(COD_{in} - COD_{out})/M \cdot Q_{in} \cdot e \cdot F} \cdot 100$$

where  $I$  is the circulating electrical current (A),  $M$  is the weight of 1 mol of COD ( $32 \text{ g mol}^{-1}$ ),  $Q_{in}$  is the influent flow rate ( $\text{L d}^{-1}$ ),  $e$  is the number of mol of electrons exchanged per mol of COD equivalent consumed ( $8 \text{ mol mol}^{-1}$ ), and  $F$  is the Faraday constant ( $96485 \text{ C mol}^{-1} e^-$ ).

- Cathode conversion efficiency ( $CC_e$ , %), also known as cathodic efficiency. It represents the ratio of  $\text{H}_2/\text{CH}_4$  recovery to the maximum theoretical production if all the electronic charges that arrive to the cathode were converted to  $\text{H}_2/\text{CH}_4$ :

$$CC_e = \frac{P \cdot Q_{H_2/CH_4} \cdot V_a / R \cdot T \cdot e \cdot F}{\int_0^{86400} I dt} \cdot 100$$

where P is the pressure in the cathodic chamber (P = 1 atm);  $Q_{H_2/CH_4}$  is the  $H_2/CH_4$  flow rate ( $L H_2/CH_4 L_a^{-1} d^{-1}$ );  $V_a$  is the anode volume (L); R is the ideal gas constant ( $R=0.082 L atm K^{-1} mol^{-1}$ ); T is the temperature (K); e is the number of mol of electrons exchanged per mol of hydrogen (2 mol  $mol^{-1}$ ) or methane (8 mol  $mol^{-1}$ ).

- Specific power consumption ( $E_{cons}$ , Wh  $g^{-1}$ -COD) is the electrical energy supplied to the MEC relative to the amount of organic matter consumed, and is calculated as:

$$E_{cons} = \frac{\int_0^{86400} V_{app} \cdot I dt}{(COD_{in} - COD_{out})/M \cdot Q_{in}}$$

where  $V_{app}$  is the applied voltage (V); I is the circulating electrical current (A);  $Q_{in}$  is the influent flow rate ( $L d^{-1}$ ); M is the weight of 1 mol of COD (32 g  $mol^{-1}$ ) and  $COD_{in}$  and  $COD_{out}$  are the COD concentration of MEC influent and effluent respectively.

- Specific energy production ( $E_{prod}$ , Wh  $g^{-1}$ -COD) is the energy produced by the MEC in terms of hydrogen and methane relative to the amount of organic matter consumed, and is calculated as:

$$E_{prod} = \frac{3 \cdot 10^3 Wh m_{H_2/CH_4}^3 (Q_{H_2} + Q_{CH_4})}{(COD_{in} - COD_{out})/M \cdot Q_{in}}$$

where  $Q_{H_2}$  is the hydrogen flow rate ( $L H_2 L_a^{-1} d^{-1}$ );  $Q_{in}$  is the influent flow rate ( $L d^{-1}$ );  $Q_{CH_4}$  is the methane flow rate ( $L CH_4 L_a^{-1} d^{-1}$ ); M is the weight of 1 mol of COD (32 g  $mol^{-1}$ ) and  $COD_{in}$  and  $COD_{out}$  are the COD concentration of MEC influent and effluent respectively.

- Net energy consumption, calculated as the difference between power consumption and energy production.

## 1.2.4. Factors affecting performance of BESs

### 1.2.4.1. Physicochemical factors

- Reactor design: this parameters influences on process performance and capital investment. The most common reactor types are H-shaped two-chamber reactors and cube-shaped two-chamber reactors (Sun et al., 2014). H-shaped reactors usually produce low power density as a result of long electrode spacing and small membrane surface area. However, due to their easy operation and high stability, this design is widely used for lab-scale tests (Logan et al., 2006). In contrast, cube-shaped two-chamber reactors are more efficient for power generation (Hays et al., 2011). It is important, though, to highlight that two-chambered BESs increase the cost of the device. As a result, single-chambered BESs have also been an important focus of research (Liu and Logan, 2004).
- Electrode materials: the synergistic relationship between the anode and the exoelectrogens affects significantly the bioelectrocatalytic process. Therefore, it is necessary that electrode material fulfills a series of requirements in order to optimize the performance of the system (Venkata-Mohan et al., 2014). The most versatile electrode material is carbon, as it is conductive, biologically compatible, chemically stable, available for bacterial colonization, and of low cost (Sun et al., 2014), which make it the most suitable for anode construction. However, carbon electrodes present high overpotential losses due to low redox reaction: therefore, coating carbon electrodes with Pt or using different electrode materials for cathodes (e.g. stainless steel) have been proposed as alternatives (Escapa et al., 2016).
- Electrode spacing: ohmic losses might be caused by the internal resistance produced by the electrolyte. As a result, electrode spacing is a key parameter affecting reactor performance. As stated above, ohmic resistance can be decreased by reducing the inter-electrode separation (Janicek et al., 2014).
- Surface area to volume ratio: a larger surface area will provide more space for the microbial adhesion, resulting in increased electron transfer rates (Venkata-Mohan et al., 2014).

#### 1.2.4.2. Biological factors

- **Inoculum:** biological inoculum is the main factor affecting a bioelectrochemical reaction. As already stated, inoculum might be of pure or mixed nature, and must be rich in exoelectrogenic species, as it has been reported that the enrichment of electrochemically active bacteria on the anode surface results in high power densities (Aelterman et al., 2008; Wang and Han, 2009). Some pure-culture approaches with different bacterial species are summarized in Table 1.2. Mixed cultures are more prone to the establishment of synergies between different species and their lower cost allows for a better economic viability, which makes them more feasible for BESs operation when treating industrial or domestic wastewater streams.

**Table 1.2.** Different microorganisms used as pure cultures in bioelectrochemical reactors.

| Microorganism                        | Substrate            | Reactor configuration | Reference                  |
|--------------------------------------|----------------------|-----------------------|----------------------------|
| <i>Shewanella oneidensis</i><br>MR-1 | Lactate              | Dual chamber          | Manohar and Mansfeld, 2009 |
| <i>Geobacter sulfurreducens</i>      | Sodium fumarate      | Single chamber        | Dumas et al., 2008         |
| <i>Escherichia coli</i>              | Synthetic medium     | Single chamber        | Zhang et al., 2006         |
| <i>Clostridium acetobutylicum</i>    | Paper industry<br>WW | Dual chamber          | Mathuriya and Sharma, 2009 |
| <i>Pseudomonas</i> sp. C27           | Synthetic WW         | Dual chamber          | Lee et al., 2012           |

- **Biofilm:** biofilm formation rate from exoelectrogenic population also affects electrogenic characteristics of the BES. As a result of solute diffusion, substrate is only available at the outer layers of the biofilm, whereas only bacterial cells at its innermost layer will be in direct contact with the electrode. Therefore, an optimum

biofilm thickness will be necessary for power output optimization (Wang and Han, 2009; Venkata-Mohan et al., 2014).

- Mediators: given that mediators play a key role in extracellular electron transfer (see above) and allow to transfer the electrons in a faster way compared to bacterial mechanisms (Omeroglu and Sanin, 2016), external addition of either synthetic or naturally produced mediators (such as phenazines or quinones) would allow for an enhancement of electrogenic performance of BESs.

### 1.2.4.3. Operational parameters

- pH: variations of pH are one of the most important parameters affecting BESs performance. Substrate degradation in BESs has been observed to be higher under neutral operating conditions (pH in the range of 6.25 to 6.50) (Raghavulu et al., 2009; Martin et al., 2010). In general, electrogenic microorganisms require a pH near neutrality in order to optimize their growth rates (Puig et al., 2010). Although pH tends to decrease as a result of continuous BESs operation, buffer addition (mainly based on phosphate and carbonate compounds) has been tested to reduce variations in pH (Nam et al., 2010). However, it must be noted that the use of buffers may represent a significant increase in operating costs.
- Temperature: changes in temperature have a strong influence on the system since they affect mass transfer rates and thermodynamic parameters, as well as microbial community composition (Oliveira et al., 2013). In general, an enhancement in power output and COD removal was observed with an increase in temperature, an effect that might be related to a reduction of ohmic resistance (due to higher conductivity of the electrolyte) and also to a promotion of microbial metabolism (Larrosa-Guerrero et al., 2010; Patil et al., 2010). However, it has also been suggested that higher temperatures could foster the growth of non-electrogenic populations, affecting the system negatively (Michie et al., 2011). Therefore, further research is necessary in order to clarify the role of temperature in BESs.
- Electrolyte ionic strength: as stated before, ionic strength of the electrolyte is inversely related to ohmic losses. Therefore, when the BES is fed with low

conductivity wastewater streams (i.e. domestic WW), electrogenic performance will be disfavored (Rozendal et al. 2008). In order to overcome this effect, Min et al. (2008) found that power density was 4 times higher after phosphate buffer addition to the medium. Other solutions, such as reducing electrode spacing, have also been tested (Logan et al., 2006).

- Applied voltage: as previously stated, H<sub>2</sub> evolution reaction needs a voltage between 0.135 and 0.25 V to occur (Cheng and Logan, 2007; Rozendal et al., 2007). In addition, it has been tested that, in the range of 0.4-1.2 V, substrate removal and microbial electrosynthesis processes are proportional to V<sub>app</sub> (Tartakovsky et al., 2009; Lu et al., 2009; Escapa et al., 2009). On the other hand, at V<sub>app</sub> > 1.2 V no effect was observed, suggesting that beyond this V value electron transfer was restricted as a result of metabolic limitations (Tartakovsky et al., 2008).
- Type of substrate: this factor is of major importance, as it serves as the organic fuel for the process and its composition and characteristics affect the composition and structure of the microbial community (Chae et al., 2009; Pant et al., 2010). Different kinds of substrate have been tested: mixed substrates (e.g. domestic or industrial wastewater streams); pure and non-fermentable substrates, such as acetate; or pure and fermentable substrates, such as glucose or even more complex compounds (cellulose) (Sun et al., 2014). In general terms, the more difficulties for degradation, the more diverse microbial community will establish in the anode, as more complex metabolic pathways will be involved (Thygesen et al., 2011).
- Organic loading rate (OLR): an enhancement of power generation and substrate degradation has been observed as OLR increases, as a result of the availability of higher substrate concentrations (Venkata-Mohan et al., 2009) and a reduced internal resistance of the cell because of an improvement of ionic strength in the medium (Martin et al., 2010; Velvizhi et al., 2012). However, it has also been reported that if the OLR is too high, competitive processes between electrogens and other types of bacteria would lead to a greater organic matter removal not related to current generation (Oliveira et al., 2013). This would result in a decrease in coulombic efficiency.

### 1.2.5. BESs for H<sub>2</sub> production

As a result of its properties, hydrogen gas has great potential as an alternative fuel for the future. Hydrogen gas is a clean energy carrier, as its oxidation does not contribute to greenhouse gas emissions (the only byproduct of H<sub>2</sub> combustion is H<sub>2</sub>O) (Ramos et al., 2012). It is also a highly efficient energy carrier: it has a higher energy content per unit weight (120 MJ kg<sup>-1</sup>) than gasoline (44 MJ kg<sup>-1</sup>) or ethanol (26.8 MJ kg<sup>-1</sup>) (Kadier et al., 2016). However, it also has some disadvantages. An efficient and cost-effective storage and distribution technology for H<sub>2</sub> gas has yet to be developed. Currently, H<sub>2</sub> storage is achieved by means of gas compression at 700 bar, a process requiring up to 20% of the energy content of H<sub>2</sub> (Edwards et al., 2008). It can also be stored at liquid phase at cryogenic temperatures, but this is even a more energy-intensive process (Edwards et al., 2008). Nevertheless, great research efforts are being made in order to overcome these obstacles and help to promote the use of H<sub>2</sub> gas as a fuel.

Although H<sub>2</sub> gas can be produced by many processes (e.g. steam reforming, dark fermentation, photofermentation, etc.), we will focus on H<sub>2</sub> production by means of BESs. Since the principle of H<sub>2</sub> production in MECs was first demonstrated (Liu et al., 2005b; Rozendal et al., 2006), efficiencies of this process have improved considerably as a result of continuous investigations. The development of single-chamber MECs lacking membrane enhanced H<sub>2</sub> production with lower energy requirements, reaching a value of 3.12 m<sup>3</sup> H<sub>2</sub> m<sup>-3</sup> d<sup>-1</sup> using a platinum-based cathode at an applied potential of 0.8 V (Call and Logan, 2008). However, the absence of a polymeric membrane presents also disadvantages, as it promotes the growth of hydrogen scavengers, such as hydrogenotrophic methanogens (Lee et al., 2009) or homoacetogens (Parameswaran et al., 2011). As this leads to a dramatic reduction in hydrogen production efficiencies, many strategies have been proposed to suppress methanogenic activity, such as low pH or temperature conditions or the use of 2-bromoethanesulfonate, an inhibitor of methanogenesis (Chae et al., 2010). In contrast to the single-chamber approach, Jeremiassé et al. (2010) reported a maximum H<sub>2</sub> production of 50 L L<sub>a</sub><sup>-1</sup> d<sup>-1</sup> in a two-chamber configuration, using a nickel foam electrode at an applied voltage of 1V. Nevertheless, a decrease in production rates over time was reported. The use of low cost materials and alternative catalysts have also proved useful for making this technology more cost-effective (Selembó et al., 2009).



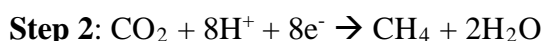
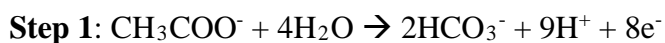
### 1.2.6. BESs for CH<sub>4</sub> production

As stated above, H<sub>2</sub> production in membraneless MECs is usually curbed as a result of methanogens proliferation, a phenomenon which is difficult to revert (Rader and Logan, 2010). The discovery that *Methanosaeta* and *Methanosarcina* (often the most abundant methanogenic species in anaerobic digesters) can accept electrons via direct interspecies electron transfer from *Geobacter metallireducens* (Rotaru et al., 2014) complicates the solution to this problem. However, it must be noted that the lack of ion exchange membrane in MEC will decrease capital costs of the device, and that CH<sub>4</sub> production in a MEC may also be more robust than H<sub>2</sub> production (Clauwaert and Verstraete, 2008).

Methane presents a lower energy content per unit of weight than H<sub>2</sub> (50 vs. 120 MJ kg<sup>-1</sup>, respectively) (Kadier et al., 2016). However, it is easier to manage, especially regarding storage and transportation issues. It must be highlighted that, being the main product of the anaerobic digestion process, technologies for CH<sub>4</sub> management are more developed, as AD has been a well-established technology for long. Moreover, CH<sub>4</sub> can be distributed by means of the existing natural gas infrastructure (Siegert et al., 2015). Finally, if produced from waste streams, the net carbon footprint associated to CH<sub>4</sub> is neutral, in comparison to that of fossil fuels. As a result of these characteristics, CH<sub>4</sub> production appears as a viable alternative fuel.

Initially, CH<sub>4</sub> production in BESs was observed by non-electrochemical mechanisms (via acetoclastic processes from acetate (Call and Logan, 2008), or via hydrogenotrophic methanogenesis (Clauwaert et al., 2008) from H<sub>2</sub>). In recent years, a new process for CH<sub>4</sub> production by means of BESs has been described, known as electromethanogenesis (Cheng et al., 2009). One of the main advantages of electromethanogenesis is that it can proceed at lower temperatures than anaerobic digestion (Bajracharya et al., 2016).

If electromethanogenesis is to be carried out with acetate as carbon source, it consists of two steps. First, acetate is degraded to carbon dioxide, with 8 mol of electrons being released. In the second step, carbon dioxide undergoes a reduction process in the cathode, where it combines with the previously-released electrons, leading to methane formation.



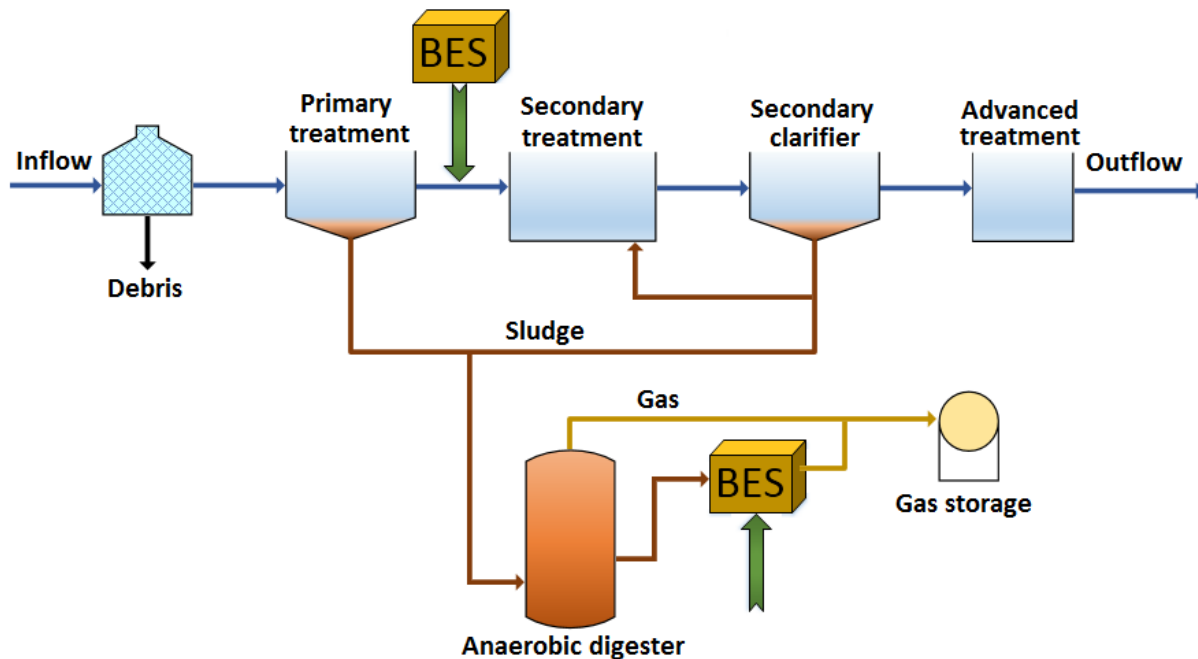
Using a graphite-based electrode, Clauwaert and Verstraete (2008) observed bioelectrochemical CH<sub>4</sub> production in a single-chambered MEC: this study reported a maximum methane production rate of  $0.75 \pm 0.12 \text{ L L}^{-1} \text{ MEC d}^{-1}$  (which represented  $86 \pm 14\%$  of acetate to methane conversion) at an applied voltage of  $-0.8 \text{ V}$  (vs. standard hydrogen electrode, SHE). When working without voltage application, CH<sub>4</sub> production data dramatically decreased to  $0.17 \pm 0.06 \text{ L L}^{-1} \text{ MEC d}^{-1}$  ( $47 \pm 17\%$  of acetate to methane conversion). With an exoelectrogen-based bioanode and a methanogen-based biocathode, Villano et al. (2011) reached a cathodic conversion efficiency of 65% at an applied voltage of  $0.5 \text{ V}$  vs. SHE (CH<sub>4</sub> production rate of  $0.018 \text{ L L}^{-1} \text{ d}^{-1}$ ). Higher cathodic efficiency data (up to 84%) were achieved at an applied voltage of  $0.2 \text{ V}$  vs. SHE (Zeppilli et al., 2015). Bioelectrochemical CH<sub>4</sub> production was also observed in other reports (Sasaki et al., 2011), while showing differences in methanogenic populations depending on voltage application.

### 1.2.7. BESs for wastewater treatment

Spanish wastewater (WW) treatment sector has grown in the last years, with more than 2500 wastewater treatment plants (WWTPs) in operation (MAGRAMA-DGA, 2010). Energetic requirements in WWTPs stand for more than 50% of operating costs (Albadalejo and Trapote, 2009). As a whole, this sector treats  $3000 \text{ hm}^3$  of WW per year, requiring  $2672 \text{ GWh year}^{-1}$  of energy input. This represents about 1% of the total electricity consumption in Spain (IDAE, 2010). However, domestic WW has been reported to contain an important amount of chemical energy stored in the form of dissolved organic matter (Heidrich et al. (2010) have estimated  $17.8 \text{ kJ g}^{-1}$ -COD). Therefore, it can be stated that, if recovered properly, this energy could help to reduce the energetic demands of WWTPs.

Although anaerobic digestion is one of the technologies used for WW treatment, this process presents inefficiencies when using substrates with a low organic charge, such as WW (Rittmann and McCarty, 2001). As an alternative, the use of BESs is particularly efficient with low strength WW and can operate at relatively low temperatures (Villano et al., 2013). Moreover, sludge production with BESs ( $0.02\text{-}0.22 \text{ g-COD}_{\text{biomass}} \text{ g-COD}^{-1}$ ) is much lower than that of conventional aerobic treatments ( $0.53 \text{ g-COD}_{\text{biomass}} \text{ g-COD}^{-1}$ ), as stated by Clauwaert et al. (2008), resulting in an easier management of sludge throughout the process.

In a conventional WWTP (Figure 1.5), the first step of the treatment is a *preliminary stage*, where the WW is screened to remove large debris in order to avoid clogging and protect pumps and equipment throughout the rest of the process. Afterwards, the WW undergoes a *primary treatment*, consisting in the decantation of all particles that are settable. About 60% of total suspended solids are removed by precipitation. The *secondary treatment* involves the reduction of contaminants by an aerobic biological treatment. As a result of this treatment, up to 90% of organic matter in WW may be removed. In a secondary clarifier, bacterial biomass is separated and recirculated to the secondary treatment tank. Occasionally, an *advanced treatment* stage is programmed afterwards for nitrogen, phosphorus or pathogens removal. Finally (*residual management* stage), the solids recovered along the process are collected, stabilized (often through the anaerobic digestion process) and disposed of properly.



**Figure 1.5.** Schematic representation of a conventional WWTP. The green arrows mark the possible locations for the integration of BESs in the system.

The integration of BESs in real WWTPs has been previously discussed (Rosenbaum et al., 2010b). One of the options is the replacement of the biological (secondary) treatment. However, most of the studies evaluating BESs fed with real domestic WW achieved a COD removal of 40-80% (Liu et al., 2004; Escapa et al., 2012; Heidrich et al., 2013). These limited COD degradation rates might not accomplish the legal limits established in terms of organic matter concentrations in the effluents. Even so, some experiences regarding practical applications of BESs at real WWTPs have been tested. For instance, two tubular MFCs (4 L each) have been operated at an in situ location on a WWTP, treating effluent from the primary settling tanks (Zhang et al., 2013).

Other approaches at larger scale have also been investigated. For example, Dong et al. (2015) evaluated electricity production in a 90-L stackable MFC fed with brewery WW, achieving efficient WW treatment with zero energetic input. Another recent study (Wu et al., 2016) with a 72-L stackable MFC also gave good results when fed with synthetic WW in terms of electrogenic performance and COD removal. Besides, evaluation of a 120-L MEC for domestic WW treatment (Heidrich et al., 2014) also obtained promising results for the development and practical implementation of MECs at pilot scale.

### **1.2.8. Integrating conventional digestion technologies and BESs**

The ability of BESs to treat a wide range of different substrates allow for multiple alternative uses of these devices. Given the special capacity of BESs to treat non-fermentable rich waste streams (such as VFA) (Ghimire et al., 2015), the integration of dark fermentation and BESs is particularly profitable, as dark fermentation effluents have high VFA content (Yang et al., 2013). Because of this, the possibility of using MECs as a post-treatment for biological processes has been considered. For example, Liu et al. (2012) used the VFA-rich effluent of dark fermentation of waste activated sludge for H<sub>2</sub> production in a MEC reporting a H<sub>2</sub> yield of 1.2 mL H<sub>2</sub> mg<sub>COD</sub><sup>-1</sup> and a H<sub>2</sub> production rate of 120 mL H<sub>2</sub> g<sub>VSS</sub><sup>-1</sup>d<sup>-1</sup>. Lu et al. (2009) obtained 700 mL H<sub>2</sub> L<sup>-1</sup> d<sup>-1</sup> at the dark fermentation stage of molasses and 1410 mL H<sub>2</sub> L<sup>-1</sup> d<sup>-1</sup> when treating the ethanol and VFA-rich effluent with BESs. In another study, after achieving a production 1.73 m<sup>3</sup> H<sub>2</sub> m<sup>-3</sup> d<sup>-1</sup> from the DF of corn stalk, Li et al. (2014) reported 3.43 m<sup>3</sup> H<sub>2</sub> m<sup>-3</sup> d<sup>-1</sup> in the BESs stage, while Lalaurette et al. (2009) obtained a 6-fold increase in global H<sub>2</sub> yield after coupling dark fermentation of corn stover and subsequent treatment of the effluent by BESs.

In addition, there is also the option of integrating anaerobic digestion and BESs, which opens the possibility of an improvement of performance when digesting the solids removed from the wastewater stream at the WWTP (see Figure 1.5). In contrast to the sequential model investigated for dark fermentation, a combined approach in the same reactor has been evaluated for the AD-BESs integration. This approach consists in directly introducing electrogenic microbiota (attached to electrodes) in the anaerobic reactor and presents the advantage of reducing the operational costs and the energy losses of the global process, as well as maximizing substrate utilization (ElMekawy et al., 2014). Apart from enhancing organic matter removal, integration of AD and BESs may also contribute to stabilize the digestion process, as the consumption of the excess of VFA by electrogenic microorganisms could prevent its collapse. This integrative configuration has been tested for treating different substrates: dye WW (Cui et al., 2014), waste activated sludge (Asztalos et al., 2015) or sucrose-based synthetic WW (Zhang et al., 2015).

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# *Chapter 2*

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## *Scope of the Thesis*



## 2.1. OBJECTIVES

The main objective of this thesis is the evaluation and analysis of the integration of bioelectrochemical systems and conventional bioprocessing technologies (such as dark fermentation and anaerobic digestion) for wastewater treatment. This study was performed both with industrial (cheese whey) and domestic wastewater streams.

In order to achieve this main objective, the operating conditions that optimize these bioprocesses were established, and two different integrative approaches (one-stage and two-stage configurations) were considered for the study. Thus, several research objectives were assessed:

- i) identifying the optimal nitrogen and lactose concentrations for dark fermentation of a synthetic feeding by means of response surface methodology (RSM), as well as the optimal substrate/inoculum ratio for dark fermentation of cheese whey and the most favorable acetate/lactate ratio for bioelectrocatalytic performance.
- ii) evaluating the response of a two-stage system integrated by dark fermentation and bioelectrochemical systems for the treatment of cheese whey.
- iii) studying the performance of bioelectrochemical systems introduced in an anaerobic reactor where the digestion process is prone to undergo acidic inhibition as a result of high organic content in the substrate.
- iv) assessing the benefits of using electromethanogenic processes with MEC electrodes to assist anaerobic digestion when treating low-strength domestic wastewater.
- v) exploring different strategies for the improvement of bioelectrocatalytic performance of exoelectrogenic bacteria: development of selective enrichment and mutagenesis induction protocols.

## 2.2. THESIS OUTLINE

Bioelectrochemical systems (BESs) have recently developed as a technology for biological waste treatments. Moreover, BESs allow to reduce the energy consumption for these treatments, since they can convert directly the energy content in the organic matter into valuable products

(such as H<sub>2</sub> or CH<sub>4</sub>). However, as BESs are a relatively new technology, many aspects regarding the feasibility of their integration with dark fermentation or anaerobic digestion have yet to be explored.

The scope of this PhD thesis is to investigate the potential of BESs as a technology for industrial and domestic wastewater treatment, as well as evaluating their integration with more traditional biological treatment technologies.

In **Chapter 1**, a general overview is presented, including a description of the characteristics of conventional bioprocessing technologies as well as bioelectrochemical systems, and their role in the field of industrial and domestic wastewater treatment.

In **Chapter 2**, the objectives of the thesis and the thesis outline are presented.

In **Chapter 3**, the materials and methodology used are described.

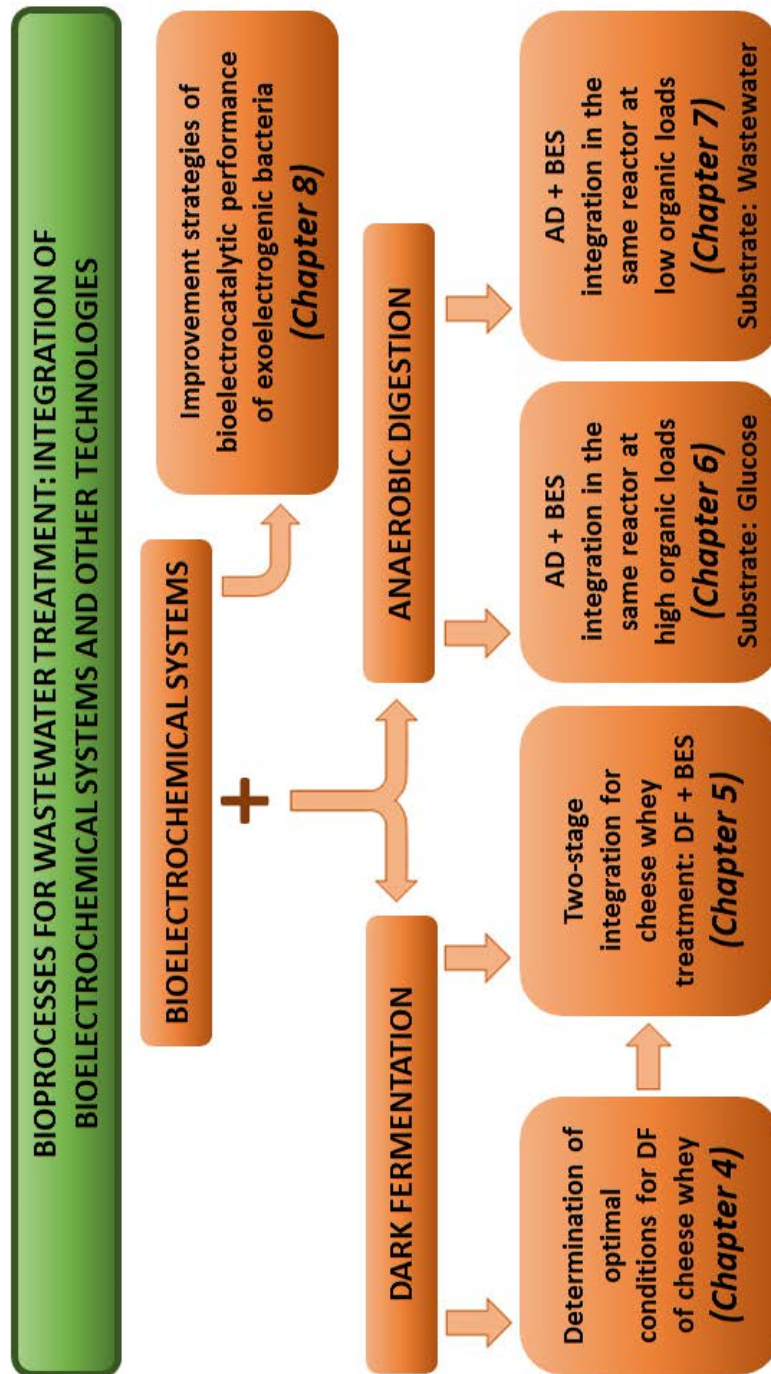
In **Chapter 4**, the determination of the optimal nitrogen concentrations for H<sub>2</sub> production through dark fermentation from a lactose-based synthetic influent was performed. These results were the starting point for the achievement of a successful integrative configuration for dark fermentation of cheese whey and bioelectrochemical systems. In **Chapter 5** the optimum substrate/inoculum ratio for dark fermentation of cheese whey and the most favorable acetate/lactate ratio for bioelectrocatalytic performance were identified. Based on these conditions, the potential of a two-stage integrative approach of both technologies (dark fermentation + biocatalyzed electrolysis) was explored for the treatment of cheese whey.

In **Chapter 6**, the integration, in a single reactor, of anaerobic digestion and biocatalyzed electrolysis was evaluated. Although this approach has been tested before, in this case the reactor was subjected to high concentrations of substrate, so that the effect of this combined approach could be studied under inhibition of anaerobic digestion by acidification. On the other hand, in **Chapter 7**, the benefits of using BESs to assist anaerobic methanogenesis when treating low-charge domestic wastewater were investigated. In this case, the low organic strength in the feeding allowed to test the effect of the integrative configuration under conditions in which AD presents inefficiencies.



**Chapter 8** presents a summary of the research experiences done during the short-term internships that I performed along my thesis in European research Centers.

In **Chapter 9**, general conclusions are presented, and **Chapter 10** consists of a global summary of this PhD thesis.



*Figure 2.1. General overview of this PhD thesis.*



# *Chapter 3*

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## *Materials and Methodology*



In this section, a description of the inocula and raw substrates used throughout the tests is presented (dry sludge, digested sludge, cheese whey and domestic wastewater). The operating conditions for each experimental set-up are shown, as well as the characteristics of the bioreactors used. Finally, the analytical methods followed are also detailed.

## 3.1. INOCULA

### 3.1.1. Dry sludge (biosolid pellets)

For the experiments carried out in **Chapter 4** (both for batch and semi-continuous fermentation tests), biosolid pellets of dry sludge (Figure 3.1) were used as inoculum. These pellets were obtained from the WWTP of León. Sludge was dehydrated on the first term, and dried at 110-115 °C afterwards, reaching a final TS content around 90%. Chemical characteristics of these pellets are presented on Table 3.1.



*Figure 3.1. The biosolid pellets of dry sludge used as inoculum.*

### 3.1.2. Digested sludge

Digested sludge obtained from the WWTP of León was used as inoculum for degradative processes in **Chapters 5 and 6**. This facility has two digesters (volume of 4000 m<sup>3</sup> each) operating

at 35 °C. Different stocks of digested sludge were used for each chapter. Chemical characterization of both digested sludge stocks is shown on Table 3.1.

**Table 3.1.** Characterization of the inocula used in **Chapter 4** (biosolid pellets, BP<sub>4</sub>), **Chapter 5** (digested sludge, DS<sub>5</sub>) and **Chapter 6** (digested sludge, DS<sub>6</sub>) (n.d.: no data).

|   | <b>BP<sub>4</sub></b>    | <b>DS<sub>5</sub></b> | <b>DS<sub>6</sub></b> |
|---|--------------------------|-----------------------|-----------------------|
| <b>Total solids (g L<sup>-1</sup>)</b>    | 936.4 ± 0.7 <sup>a</sup> | 24.0 ± 0.6            | 16.7 ± 0.8            |
| <b>Volatile solids (g L<sup>-1</sup>)</b> | 616.1 ± 0.6 <sup>a</sup> | 11.2 ± 0.4            | 11.9 ± 0.6            |
| <b>Organic matter (%)</b>                 | 44.70                    | 1.25                  | 1.12                  |
| <b>Ca<sup>2+</sup> (ppm)</b>              | 19                       | 116 ± 15              | 576 ± 17              |
| <b>Mg<sup>2+</sup> (ppm)</b>              | 4                        | 399 ± 62              | 108 ± 3               |
| <b>Na<sup>+</sup> (ppm)</b>               | 460                      | 131 ± 31              | 48 ± 1                |
| <b>K<sup>+</sup> (ppm)</b>                | n.d.                     | 20 ± 2                | 232 ± 7               |
| <b>Fe (ppm)</b>                           | 12                       | 404 ± 13              | 263 ± 7               |
| <b>Mn (ppm)</b>                           | 133                      | 6 ± 0                 | 3 ± 0                 |
| <b>Cu (ppm)</b>                           | 469                      | 9 ± 0                 | 3 ± 0                 |
| <b>Zn (ppm)</b>                           | 801                      | 41 ± 0                | 17 ± 1                |
| <b>Al (ppm)</b>                           | 17                       | 320 ± 0               | n.d.                  |
| <b>Cd (ppb)</b>                           | 790                      | 46 ± 1                | n.d.                  |
| <b>Cr (ppb)</b>                           | 84                       | 1670 ± 5              | n.d.                  |
| <b>Ni (ppb)</b>                           | 54                       | 635 ± 2               | n.d.                  |

<sup>a</sup>Results in g kg<sup>-1</sup>

## 3.2. SUBSTRATES

### 3.2.1. Cheese whey

The only raw substrate used for dark fermentation or anaerobic digestion processes was cheese whey (synthetic salt solutions were also used as substrate in some experiments – their composition can be found in the corresponding chapter). Cheese whey was obtained from a dairy industry placed in Zamora (Spain), by concentrating a diluted lactose-rich stream via reverse

osmosis. Two different stocks of cheese whey were used as substrate, CW<sub>4</sub> (used in **Chapter 4**) and CW<sub>5</sub> (used in **Chapter 5**). Their composition is presented in Table 3.2.

**Table 3.2.** Characterization of the cheese whey used in **Chapter 4** (CW<sub>4</sub>) and **Chapter 5** (CW<sub>5</sub>) (n.d.: no data).

|   | CW <sub>4</sub> | CW <sub>5</sub> |
|---|-----------------|-----------------|
| <b>Total solids (g L<sup>-1</sup>)</b>                  | 166             | 126.8 ± 8.6     |
| <b>Volatile solids (g L<sup>-1</sup>)</b>               | 152             | 116.8 ± 7.8     |
| <b>COD (g L<sup>-1</sup>)</b>                           | 176             | 122.1 ± 5.6     |
| <b>Lactose (g L<sup>-1</sup>)</b>                       | 154             | 103.4 ± 2.1     |
| <b>Lactate (g L<sup>-1</sup>)</b>                       | 3.1             | 3.0 ± 1.2       |
| <b>N Kjeldahl (mg L<sup>-1</sup>)</b>                   | 613             | 1200 ± 26       |
| <b>N-NH<sub>4</sub><sup>+</sup> (mg L<sup>-1</sup>)</b> | 58              | 108 ± 3         |
| <b>Cl<sup>-</sup> (mg L<sup>-1</sup>)</b>               | 2486            | 981 ± 9         |
| <b>SO<sub>4</sub><sup>2-</sup> (mg L<sup>-1</sup>)</b>  | 562             | 430 ± 15        |
| <b>NO<sub>3</sub><sup>-</sup> (mg L<sup>-1</sup>)</b>   | <50             | n.d.            |
| <b>PO<sub>4</sub><sup>3-</sup> (mg L<sup>-1</sup>)</b>  | 2761            | 1455 ± 89       |
| <b>Ca<sup>2+</sup> (mg L<sup>-1</sup>)</b>              | 820             | 1260 ± 139      |
| <b>Mg<sup>2+</sup> (mg L<sup>-1</sup>)</b>              | n.d.            | 783 ± 68        |
| <b>Na<sup>+</sup> (mg L<sup>-1</sup>)</b>               | 3191            | 181 ± 19        |
| <b>K<sup>+</sup> (mg L<sup>-1</sup>)</b>                | 833             | 525 ± 18        |

### 3.2.2. Domestic wastewater

Domestic wastewater collected from the WWTP of León was used both as the inoculum and the substrate for the bioelectrocatalytic study in **Chapter 7**. Five different wastewater stocks were used for the different operating conditions in **Chapter 7**. Their chemical composition is detailed in Table 3.3.

**Table 3.3.** Characterization of the five sets of domestic wastewater used for **Chapter 7**.

|  | Domestic wastewater (WW) |             |             |             |             |
|--|--------------------------|-------------|-------------|-------------|-------------|
|  | HRT 4 h                  | HRT 8 h     | HRT 12 h    | HRT 24 h    | Batch       |
| <b>TSS (mg L<sup>-1</sup>)</b>           | 41.0 ± 1.0               | 45.8 ± 0.3  | 55.3 ± 1.8  | 53.8 ± 1.3  | 55.0 ± 0.5  |
| <b>VSS (mg L<sup>-1</sup>)</b>           | 9.8 ± 0.3                | 9.3 ± 0.3   | 10.0 ± 2.0  | 9.8 ± 0.8   | 10.8 ± 0.8  |
| <b>TOC (mg L<sup>-1</sup>)</b>           | 22.7 ± 1.4               | 24.6 ± 1.3  | 32.7 ± 1.6  | 34.0 ± 1.0  | 89.4 ± 11.9 |
| <b>Total N (mg L<sup>-1</sup>)</b>       | 19.7 ± 0.9               | 21.0 ± 1.0  | 20.5 ± 1.4  | 20.9 ± 1.2  | 59.4 ± 3.0  |
| <b>NH<sub>4</sub><sup>+</sup> (ppm)</b>  | 18.9 ± 5.1               | 20.9 ± 5.4  | 20.0 ± 6.2  | 19.1 ± 5.2  | 21.5 ± 4.9  |
| <b>pH</b>                                | 7.08 ± 0.12              | 7.22 ± 0.12 | 7.01 ± 0.06 | 7.13 ± 0.10 | 7.16 ± 0.14 |
| <b>Conductivity (µS cm<sup>-1</sup>)</b> | 504 ± 10                 | 486 ± 12    | 498 ± 12    | 496 ± 12    | 492 ± 10    |
| <b>COD (mg L<sup>-1</sup>)</b>           | 65 ± 2                   | 67 ± 3      | 77 ± 3      | 78 ± 3      | 188 ± 20    |
| <b>Acetate (mg L<sup>-1</sup>)</b>       | 36 ± 6                   | 27 ± 2      | 33 ± 6      | 29 ± 5      | 27 ± 1      |

### 3.3. EXPERIMENTAL SET-UP

#### 3.3.1. Small scale fermentation/biodegradability tests

Erlenmeyer flasks with a total volume of 250 mL were used for batch and semi-continuous dark fermentative processes of cheese whey (**Chapters 4 and 5**), as well as for biodegradability tests of glucose (see **Chapter 6**). These tests were performed at  $35 \pm 1$  °C (mesophilic conditions) by means of a thermostatic bath (Figure 3.2). The flasks were equipped with magnetic stirrers, and gas production data were recorded by displacement of water or an acid saline solution.





*Figure 3.2. View of the small-scale fermentation/biodegradability tests.*

### 3.3.2. Medium scale fermentation/anaerobic digestion tests

Medium scale dark fermentation (**Chapter 4**) or anaerobic digestion tests (**Chapter 6**) were performed in 3-L methacrylate reactors (Figure 3.3). Depending on the conditions of the experiment, these reactors worked in batch or in semi-continuous mode. As in the small scale tests, medium scale reactors were continuously stirred by means of regulated RZR2020 stirrers (Heidolph GmbH, Schwabach, Germany). The systems were equipped with a thermostatic jacket to keep constant the temperature inside the reactors.



*Figure 3.3. The 3-L methacrylate reactors used for the different experiments throughout the thesis.*

#### 3.3.3. Membrane unit

A membrane unit (Series 3000/3B, Scherzinger Pump Technology, Furtwangen, Germany) was used for the filtration of the supernatant from dark fermentation of cheese whey in **Chapter 5**. This device consists on a feeding tank with a working volume of 500 mL, a vacuum pump, a manometer, and several safety and regulation valves (Figure 3.4). Filtration surface in the membrane unit was 28 cm<sup>2</sup>. A polyvinylidene fluoride membrane (RM UV150T 1016, Microdyn-Nadir GmbH, Wiesbaden, Germany) was used for the filtration process, working under a pressure between 1-2 bar.



*Figure 3.4. Membrane unit and PVDF membrane used for filtration in Chapter 5.*

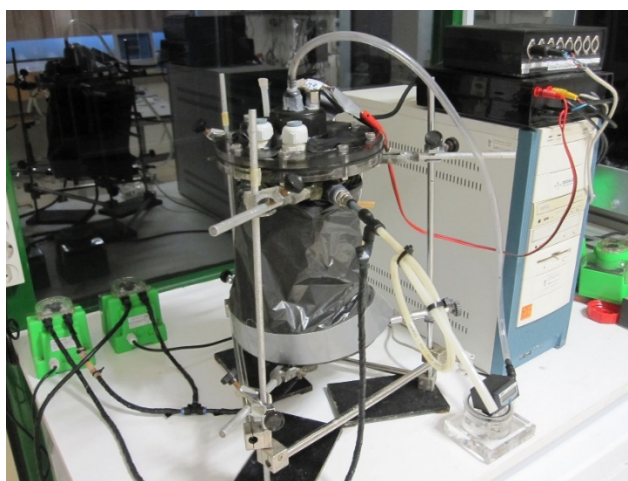
#### 3.3.4. Small scale microbial electrolysis cell

A series of polycarbonate plates was used to construct a membrane-less microbial electrolysis cell (MEC) for the test in **Chapter 5**. Rubber separators between the plates allowed for a gas-tight space inside the MEC. Two 0.6-mm-thick pieces of polyester cloth divided the MEC in two compartments with a volume of 50 mL each. The anode consisted on two layers of carbon felt with a surface of 50 cm<sup>2</sup> each (SIGRATHERM soft felt GFD 2, SGL Carbon Group, Wiesbaden, Germany). The cathode was a gas diffusion electrode (SIGRACET GDL 25 BC, SGL Carbon Group, Wiesbaden, Germany) containing electrodeposited Ni particles (550 mg cm<sup>-2</sup>). The inter-electrode separation was set to 1 mm. The polyester cloth avoided any electrical contact between the anode and the cathode. A 10 x 5 cm heating plate (5 W power) was placed on the anodic side of the cell in order to control the temperature at 25 ± 1 °C by means of a temperature controller (National Instruments PCI-6221). The MEC had connections for gas exits and feeding

in-lets. Gas production was measured by means of bubble counters connected to a U-tube glass and interfaced with a data acquisition system. A  $16\ \Omega$  resistor was added to the circuit for on-line current measurements at 60 min intervals using a data acquisition system (National Instruments PCI-6221). The same interval was used to record gas flow rates. An adjustable DC power supply (BK PRECISION 9120) maintained the applied voltage at the pre-set level ( $V_{\text{app}} = 1\ \text{V}$ ).

### 3.3.5. Medium scale microbial electrolysis cell

For the experiment in **Chapter 7**, a continuous flow, single-chamber, membraneless MEC with a total volume of 3 L was used (Figure 3.5). The reactor was equipped with connections for gas exits and liquid entries and exits, and it was covered to prevent algae growth. The electrolytic module (anode + cathode) was submerged in the feeding stock. The anode consisted on one layer of carbon felt (SIGRATHERM, see above) with dimensions of 210 x 100 mm, while the cathode was a stainless steel plate (with the same dimensions). As in the small scale MEC, a polyester cloth was placed between the anode and cathode to avoid any electrical contact (the inter-electrode separation was set to 1 mm). Gas production was measured with an MGC-1 milli-gas counter (Ritter Co, Bochum, Germany). Temperature was controlled at  $21 \pm 2\ ^\circ\text{C}$ . The culture medium in the reactor was mixed with an external recirculation loop using two peristaltic pumps (Dosiper C1R; León, Spain) operating in series (recirculation rate of  $9\ \text{L h}^{-1}$ ). An adjustable DC power supply (BK PRECISION 9120) maintained the applied voltage at the pre-set level ( $V_{\text{app}} = 1\ \text{V}$ ). Current measurements (as well as the recording of gas flow rates) were performed at 60 min intervals with a data acquisition system (see above).



*Figure 3.5. The MEC used for continuous test in Chapter 7.*

#### **3.4. ANALYTICAL TECHNIQUES**

##### **3.4.1. Total solids and volatile solids**

Total solids (TS) and volatile solids (VS) were determined following the APHA methods (2005). Crucibles for solids determination were previously dried and weighed. A sample volume of 10 mL is weighed on the crucible and dried at 105 °C, then cooled in a moisture-less environment. After a new weight measurement, the amount of TS in the sample can be calculated. In order to obtain the VS content, these crucibles were put at 550 °C for 60 min and weighed again. VS content was the difference between the last two weight values.

##### **3.4.2. Total suspended solids and volatile suspended solids**

Total suspended solids (TSS) and volatile suspended solids (VSS) were determined following the APHA methods (2005). Samples were filtered through a weighed standard glass-fiber filter. The filter was dried afterwards at 105 °C and weighed. The amount of TSS was measured from the increase in weight of the filter. In order to obtain the VSS content, the same filter was incinerated at 550 °C for 30 min. The weight loss after this step represents the VSS amount in the sample.

##### **3.4.3. pH and conductivity**

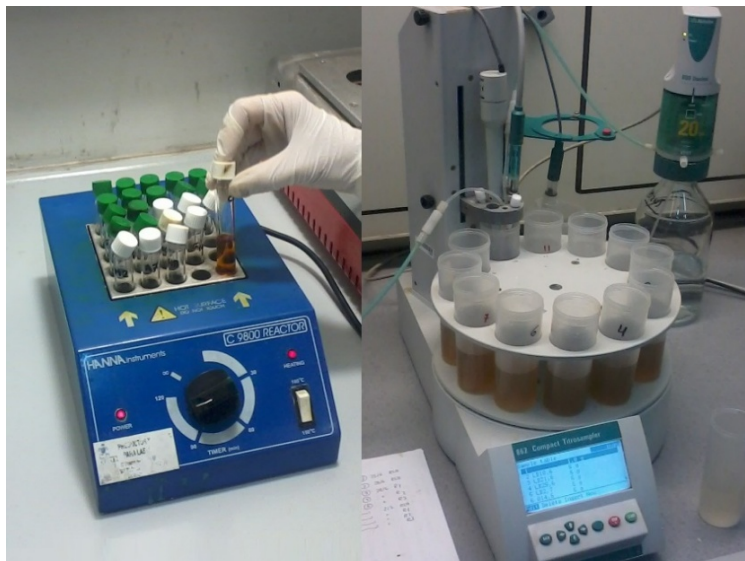
pH values of the influents and effluents were measured using a pH meter (GLP 22, Crison Instruments, S.A.). The conductivity was measured with a conductivity meter (sension™156 Multiparameter, Hach Co., Loveland, CO, USA).

##### **3.4.4. Organic matter**

Organic matter was measured in accordance with the Walkley-Black method (Walkley & Black, 1934). This method consists on the oxidation of the organic matter by digesting the sample for 30 min in a mixture of H<sub>2</sub>SO<sub>4</sub> and K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> 1N. A volumetric titration with ammonium iron (II) sulfate 0.5N (Mohr's salt) and diphenylamine as indicator is done afterwards. Organic carbon was measured from the amount of dichromate taking part in the reaction. Organic matter was calculated considering that 58% of organic matter is organic carbon (Primo & Carrasco, 1973).

### 3.4.5. Chemical oxygen demand

Chemical oxygen demand (COD) was measured following the APHA methods (2005). Samples were digested at 150 °C for 2 hours in a Hanna C9800 reactor, in a medium with H<sub>2</sub>SO<sub>4</sub> and an excess of K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>. Silver sulfate (Ag<sub>2</sub>SO<sub>4</sub>) was the catalyst of this reaction. After the digestion process, the non-reacted dichromate was titrated with Mohr's salt using a Metrohm 862 Compact Titrator (Figure 3.6).



*Figure 3.6. Equipment for the COD analysis.*

### 3.4.6. Total organic carbon

Total organic carbon (TOC) was measured using a total organic carbon analyzer (multi N/C 3100, AnalytikJena).

### 3.4.7. Nitrogen Kjeldahl

Total nitrogen content was measured by the Kjeldahl method (MAPA, 1994). This value contains both organic nitrogen and ammonium. Sample was digested in the presence of H<sub>2</sub>SO<sub>4</sub> at 400 °C for 35 min. As a result, nitrogen becomes part of ammonium sulfate. This (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> is distilled in a distillation unit UDK 140 (VELP SCIENTIFICA, Italy), then the NH<sub>4</sub><sup>+</sup> is mixed with boric acid (4%) and determined by titration with HCl 0.5N at a pH = 4.8.

#### 3.4.8. Ammonium

Ammonium nitrogen ( $\text{NH}_4^+$ ) was measured in accordance with the APHA methods (2005), by means of an ion-selective electrode (781 pH/Ion Meter, Metrohm AG, Herisau, CH).

#### 3.4.9. Analysis of chemical elements

The analysis of chemical elements was performed digesting 0.3 g of dried sample in 10 mL of nitric acid at 65% in a microwave oven at 100 °C for 5 min and subsequently at 190 °C for 30 min. A Perkin Elmer Optima 2000 DV inductively coupled plasma atomic emission spectrometer (ICP-AES) was used for the analysis of P, Na, K, Ca and Mg. Heavy metals were analyzed using ICP-MS (mass spectrometry) Nex-ION 300D Perkin Elmer. Anions were analyzed by capillary electrophoresis Agilent G1600.

#### 3.4.10. Gas chromatography

Gas composition was analyzed by means of a gas chromatograph (Varian CP-3800 GC) equipped with a thermal conductivity detector (TCD).  $\text{CH}_4$ ,  $\text{CO}_2$ ,  $\text{N}_2$ ,  $\text{H}_2$  and  $\text{O}_2$  were separated with a four-meter-long packed column (HayeSep Q 80/100) followed by a one-meter-long molecular sieve column (Molecular Sieve 13x80/100 Mesh, 1.0 m x 1/8'' x 2.0 m). The carrier gas was helium, and the operating conditions were 331 kPa and 50 °C.

#### 3.4.11. Volatile fatty acids

Volatile fatty acids (VFA) content was measured using the same gas chromatograph using the autosampler (Varian Chrompack 8-200), and with a flame ionization detector (FID) equipped with a Nukol capillary column (30 m x 0.25 mm x 0.25  $\mu\text{m}$ ) from Supelco Analytical (Bellefonte, PA, USA). VFA samples were previously prepared by centrifugation (3500 rpm, 10 min) and the supernatant was filtered with cellulose filters (0.45  $\mu\text{m}$ ). The carrier gas was helium. The injector and detector temperatures were 220 °C and 250 °C, respectively. The oven temperature was 150 °C during the first 3 min of process and raised to 180 °C at an increasing rate of 10 °C  $\text{min}^{-1}$ . The calibration was done with a mixture of standard VFA from Supelco (C2-C7). The detection limit for VFA analysis was 5.0  $\text{mg L}^{-1}$ .

#### **3.4.12. Alcohols**

Ethanol and 1,3-propanediol were analyzed using a gas chromatograph Perkin Elmer Autosystem XL equipped with an FID and a capillary column Varian WCOT fused silica (50 m x 0.25 mm x 0.2 µm) CP-WAX 57CB. The carrier gas was helium. The temperature of the oven was set at 60 °C for 2 min, and thereafter increased to 180 °C (5 °C min<sup>-1</sup>). Injector and detector temperatures were 250 °C.

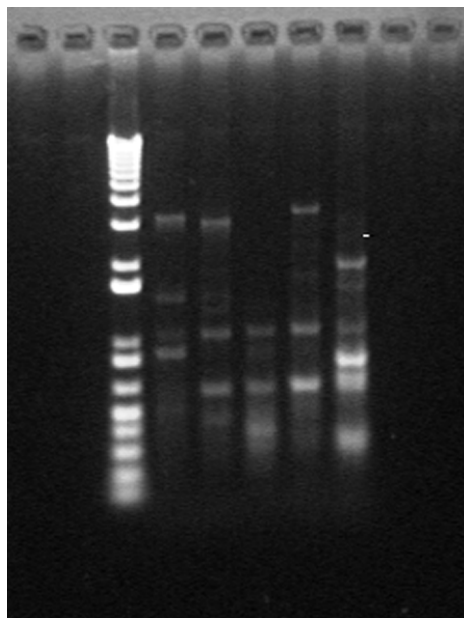
#### **3.4.13. High pressure liquid chromatography (HPLC)**

Lactose and lactic acid were analyzed using a liquid chromatograph (Waters 2690 Alliance HPLC) equipped with a refractive index detector. The column used was Varian PL Hi-Plex (300 mm x 7.7 mm x 8 mm). The temperature of the oven was 60 °C and 40 °C for the detector. Sulfuric acid of 5 mM was used as the mobile phase with a volume rate of 0.4 mL min<sup>-1</sup>.

#### **3.4.14. Microbial community analysis**

PowerSoil™ DNA Isolation Kit (Mo Bio Laboratory Inc., USA) was used for DNA extraction. The 16S rDNA fragments were amplified by PCR (Polymerase Chain Reaction). The region corresponding to positions 357 and 518 in the 16S rDNA of *Escherichia coli* was PCR-amplified using the forward primer EUB357f (5'-CCTACGGGAGGCAGCAG-3') with a GC clamp (5'-CGCCCGCCGCGCCCGCGCCCGGCCCGCCCGCCCGCCCGCCCGCCCGCCCG-3') at the 5' end to stabilize the melting behavior of the DNA fragments and the reverse primer UNIV518r (5'-ATTACCGCGGCTGCTGG-3'). PCR amplification was conducted in an automated thermal cycler (GeneAmp PCR System 9700, Applied Biosystem, USA). PCR products were electrophoresed on 1% (wt:vol) agarose gel in 1x TAE for 30 min for 80 V, and then checked with GelRed™ Nucleic Acid Gel Stain (Biotium Inc, USA) to confirm the amplification (Figure 3.7). DGGE (Denaturing Gradient Gel Electrophoresis) was carried out using the Dcode™ Universal Mutation Detection System (BioRad, California, USA) in accordance with the manufacturer's instructions.



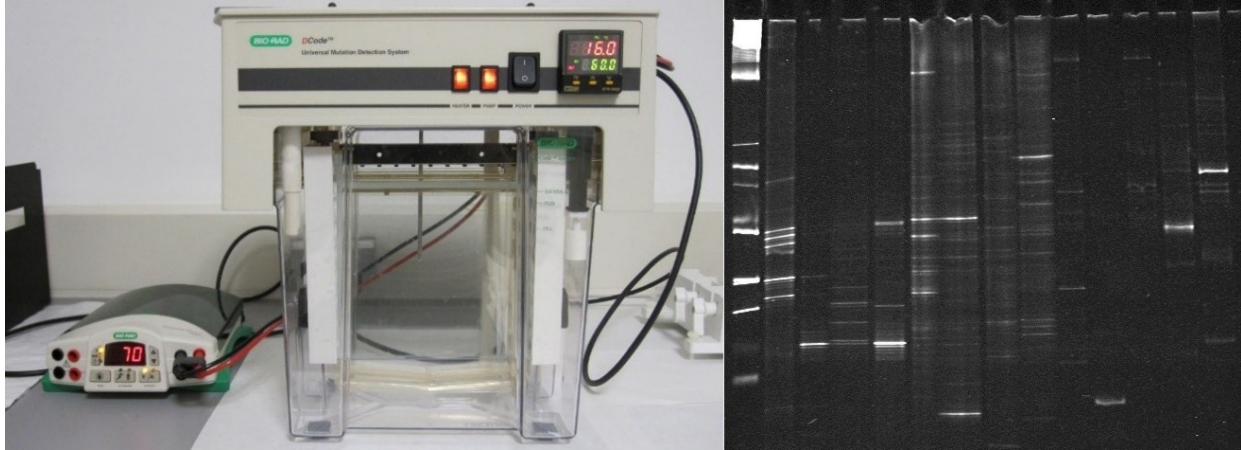


*Figure 3.7. View of an agarose gel.*

PCR products were electrophoresed in 1x TAE buffer for 480 min at 70 V and 60 °C on polyacrylamide gel (7.5%) containing a linear gradient ranging from 40% to 60% denaturant (urea and formamide) (Figure 3.8). After electrophoresis, polyacrylamide gel was stained with GelRed™ Nucleic Acid Gel Stain for 30 min, and then visualized on UV transilluminator. The bands were cut out from DGGE polyacrylamide gel for 16S rDNA sequencing. DNA fragments from the excised bands were PCR-amplified with the forward primer EUB357f without a GC clamp and the reverse primer UNIV518r. After PCR amplification, PCR products were purified using a Kit Nucleic Acid and Protein Purification MachereyeNagel (Clontech, USA). All the strands of the purified PCR products were sequenced with primers EUB357f by ABI PRISM Big Terminator Cycle Sequencing Kit and Amersham MegaBace DNA sequencer (GE Healthcare, USA) in accordance with the manufacturer's instructions. Sequence data were analyzed with Chromas Lite 2.01 software (Ibis Bioscience, Carlsbad, CA, USA) and submitted to the non-redundant nucleotide database at GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>).

These sequences were further aligned with the closest matches found in the GenBank database with the CLUSTALW function of Molecular Evolutionary Genetics Analysis package (MEGA).





*Figure 3.8. DGGE equipment and one of the DGGE gels obtained.*

### 3.5. REFERENCES

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# *Chapter 4*

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## *Biohydrogen production from lactose: influence of substrate and nitrogen concentration*

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*Chapter prepared according to the guidelines of Environmental Technology*



**Abstract**

Hydrogen produced from renewable sources may be considered the energy vector of the future. However, reducing process costs is imperative in order to achieve this goal. In the present research, the effect of nitrogen (N), initial pH and substrate content for starting up the dark fermentative process was studied using the response surface methodology. Anaerobic digested dried sludge (biosolid pellets) was used as the inoculum. Synthetic wastewater was used as the substrate in batch reactors. A decrease in H<sub>2</sub> production was observed with the increase in N and lactose concentrations. This drop was considerably greater when the concentration of lactose was at its lower level. Although the increase in lactose concentration results in a lower H<sub>2</sub> production, the effect of N on the response is attenuated at higher levels of lactose. On the other hand, the effect of initial pH on the fermentation system was not significant. The evaluation on the process under semi-continuous conditions was performed using anaerobic sequencing batch reactors (ASBRs). The process was evaluated at different C/N ratios using synthetic wastewater. Results showed higher hydrogen yields with the gradual decrease in nitrogen content. The addition of cheese whey to the ASBR resulted in a H<sub>2</sub> production rate of 0.18 L H<sub>2</sub> L<sup>-1</sup> d<sup>-1</sup>.



#### 4.1. INTRODUCTION

Recent research activities have focused extensively on alternative fuels with the aim of reducing the use of fossil fuels and the external dependency on this type of energy resources. Hydrogen ( $H_2$ ) has been suggested as the energy carrier of the future, serving as a medium through which primary energy can be stored, transported and utilized in different applications. [1] There are several ways of producing  $H_2$ . However, biological methods are preferable because they offer the possibility of using renewable sources as the use of sunlight,  $CO_2$ , organic materials like carbohydrate-rich wastes and in recent years the use of lignocellulosic and algae biomass, [2, 3] thus bringing out new opportunities. Among biological processes to obtain  $H_2$ , dark fermentation is the only one with a prospect of being rapidly scaled up owing to its similarity to the well-known anaerobic digestion process. [4]

$H_2$  production from renewable sources may be considered to have a positive impact on the environment, although some other aspects should be taken into account such as the efficiency of producing, storing and distributing this gas. Therefore, investigations towards finding new, cheaper methods to produce  $H_2$  are of great relevance. The dark fermentation process has been proposed as a cheap method for obtaining  $H_2$  when waste materials are used as substrates. [5, 6] This process has been extensively studied by different authors using carbohydrate-rich wastewaters, food wastes and agricultural wastes among others, [2, 7-9] reporting that carbohydrate-rich substrates are preferable to protein- and lipid-rich substrates. [10] In addition, several factors are of great relevance to the long-term stability of the process, especially when the use of mixed microflora is the choice for obtaining  $H_2$ -producing microorganisms as a means for reducing operating costs. Among these factors, the application of pre-treatments, operating conditions, the presence of inhibitory substances and the C/N ratio present an important effect on  $H_2$  yields.

The effect of nutrient addition on fermentative  $H_2$  production has been studied by several authors. [11-13] However, most experiments were carried out under batch conditions. There is still a need to identify the increase obtained in  $H_2$  production rate (HPR) under continuous conditions since any addition of nutrients translates into an increase in operating costs. When carbohydrate-rich wastes are used as substrate, a nutrient-deficient condition may be established by running the process over an extended period. Nitrogen is usually the nutrient that is added to

the process either as an ammonium salt or urea. This is the case when considering the biological treatment of cheese whey (CW). This waste is the residual liquid remaining after the precipitation and removal of milk casein during cheese-making. CW represents about 85-95% of the milk volume and retains 55% of milk nutrients, such as lactose, soluble proteins, lipids and mineral salts. [14] Biological oxygen demand (BOD<sub>5</sub>) and chemical oxygen demand (COD) values ranges between 40-60 and 50-80 kg m<sup>-3</sup>, respectively, and the BOD<sub>5</sub>/COD ratio is commonly higher than 5. [15]

H<sub>2</sub> production from CW by dark fermentation may be an interesting way to achieve the valorization of this liquid stream. CW has been reported as a suitable substrate for the dark fermentation process due to its high content in carbohydrates, [16-18]. Several studies have reported on the use of CW as the substrate. Many of these studies were carried out using continuously stirred tank reactors (CSTRs), [19]. This is the case of the study performed by Venetsaneas et al. [20]. These authors evaluated the fermentative H<sub>2</sub> process at 35 °C with a hydraulic retention time (HRT) of 24 h and an organic loading rate (OLR) of 60 g COD L<sup>-1</sup> d<sup>-1</sup>. As result, they reported a yield of 1.9 L H<sub>2</sub> L<sup>-1</sup> CW (0.61 ± 0.04 mol H<sub>2</sub> mol<sup>-1</sup> glucose consumed). Lower HRTs were tested by de Amorim et al. [21] also at mesophilic regimen (37 °C). In this case, these authors reported a H<sub>2</sub> yield of 2.8 mol H<sub>2</sub> mol<sup>-1</sup> lactose at an HRT of 6 h (OLR of 138.6 g lactose L<sup>-1</sup> d<sup>-1</sup>). Higher yields have been obtained under thermophilic regimen achieving a maximum value of 5.1 mol H<sub>2</sub> mol<sup>-1</sup> lactose. [22] Due to the high influence of nutrients and C/N ratio on the dark fermentation process, further experimentation should be carried out in an attempt to improve the knowledge of the process and to increase H<sub>2</sub> yields under continuous conditions.

The present research studied the start-up stage of the dark fermentation process at different C/N ratios and lactose content using the response surface methodology. A second study was performed with the aim of assessing the influence of nitrogen content on H<sub>2</sub> production under continuous conditions using synthetic wastewater and CW as substrates.



## 4.2. MATERIAL AND METHODS

### 4.2.1. Response surface methodology

A three-factor central composite design was performed using as factors the following: initial pH, N concentration and lactose content (denoted as  $X_1$ ,  $X_2$  and  $X_3$ , respectively). The distance of the axial points to the center of the experiment was set at 1.682 (codified units). The results obtained from the experiments were used to fit a second-order response surface to analyze the effect of the different factors on  $H_2$  production. The response was analyzed by multiple regression by means of the least-square method to fit the following model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where  $Y$  is the response variable and  $\beta$  are the regression coefficients. The degree of fitting of the model to the experimental values was evaluated by the coefficient of determination  $R^2$  and  $R^2_{adj}$ . The  $p$ -values were used to indicate the significant model terms ( $p < .05$ ). The fitting process was carried out by using MATLAB R12.

### 4.2.2. Experimental set-up

#### 4.2.2.1. Substrates

Fermentation tests were studied using a synthetic solution. This solution was prepared by the addition of lactose and  $NH_4Cl$  (as C and N sources, respectively) and a mineral medium. [23] CW was obtained from a local cheese manufacturer. Reverse osmosis was used to separate the lactose-rich effluent, in order to obtain a concentrated waste stream. The chemical characterization of CW is presented in Table 4.1.

#### 4.2.2.2. Batch fermentation tests

Batch fermentation tests were carried out using a synthetic solution as the substrate. Biosolid pellets were used as inoculum. These pellets were obtained from the wastewater treatment plant of León. The use of biosolid pellets has been reported as a cheap and proper method to obtain a source of  $H_2$ -producing microorganisms. [24] The total solid (TS) and volatile solid (VS) content of the pellets were  $936.4 \pm 0.7$  and  $616.1 \pm 0.6$  g  $kg^{-1}$ , respectively. The fermentation system was

inoculated with 4 g of pellets. The chemical characterization of the inoculum is shown in Table 4.1.

**Table 4.1.** Chemical characteristics of CW and inoculum (biosolid pellets).

| CW  |      | Biosolid pellets   |      |
|---|------|--------------------|------|
| TS (g L <sup>-1</sup> )                             | 166  | N Kjeldahl (%)     | 5.7  |
| VS (g L <sup>-1</sup> )                             | 152  | Organic matter (%) | 44.7 |
| COD (g L <sup>-1</sup> )                            | 176  | Organic C (%)      | 25.9 |
| Lactose (g L <sup>-1</sup> )                        | 154  | P (ppm)            | 18   |
| Lactic acid (g L <sup>-1</sup> )                    | 3.1  | Ca (ppm)           | 19   |
| Alkalinity (mg L <sup>-1</sup> )                    | 1260 | Mg (ppm)           | 4    |
| N Kjeldahl (mg L <sup>-1</sup> )                    | 613  | Na (ppm)           | 460  |
| NH <sub>4</sub> -N(mg L <sup>-1</sup> )             | 58   | Fe (ppm)           | 12   |
| Cl <sup>-</sup> (mg L <sup>-1</sup> )               | 2486 | Mn (ppm)           | 133  |
| SO <sub>4</sub> <sup>2-</sup> (mg L <sup>-1</sup> ) | 562  | Cu (ppm)           | 469  |
| NO <sub>3</sub> <sup>-</sup> (mg L <sup>-1</sup> )  | < 50 | Zn (ppm)           | 801  |
| PO <sub>4</sub> <sup>3-</sup> (mg L <sup>-1</sup> ) | 2761 | Al (ppm)           | 17   |
| Na (mg L <sup>-1</sup> )                            | 3191 | Cd (ppb)           | 790  |
| K (mg L <sup>-1</sup> )                             | 833  | Cr (ppb)           | 84   |
| Ca (mg L <sup>-1</sup> )                            | 820  | Ni (ppb)           | 54   |

Batch experiments were performed on 250 mL Erlenmeyer flasks equipped with magnetic stirrers. Erlenmeyer flasks were connected to bottle gas meters to collect gas by displacement of a saline solution. Gas analysis was performed (H<sub>2</sub>, CH<sub>4</sub> and CO<sub>2</sub>) three times per week. All systems were run under mesophilic conditions (35 ± 1 °C). The duration was based on the total stoppage of gas production. Liquid samples were collected for measurement of COD, volatile fatty acids (VFAs), ethanol, 1,3-propanediol and lactic acid.

Factors studied were initial pH ( $X_1$ ) from 4 to 6 units, N concentration ( $X_2$ ) from 0.5 to 10 g N L<sup>-1</sup> and lactose concentration ( $X_3$ ) from 2 to 90 g L<sup>-1</sup>. Either phosphoric acid (solution 0.1 M) or alkaline solution (mixture of NaHCO<sub>3</sub>, KH<sub>2</sub>PO<sub>4</sub> and KOH in a proportion of 1:1:1 (weight)

with a TS concentration of 60 g L<sup>-1</sup>) was used to set the initial pH of reactors. No further control of pH was carried out during the course of the fermentation. Table 4.2 shows the experimental set-up.

*Table 4.2. Factors and levels of the experimental design.*

| <b>Run</b>           | <b>X<sub>1</sub> = pH</b> | <b>X<sub>2</sub> = N (g L<sup>-1</sup>)</b> | <b>X<sub>3</sub> = Lactose (g L<sup>-1</sup>)</b> |
|----------------------|---------------------------|---|---|
| <i>First order</i>   |                           |   |   |
| 1                    | 6.4                       | 8.1   | 72.2  |
| 2                    | 4.61                      | 8.1   | 72.2  |
| 3                    | 6.4                       | 2.4   | 72.2  |
| 4                    | 4.61                      | 2.4   | 72.2  |
| 5                    | 6.4                       | 8.1   | 19.8  |
| 6                    | 4.61                      | 8.1   | 19.8  |
| 7                    | 6.4                       | 2.4   | 19.8  |
| 8                    | 4.61                      | 2.4   | 19.8  |
| <i>Axial points</i>  |                           |   |   |
| 9                    | 7                         | 5.25  | 46  |
| 10                   | 4                         | 5.25  | 46  |
| 11                   | 5.5                       | 10  | 46  |
| 12                   | 5.5                       | 0.5   | 46  |
| 13                   | 5.5                       | 5.25  | 90  |
| 14                   | 5.5                       | 5.25  | 2   |
| <i>Central point</i> |                           |   |   |
| 15                   | 5.5                       | 5.25  | 46  |
| 16                   | 5.5                       | 5.25  | 46  |
| 17                   | 5.5                       | 5.25  | 46  |
| 18                   | 5.5                       | 5.25  | 46  |
| 19                   | 5.5                       | 5.25  | 46  |
| 20                   | 5.5                       | 5.25  | 46  |

##### 4.2.2.3. Semi-continuous fermentation tests

The fermentation was carried out under semi-continuous operation at mesophilic conditions ( $35 \pm 1$  °C). Fermentation systems were initially evaluated in three reactors working with a synthetic solution prepared with lactose, mineral medium (as in the previous experiments) and different concentrations of N. The selection of N content was based on results obtained from batch fermentation tests. The feeding solution was prepared with a lactose concentration of 36 g L<sup>-1</sup> and varying contents of N (0.5, 1.4 and 2.3 g N L<sup>-1</sup>). These assays were denoted as T1, T2 and T3.

Erlenmeyer flasks with a working volume of 250 mL were used as reactors and inoculated with 4 g of pellets. Feeding was done once a day, along with pH adjustment to 5.5 units. Reactors were fed with 100 mL d<sup>-1</sup> of the synthetic solution (HRT of 2.5 d). Biomass sedimentation was allowed for 30 min previously to each feeding procedure. Gas and liquid samples were taken twice a week for the analysis of gas composition, VFA concentration, TS and VS content.

A second experiment was performed using an anaerobic sequencing batch reactor (ASBR). The reactor had a working volume of 3 L and was run under the semi-continuous mode. The temperature was kept at  $35 \pm 1$  °C. The agitation was made by a mechanical stirrer Heidolph RZR1. The pH of the fermentation process was adjusted daily at 5.5 units. This reactor was inoculated with 36 g of biosolid pellets. The reactor was daily fed with 1 L (HRT of 3 d) of synthetic solution. The feeding process consisted of 4 cycles regularly spaced along a day. Peristaltic pumps (Dosiper C1R) were used for this purpose. Biomass sedimentation was allowed during 40 min before each feeding cycle. The concentration of N in the synthetic solution was initially set at 1.4 g L<sup>-1</sup> (period P1), and subsequently decreased to 0.9 and 0.5 g L<sup>-1</sup> (periods P2 and P3). This study lasted 35 days. Subsequently, the reactor was fed with CW (lactose concentration of 36 g L<sup>-1</sup>) during 20 days (period P4).

##### 4.2.2.4. Analytic techniques

N content, TS, VS, organic matter, gas composition and VFAs were analyzed as previously described. [25] Ethanol and 1,3-propanediol were analyzed using a gas chromatograph Perkin Elmer Autosystem XL equipped with an FID and a capillary column Varian WCOT fused silica (50 m x 0.25 mm x 0.2 µm) CP-WAX 57CB. The carrier gas was helium. The temperature of the

oven was set at 60 °C for 2 min, and thereafter increased to 180 °C (5 °C min<sup>-1</sup>). Injector and detector temperatures were 250 °C. Lactose and lactic acid were analyzed using a liquid chromatograph (Waters 2690 Alliance HPLC) equipped with a refractive index detector. The column used was Varian PL Hi-Plex (300 mm x 7.7 mm x 8 µm). The temperature of the oven was 60 °C and for the detector was 40 °C.

The analysis of chemical elements was performed digesting 0.3 g of dried sample in 10 mL of nitric acid at 65% in a microwave oven at 100 °C for 5 min and subsequently at 190 °C for 30 min. A Perkin Elmer Optima 2000 DV inductively coupled plasma atomic emission spectrometer (ICP-AES) was used for the analysis of P, Na, K, Ca and Mg. Heavy metals were analyzed using ICP-MS (mass spectrometry) Nex-ION 300D Perkin Elmer. Anions were analyzed by capillary electrophoresis Agilent G1600.

### 4.3. RESULTS AND DISCUSSION

#### 4.3.1. Batch fermentation tests

Table 4.3 shows results of gas production and analysis of the liquid phase obtained from batch tests. The response fitted to the polynomial model was H<sub>2</sub> production. The equation found to describe the influence of the three factors on the bio-H<sub>2</sub> production was

$$Y = 305.2 - 128.16X_1 - 78.1X_2 + 29.1X_3 + 0.14X_1X_2 - 5.9X_1X_3 + 0.91X_2X_3 + 29.1X_1^2 + 0.93X_2^2 - 0.012X_3^2 \quad (2)$$

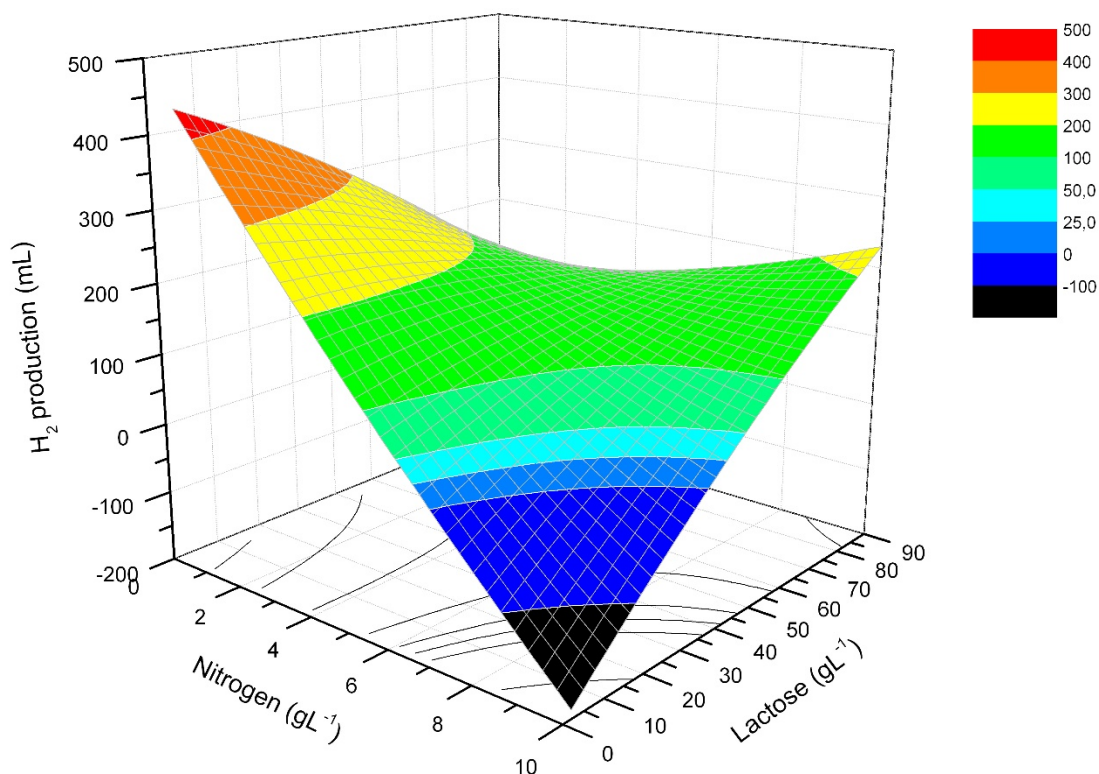
The eigenvalues were  $\lambda_1 = 29.44$ ,  $\lambda_2 = -0.45$ ,  $\lambda_3 = 1.07$ . The different signs indicate a saddle point. Figure 4.1 shows a three-dimensional graph of the quadratic model. A decrease in H<sub>2</sub> production was observed with the increase in N and lactose concentrations. This drop was considerably greater when the concentration of lactose was at its lower level. The increase in lactose concentration also resulted in lower H<sub>2</sub> production, but the effect of N attenuated the decrease experienced by the response. Results suggest that the best operability area should be placed on the lower values of lactose and N concentration. Although the model indicated a maximum out of the experimental region, testing at higher lactose concentrations was not

#### 4. BIOHYDROGEN PRODUCTION FROM LACTOSE

considered because of the implication of operating out of the range given by the physical separation process for obtaining CW.

*Table 4.3. Results of gas production and chemical analysis from batch tests.*

| Run | Gas<br>(mL) | H <sub>2</sub> yield<br>(mL g <sup>-1</sup> <sub>lactose</sub> ) | Ethanol<br>(mg L <sup>-1</sup> ) | 1,3-Prop<br>(mg L <sup>-1</sup> ) | Lactic<br>(mg L <sup>-1</sup> ) | Acetic<br>(mg L <sup>-1</sup> ) | Propionic<br>(mg L <sup>-1</sup> ) | Butyric<br>(mg L <sup>-1</sup> ) |
|-----|-------------|--|----------------------------------|-----------------------------------|---------------------------------|---------------------------------|------------------------------------|----------------------------------|
| 1   | 3           | 0  | 0                                | 58                                | 0                               | 35                              | 70                                 | 142                              |
| 2   | 1049        | 5  | 2400                             | 88                                | 0                               | 339                             | 34                                 | 190                              |
| 3   | 16          | 0  | 300                              | 0                                 | 300                             | 653                             | 35                                 | 104                              |
| 4   | 1564        | 6.2  | 4200                             | 21                                | 1200                            | 356                             | 0                                  | 1179                             |
| 5   | 183         | 5.3  | 400                              | 0                                 | 0                               | 666                             | 0                                  | 0                                |
| 6   | 1           | 0  | 0                                | 86                                | 0                               | 206                             | 211                                | 745                              |
| 7   | 1395        | 23.5   | 3400                             | 0                                 | 800                             | 335                             | 0                                  | 941                              |
| 8   | 887         | 13.8   | 2600                             | 0                                 | 800                             | 281                             | 0                                  | 0                                |
| 9   | 171         | 0.7  | 300                              | 0                                 | 0                               | 1146                            | 0                                  | 56                               |
| 10  | 1236        | 6.3  | 3400                             | 0                                 | 400                             | 786                             | 0                                  | 0                                |
| 11  | 57          | 0.7  | 100                              | 18                                | 200                             | 588                             | 0                                  | 0                                |
| 12  | 598         | 4.2  | 100                              | 0                                 | 0                               | 1068                            | 114                                | 1394                             |
| 13  | 358         | 1.4  | 300                              | 0                                 | 0                               | 585                             | 0                                  | 80                               |
| 14  | 164         | 8.2  | 300                              | 0                                 | 200                             | 602                             | 0                                  | 12                               |
| 15  | 398         | 3.6  | 0                                | 0                                 | 0                               | 712                             | 0                                  | 801                              |
| 16  | 217         | 2  | 100                              | 0                                 | 0                               | 429                             | 0                                  | 0                                |
| 17  | 134         | 1.5  | 100                              | 72                                | 200                             | 402                             | 0                                  | 349                              |
| 18  | 160         | 2.9  | 0                                | 0                                 | 0                               | 536                             | 120                                | 450                              |
| 19  | 1078        | 5.2  | 150                              | 0                                 | 200                             | 685                             | 0                                  | 360                              |
| 20  | 408         | 4.4  | 0                                | 0                                 | 150                             | 430                             | 50                                 | 420                              |



**Figure 4.1.** Response surface plot of the quadratic model for the production of  $H_2$  at  $X_1$  (pH) at 5.5 units.

Table 4.4 shows results obtained from the multiple regression analysis. The concentration of lactose was the only factor presenting a significant effect on the response ( $p$ -value < .05). The interaction effect between N and lactose concentration also presented a  $p$ -value < .05. The significant effect of this interaction is mainly influenced by the strong dependence of the response on lactose concentration.

VFA and alcohols were also measured at the end of the batch fermentation tests. These responses could not be adjusted to a polynomial equation, since  $R^2$  values obtained for total VFA and ethanol demonstrated lack of fit. However, maximum values of VFA content were obtained for N at its lower levels. Working under similar nitrogen levels, Salerno et al. [26] observed a slight decrease in biogas production with the increase in ammonia concentration. These authors also observed that the influence of nitrogen content was higher on the biogas production rate than

on the cumulative biogas production, with an increase in the lag phase during the fermentation. This extended lag phase was also observed at the start-up of those runs with a nitrogen content over 5.25 g N L<sup>-1</sup>, while an immediate biogas production was observed for tests at 0.5 g N L<sup>-1</sup> (run 12) and 2.4 g N L<sup>-1</sup> (runs 4, 7 and 8).

**Table 4.4.** Estimated regression coefficients for H<sub>2</sub> production.

| Term constant                 | Coefficient | Error  | <i>p</i> -value |
|-------------------------------|-------------|--------|-----------------|
| $\beta_0$                     | 305.18      | 925.92 | 0.7485          |
| $\beta_1$                     | -128.16     | 301.28 | 0.6796          |
| $\beta_2$                     | -78.07      | 69.26  | 0.2860          |
| $\beta_3$                     | 29.07       | 7.49   | 0.0031          |
| $\beta_{12}$                  | 0.14        | 11.03  | 0.9899          |
| $\beta_{13}$                  | -5.88       | 1.20   | 0.0006          |
| $\beta_{23}$                  | 0.91        | 0.38   | 0.0361          |
| $\beta_{11}$                  | 29.15       | 26.30  | 0.2936          |
| $\beta_{22}$                  | 0.93        | 2.62   | 0.7300          |
| $\beta_{33}$                  | -0.01       | 0.03   | 0.7044          |
| <i>Statistical parameters</i> |             |        |                 |
| R <sup>2</sup>                |             |        | 0.842           |
| Adj R <sup>2</sup>            |             |        | 0.700           |
| Error                         |             |        |                 |
| Sum of squares                |             |        | 63,315.47       |
| Mean square                   |             |        | 6331.54         |
| <i>F</i> statistic            |             |        | 5.920           |
| Prob > <i>F</i>               |             |        | 0.0051          |

Biogas production and composition of data reported for the central point presented high variability which can be explained by the use of biosolid pellets. This also may explain the wide range of values for the lag phase and as a consequence it is reasonable to assume that the quantity



of active microflora may as well be presented in variable amounts. Despite this fact, higher H<sub>2</sub> production was observed at lower values of N content.

With regard to the effect of pH on the response, this factor presented no significant effect. Acidification of the substrate occurred naturally during the fermentation because the pH was not controlled. Batch tests set at high pH values presented an initial decrease in pH thus reaching optimum values for attaining H<sub>2</sub> activity. A further acidification of the systems due to the increase in VFA concentration resulted in lower values of pH until reaching an inhibitory state that caused the stoppage of the fermentative stage. Results were in agreement with those reported by Davila-Vazquez et al. [27] who also evaluated different initial pH values using RSM in fermentative H<sub>2</sub> producing reactors under batch conditions. The relevance of this result is associated with the fact that regulating the pH of the feeding substrate is not essential for starting-up H<sub>2</sub> producing systems especially when considering the treatment of influents with high sugar content obtained from alkaline pre-treatment processes. However, it should be observed that those experiments with low initial pH presented higher content of ethanol. With regard to the production of 1,3-propanediol, the amount produced of this compound was negligible.

#### 4.3.2. Semi-continuous fermentation tests

Semi-continuous fermentation tests were carried out at three different N levels due to the high variability in gas production observed in batch assays. The concentration of N was selected based on results obtained from previous tests. Main parameters regarding these tests are presented in Table 4.5. A nil H<sub>2</sub> gas production was observed for T1 (the lowest value of N concentration). T2 and T3 presented a similar lag phase (Figure 4.2). Once active microflora was attained in the fermentation systems, these reactors were capable of maintaining the fermentative producing stage for a similar period, with T2 presenting a higher gas production. This resulted in higher H<sub>2</sub> production. Methane production was not detected in any of the tests (detection limit of the GC equipment was 1%).

Mean VFA values obtained during the period of active H<sub>2</sub> production for T2 and T3 experiments are also presented in Table 4.5. Acetic acid was the only organic acid found in the T1 system, whereas a mixture of acetic and butyric was predominantly present in T2 and T3, with lower values of caproic acid. Free VFA values rose during the first 10 days, reaching a peak

#### 4. BIOHYDROGEN PRODUCTION FROM LACTOSE

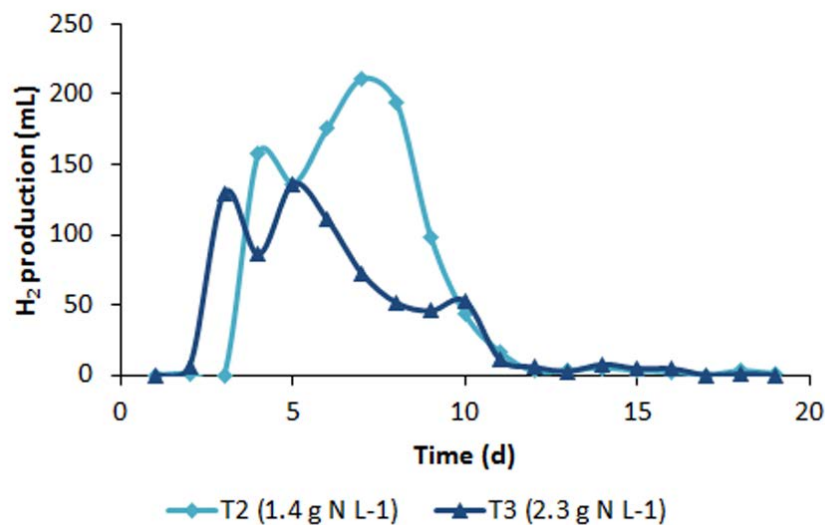
concentration and decreasing thereafter. This peak value was 10988 mg L<sup>-1</sup> (183 mM) for T1, 5657 mg L<sup>-1</sup> (73 mM) for T2 and 5774 mg L<sup>-1</sup> (89 mM) for T3. Although the H<sub>2</sub>-producing microflora was active when submitted to VFA values higher than 19 mM (threshold previously described by Van Ginkel and Logan [28]), H<sub>2</sub> yields here obtained were lower than those reported in the literature. [26, 29]

**Table 4.5.** Results from the semi-continuous tests using Erlenmeyer flasks and ASBR (3L).

| <b>Erlenmeyer flasks</b>                              | <b>T1</b>   | <b>T2</b>   | <b>T3</b>   |           |
|---|-------------|-------------|-------------|-----------|
| Total biogas (mL)                                     | 141         | 2809        | 1757        |           |
| H <sub>2</sub> (mL)                                   | 0           | 1048        | 727         |           |
| OLR (g L <sub>r</sub> <sup>-1</sup> d <sup>-1</sup> ) | 12.75       | 12.75       | 12.75       |           |
| HPR (L L <sub>r</sub> <sup>-1</sup> d <sup>-1</sup> ) |             | 0.58        | 0.34        |           |
| H <sub>2</sub> yield (L kg <sup>-1</sup> VS)          |             | 45.6        | 26.9        |           |
| Acetic (mg L <sup>-1</sup> )                          | 3316 ± 2111 | 1530 ± 624  | 2533 ± 1448 |           |
| Butyric (mg L <sup>-1</sup> )                         | 0           | 2648 ± 1571 | 2172 ± 1093 |           |
| Caproic (mg L <sup>-1</sup> )                         | 0           | 355 ± 234   | 448 ± 189   |           |
| VFA total (mg L <sup>-1</sup> )                       | 3316 ± 2111 | 4445 ± 2055 | 5041 ± 718  |           |
| <b>ASBR (3 L)</b>                                     | <b>P1</b>   | <b>P2</b>   | <b>P3</b>   | <b>P4</b> |
| Gas production (mL d <sup>-1</sup> )                  | 1073        | 1195        | 2052        | 3308      |
| H <sub>2</sub> production (mL d <sup>-1</sup> )       | 198         | 200         | 425         | 539       |
| OLR (g L <sub>r</sub> <sup>-1</sup> d <sup>-1</sup> ) | 11.30       | 11.30       | 11.30       | 12.28     |
| HPR (L L <sub>r</sub> <sup>-1</sup> d <sup>-1</sup> ) | 0.07        | 0.07        | 0.14        | 0.18      |
| H <sub>2</sub> yield (L kg <sup>-1</sup> VS)          | 5.81        | 5.88        | 12.51       | 14.64     |

Note: HPR, H<sub>2</sub> production rate

The theoretical yield for H<sub>2</sub> production is higher within the acetic production route. However, the production of butyric acid is more favorable for the cell, as the energy released by each mole of glucose is larger than the energy released in the degradation of glucose to acetate. In practice, butyric fermentation carried out by *Clostridium* sp., is considered to be the most effective H<sub>2</sub> production pathway.[30]The highest butyric acid production was obtained for T2 (1.4 g N L<sup>-1</sup>), which would also explain the higher H<sub>2</sub> production attained by this system.



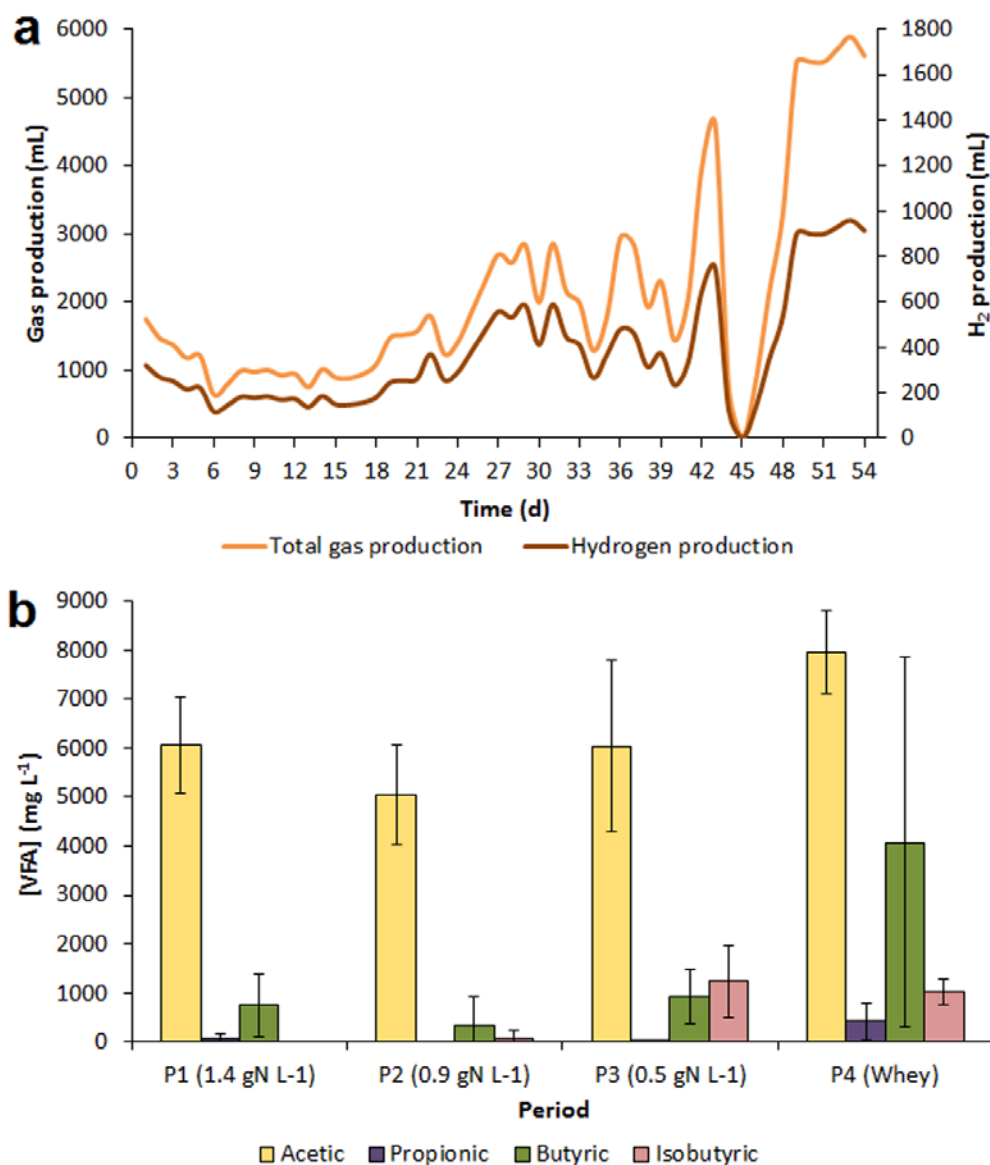
**Figure 4.2.** Evolution of total gas production for the semi-continuous fermentation tests in 250 mL Erlenmeyer flasks.

The second experimental stage was evaluated in a 3 L ASBR. The experiment consisted of a gradual reduction of N added to the culture, starting from the best result obtained on the previous tests (T2). Evolution of gas production along the experiment is shown in Figure 4.3a and Table 4.5. As N concentration was reduced, an increase in gas production was observed to a 150% and 200% on the period at 0.9 and 0.5 g N L<sup>-1</sup>, respectively. In addition, the content of H<sub>2</sub> was higher for the period with lower N concentration. When using CW as the substrate, H<sub>2</sub> production rose significantly even though lactose contents were similar (36 g L<sup>-1</sup>). During the set of experiments using Erlenmeyer flasks, the pH adjustment was performed once a day. The main consequence of this is that microflora was submitted to both high pH and osmotic pressure variations at one particular moment each day. On the other hand, when using the 3 L ASBR, the feeding procedure was divided into four feeding cycles day<sup>-1</sup>. This fact results in microbial consortia suffering from less osmotic stress, which could explain higher stability observed in these later system.

VFA evolution of this second stage is presented in Figure 4.3b. Acetic acid production was approximately constant during the three periods. On the other hand, butyric and isobutyric acids appeared mainly when N content was at its lowest level. Interestingly, no caproic acid was produced during this experimental stage. The concentration of propionic acid, which is associated with low H<sub>2</sub> yields, was almost negligible during the whole experiment. As for P4, the rise in H<sub>2</sub>

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production caused an increase in VFA formation. CW has a low content in N ( $0.6 \text{ g N}_{\text{Kjeldahl}} \text{ L}^{-1}$ ,  $\text{NH}_4^+$  values being below  $0.06 \text{ g L}^{-1}$ ), which could enhance the formation of butyric and isobutyric acids during this stage. However, the increase in VFA concentration caused a posterior drop in  $\text{H}_2$  production (day 45). As a result, propionic acid formation was the prevailing fermentation pathway. The cease of microbial activity led to a decrease in VFA due to the replacement of the reactor liquor by the feeding solution. This effect also reduced the imbalance of proton gradient across the cell membrane and thus reactivated  $\text{H}_2$  production.



**Figure 4.3.** Evolution of a) total gas and  $\text{H}_2$  production and b) VFA production throughout the four periods (P1, P2, P3 and P4) of the experiment using an ASBR (3L).

Isobutyrate formation follows a pathway starting from  $\alpha$ -ketoisovalerate. The genera *Clostridium* and *Lactococcus* carry out  $\alpha$ -ketoisovalerate oxidative decarboxilation, until its conversion to isobutyrate, producing NADH + H<sup>+</sup> and ATP (Ehrlich pathway). [31, 32]  $\alpha$ -Ketoisovalerate may appear as a result of two different pathways: it is a by-product of valine transamination and also an intermediate of the valine biosynthesis pathway. [33] Under dark fermentative H<sub>2</sub> production, *Clostridium* is one of the microbes responsible for valine biosynthesis. As many of the amino-acid biosynthesis pathways, valine biosynthesis also requires a transamination step, mainly from glutamine. Glutamine biosynthesis requires NH<sub>4</sub><sup>+</sup>, therefore it can be hypothesized that a decrease in NH<sub>4</sub><sup>+</sup> concentration (as in P2 and P3) may reduce glutamine biosynthesis rate in *Clostridium*, and valine biosynthesis as a consequence. Thus, a valine biosynthesis intermediate (such as  $\alpha$ -ketoisovalerate) must be degraded to isobutyrate in order to prevent its accumulation. As for P4, the low nitrogen content of CW may be the explanation for the lower content of isobutyrate (1244 mg L<sup>-1</sup> of isobutyric acid in P3 and 766 mg L<sup>-1</sup> in P4).

#### 4.4. CONCLUSIONS

Results demonstrated the feasibility of using biosolid pellets for obtaining H<sub>2</sub>-producing microflora under different conditions tested. The H<sub>2</sub> yield obtained was 23.5 mL g<sup>-1</sup> lactose at an N concentration of 2.4 g L<sup>-1</sup>. The increase in N content negatively affected the H<sub>2</sub> yield, suggesting that the best results appear at low values of N and lactose concentrations. When evaluating the combined effect of N and lactose concentration, this negative effect observed with the increase in N concentration was attenuated by the increase in substrate concentration. In addition, acidification of substrate was attained disregarding the initial pH conditions.

Semi-continuous operation was tested using an ASBR. An increase in H<sub>2</sub> production was observed whenever N content was gradually reduced. The operation at 0.5 g N L<sup>-1</sup> resulted in a production of 0.14 L H<sub>2</sub> L<sub>r</sub><sup>-1</sup> d<sup>-1</sup> when using synthetic wastewater as the substrate. The addition of CW to the reactor caused instabilities but higher production was observed (0.18 L H<sub>2</sub> L<sup>-1</sup> d<sup>-1</sup>).

### **Disclosure statement**

No potential conflict of interest was reported by the authors.

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# *Chapter 5*

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*A two-stage process for hydrogen production from cheese whey: Integration of dark fermentation and biocatalyzed electrolysis*

**International Journal of Hydrogen Energy, 40, 168-175 (2015)**  
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*Chapter prepared according to the guidelines of the International Journal of Hydrogen Energy*



### **Abstract**

This paper explores the potential of a two-stage process (fermentative + biocatalyzed electrolysis) to reduce the organic load of an industrial waste stream (cheese whey) in parallel with hydrogen production. Overall, the combined process helped to significantly reduce the chemical oxygen demand (COD) of the effluent, while producing hydrogen at a maximum yield of 94.2 L H<sub>2</sub> kg<sub>vs</sub><sup>-1</sup>. The low pH of the fermentative effluent fed into the bioelectrochemical reactor helped to control methanogenic and homoacetogenic activity during the second stage of the treatment. However, this acid stream needed to be diluted and amended with salts and acetate to avoid the collapse of hydrogen production rate. Therefore, practical application of a two stage process for the treatment of cheese whey would require the existence of a secondary waste stream for dilution of the acidified effluent, thus balancing its nutrients composition prior to feeding into the bioelectrochemical system.



## 5.1. INTRODUCTION

The energy-water nexus has received a growing interest in the past decades as the human population pressure on global resources intensifies. The main idea behind this concept is that water is needed to produce energy, energy is needed to manage water, and both activities are mutually intertwined. As a result, all of the power generation technologies (even solar and wind power) have an associated water footprint. For instance, in 2005 in Spain, water requirements for thermal energy, electricity and transportation fuels amounted to 8.5 km<sup>3</sup>, representing 20% of the total water withdraw for human use [1]. Moreover, the water needed for fuels production (hydrocarbons and nuclear fuels) usually becomes polluted with organics, salts, solids, heavy and radioactive metals, microorganisms, etc., thus demanding relatively sophisticated treatments before discharging into the environment.

Likewise, water management systems and technologies have their associated energy footprint. Water must be collected, treated, pumped and disposed, all of these procedures requiring energy. For the particular case of wastewater treatment, this activity demanded in 2009 in Spain about 2476 GWh year<sup>-1</sup> representing around 1% of the national electricity consumption [2-4].

To break this mutual dependence and therefore ensure the environmental (and economical) sustainability of water and energy management, new technologies and procedures would need to be developed. For wastewater treatment in particular, this would mean less energy-intensive or even net-energy-production technologies, which could be attained if we could make use of the energy content of the organic matter dissolved into the wastewater [5]. In this paper we will focus on fermentative hydrogen production (FHP) and biocatalyzed electrolysis, two biologically-based technologies devised for wastewater treatment that convert the potential energy stored in the organics into H<sub>2</sub>. FHP is a well-known technology easy to scale up, that produces a biogas containing primarily hydrogen and carbon dioxide. However, its practical application is hampered by an important drawback: the maximal H<sub>2</sub> yield is limited to 33% of the theoretical maximum based on stoichiometric conversion of glucose [6]. As a result, investigations have been fostered during the last decades towards finding new methods for maximizing the production of H<sub>2</sub>. In 2005 biocatalyzed electrolysis emerged as a complementary technology to fermentative processes, allowing to recover up to 90% of the energy content in the substrate [7]. Biocatalyzed electrolysis takes place in a special type of electrochemical reactor known as microbial electrolysis cell (MEC),

that make use of electrogenic bacteria to convert a wide range of organic compounds into H<sub>2</sub> under anaerobic conditions and with the aid of a certain electrical input [8-11].

Effluent from cheese factories represents a suitable substrate for hydrogen recovery in a combined FHP-MEC process. Cheese whey (CW), the effluent remaining after the precipitation and removal of milk casein during cheese-making, represents about 85-95% of the milk volume, having an index of biodegradability expressed in terms of biological and chemical oxygen demand (BOD<sub>5</sub>:COD ratio) in the range 0.4-0.8 [12], and retaining 55% of milk nutrients such as lactose, soluble proteins, lipids, and mineral salts [13]. In this study we investigated the feasibility of an integrated FHP-MEC process for CW treatment and hydrogen recovery, trying to identify both the optimum rate of CW:inoculum during the FHP treatment and the requirements of the FHP effluent for being fed to the MEC post-treatment.

## 5.2. MATERIAL AND METHODS

### 5.2.1. Inoculum and substrate for the fermentation process

CW was obtained from a cheese facility located in Zamora (Spain) by concentrating a dilute lactose-rich stream via reverse osmosis. The inoculum for the fermentation process consisted of digested sludge obtained from the wastewater treatment plant (WWTP) of the city of León (Spain). The chemical characteristics of the inoculum and substrate are presented in Table 5.1. FHP tests were performed under batch conditions using different ratios of substrate (CW) and inoculum (I) (referred to as CW:I, and defined as the proportion of volatile solids (VS) between the CW and the I). These ratios were gradually increased from 1.25 to 15.7 to prevent acid build-up that may cause the inhibition of the process.

Batch experiments were performed under mesophilic conditions ( $35 \pm 1$  °C) using 250 mL Erlenmeyer flasks equipped with magnetic stirrers. Gas production was recorded by displacement of an acid and saline solution, and data were normalized to standard temperature (0 °C) and pressure (760 mmHg). Biogas and liquid samples were regularly taken and analyzed (see below). FHP performance was characterized using the cumulative H<sub>2</sub> production (mL) within the first 24 h of the batch tests, the volumetric H<sub>2</sub> production rate per liter of reactor and hour (mLH<sub>2</sub> L<sub>r</sub><sup>-1</sup> h<sup>-1</sup>) and the H<sub>2</sub> yield (L H<sub>2</sub> kg<sub>vs</sub><sup>-1</sup>). At the end of the fermentative tests, the effluent obtained from



the trial with the highest H<sub>2</sub> production was collected and used as the feeding solution for the MEC. The content of the Erlenmeyer flask was centrifuged (using SIGMA 2-16P centrifuge, 20 min, 7800 rpm) and the supernatant was filtered by means of a vacuum filter (Series 3000/3B, Scherzinger Pump Technology, Furtwangen, Germany) with a polyvinylidene fluoride membrane (RM UV150T 1016, Microdyn-Nadir GmbH, Wiesbaden, Germany).

*Table 5.1. Chemical characterization of the inoculum and the CW.*

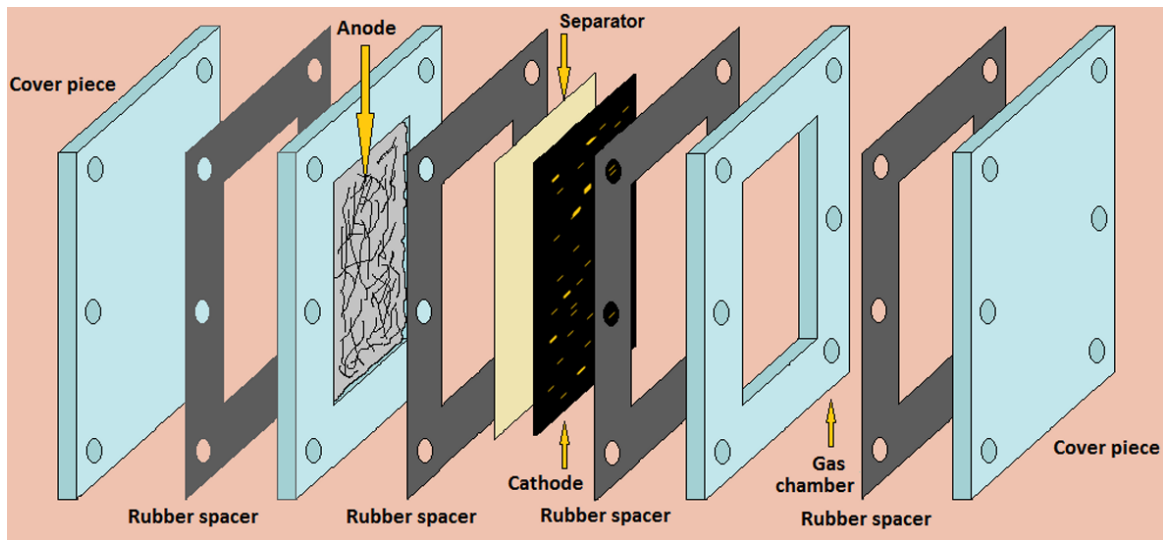
| <b>Parameter</b>                                       | <b>Inoculum</b> | <b>CW</b>   |
|--|-----------------|-------------|
| <b>TS (g L<sup>-1</sup>)</b>                           | 24.0 ± 0.6      | 126.8 ± 8.6 |
| <b>VS (g L<sup>-1</sup>)</b>                           | 11.2 ± 0.4      | 116.8 ± 7.8 |
| <b>COD (g O<sub>2</sub> L<sup>-1</sup>)</b>            | 29.5 ± 1.4      | 122.1 ± 5.6 |
| <b>Lactose (g L<sup>-1</sup>)</b>                      | –               | 103.4 ± 2.1 |
| <b>Organic matter (%)</b>                              | 1.25            | –           |
| <b>Organic carbon (%)</b>                              | 0.73            | –           |
| <b>Acetate (mg L<sup>-1</sup>)</b>                     | 2 ± 0           | 246 ± 57    |
| <b>Lactate (mg L<sup>-1</sup>)</b>                     | 252 ± 14        | 3016 ± 123  |
| <b>Alkalinity (mg CaCO<sub>3</sub> L<sup>-1</sup>)</b> | 1.1 ± 0.1       | 1.8 ± 0.2   |
| <b>pH</b>  | 7.34 ± 0.02     | 4.68 ± 0.04 |
| <b>N-NH<sub>4</sub> (mg L<sup>-1</sup>)</b>            | 578 ± 12        | 108 ± 3     |
| <b>N Kjeldahl (mg L<sup>-1</sup>)</b>                  | 1300 ± 28       | 1200 ± 26   |
| <b>Cl<sup>-</sup> (ppm)</b>                            | 38 ± 2          | 981 ± 9     |
| <b>SO<sub>4</sub><sup>2-</sup> (ppm)</b>               | 253 ± 12        | 430 ± 15    |
| <b>PO<sub>4</sub><sup>3-</sup> (ppm)</b>               | 726 ± 74        | 1455 ± 89   |
| <b>Na<sup>+</sup> (ppm)</b>                            | 131 ± 31        | 181 ± 19    |
| <b>Ca<sup>2+</sup> (ppm)</b>                           | 116 ± 15        | 1260 ± 139  |
| <b>Mg<sup>2+</sup> (ppm)</b>                           | 399 ± 62        | 783 ± 68    |
| <b>K<sup>+</sup> (ppm)</b>                             | 20 ± 2          | 525 ± 18    |

## 5.2.2. Microbial electrolysis cell (MEC)

### 5.2.2.1. MEC design and operation

A continuous-flow, membrane-less MEC constructed with a series of polycarbonate plates was used in this study (Figure 5.1). The cell was equipped with connections for gas exits and liquid entries and exits. Both the anodic compartment and the gas collection chamber had a volume of 50 mL. The anodic compartment contained two layers of 5 mm thick carbon felt of 100 x 50 mm (SIGRATHERM soft felt GFD 2, SGL Carbon Group, Wiesbaden, Germany). The cathode consisted of a gas diffusion electrode (SIGRACET GDL 25 BC, SGL Carbon Group, Wiesbaden, Germany) containing electrodeposited Ni particles (550 mg cm<sup>-2</sup>). Two 0.6-mm-thick pieces of polyester cloth were sandwiched between the anodic and the H<sub>2</sub> collection compartments to avoid any electrical contact between the two electrodes. The inter-electrode separation was set to 1 mm.

The temperature was set at 25 °C using a thermocouple probe placed in the anodic chamber, a temperature controller (National Instruments PCI-6221) and a 10 x 5 cm heating plate (5 W power) placed on the anodic side of the cell. The gas produced was measured with the help of bubble counters connected to a U-tube glass and interfaced with a data acquisition system. A 16 Ω resistor was added to the circuit for on-line current measurements at 60 min intervals using a data acquisition system (National Instruments PCI-6221). The same interval was used to record gas flow rates. An adjustable DC power supply (BK PRECISION 9120) maintained the applied voltage at the pre-set level ( $V_{\text{app}} = 1 \text{ V}$ ).



**Figure 5.1.** Architecture of the polycarbonate MEC used in this study.

### 5.2.2.2. Inoculation and feeding process of the MEC

The MEC was inoculated with the effluent of a domestic-wastewater-fed MEC operated in continuous mode for more than one year. Once the start-up period was accomplished, the MEC system was fed with a synthetic solution containing acetate as carbon source.

During the first experimental phase (Section Effect of lactate addition over H<sub>2</sub> production), the MEC was fed with a synthetic substrate, and an infusion pump (model NE-1000, New Era Pump Systems Inc., USA) was used to pump the carbon source (lactate and/or acetate) solution at the required organic loading rate (OLR). Dilution water was fed by using a MasterFlex peristaltic pump which provided a hydraulic retention time (HRT) of 10 h. Feeding solutions were filtered sterilized and stored at 4 °C. Deionized water was used in the preparation of these solutions and chemical reagents were of analytical grade. A liter of nutrient stock solution contained: 6.2 g of KCl, 1.7 g of KH<sub>2</sub>PO<sub>4</sub>, 2.7 g of K<sub>2</sub>HPO<sub>4</sub>, 0.8 g of NH<sub>4</sub>Cl, 0.3 g of yeast extract, 0.1 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g of CaCl<sub>2</sub>·2H<sub>2</sub>O and 1 mL of a trace metal solution. A liter of this trace metal solution contained: 2 g of FeCl<sub>2</sub>·4H<sub>2</sub>O, 0.5 g of MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.03 of CuCl<sub>2</sub>, and 0.05 g for each of the following components: H<sub>3</sub>BO<sub>3</sub>, ZnCl<sub>2</sub>, (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, AlCl<sub>3</sub>, CoCl<sub>2</sub>·6H<sub>2</sub>O, NiCl<sub>2</sub>.

In the second experimental phase (Section Effect of acidified CW over MEC performance), the raw filtered supernatant (CW-R) obtained from the FHP tests (batch experiments described in Section Inoculum and substrate for the fermentation process) was diluted eight times and fed to the MEC at a constant HRT of 10 h in four different conditions: unamended (CW-D), amended with acetate (CW-DA), amended with salts (CW-DS) and amended with acetate and salts (CW-DAS). Table 5.2 shows the chemical characterization of all these effluents fed to the MEC.

MEC performance was characterized using the volumetric H<sub>2</sub> production rate per liter of anode (L H<sub>2</sub> L<sub>a</sub><sup>-1</sup> d<sup>-1</sup>), the H<sub>2</sub> yield (L H<sub>2</sub> kg<sub>vs</sub><sup>-1</sup>) and the coulombic efficiency (%) which were calculated in accordance with Escapa et al. [14].

### 5.2.3. Analytical measurements

Nitrogen content, total solids (TS), volatile solids (VS), chemical oxygen demand (COD), gas composition and volatile fatty acids (VFAs) were analyzed as described in Martínez et al. [15]. The analysis of chemical elements was performed using 0.3 g of dried sample digested in 10 mL

of nitric acid at 65% in a microwave oven at 100 °C for 5 min and subsequently, at 190 °C for 30 min. A Perkin Elmer Optima 2000 DV inductively-coupled plasma atomic emission spectrometer (ICP-AES) was used for the determination of P, Na, K, Ca, and Mg.

Lactose and lactic acid were analyzed using a liquid chromatograph (Waters 2690 Alliance HPLC) equipped with a refractive index detector. The column used was Varian PL Hi-Plex (300 mm x 7.7 mm x 8 mm). The temperature of the oven was 60 °C and 40 °C for the detector. Sulfuric acid of 5 mM was used as the mobile phase with a volume rate of 0.4 mL min<sup>-1</sup>.

**Table 5.2.** Composition of 70:30 synthetic solution, and of the different CW solutions fed to the MEC: a) CW-R = raw supernatant of CW; b) CW-D = 8-time diluted CW-R; c) CW-DA = CW-D after addition of acetate; d) CW-DS = CW-D after addition of salts; e) CW-DAS = CW-D after addition of acetate and salts.

|  | <b>70:30<br/>Synthetic<br/>solution</b> | <b>CW-R</b> | <b>CW-D</b> | <b>CW-DA</b> | <b>CW-DS</b> | <b>CW-DAS</b> |
|--|---|-------------|-------------|--------------|--------------|---------------|
| <b>Acetate (mM)</b>                                    | 9.8                                     | 15.5 ± 0.1  | 1.9 ± 0.0   | 9.7 ± 0.1    | 1.9 ± 0.0    | 9.7 ± 0.1     |
| <b>Lactate (mM)</b>                                    | 4.3                                     | 33.3 ± 0.1  | 4.2 ± 0.0   | 4.2 ± 0.0    | 4.2 ± 0.0    | 4.2 ± 0.0     |
| <b>K<sup>+</sup> (mg L<sup>-1</sup>)</b>               | 4350                                    | 344 ± 12    | 43 ± 1      | 43 ± 1       | 4893 ± 26    | 4893 ± 26     |
| <b>Cl<sup>-</sup> (mg L<sup>-1</sup>)</b>              | 3531                                    | 208 ± 15    | 26 ± 2      | 26 ± 2       | 3530 ± 35    | 3530 ± 35     |
| <b>PO<sub>4</sub><sup>3-</sup> (mg L<sup>-1</sup>)</b> | 2659                                    | 1200 ± 58   | 150 ± 7     | 150 ± 7      | 2659 ± 74    | 2659 ± 74     |

#### 5.2.4. Microbial community analysis

PowerSoil™ DNA Isolation Kit (Mo Bio Laboratory Inc., USA) was used for DNA extraction. The 16S rDNA fragments were amplified by PCR (Polymerase Chain Reaction). The region corresponding to positions 357 and 518 in the 16S rDNA of Escherichia coli was PCR-amplified using the forward primer EUB357f (5'-CCTACGGGAGGCAGCAG-3') with a GC clamp (5'-CGCCCGCCGCGCCCGCGCCCGGCCCGCCGCCCCCGCCCC-3') at the 5' end to stabilize the melting behavior of the DNA fragments and the reverse primer UNIV518r (5'-ATTACCGCGGCTGCTGG-3'). PCR amplification was conducted in an automated thermal cycler (GeneAmp PCR System 9700, Applied Biosystem, USA). PCR products were

electrophoresed on 1% (wt:vol) agarose gel in 1x TAE for 30 min for 80 V, and then checked with GelRed™ Nucleic Acid Gel Stain (Biotium Inc, USA) to confirm the amplification. DGGE (Denaturing Gradient Gel Electrophoresis) was carried out using the Dcode™ Universal Mutation Detection System (BioRad, California, USA) in accordance with the manufacturer's instructions.

PCR products were electrophoresed in 1x TAE buffer for 480 min at 70 V and 60 °C on polyacrylamide gel (7.5%) containing a linear gradient ranging from 40% to 60% denaturant (urea and formamide). After electrophoresis, polyacrylamide gel was stained with GelRed™ Nucleic Acid Gel Stain for 30 min, and then visualized on UV transilluminator. The bands were cut out from DGGE polyacrylamide gel for 16S rDNA sequencing. DNA fragments from the excised bands were PCR-amplified with the forward primer EUB357f without a GC clamp and the reverse primer UNIV518r. After PCR amplification, PCR products were purified using a Kit Nucleic Acid and Protein Purification MachereyeNagel (Clontech, USA). All the strands of the purified PCR products were sequenced with primers EUB357f by ABI PRISM Big Terminator Cycle Sequencing Kit and Amersham MegaBace DNA sequencer (GE Healthcare, USA) in accordance with the manufacturer's instructions. Sequence data were analyzed with Chromas Lite 2.01 software (Ibis Bioscience, Carlsbad, CA, USA) and submitted to the non-redundant nucleotide database at GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>).

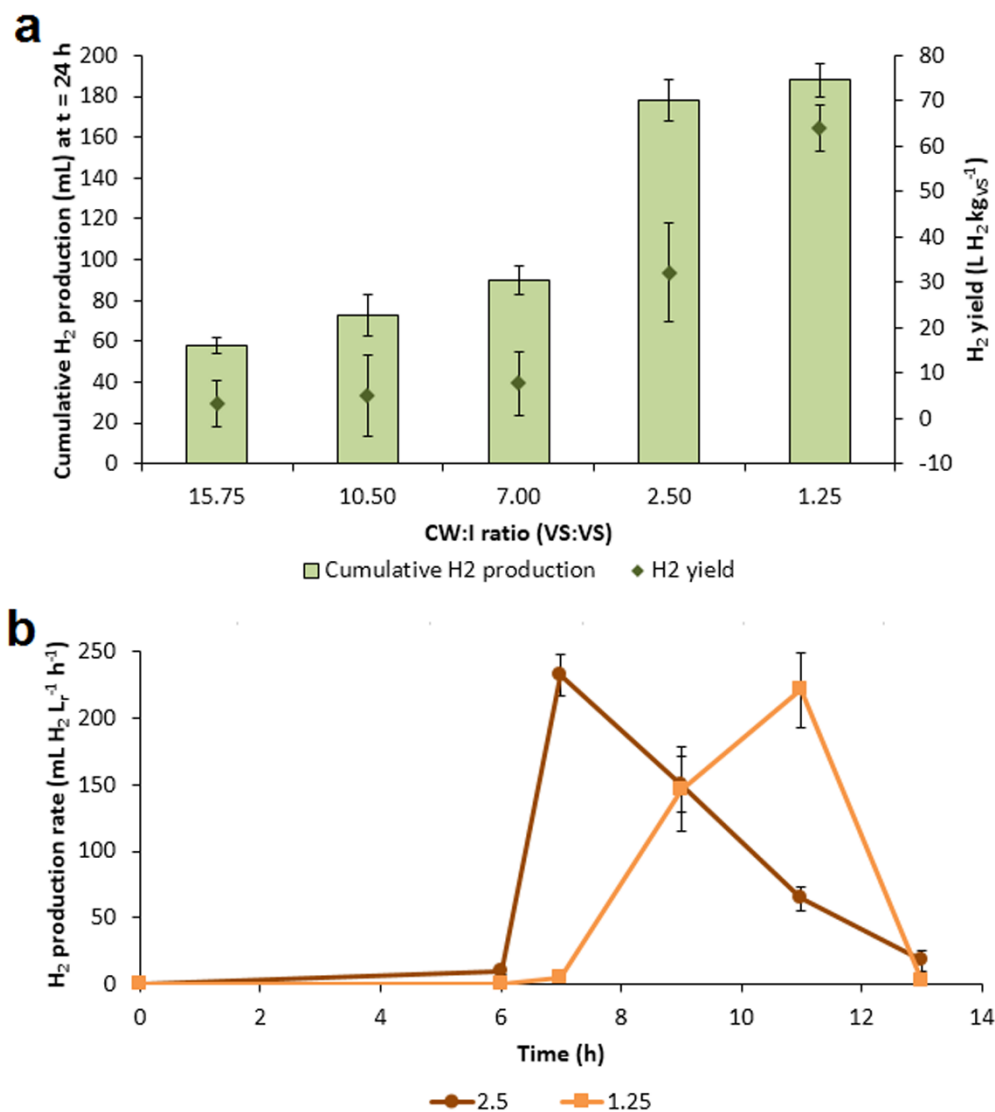
These sequences were further aligned with the closest matches found in the GenBank database with the CLUSTALW function of Molecular Evolutionary Genetics Analysis package (MEGA).

### **5.3. RESULTS AND DISCUSSION**

#### **5.3.1. Selection of the optimum CW:I ratio during the FHP**

Five duplicates batch fermentation tests were performed at CW:I ratios of 1.25, 2.50, 7.00, 10.50 and 15.75 (VS:VS). All tests presented an initial lag phase lasting 4-7 h, and in all cases hydrogen production stopped completely after 24 h as pH dropped below 4. Total cumulative H<sub>2</sub> production (Figure 5.2a) increased with decreasing the proportion of CW in the feeding, reaching its maximum at CW:I ratios of 2.5 and 1.25, thus suggesting the appearance of saturating conditions (i.e. further reductions in the CW:I ratio would probably not improve the hydrogen

production). H<sub>2</sub> yield also increased with reducing the proportion of CW, although no saturating conditions were observed. Similar COD removal rates were found in all cases (nearly 80%, data not shown).



**Figure 5.2.** a) Cumulative H<sub>2</sub> production and H<sub>2</sub> yields for batch fermentation tests; b) H<sub>2</sub> production rates for the CW:I ratios at 2.5 and 1.25.

Considering these results, the selection of the optimum CW:I is not straightforward: while the CW:I ratio of 1.25 allowed to extract more hydrogen from the substrate, larger amounts of wastewater might be treated at a CW:I ratio of 2.5. Therefore, to make a choice we also took into account the rates at which hydrogen was produced and the length of the lag phases for the CW:I

ratios of 1.25 and 2.50 (Figure 5.2b). The CW:I ratio of 2.5 presented a lag phase of approximately 6 h, and H<sub>2</sub> production rate reached its maximum at ~7 h. In contrast, at CW:I of 1.25 a slightly larger lag phase was observed and the H<sub>2</sub> production rate reached its maximum at about 11 h. Therefore 2.5 was selected as the optimum CW:I ratio because it allows a relatively high energy extraction from the substrate at fair rates of substrate treatment. Moreover, the filtered supernatant obtained from this CW:I ratio was used as a raw feeding for the MEC during the second experimental phase (see Section Effect of acidified CW over MEC performance).

VFAs and lactic acid analysis (Table 5.3) showed that, as expected, lactate was the most abundant product of the fermentation.

**Table 5.3.** VFA and lactate concentrations during FHP test at CW:I ratio of 2.5.

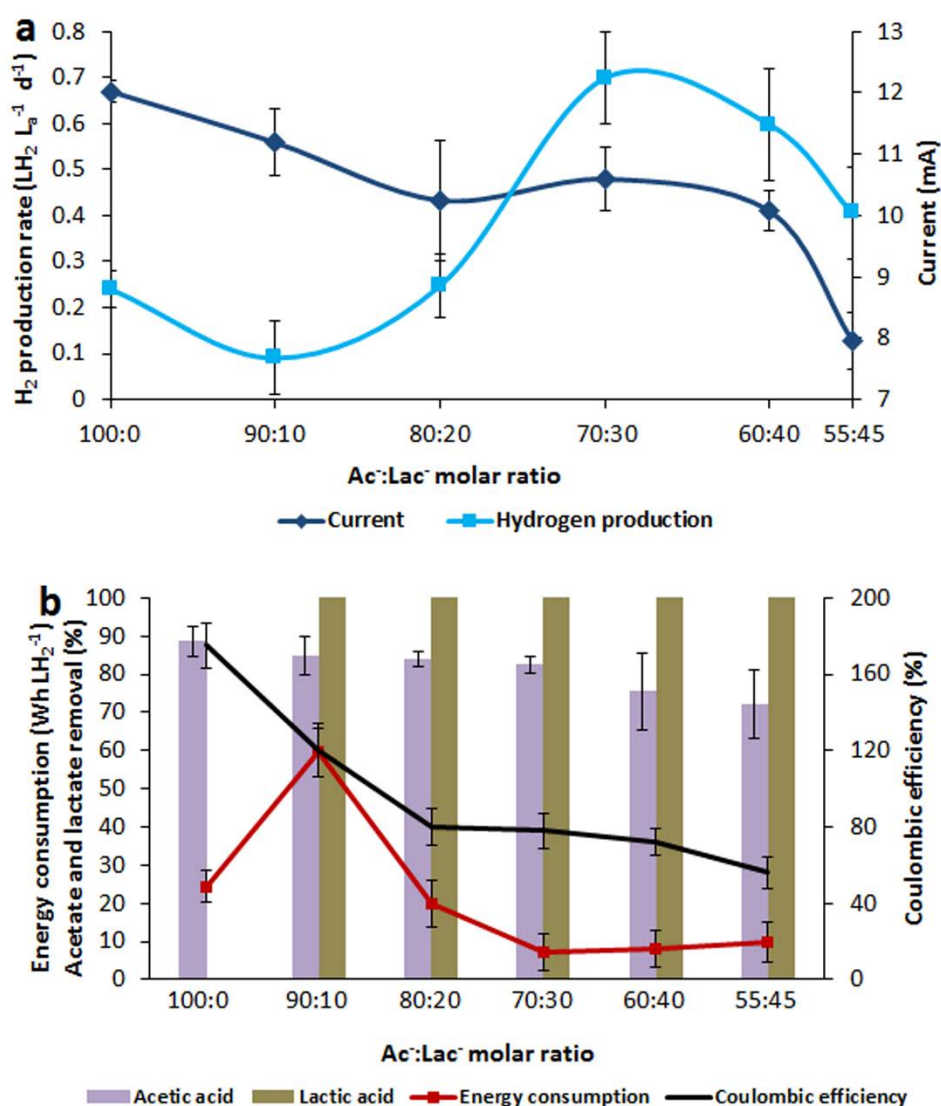
| <b>Time (h)</b> | <b>Acetate (mM)</b> | <b>Propionate (mM)</b> | <b>Butyrate (mM)</b> | <b>Lactate (mM)</b> |
|-----------------|---------------------|------------------------|----------------------|---------------------|
| <b>0</b>        | 1.9 ± 0.2           | 0.0 ± 0.0              | 0.0 ± 0.0            | 7.5 ± 1.2           |
| <b>7</b>        | 6.5 ± 0.2           | 0.9 ± 0.0              | 0.7 ± 0.0            | 28.1 ± 3.4          |
| <b>9</b>        | 9.3 ± 0.3           | 1.6 ± 0.1              | 2.2 ± 0.1            | 36.4 ± 3.2          |
| <b>11</b>       | 10.5 ± 0.4          | 0.8 ± 0.1              | 3.8 ± 0.1            | 48.6 ± 6.3          |
| <b>13</b>       | 13.0 ± 0.5          | 1.1 ± 0.0              | 4.7 ± 0.2            | 64.2 ± 8.5          |
| <b>24</b>       | 12.9 ± 0.4          | 0.9 ± 0.1              | 3.7 ± 0.3            | 99.9 ± 9.6          |

### 5.3.2. Performance of MEC system

#### 5.3.2.1. Effect of lactate addition over H<sub>2</sub> production

The tests were performed on a 50 mL MEC that had been operated for more than 30 days with a synthetic solution containing acetate (Ac<sup>-</sup>) as the only carbon source. It was observed that at OLRs above ~2 g Ac<sup>-</sup> L<sub>a</sub><sup>-1</sup> d<sup>-1</sup> (HRT = 10 h), the electrical current saturated (results not shown) and further increases in the OLR did not improve current density nor hydrogen production. This OLR corresponded to an acetate concentration in the feeding medium of 14.2 mM, therefore being selected as the molar concentration of the carbon source (acetate + lactate) throughout this first set of tests (HRT = 10 h).

Once electrical current and H<sub>2</sub> production rates stabilized, lactate was gradually incorporated in the feeding and the pH was adjusted to  $5.0 \pm 0.4$  to simulate the physicochemical conditions of the effluent of the FHP. Electrical current and hydrogen production declined substantially when the relative molar fraction of acetate and lactate in the feeding (Ac<sup>-</sup>:Lac<sup>-</sup>) was set at 90:10 (Figure 5.3a). This was attributed to the need of the electrogenic microorganisms to adapt to the new feeding conditions (the presence of lactate and low pH). Subsequent reductions in acetate molar fraction stabilized electrical current at 10 mA. In contrast, hydrogen production experienced a notable improvement accompanied by a sharp decline in methane concentration in



**Figure 5.3.** Evolution of: a) Current and H<sub>2</sub> production; b) Acetic and lactic acids removal, Coulombic efficiency and energy consumption for the different Ac<sup>-</sup>:Lac<sup>-</sup> ratios tested.



the off-gas, which might be related to the inhibitory effect of low pH on the methanogenic activity (Table 5.4). Since methane formation in membrane-less MECs is primarily associated with hydrogen consumption by methanogens [16], a diminished methanogenic activity would explain a higher hydrogen recovery in the off gas. At lactate molar proportions of 50% (Ac<sup>-</sup>:Lac<sup>-</sup> 50:50) and above, the performance of MEC declined sharply, stopping H<sub>2</sub> production and current (results not shown).

*Table 5.4. pH and gas composition results for the different Ac<sup>-</sup>:Lac<sup>-</sup> ratios tested.*

| <b>Time (h)</b>         | <b>100:0</b> | <b>90:10</b> | <b>80:20</b> | <b>70:30</b> | <b>60:40</b> | <b>55:45</b> |
|-------------------------|--------------|--------------|--------------|--------------|--------------|--------------|
| <b>pH influent</b>      | 8.50 ± 0.12  | 5.75 ± 0.08  | 5.43 ± 0.21  | 4.99 ± 0.09  | 4.75 ± 0.12  | 4.60 ± 0.06  |
| <b>pH effluent</b>      | 7.47 ± 0.06  | 7.10 ± 0.14  | 6.78 ± 0.17  | 6.48 ± 0.19  | 6.10 ± 0.10  | 6.53 ± 0.15  |
| <b>Log mean pH</b>      | 7.97         | 6.40         | 6.08         | 5.70         | 5.40         | 5.51         |
| <b>% H<sub>2</sub></b>  | 82.9 ± 2.4   | 80.9 ± 1.3   | 78.6 ± 0.8   | 95.7 ± 1.9   | 74.0 ± 2.1   | 73.3 ± 2.4   |
| <b>% CH<sub>4</sub></b> | 17.2 ± 0.1   | 19.1 ± 1.8   | 21.4 ± 2.1   | 4.3 ± 0.1    | 0.0 ± 0.0    | 0.7 ± 0.0    |
| <b>% CO<sub>2</sub></b> | 0.0 ± 0.0    | 0.0 ± 0.0    | 0.0 ± 0.0    | 0.0 ± 0.0    | 26.0 ± 0.0   | 26.0 ± 0.0   |

The occurrence of coulombic efficiencies above 100% (Figure 5.3b) strongly suggests the existence of significant hydrogen recycling, a phenomenon that usually takes place in membrane-less reactors and consists of the anodic reoxidation of part of the hydrogen produced at the cathode. Its main effect is the appearance of a parasitic electrical current that does not result in a net hydrogen production or substrate consumption and only increases artificially the energy consumption. Ruiz et al. [17], claimed that in an acetate-fed membrane-less MEC about 2/3 of the hydrogen recycled was attributable to homoacetogenic activity, which uses hydrogen as the electron source to produce acetate that is then consumed by the anode respiring bacteria. Interestingly, as the molar fraction of lactate increased and the logarithmic mean of the pH inside the anodic chamber declined, the coulombic efficiency dropped accordingly (Table 5.4, Figure 5.3b). This could be related to a metabolic change in the anode respiring bacteria but also to a reduced activity of homoacetogenic bacteria. In fact, and although it is an issue not well studied yet, Luo et al. [18] found that a pH of 5.5 might inhibit homoacetogenic activity. Fig. 3b shows the energy consumption per unit of hydrogen produced at the different Ac<sup>-</sup>:Lac<sup>-</sup> ratios. Except for

the Ac<sup>-</sup>:Lac<sup>-</sup> 90:10, the energy consumption decreased with the mean pH inside the reactor, confirming the existence of a significant hydrogen recycling as pH increases affecting severely the performance of the MEC. Therefore, low pH inside the MEC reactor not only improved hydrogen recovery via reduced methanogenic activity, but might also help to curb the hydrogen recycling and improve the overall performance of the MEC. Nevertheless this is an issue that requires further investigation and needs to be better understood.

The amount of lactate in the effluent of the MEC was always below the detection limit of the HPLC, revealing a total degradation of lactate (Figure 5.3b), although metabolic pathways are still not clear. Direct current production from lactate oxidation is possible in bioelectrochemical reactors [19], yet intermediate lactate conversion to acetate or ethanol cannot be ruled out [20]. As many other microorganisms, *Geobacter* presents the enzyme D-Lactate dehydrogenase (1.1.1.28), a reversible enzyme catalyzing the formation of pyruvate from lactate at high lactate concentrations. Pyruvate produced from lactate may be degraded to acetate afterwards, which would explain the decreasing removal rate of acetate as lactate molar fraction increases.

### 5.3.2.2. Effect of acidified CW over MEC performance

As shown in Figure 5.3, 70:30 is the molar fraction of acetate and lactate that optimizes the MEC performance, therefore been selected and fixed as the composition of the carbon source in the feeding solution during this second set of tests.

Once the performance of the MEC stabilized in terms of current and hydrogen production, the feeding solution was switched to the diluted effluent obtained from the CW fermentation (CW:I = 2.5) amended with acetate (CW-DA) to provide a ratio Ac<sup>-</sup>:Lac<sup>-</sup> similar to the optimum found in the previous section (Figure 5.4). This caused a gradual decrease in current and hydrogen production that collapsed when acetate was totally removed from the feeding (CW-D). To recover previous performance it was needed to restore the initial synthetic feeding solution, requiring 3-5 days for current density to regain 10 mA.

Since the CW fermented effluent was 8 times diluted before being fed to the MEC, it was hypothesized that the decline and collapse observed was related to the lack of salts. To test this hypothesis, we added some salts to the feeding to provide a concentration similar to that of the synthetic feeding. This new amended effluent (CW-DAS) was fed to the MEC with no observable

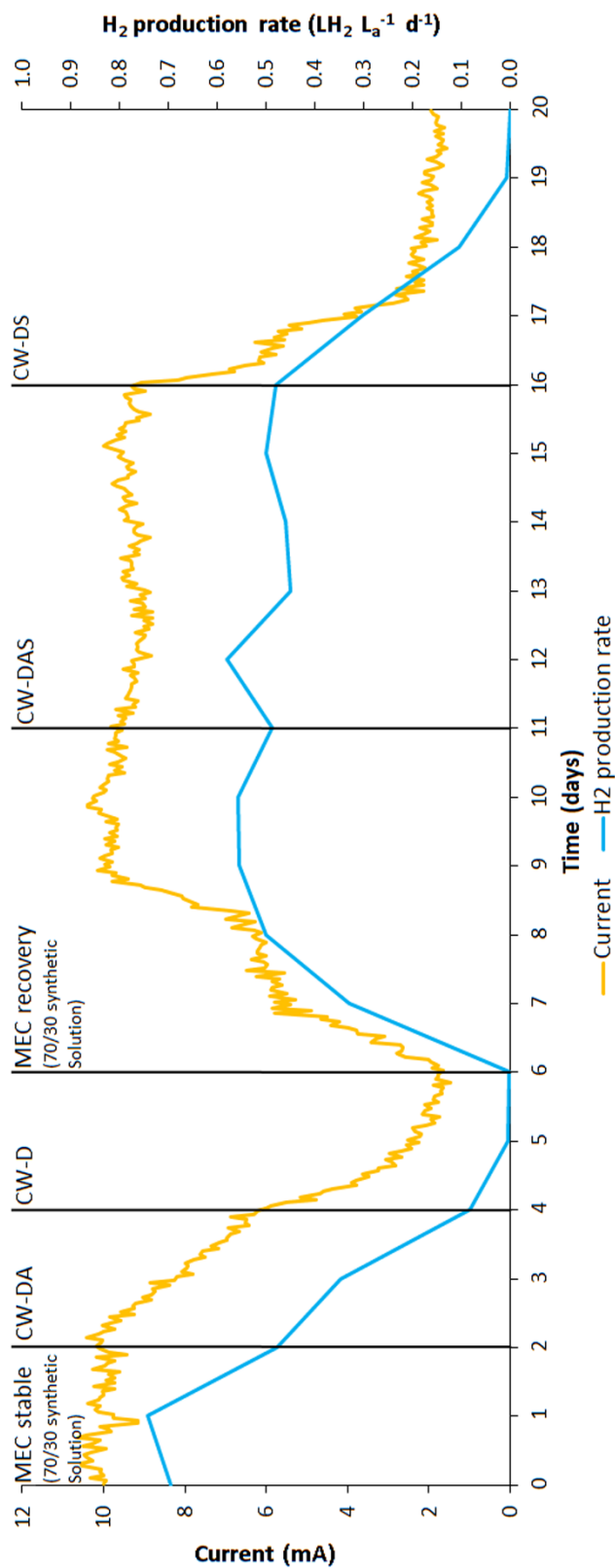


Figure 5.4. Evolution of current and H<sub>2</sub> production with the different substrates fed to the MEC.

changes in the current or H<sub>2</sub> production rate, achieving a COD removal rate of 82%. In the final stage of this experimental section, acetate was removed from the feeding solution (CW-DS), which caused a sharp decline in the electrical current leading hydrogen production to a collapse. Since other authors have reported safe use of lactate as the only carbon source in MFCs and MECs at concentrations of 10-20mM [19, 21], it seems that acetate is playing a crucial role to sustain the electrogenic activity in our MEC. In any case, regardless of the presence of acetate or salts in the feeding, the concentration of lactate in the effluent was below the detection limit of the HPLC, suggesting fast degradation inside the anodic chamber.

### 5.3.3. Prospects for CW treatment through a two-stage process (FHP + MEC)

During the FHP experimental phase, the 250 mL Erlenmeyer flasks produced  $178 \pm 2$  mL of H<sub>2</sub> within the first 11 h (at a CW:I ratio of 2.5), which represents a volumetric H<sub>2</sub> production of  $0.7 \text{ L H}_2 \text{ L}_r^{-1}$ . 90% of the volume of the effluent was obtained as supernatant after centrifugation and fed to the MEC where yielded a total hydrogen production of 1.5 L H<sub>2</sub> (HRT of 10 h, and H<sub>2</sub> production rate of  $0.5 \text{ L H}_2 \text{ L}_a^{-1} \text{ d}^{-1}$ ). Therefore, the global hydrogen production from the original 200 mL of CW in the FHP and MEC amounted to 2.2 L H<sub>2</sub>, corresponding to a H<sub>2</sub> yield of  $94.2 \text{ L H}_2 \text{ kg}_{\text{vs}}^{-1}$ , which is well above the H<sub>2</sub> yields of CW fermentation reported in literature ( $47.9\text{-}78.2 \text{ L H}_2 \text{ kg}_{\text{COD}}^{-1}$ ) [13,22]. However, we have to keep in mind that to achieve this H<sub>2</sub> yield the FHP effluent needed to be diluted and amended with salts and acetate before being fed to the MEC and therefore, some of the H<sub>2</sub> thus produced would correspond to the externally-added acetate. Hence, and from a practical point of view, the use of a MEC as second stage treatment to the FHP of CW might not be economically or environmentally feasible. Nevertheless, other waste streams produced in the facility could help in these circumstances. For instance, the effluent from the FHP of CW could be diluted with the cleaning and sanitary sewer wastewaters or even with process effluents from nearby industries thus obtaining a feeding to MEC with a composition similar to that that optimized its performance.

### 5.3.4. Microbial community analysis

DNA samples collected from the anode of the MEC were analyzed at the end of the experiment. As expected, electrogenic bacteria such as *Geobacter thiogenes* and *Shewanella*

*oneidensis* were found to be attached to the carbonous anode, suggesting they were the main responsible for electricity production. In contrast, the microbial communities present in MEC effluents changed throughout the different stages of the experiment (Table 5.5). During the first stage (100:0 Ac<sup>-</sup>:Lac<sup>-</sup>), a microbial population based on the genera *Comamonas* (phylum  $\beta$ -Proteobacteria) and *Streptomyces* (phylum Actinobacteria) dominated in the electrolytic device. However, when the feeding solution was switched to 70:30 (Ac<sup>-</sup>:Lac<sup>-</sup>), *Bacillus* and *Lactobacillus* (phylum Firmicutes), and *Vibrio* and *Pseudomonas* (phylum  $\gamma$ -Proteobacteria) prevailed as the dominating genera. All these genera, as well as *Comamonas* and *Streptomyces* have been reported to have the enzyme D-Lactate dehydrogenase [23-25], which has a role in the conversion of lactate to acetate.

**Table 5.5.** DGGE 16S rDNA band identifications.

| Stage   | GenBank closest match               | Identity (%) | Class               |
|---|-------------------------------------|--------------|---------------------|
| <b>Synthetic solution<br/>(100:0, Ac<sup>-</sup>:Lac<sup>-</sup>)</b> | <i>Comamonas aquatica</i>           | 92           | Betaproteobacteria  |
|   | <i>Comamonas denitrificans</i>      | 89           | Betaproteobacteria  |
|   | <i>Comamonas kerstersii</i>         | 96           | Betaproteobacteria  |
|   | <i>Streptomyces viridosporus</i>    | 94           | Actinobacteria      |
|   | <i>Streptomyces rochei</i>          | 83           | Actinobacteria      |
| <b>Synthetic solution<br/>(70:30, Ac<sup>-</sup>:Lac<sup>-</sup>)</b> | <i>Bacillus ginsengihumi</i>        | 88           | Bacilli             |
|   | <i>Bacillus subtilis</i>            | 88           | Bacilli             |
|   | <i>Pseudomonas fluorescens</i>      | 90           | Gammaproteobacteria |
|   | <i>Pseudomonas monteilii</i>        | 92           | Gammaproteobacteria |
|   | <i>Vibrio areninigræ</i>            | 95           | Gammaproteobacteria |
|   | <i>Lactobacillus acidophilus</i>    | 88           | Bacilli             |
| <b>CW-DAS</b>   | <i>Lactobacillus perolens</i>       | 100          | Bacilli             |
|   | <i>Clostridium aurantibutyricum</i> | 86           | Clostridia          |
|   | <i>Clostridium beijerinckii</i>     | 99           | Clostridia          |
|   | <i>Shewanella oneidensis</i>        | 89           | Gammaproteobacteria |
|   | <i>Geobacter thiogenes</i>          | 89           | Deltaproteobacteria |

Finally, when the MEC was fed with the diluted supernatant of the FHP amended with salts and acetate (CW-DAS), the genus *Clostridium* was detected. As *Clostridium* is one of the microorganisms involved in FHP, it is not surprising that some spores may have entered the electrocatalytic device with the feeding inflow.

#### 5.4. CONCLUSIONS

A two-stage process (fermentative + biocatalyzed electrolysis) was evaluated for H<sub>2</sub> production and organics removal from an industrial waste stream (cheese whey). In the first stage, the cheese whey was fermented under batch conditions producing 0.7 L H<sub>2</sub> L<sub>r</sub><sup>-1</sup> and obtaining an acidified effluent. This effluent was used as a feeding for the second stage process. Dilution and amendment of this acid stream with salts and acetate was needed before being fed into the bioelectrochemical system. The H<sub>2</sub> production rate in this second stage amounted to 0.5 L H<sub>2</sub> L<sub>a</sub><sup>-1</sup> d<sup>-1</sup>. The low pH of the fermentation effluent seemed suitable to mitigate methanogenic activity and prevent homoacetogenic H<sub>2</sub> consumption in the bioelectrochemical system, therefore improving the overall performance.

#### Acknowledgements

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# *Chapter 6*

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*Mitigation of VFAs build-up  
during AD overload  
through the integration  
of bioelectrochemical  
electrodes*

**Submitted to Waste Management**  
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*Chapter prepared according to the guidelines of Waste Management*



**Abstract**

Both anaerobic digestion and bioelectrochemical systems have a great potential as a technology for energy recovery from waste streams, and their mutual integration can help to overcome some of the hurdles associated to them. However, their implementation in the same reactor has not been widely evaluated yet. In this study we explore the benefits of integrating an electrogens-enriched electrode inside a batch-operating anaerobic digester undergoing inhibition by excessive VFA accumulation. An increased CH<sub>4</sub> production at the early stages of the operational periods for the electrode-containing reactors was observed. Besides, the presence of the electrodes resulted in a delay in VFA build-up, indicating an improvement in VFA removal. Interestingly, these effects seemed to be based on biomass proliferation rather than electrochemical activity.



## 6.1. INTRODUCTION

Due to its flexibility and robustness as a technology, anaerobic digestion (AD) has become the main biological process for biogas production from wastewater (Carlsson et al., 2012). Besides, AD appears as an alternative to aerobic processes for high-strength organic wastewater treatment, due to lower energy demands as it does not require aeration (Martin et al., 2011). As a result, the number of anaerobic digesters installed world-wide has recently increased (Tartakovsky et al., 2011).

However, AD also presents vulnerabilities. When the composition of the wastewater stream is based on simple organic molecules, the high sensitivity of methanogenic microbiota to acidic conditions and its slow growth rate may result on the accumulation of volatile fatty acids (VFA). Acetate production from  $H_2$  by homoacetogens (Angenent et al., 2004) also contributes to VFA accumulation, subsequently causing a collapse of the anaerobic process (Ward et al., 2008). Hence, the improvement of VFA degradation rates becomes a key issue for maintaining stable operation in anaerobic systems (Zhang et al., 2013).

Bioelectrochemical systems (BESs) represent a more recently developed alternative to conventional wastewater treatments (Sleutels et al., 2012). Microbial electrolysis cells (MECs) in particular are one of the most promising bioelectrochemical devices (Zhang & Angelidaki, 2014). In MECs, exoelectrogenic microorganisms, such as *Geobacter* or *Shewanella*, participate in the degradation of organic matter, which with the aid of a small electrical input can be converted into valuable products (e.g.  $H_2$  or  $CH_4$ ) in an anaerobic environment. MECs can work with a wide range of substrates, and VFA-rich waste streams represent an ideal feeding, since most of this organic compounds can be readily converted to electricity by electrogens. This ability has been exploited to explore the possibility of using MEC as a post-treatment for biological process, especially fermentative processes, where due to thermodynamical limitations the concentrations of VFAs is usually high (Yang et al., 2013; Moreno et al., 2015).

Moreover, fermentative processes and BES have been integrated within the same reactor, which represents a significant improvement since it avoids the need of two separate stages, reduces the operational costs of the processes and energy losses and maximizes substrate utilization (ElMekawy et al., 2014). This combined approach has been evaluated for the treatment of different substrates, such as dye wastewater (Cui et al., 2014), waste activated sludge (Asztalos et al., 2015;

Liu et al., 2016), molasses (De Vrieze et al., 2014) and synthetic wastewaters (Zhang et al., 2015; Cai et al., 2016). However, these studies have been performed at non-inhibitory organic loads, and offer no information on how BES/AD integration behaves when VFAs start to accumulate. This is an important issue since the integration of fermentative and bioelectrocatalytic processes may contribute to the stabilization of the anaerobic digester (through the consumption of the excess of VFA by electrogens). To our knowledge this integrative configuration has not been evaluated at inhibitory VFA concentrations.

In this work we integrate an electrogens-enriched electrode within an anaerobic digester where the digestion process is prone to undergo acidic inhibition. We assess the impact of the integrated electrode on the evolution of the process in terms of VFAs profiles, electric charge and CH<sub>4</sub> production. We also propose several adaptation strategies for the electrogenic anodophilic biomass to the conditions inside the digester.

## **6.2. MATERIAL AND METHODS**

### **6.2.1. Reactor design**

As stated above, the aim of this study was to describe the role of the integration of electrogens-enriched electrodes in an anaerobic reactor where the AD process is undergoing inhibition because of an excessive VFA accumulation. In order to define these acidic inhibition conditions, biodegradability tests of glucose with inoculum were prepared in duplicate and evaluated. Digested sludge from the WWTP of León, Spain, was used as the inoculum. This sludge presented 1.7% TS and 1.2% VS (see Table 6.1 for further characterization of the inoculum). Glucose was the substrate for anaerobic digestion. In order to achieve inhibitory acidification conditions, we tried a VS-substrate/VS-inoculum ratio of 2, based on previous experiences in our lab. We also tested the VS-substrate/VS-inoculum ratio of 0.25 to quantify the differences between both ratios in terms of acidification. The biodegradability tests were carried out in continuously-stirred 250-mL Erlenmeyer flasks. A thermostatic bath was used to maintain the temperature at 25 °C.

The integration of anaerobic digestion and bioelectrochemical systems was done in 3-L methacrylate reactors. A glucose solution was mixed with the digested sludge so as to maintain



the defined VS-substrate/Vs-inoculum ratio. The reactors worked in batch mode, and total volume in the reactor was 2 L. The systems were continuously-stirred and maintained at 25 °C by means of a thermostatic jacket. Three reactors were used for the experiment. The reactors were equipped with connections for gas exits and liquid entries and exits, and two of them contained the electrolytic modules (anode + cathode) submerged in the feeding stock. Reactor AD (anaerobic digestion) consisted on a conventional anaerobic reactor, and was used as the control for the others. The other anaerobic digestion reactors were equipped with two pairs of biological electrodes. One of them worked in open-circuit mode (OC reactor), whereas a voltage of 0.9 V was applied to the other one (closed-circuit mode, CC reactor).

**Table 6.1.** Characterization of the inoculum used in this experiment.

| Parameter   | Inoculum      |
|---|---------------|
| pH  | 7.36          |
| Organic matter (%)                                | 1.12 ± 0.05   |
| Total nitrogen (%)                                | 0.21 ± 0.01   |
| C/N ratio   | 3.15          |
| Total solids (g L <sup>-1</sup> )                 | 16.71 ± 0.80  |
| Volatile solids (g L <sup>-1</sup> )              | 11.93 ± 0.59  |
| NH <sub>4</sub> <sup>+</sup> (g L <sup>-1</sup> ) | 1.13 ± 0.05   |
| Alkalinity (g-CaCO <sub>3</sub> L <sup>-1</sup> ) | 2.32 ± 0.10   |
| VFA (g L <sup>-1</sup> )                          | 0.50 ± 0.001  |
| Acetate (g L <sup>-1</sup> )                      | 0.030 ± 0.001 |
| PO <sub>4</sub> <sup>3-</sup> (ppm)               | 440 ± 13      |
| Ca <sup>2+</sup> (ppm)                            | 576 ± 17      |
| Mg <sup>2+</sup> (ppm)                            | 108 ± 3       |
| K <sup>+</sup> (ppm)                              | 232 ± 7       |
| Na <sup>+</sup> (ppm)                             | 48.4 ± 1.0    |
| Mn (ppm)  | 3.4 ± 0.1     |
| Fe (ppm)  | 263 ± 7       |
| Cu (ppm)  | 3.4 ± 0.1     |
| Zn (ppm)  | 17.1 ± 0.5    |

### 6.2.2. Electrode preparation and operation

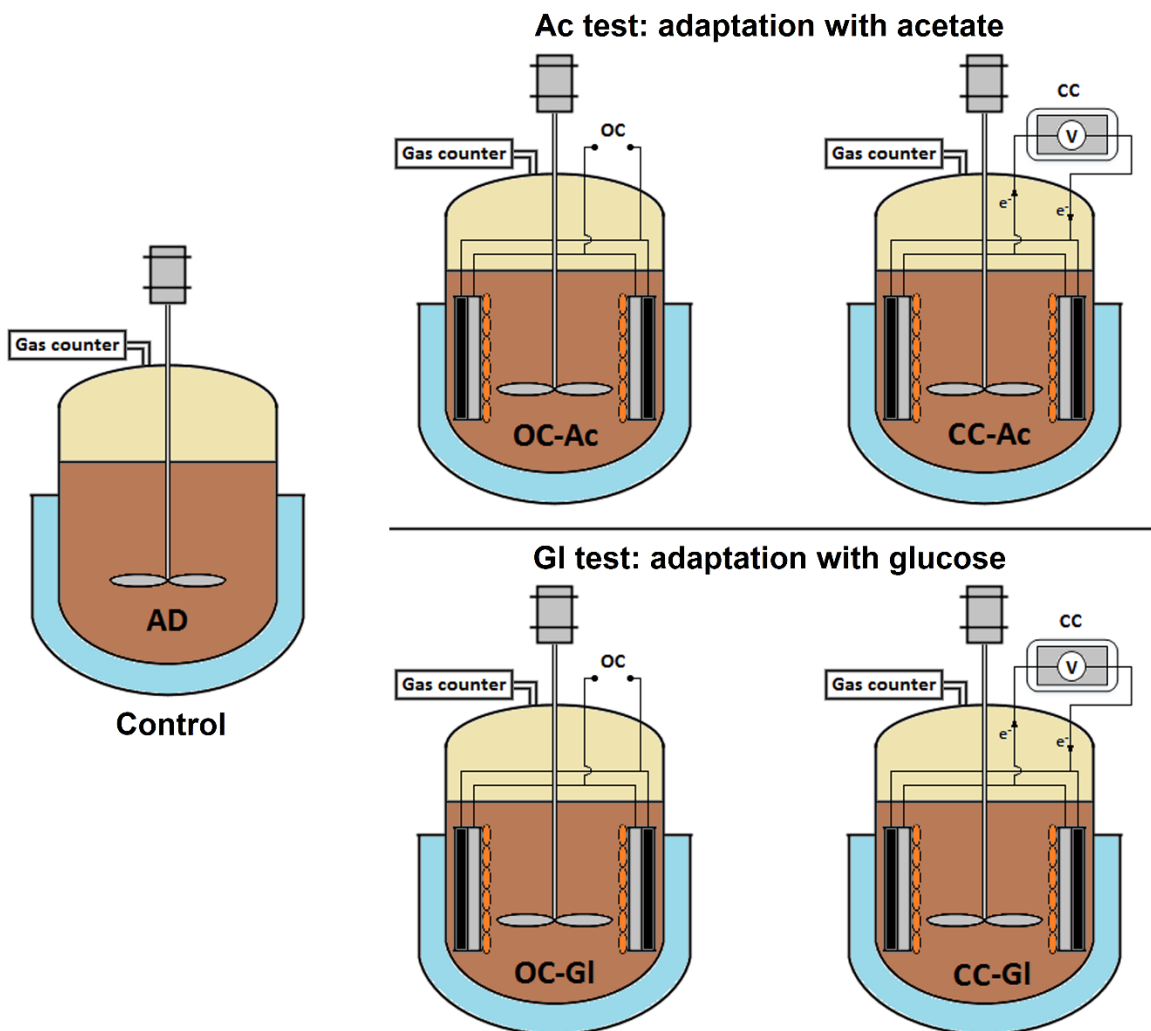
Electrodes for reactors OC and CC were prepared with two layers of carbon felt 1-cm-thick (Sigratherm soft felt GFD 2, SGL Carbon Group, Wiesbaden, Germany) as the anode; the cathode was a titanium electrode, both with dimensions of 100 x 100 mm. One 0.6-mm-thick piece of polyester cloth was sandwiched between the anode and the cathode to avoid any electrical contact between the two electrodes. The inter-electrode separation was set to 1 mm.

Evaluation of anaerobic digestion-bioelectrochemical systems integration performance was done under different protocols to adapt electrogens to system conditions: in a first approach, the electrodes had been previously acclimatized with an applied voltage of 0.9 V in a salt solution. The composition of this salt solution has been defined elsewhere (Moreno et al., 2016). The carbon source was sodium acetate (500 mg-Ac L<sup>-1</sup>). All solutions were filter sterilized and stored at 4 °C to prevent microbial growth. Distilled water was used for solution preparation, and the chemicals and reagents used were of analytical grade. At the next approach tested electrodes were acclimatized with the same digested sludge used in the reactors, again with sodium acetate as carbon source (500 mg-Ac L<sup>-1</sup>), and at 0.9 V (acetate test). Acetate was chosen for the adaptation protocol as it is considered one of the preferred substrates for electrogenic populations. Finally, another pre-acclimation protocol tested consisted on the electrodes being pre-adapted to the glucose in the reactor: in order to do so, the same digested sludge was used, but the carbon source was a growing concentration of glucose, with no acetate (glucose test). Therefore, in this case adaptation and operating conditions were as similar as possible. As in the previous pre-adaptation protocols, a voltage of 0.9 V was applied. Acetate (Ac) and glucose (Gl) tests were performed in duplicate. In this text we refer to each experiment condition as follows: AD for the anaerobic digestion (control) reactor; OC-Ac and CC-Ac for the open-circuit and applied voltage reactors pre-adapted with acetate, respectively; and finally, OC-Gl and CC-Gl for the open-circuit and applied voltage reactors pre-adapted with glucose, respectively (Figure 6.1). The nomenclature “electrode-integrated digesters” is also used throughout the text to refer to OC-Ac, CC-Ac, OC-Gl and CC-Gl as a whole. As explained below, the initial test presented poor results, therefore it is not represented in Figure 6.1.

A 16 Ω resistor was added to the circuit for on-line current measurements at 10 min intervals using a data acquisition system (National Instruments PCI-6221). The same interval was

used to record gas flow rates, which was measured with an MGC-1 milli-gas counter (Ritter Co, Bochum, Germany). An adjustable DC power supply (BK Precision 9120) maintained the applied voltage at the pre-set level ( $V_{app} = 0.9V$ ).

Integrated systems performance was assessed in terms of: (i) specific  $CH_4$  production ( $L-CH_4 \text{ kg-SV}^{-1}$ ); (ii) current production (A); and (iii) VFA removal rates (%).



**Figure 6.1.** Schematic view of the reactors used in this study: AD (conventional AD), OC-Ac and OC-Gl (open-circuit mode pre-adapted with acetate and glucose, respectively) and CC-Ac and CC-Gl ( $V_{app} = 0.9 V$ , pre-adapted with acetate and glucose, respectively).

### **6.2.3. Analytical measurements and calculations**

Gas composition and volatile fatty acids (VFA) were analyzed as described by Martínez et al. (2012). Conductivity and pH were determined in accordance with the methods described by the APHA methods (APHA, 2005) and were regularly quantified during the anaerobic digestion/electrocatalytic process. Total solids (TS) and volatile solids (VS) were determined in accordance with the APHA methods (APHA, 2005). Total organic carbon, total carbon and total nitrogen content were determined using a total organic carbon analyzer (multi N/C 3100, AnalytikJena).

## **6.3. RESULTS AND DISCUSSION**

As stated above, the aim of this study is to assess the impact of integrating an electrogens-enriched electrode within an anaerobic digester where the digestion process is prone to undergo acidic inhibition. These acidic conditions were forced by imposing a high substrate (glucose) concentration, and thus, preliminary tests were performed to determine the most appropriate ratio VS-substrate/Vs-inoculum to induce acidification (Table 6.2). Results showed that specific CH<sub>4</sub> production for ratio 0.25 was around the theoretical CH<sub>4</sub> production from glucose, whereas it decreased dramatically for ratio 2, indicating AD inhibition. Moreover, total VFA values for ratio 2 were 20 times higher than those of ratio 0.25. Therefore the ratio 2 (VS-substrate/Vs-inoculum) was selected to induce acidification throughout the rest of the experiment.

When being fed with the selected ratio, all reactors (including those that integrated the electrode) underwent rapid acidification and negligible amounts of CH<sub>4</sub> were produced. Moreover, the AD reactor (control) succeeded in recovering partially, but only after 60 h, whereas the electrode-integrated digesters did not resume CH<sub>4</sub> production. This led us to hypothesize that the poor performance observed in the electrode-based reactor could be attributed to a lack of proper adaptation of the anodic microorganisms to the operating conditions within the AD reactors (note that the anodes used in this first test were grown with a synthetic effluent using acetate as substrate). Therefore, we devised alternative adaptation strategies. The first approach was to re-inoculate and grow the bioanodes with anaerobic sludge amended with acetate. Once the current stabilized, the anodes were re-assembled and transferred to the electrode-integrated digesters: OC-

Ac (operated in open circuit) and CC-Ac (operated in applied voltage conditions). An alternative strategy was to inoculate and grow the bioanodes with anaerobic sludge but this time amended with glucose. Again, once current stabilized, these bioanodes were reassembled and transferred to the electrode-integrated digesters: OC-Gl (operated in open circuit) and CC-Gl (operated in applied voltage conditions). For more information on the acclimation procedures, we refer the reader to section 2.

In the following sections, CH<sub>4</sub> production, and VFA profiles were evaluated in order to assess the impact of integrating AD and electrogenic electrodes, as well as the effect of the pre-adaptation protocols on reactors performance. Data obtained from the test with unadapted anodes (results described above) will not be considered for the rest of the discussion.

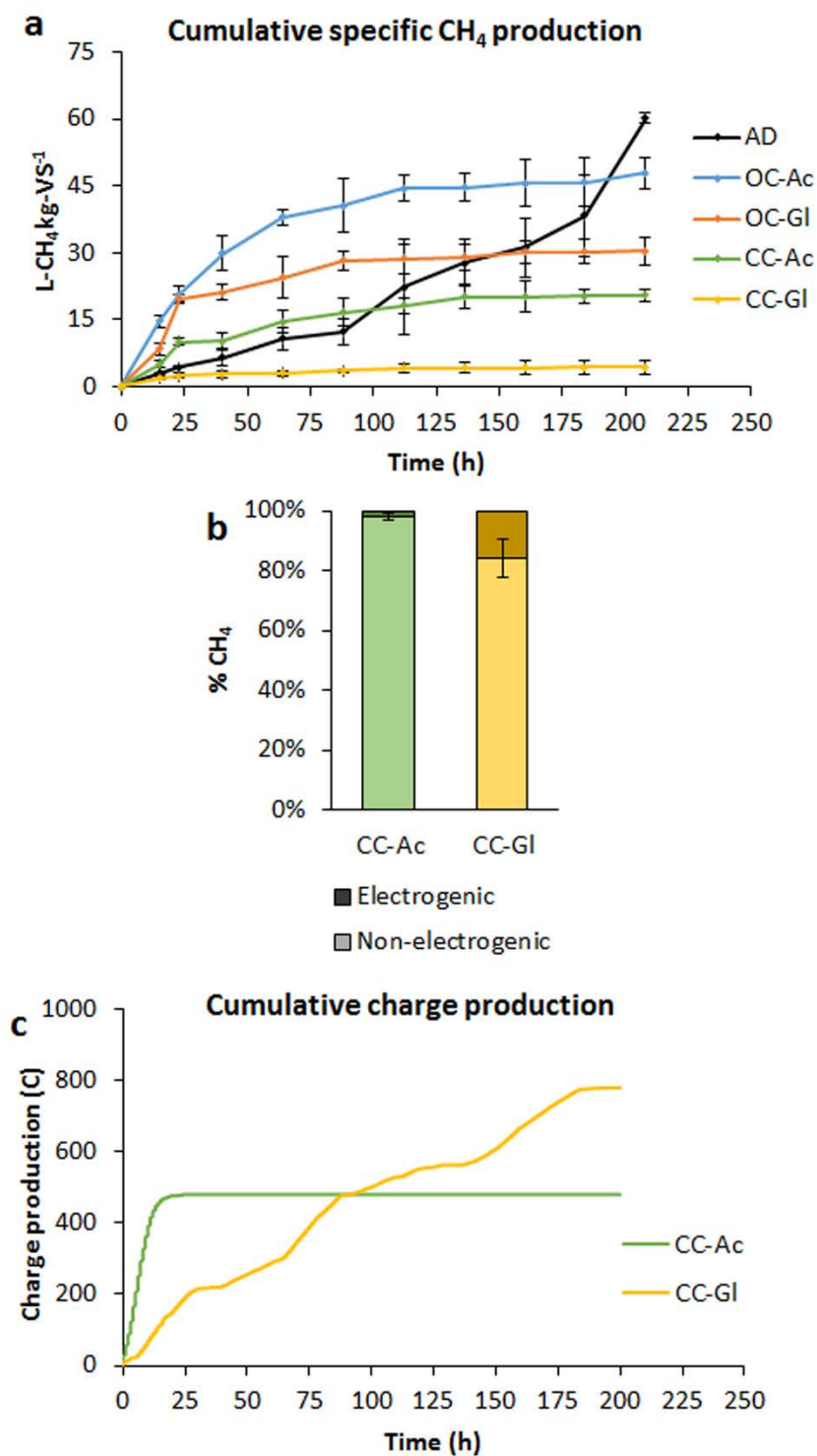
**Table 6.2.** Specific CH<sub>4</sub> production and total VFA production for glucose biodegradability tests (ratios 0.25 and 2, VS-substrate/Vs-inoculum). Production profile of acetate, butyrate and propionate are presented for ratio 2 (n.d. = no data).

| Time (h) | Specific CH <sub>4</sub> production<br>(L-CH <sub>4</sub> kgsv <sup>-1</sup> ) |         | VFA production (mg L <sup>-1</sup> ) |         |         |          |            |
|----------|--|---------|--------------------------------------|---------|---------|----------|------------|
|          | Ratio 0.25   | Ratio 2 | Ratio 0.25                           | Ratio 2 | Acetate | Butyrate | Propionate |
|          |  |         | Total                                | Total   |         |          |            |
| 0        | 0 ± 0  | 0 ± 0   | 37                                   | 42      | 36      | 6        | 0          |
| 15       | 105 ± 15   | 11 ± 3  | 225                                  | 1560    | 491     | 1043     | 18         |
| 23       | 153 ± 24   | 23 ± 2  | 170                                  | 2988    | 820     | 2139     | 24         |
| 40       | 234 ± 22   | 30 ± 10 | 240                                  | 3814    | 1190    | 2372     | 173        |
| 64       | 240 ± 15   | 34 ± 2  | 4                                    | 4513    | 1496    | 2415     | 364        |
| 88       | 245 ± 17   | 37 ± 14 | 3                                    | 5576    | 1902    | 2942     | 356        |
| 112      | 253 ± 15   | 39 ± 0  | n.d.                                 | n.d.    | n.d.    | n.d.     | n.d.       |
| 136      | 268 ± 15   | 43 ± 16 | 84                                   | 6458    | 2300    | 3280     | 415        |
| 160      | 283 ± 15   | 47 ± 16 | 6                                    | 6383    | 2298    | 3325     | 413        |
| 184      | 293 ± 25   | 48 ± 18 | n.d.                                 | n.d.    | n.d.    | n.d.     | n.d.       |
| 208      | 299 ± 15   | 50 ± 15 | n.d.                                 | n.d.    | n.d.    | n.d.     | n.d.       |

### 6.3.1. Methane production and current profiles

The reactors displayed very different behaviours from the beginning of the tests. On the one hand, specific CH<sub>4</sub> production (Figure 6.2a) was higher in the electrode-containing reactors, at least during the first 100 h of the experiments (except for the CC-Gl test). Although methane production continued to grow steadily in the AD reactor, and become stagnant in all the rest after this first stage, these results offered us the first indication of the positive role of implementing a BES in an acidified anaerobic reactor. Interestingly, methane production increased more rapidly in those reactors operated in open circuit conditions (OC-Ac and OC-Gl), suggesting that direct interspecies electron transfer (DIET) rather than cathodic methane/hydrogen production was the most probable mechanism for electron exchange in the system (Rotaru et al., 2014). In fact, the theoretical maximum amount of methane calculated from the electric charge reaching the cathode in the applied voltage reactors (Figure 6.2b), represented a small fraction if compared with the total amount of methane measured. These results suggest that the introduction of the electrodes allowed for an enhancement of methanogenic processes as a consequence of better biomass proliferation, rather than voltage application, in accordance with previous results reported by De Vrieze et al. (2014).

In spite of this low charge production, it was observed that the electrodes adapted to acetate (CC-Ac) produced charge at a faster rate during the first hours of the test, although it rapidly become stagnant (Figure 6.2c), probably as a result of a lack of proper adaptation of anodophilic microorganisms. In contrast, the glucose adapted reactors produced electrical charges at lower rate, but steadily throughout the entire tests, which may indicate that adaptation to glucose prevents osmotic shock and subsequent failure of electrogenic processes, producing more stable anodes than adaptation to acetate.

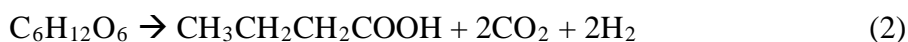


**Figure 6.2.** (a) Specific  $\text{CH}_4$  production for reactors. (b) Theoretical  $\text{CH}_4$  production by electromethanogenic processes. (c) Current consumption profiles of CC reactors.

### 6.3.2. Volatile fatty acids analysis

Figure 6.3a presents the total VFAs concentration profiles for all the reactors. It clearly shows that regardless of the operation mode of the electrogenic-based reactors, the presence of a bioanode helped to maintain low the concentration of VFAs (at least during the first 100 h of the tests) compared to AD reactor. This result agrees with the results discussed in the previous section, where methane production improved in electrogenic-based digesters both for open-circuit (OC) and close-circuit (CC) conditions.

According to our view, two possible situations can explain this attenuation in the build-up of VFAs, namely: a) no VFAs are being produced in electrode-integrated digesters, and b) VFAs are being produced but they are rapidly degraded by anodophilic microorganisms so they cannot be measured. If the first hypothesis were true, the amount of CO<sub>2</sub> production should be stoichiometrically correspondent with VFA on a theoretical basis, according to equations 1 and 2 (i.e.: if VFA levels are low, CO<sub>2</sub> production should be low as well). Conversely, if the second hypothesis were true there should be no direct correspondence between CO<sub>2</sub> production and VFA concentrations. The stoichiometry of glucose degradation reactions to acetate and butyrate is:



Therefore, to identify the most likely explanation we performed mass balances both for the AD and electrode-integrated digesters (Figure 6.4). Since all electrode-integrated digesters behaved in a similar way (i.e., attenuated VFAs accumulation), we selected the OC-Ac test as a representative for the sake of simplicity and compared it to the AD. It can be observed that, on the first stages of the experiment, CO<sub>2</sub> production in electrode-integrated digesters is much higher than the control, resulting in CO<sub>2</sub> production not being stoichiometrically correspondent with VFA production for electrode-integrated digesters. We calculated the ratio between the real CO<sub>2</sub> measured and the theoretical CO<sub>2</sub> expected from the observed VFA production. It can be seen that, for electrode-integrated reactors at the first stages of the tests, CO<sub>2</sub> production is much higher than



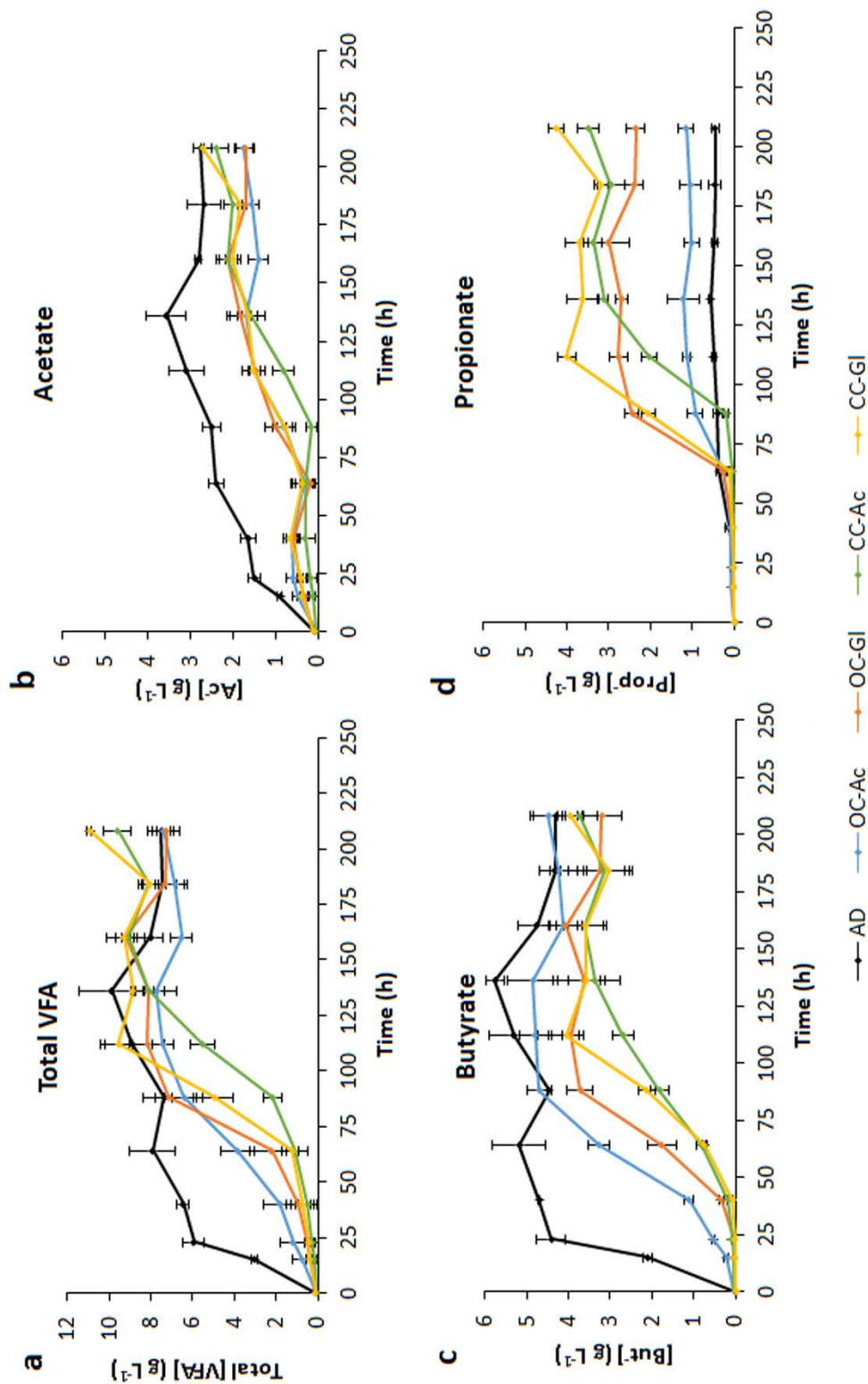
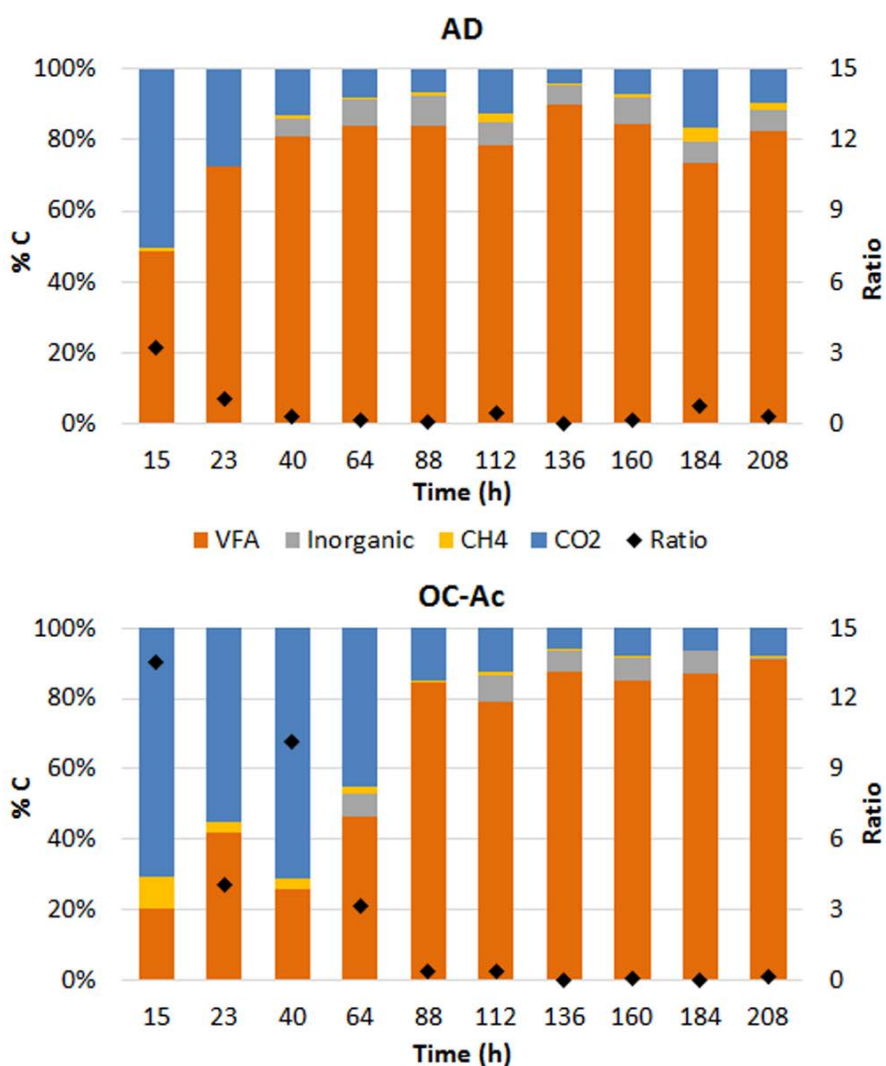


Figure 6.3. (a) Total VFA production data for all the conditions tested. (b) Acetate, (c) butyrate and (d) propionate values are also presented.

## 6. INTEGRATION OF BIOELECTROCHEMICAL ELECTRODES-ANAEROBIC DIGESTION

the theoretically expected, suggesting that more VFA are being produced, but not detected because of them probably being degraded immediately by anodophilic microorganisms, which would support the second hypothesis. In fact, the AD reactor has a better correspondence between real and theoretical production, and also higher VFA levels in absolute terms, which would be due to the absence of electrogenic population.



**Figure 6.4.** Left y-axis: proportion of degraded carbon destined to different compounds: VFA, inorganic carbon, CH<sub>4</sub>, CO<sub>2</sub>. Right y-axis: ratio between the real CO<sub>2</sub> production measured and the theoretical CO<sub>2</sub> production on a stoichiometric basis after VFA production.

From Figures 6.3b and 6.3c, we can see that the presence of anodes inside the electrode-integrated digesters helped to keep low (again, at least during the first stage of the test) the levels of butyrate, but specially the levels of acetate. Acetate (Figure 6.3b) follows a similar pattern for all the electrode-integrated digesters (regardless of the operation mode or the adaptation process), whereas much higher concentrations are present in AD reactor. This suggests that the mere presence of the electrode inside the reactor has an effect over acetate degradation rates, irrespective of voltage application. On the other hand, evolution of butyrate concentrations (Figure 6.3c) shows differences between OC and CC reactors: the OC-tests behave more similarly to the control AD reactor, whereas butyrate concentrations keep low for longer at the CC-tests. Therefore, it seems that the application of voltage to the electrode presents a certain impact on butyrate concentrations, although this effect is comparatively less relevant than the impact of the presence of the electrogens-enriched electrode.

In contrast to butyrate and acetate, propionate concentration increased more rapidly in the electrode-integrated digesters; and for most of the tests, its levels were well above those found in the AD reactor (Figure 6.3d). It is important to note that propionate begins to accumulate coinciding more or less in time with the stagnation of methane production, which suggests that the poor performance observed in the electrode-integrated digesters is most probably connected to propionate build-up. In fact, and as reported by Ma et al. (2009), accumulation of propionate to values higher than  $1.5 \text{ g L}^{-1}$  results in the inhibition of  $\text{CH}_4$  production. Interestingly, OC-Ac, the reactor with lower levels of propionate (aside from the control reactor) also produced more methane (Figure 6.2a), which further supports the strong relation between propionate concentration and reactor performance.

Figure 6.3d also shows that acetate adapted bio electrodes seem to delay and even reduce the levels of propionate accumulation in comparison to glucose adapted systems. On the other hand, glucose pre-growth allowed for better electrogenic characteristics, which indicated that adaptation of electrogens to operational conditions prior to the introduction of the electrodes would allow for a better response.

As a whole, it can be stated that the integration of electrogens-enriched electrodes inside an anaerobic reactor undergoing inhibitory acidification conditions results in an increase of  $\text{CH}_4$  production and a delay in VFA accumulation during the initial stage of the operation.

### **6.3.3 Outlook and future perspectives**

In view of the results here presented, it can be said that the integration of an electrogens-enriched electrode has some positive effects on the performance of an overloaded digester. From an operational point of view, the fact that CH<sub>4</sub> production is increased and VFA levels are maintained low during the first 100 h of the test provide a wide range of alternatives when working in continuous mode at short HRTs (so as to prevent VFA build-up). Thus, further studies in continuous mode have to be tested in order to evaluate the effect of HRT over the response. Moreover since the ratio between the electrodes surface and the reactor volume in our experiments has not been optimized, there might be space for improvement: it is reasonable to think that an increment in electrodes surface area would bring an improved performance (more biogas production and faster VFAs consumption) as a result of a higher presence of anodophilic microorganisms.

However, there still remains the question why propionate production and accumulation is more intense in the hybrid reactors. Given that high propionate concentrations may inhibit AD processes and cause system failure, future investigations should be oriented to identify the mechanisms behind this propionate build-up in electrode-based reactors, and develop strategies to avoid its accumulation. In this context, a mixed adaptation process both with acetate and glucose appears as a suitable alternative. Finally, testing new adaptation protocols with different raw substrates instead of glucose would be of particular importance, in the light of the impact of the bioanodes adaptation procedure in the performance of the digestion process.

## **6.4. CONCLUSIONS**

In this study, we assessed the feasibility of integrating anaerobic digestion and bioelectrochemical technologies. Results revealed that the introduction of electrogens-enriched electrodes in a batch-mode anaerobic digester prone to undergo acidic inhibition allows for the improvement of the AD process during the initial stage of the operation, by delaying VFA build-up, as well as enhancing CH<sub>4</sub> production. This amelioration seems to be based on the presence of the electrode as a solid support for biomass proliferation rather than electrochemical activity. However, after 100 hours of operation propionate concentrations started to increase dramatically

in the electrode-integrated digesters and led to the collapse of the systems (and a complete stoppage of methanogenic processes as a consequence). In order to prevent propionate accumulation and obtain a sustainable improvement in AD process, the design of pre-adaptation protocols for bacteria is advisable.

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# *Chapter 7*

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*Domestic wastewater treatment in parallel with methane production in a microbial electrolysis cell*

**Renewable Energy, 93, 442-448 (2016)**

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*Chapter prepared according to the guidelines of Renewable Energy*



**Abstract**

Microbial electrolysis cells (MECs) have great potential as a technology for wastewater treatment in parallel to energy production. In this study we explore the feasibility of using a low-cost, membraneless MEC for domestic wastewater treatment and methane production in both batch and continuous modes. Low-strength wastewater can be successfully treated by means of an MEC, obtaining significant amounts of methane. The results also suggest that hydrogenotrophic methanogenesis reduce the incidence of homoacetogenic activity, thus improving the overall MEC performance. However, gas production rates are low and important aspects such as methane solubility in water still remain a challenge. Overall, MECs can offer competitive advantages not only for low-strength wastewater treatment but also as an aid to anaerobic methane production by improving the chemical oxygen demand (COD) removal and methane production rates.



## 7.1. INTRODUCTION

Domestic wastewater (WW) usually requires treatment before being discharged into the environment. Activated sludge, a widespread WW treatment process, is an energy-intensive technology that demands ~1% of the total Spanish electrical energy consumption [1]. WW may contain significant amounts of chemical energy stored in the dissolved organics (some authors have estimated 17.8 kJ g<sup>-1</sup>-COD [2]) that, if appropriately recovered, could improve the energy balance of the process. This residual energy content can be exploited by means of biological treatments, among which anaerobic digestion (AD) is preminent. AD has proved to be a reliable, flexible and robust technology to treat and valorize a wide range of organic waste streams, but it usually fails to treat low-strength WW.

Microbial electrolysis cells (MECs) are a leading edge bio-based technology that has recently emerged as an alternative to conventional processes for organic waste treatment and energy production. In contrast to AD, MECs can treat diluted wastewater streams at relatively low temperatures [3], and have already delivered promising results in the laboratory [4] and in studies at semi-pilot [5] and pilot scales [6]. MECs are usually aimed at producing hydrogen gas (as opposed to the methane produced by AD), a fuel with a high gravimetric energy content and an important feedstock in many industrial processes. However the production of hydrogen presents numerous challenges (especially when treating low-strength WW) that jeopardize the upscaling process [7]. Indeed, if the hydrogen produced at the cathode is not readily evacuated it can be re-oxidized at the anode, a phenomenon known as hydrogen recycling, which deteriorates the performance of the MEC by limiting COD removal and reducing energy recovery [8]. In addition, cathodic hydrogen can be converted to acetate by homoacetogenic microorganisms resulting in a phenomenon with similar consequences to hydrogen recycling (i.e. limited COD removal and energy recovery [9, 10]). These difficulties could easily be overcome by using polymer membranes to separate the anolyte from the catholyte. However, polymer membranes are usually expensive, make the reactor design more complex, and increase the energy usage of the MEC, which finally impacts the capital and operational costs and threatens the prospect of practical application of this technology.

Aside from interfering in the bioelectrochemical operation of the MEC, hydrogen management presents additional challenges. For instance, due to its low volumetric energy content,

if hydrogen is not used *in situ* in the WW treatment facility, it would require intensive compression for its transportation, an operation that demands a substantial amount of energy [11]. Moreover, the equipment required for compression is usually expensive [11], and may not always be justified for the low hydrogen productivity of MECs [12]. Finally, the hydrogen produced in membraneless MECs is usually mixed with significant amounts of methane, which complicates its use as a feedstock for high-added-value industrial applications (that usually require high purity hydrogen), thus restricting its use for energy valorization. This contamination is hard to avoid since methanogenesis cannot be successfully suppressed once it becomes dominant in an MEC [13]. Moreover, it has recently been discovered that *Methanosaeta* and *Methanosarcina*, often the most abundant methanogens in anaerobic digesters, can accept electrons via direct interspecies electron transfer from *Geobacter metallireducens* [14-16], which increases the difficulty of finding a satisfactory solution for hydrogen contamination with methane.

Rather than avoiding its production, methane might prove to be a suitable alternative to hydrogen whenever the aim is to produce a reliable fuel from WW. If cathodic hydrogen can be converted to methane as soon as it is produced, or even if the electrons that arrive at the cathode could be used directly to reduce carbon dioxide to methane, the difficulties associated with homoacetogenic activity and the hydrogen recycling phenomenon would be ameliorated, if not completely removed, thanks to the presence of hydrogenotrophic methanogens [9]. This is because the Michaelis-Menten constant for hydrogenotrophic methanogens is significantly lower than for homoacetogens, and the H<sub>2</sub> threshold concentration for methanogens is also several orders of magnitude lower [17]. Moreover, methane can be produced in MECs with a relatively simple design and with the additional advantage that methane is an easier gas to manage than hydrogen.

In this study, we aimed to assess the opportunities for scalability of low-cost MECs for WW treatment and methane production, paying special attention to the problems associated with the use of low-strength WW where MECs offer a competitive advantage over aerobic WW treatments [18]. We also explore some of the benefits of using MECs to assist anaerobic methanogenesis.

## 7.2. MATERIAL AND METHODS

### 7.2.1. Influent

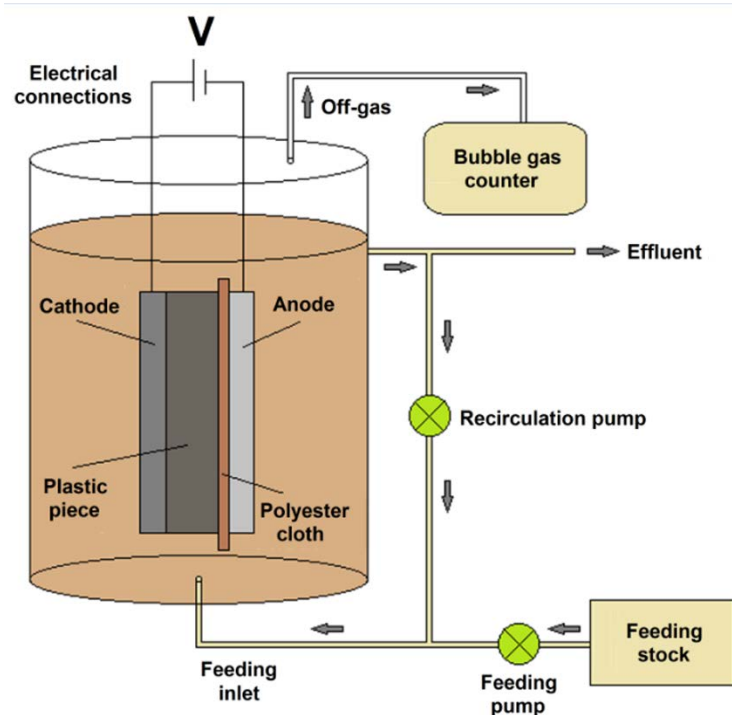
Synthetic medium (SM) and real domestic wastewater (WW) were used as influents for the MEC at different stages of the experiment. The composition of both SM and WW are described in Table 7.1. WW was collected from the municipal wastewater treatment plant of León (Spain). The chemical composition of SM was (in mg L<sup>-1</sup>): K<sub>2</sub>HPO<sub>4</sub> (14.3); KH<sub>2</sub>PO<sub>4</sub> (46.2); NH<sub>4</sub>Cl (90); NaCl (20); CaCl<sub>2</sub>·2H<sub>2</sub>O (30); MgSO<sub>4</sub>·7H<sub>2</sub>O (100); yeast extract (10); and 1 mL L<sup>-1</sup> of a trace metal solution of composition: MgCl<sub>2</sub>·6H<sub>2</sub>O (410); MnCl<sub>2</sub>·4H<sub>2</sub>O (50); FeCl<sub>2</sub>·4H<sub>2</sub>O (50); NiCl<sub>2</sub>·4H<sub>2</sub>O (12); ZnSO<sub>4</sub>·7H<sub>2</sub>O (10); CoCl<sub>2</sub> (7.7); CaCl<sub>2</sub>·2H<sub>2</sub>O (30); Al(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O (29.4); Na<sub>2</sub>SeO<sub>4</sub> (8.7); Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (2); CuSO<sub>4</sub>·5H<sub>2</sub>O (1); H<sub>3</sub>BO<sub>3</sub> (2); NaWO<sub>4</sub>·2H<sub>2</sub>O (1). The carbon source was sodium acetate (300 mg-COD L<sup>-1</sup>). All solutions were filter sterilized and stored at 4 °C to prevent microbial growth. Distilled water was used for solution preparation, and the chemicals and reagents used were of analytical grade.

**Table 7.1.** Characterization of the five sets of domestic wastewater used as influent in this study, and of the synthetic medium (ND = not determined).

|                                    | Domestic wastewater (WW) |             |             |             |             | Synthetic medium (SM) |
|------------------------------------|--------------------------|-------------|-------------|-------------|-------------|-----------------------|
|                                    | HRT 4 h                  | HRT 8 h     | HRT 12 h    | HRT 24 h    | Batch       |                       |
| TSS (mg L <sup>-1</sup> )          | 41.0 ± 1.0               | 45.8 ± 0.3  | 55.3 ± 1.8  | 53.8 ± 1.3  | 55.0 ± 0.5  | ND                    |
| VSS (mg L <sup>-1</sup> )          | 9.8 ± 0.3                | 9.3 ± 0.3   | 10.0 ± 2.0  | 9.8 ± 0.8   | 10.8 ± 0.8  | ND                    |
| TOC (mg L <sup>-1</sup> )          | 22.7 ± 1.4               | 24.6 ± 1.3  | 32.7 ± 1.6  | 34.0 ± 1.0  | 89.4 ± 11.9 | 168.8 ± 8.1           |
| Total N (mg L <sup>-1</sup> )      | 19.7 ± 0.9               | 21.0 ± 1.0  | 20.5 ± 1.4  | 20.9 ± 1.2  | 59.4 ± 3.0  | 26.5 ± 1.8            |
| NH <sub>4</sub> <sup>+</sup> (ppm) | 18.9 ± 5.1               | 20.9 ± 5.4  | 20.0 ± 6.2  | 19.1 ± 5.2  | 21.5 ± 4.9  | 36.5 ± 10.5           |
| pH                                 | 7.08 ± 0.12              | 7.22 ± 0.12 | 7.01 ± 0.06 | 7.13 ± 0.10 | 7.16 ± 0.14 | 6.49 ± 0.12           |
| Conduct. (μS cm <sup>-1</sup> )    | 504 ± 10                 | 486 ± 12    | 498 ± 12    | 496 ± 12    | 492 ± 10    | 732 ± 14              |
| COD (mg L <sup>-1</sup> )          | 65 ± 2                   | 67 ± 3      | 77 ± 3      | 78 ± 3      | 188 ± 20    | 450 ± 22              |
| Acetate (mg L <sup>-1</sup> )      | 36 ± 6                   | 27 ± 2      | 33 ± 6      | 29 ± 5      | 27 ± 1      | 330 ± 24              |

### 7.2.2. MEC design, instrumentation and operation

A continuous-flow, single-chamber, membraneless MEC, with a total volume of 3 L was used for the experiment (Figure 7.1). The reactor was equipped with connections for gas exits and liquid entries and exits, and contained the electrolytic module (anode + cathode) submerged in the feeding stock, either SM or WW. The anode consisted of one layer of carbon felt 1 cm thick (Sigratherm soft felt GFD 2, SGL Carbon Group, Wiesbaden, Germany), and the cathode was a stainless steel electrode, both with dimensions of 210 x 100 mm. One 0.6-mm-thick piece of polyester cloth was sandwiched between the anode and the cathode to avoid any electrical contact between the two electrodes. The inter-electrode separation was set to 1 mm. Prior to its introduction in the reactor, the anode was inoculated with the effluent from another WW-fed MEC that had been operating for more than a year, supplemented with 1 g L<sup>-1</sup> AcNa. Furthermore, it was pre-acclimatized with the same synthetic medium that would be used as the feeding source afterwards (SM, see above). The culture medium in the reactor was mixed with an external recirculation loop using two peristaltic pumps (Dosiper C1R; León, Spain) operating in series (recirculation rate of 9 L h<sup>-1</sup>). Two replicates of the experiment were performed, at a controlled temperature of 21 ± 2 °C.



**Figure 7.1.** Schematic representation of the laboratory set-up.



A 16  $\Omega$  resistor was added to the circuit for on-line current measurements at 60 min intervals using a data acquisition system (National Instruments PCI-6221). The same interval was used to record gas flow rates, which was measured with an MGC-1 milligas counter (Ritter Co, Bochum, Germany). An adjustable DC power supply (BK Precision 9120) maintained the applied voltage at the pre-set level ( $V_{app} = 1V$ ).

MEC voltage scans were performed by increasing the applied voltage from 0.0 V to 1.4 V in 0.07 mV s<sup>-1</sup> increments. Internal resistance ( $R_{int}$ ) was calculated as the slope of the linear section of the voltage scan plot. More details can be found elsewhere [19].

The reactor was initially fed with SM. The behavior of the MEC was studied in both batch and continuous modes. When in continuous mode, it was evaluated under hydraulic retention times (HRTs) of 4, 8, 12 and 24 h. Each set of operating conditions was maintained for at least three retention times to ensure stable conditions. Tests under an applied voltage of 1V (AV) and in open-circuit mode (OC) were carried out in batch and continuous mode to compare the electrogenic performance of the device with the potential fermentative processes occurring inside the reactor. The MEC was later fed with WW, initially in batch mode and then in continuous mode. As reported in Ref. [20], conventional anaerobic digestion (without electrodes) results in lower CH<sub>4</sub> production rates and therefore no conventional anaerobic digestion tests were here evaluated.

The MEC performance was assessed in terms of: (i) volumetric CH<sub>4</sub> production rate, both in gaseous and dissolved forms; (ii) Coulombic and Cathodic Conversion efficiency ( $C_e$  and  $CC_e$ , respectively), calculated as described by Ref. [7] and including dissolved CH<sub>4</sub> in the calculation of  $CC_e$ ; (iii) COD removal rates (%); and (iv) power consumption, energy production and net energy consumption, expressed on the basis of COD removal and calculated in accordance with [21].

### **7.2.3. Analytical measurements and calculations**

Gas composition and volatile fatty acids (VFA) were analyzed by gas chromatography as described by Ref. [22]. Dissolved methane concentrations were measured using the headspace method [23].

Chemical oxygen demand (COD), conductivity and pH were determined in accordance with the methods described by the [24] and were regularly quantified during the electrocatalytic process. COD was measured using a Metrohm 862 Compact Titrosampler. The homogenized sample was digested in the presence of dichromate at 150 °C for 2 h in a Hanna C9800 reactor. Total suspended solids (TSS) and volatile suspended solids (VSS) in WW were determined in accordance with [24]. Ammonium was determined by an ion-selective electrode (781 pH/Ion Meter, Metrohm). Total organic carbon, total carbon and total nitrogen content were determined using a total organic carbon analyzer (multi N/C 3100, AnalytikJena).

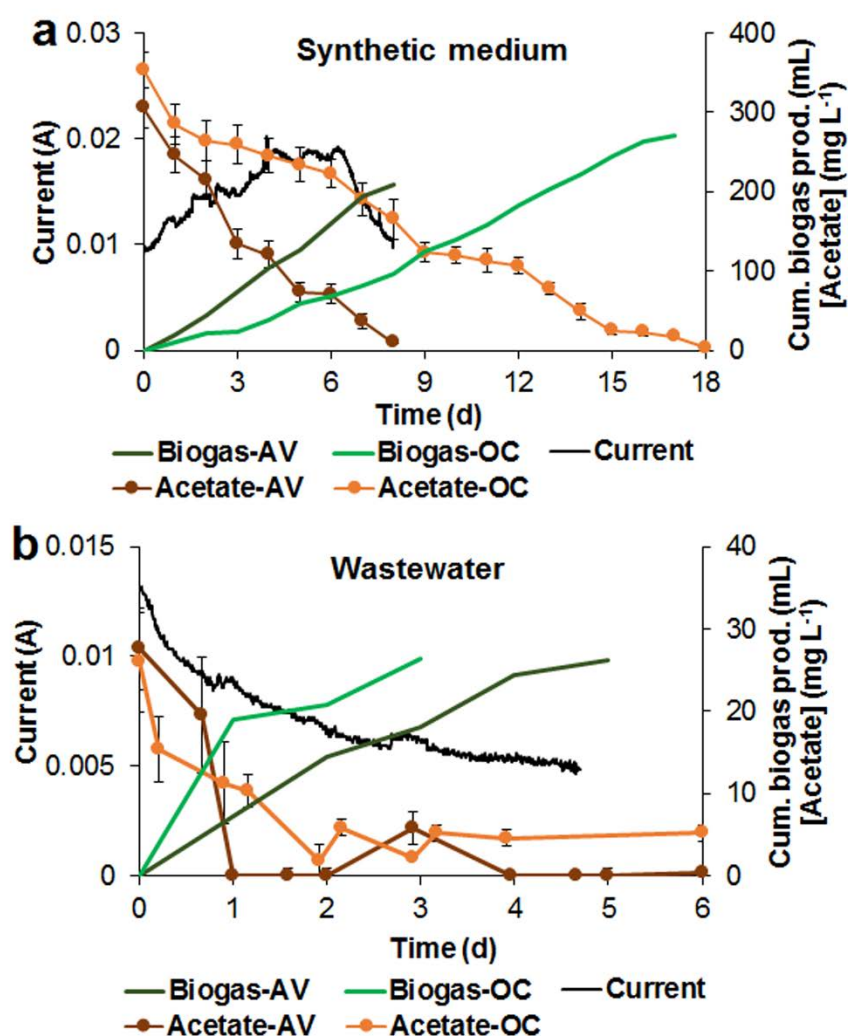
### 7.3. RESULTS AND DISCUSSION

#### 7.3.1. Performance of MEC system: batch mode

The MEC was first operated in batch mode to assess its bioelectrochemical performance using both SM and WW as substrates. When the reactor was fed with SM and the AV was set at 1V (SM-AV), it took 192 h (8 days) to consume more than  $95 \pm 1\%$  of the acetate in the electrolyte (Figure 7.2a), yielding a cumulative methane gas production of  $178 \pm 5$  mL ( $\text{CH}_4$  production rate of  $8.0 \pm 0.2$  mL- $\text{CH}_4$  L $^{-1}$  d $^{-1}$ , Table 7.2). Importantly, no  $\text{H}_2$  was detected in the off gas. This test was repeated in open-circuit conditions (SM-OC), resulting in lower rates of methane production ( $4.6 \pm 0.2$  mL- $\text{CH}_4$  L $^{-1}$  d $^{-1}$ ), no  $\text{H}_2$  in the off gas, and lower rates of acetate consumption as indicated by the more moderate slope in Figure 7.2a, requiring more than 430 h (~18 days) to remove more than  $95 \pm 1\%$  of the acetate originally present in the feed. However, cumulative methane production (gas + dissolved) by the end of the SM-OC test was ~43% higher ( $273 \pm 10$  mL in SM-OC vs.  $191 \pm 6$  mL in SM-AV), suggesting that in the SM-AV tests a significant amount of the substrate was diverted to processes other than methane production. These other processes have been labelled as “undefined COD” in the COD balance in Figure 7.3a, and can be partially explained by biomass growth. Therefore, the differences observed between the SM-AV and SM-OC tests could be attributed, to some extent, to different biomass yields between the acetoclastic and electrogenic microorganisms.

When the reactor was fed with WW in AV conditions (WW-AV, Figure 7.2b), gas production stopped after 107 h (~4.5 days) with a methane production rate of  $1.4 \pm 0.3$  mL- $\text{CH}_4$

$L_r^{-1} d^{-1}$  but only  $4 \pm 3\%$  COD removal. Operation in open-circuit conditions (WW-OC) yielded similar  $CH_4$  production rates ( $1.3 \pm 0.2 \text{ mL } CH_4 L_r^{-1} d^{-1}$ ) with an off-gas consisting largely of  $CO_2$ . Therefore, low acetate concentrations present in WW probably resulted on a sharp decrease in  $CH_4$  production parameters, in comparison with SM. Both in AV and OC conditions, a very small amount of the COD contained in WW and fed to the cell was finally converted to methane, with most of the organic matter remaining untreated (Figure 7.3a). Acetate concentration inside the reactor was analyzed regularly in the course of the experiments, revealing much faster consumption during the AV tests (Figure 7.2a and b).



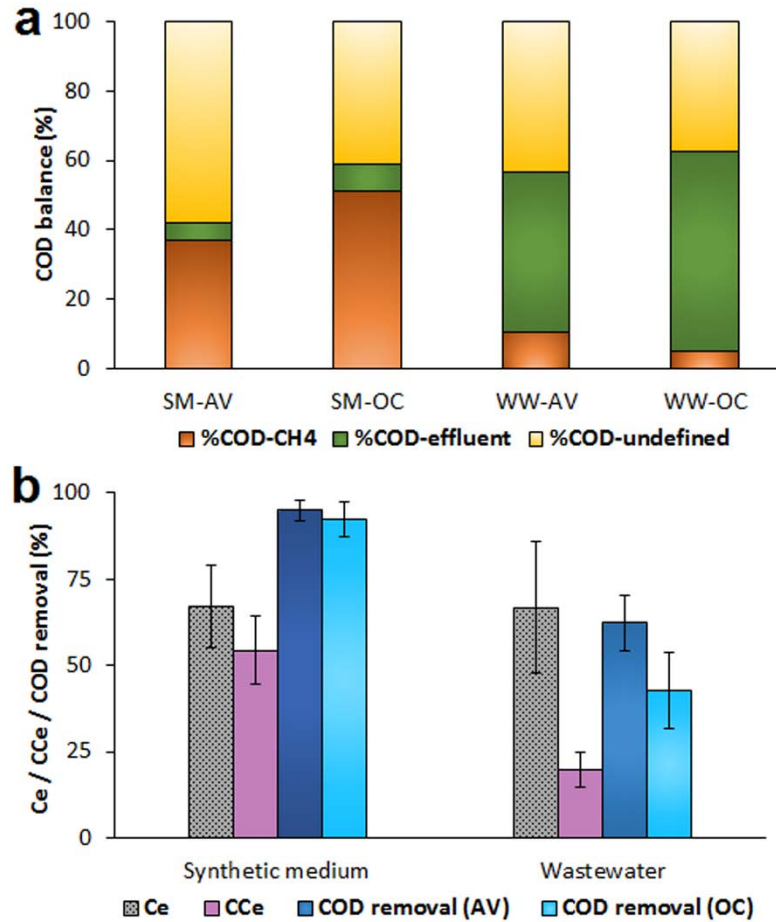
**Figure 7.2.** Profile of current production, biogas production and acetate concentration using: a) Synthetic medium (SM) and b) real domestic wastewater (WW) at IV (AV) and in open-circuit (OC), in batch mode (two replicates).

**Table 7.2.** Operational parameters of the MEC with SM and WW in batch (ND = not determined).

|   | Synthetic medium (SM) |           | Domestic WW (WW) |           |
|---|-----------------------|-----------|------------------|-----------|
|   | AV                    | OC        | AV               | OC        |
| <b>CH<sub>4</sub> production rate (mL-CH<sub>4</sub> L<sub>r</sub><sup>-1</sup> d<sup>-1</sup>)</b> | 8.0 ± 0.2             | 4.6 ± 0.2 | 1.4 ± 0.3        | 1.3 ± 0.2 |
| <b>Energy consumption (kWh kg<sup>-1</sup>-COD<sub>rem</sub>)</b>                                   | 2.25 ± 0.26           | ND        | 2.92 ± 0.71      | ND        |
| <b>Net energy consumption (kWh kg<sup>-1</sup>-COD<sub>rem</sub>)</b>                               | 0.73 ± 0.9            | ND        | 2.15 ± 0.42      | ND        |

Notably, the use of WW as a substrate resulted in lower cathodic conversion efficiency (CC<sub>e</sub>) (Figure 7.3b), meaning that a significant proportion of the electrons arriving at the cathode were not converted to methane. The cathodic reduction of oxidized compounds usually found in WW such as nitrates and sulfates might partially account for this loss [25]. Although these compounds (nitrates and sulfates) are generally present at low concentrations in WW, their impact on CC<sub>e</sub> in MECs operating at low current densities, as in this case, might be of great importance.

The configuration of our reactor as a single-chamber design makes it difficult to identify the mechanisms by which methane was produced. Undoubtedly, acetoclastic methanogenesis was present as shown in the OC tests; the question remains whether bioelectrogenic methane was generated through direct bio-electrochemical reduction of CO<sub>2</sub> [26], or whether hydrogen was produced initially and later converted to methane in a second step [27]. The absence of hydrogen in the off-gas in all tests suggests that this last mechanism did not occur to a significant extent, if at all. Therefore, direct extracellular electron transfer (EET) appears as the most likely mechanism for CH<sub>4</sub> production (electromethanogenesis) [26]. Previous studies have demonstrated that the genus *Methanobacterium* might be responsible for electromethanogenic processes [28, 29]. Microbial corrosion of the cathode represents an additional plausible mechanism to explain a proportion of the methane produced [30], although the cathode did not show visible signs of deterioration when the tests were finished.



**Figure 7.3.** a) COD balance using both a synthetic medium (SM) and real domestic wastewater (WW) as substrates at IV (AV) and in open-circuit mode (OC), in batch mode (two replicates). b) Coulombic ( $C_e$ ) and cathodic ( $C_{ce}$ ) efficiencies, and COD removal for both substrates.

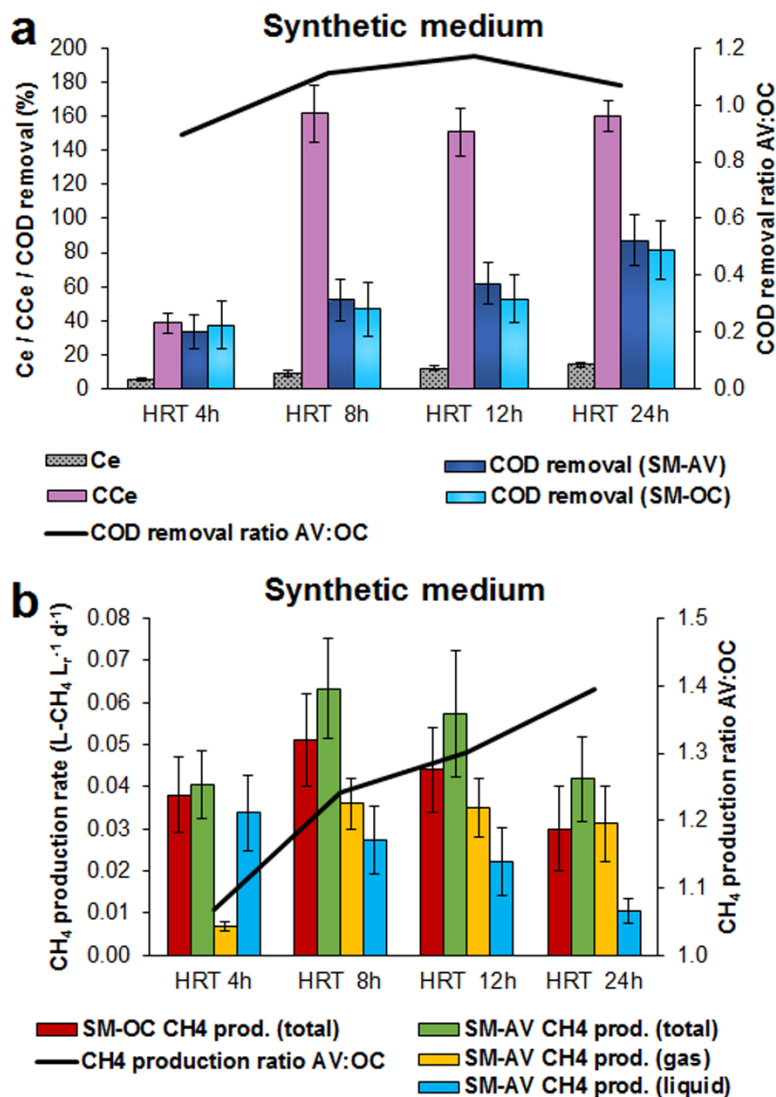
Hydrogen recycling or homoacetogenic activity cannot be ruled out. However, if these phenomena were taking place in our reactor, it was to a low extent. Several arguments and observations support this hypothesis: first, homoacetogenic proliferation and hydrogen recycling are usually limited when hydrogenotrophic methanogens are present [9]. Second, acetate concentration in our tests decreased steadily (Figure 7.2) and did not stabilize, as may happen when homoacetogenic activity is allowed [9]. Third, the relatively low  $C_e$  values observed (below 75%; Figure 7.3b) were also indicative of limited, if any, hydrogen recycling –  $C_e$ s well above 100% are usually reported in those studies where hydrogen recycling is not prevented [7, 9].

Finally, the internal resistance of the MEC (calculated from the linear section of the voltage scans using WW as a substrate, result not shown) was estimated to be  $40 \pm 11 \Omega$  ( $1900 \Omega \text{ m}^{-2}$ ), which is slightly lower than the internal resistance observed in a single-chamber WW-fed MEC for hydrogen production [4]. Nevertheless, this parameter needs to be greatly reduced to make our design profitable [31].

### 7.3.2. Performance of MEC system: continuous mode

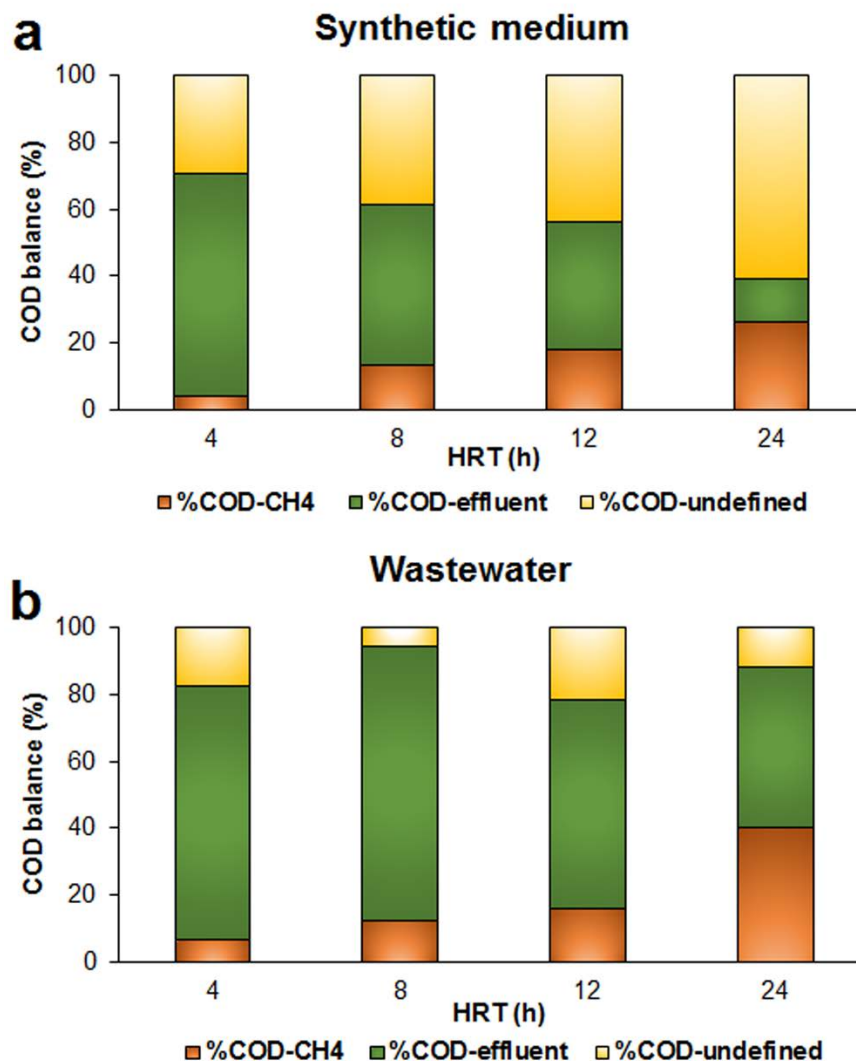
Following the batch tests, the MEC was operated in continuous mode to assess its bioelectrochemical performance within conditions similar to those usually found at wastewater treatment facilities. The MEC was operated in continuous mode at a HRT of 24 h for 9 days to promote adaptation of the microbial communities to the continuous operating conditions before the beginning of the tests.

The use of SM as substrate showed how COD removal improved, as expected, with HRT (from  $33 \pm 10\%$  at 4 h to  $87 \pm 15\%$  at 24 h; Figure 7.4a). Nevertheless, a significant amount of the acetate consumed was not converted to electricity, as revealed by the relatively low  $C_{e,s}$  shown in Figure 7.4a. These low  $C_{e,s}$  could be explained by two main reasons. First, acetoclastic microorganisms that consume acetate for non-electrogenic methane production may be present. This hypothesis is corroborated by the unusually high  $CC_e$  (above 150%) at HRTs of 8, 12 and 24 h, which revealed the existence of a source of methane other than electrogenesis. Surprisingly, with HRT at 4 h the  $CC_e$  collapsed to 39%, which was attributed to a partial washout of methanogenic microorganisms due to low HRT [32]. Second, cell maintenance and biomass proliferation of both anode-respiring bacteria (ARB) and acetoclastic microorganisms represent an alternative electron sink taking electrons from the substrate that are not subsequently converted into electricity. The COD balances in Figure 7.5a reveal that there was a significant amount of undetermined COD that might correspond to some extent to this “COD assimilation” as discussed in section 7.3.1.



**Figure 7.4.** a) Operational parameters at IV (AV) and open-circuit (OC) using synthetic medium (SM) under different HRTs. b) Total, gas-phase and dissolved  $CH_4$  production rates under different HRTs.

Figure 7.4a also illustrates the positive impact of high HRT on  $C_e$ , which can be explained by the lower acetate concentration inside the reactor as HRT increased. Indeed, low acetate concentrations should favor ARB activity to the detriment of acetoclastic activity, as ARB have a greater affinity for acetate than do acetoclastic microorganisms at low substrate concentrations [33]. Similar results have been reported elsewhere [34].



**Figure 7.5.** COD balance under different HRTs for: a) Synthetic medium (SM) and b) Real domestic WW (WW) at IV (AV).

Methane production rate declined with increasing HRT (Figure 7.4b), most likely as a result of reducing the organic loading rate. This trend did not apply at a HRT of 4 h, probably because of the partial washout of the methanogenic communities. Tests performed in OC conditions revealed that, at a HRT of 4 h, comparable amounts of methane (which in this situation can be completely attributed to acetoclastic activity) were produced in the absence of applied voltage (Figure 7.4b, black bars). The ratio between total methane production in AV and OC conditions was calculated for an estimate of the benefit of using the MEC for methane production. Figure



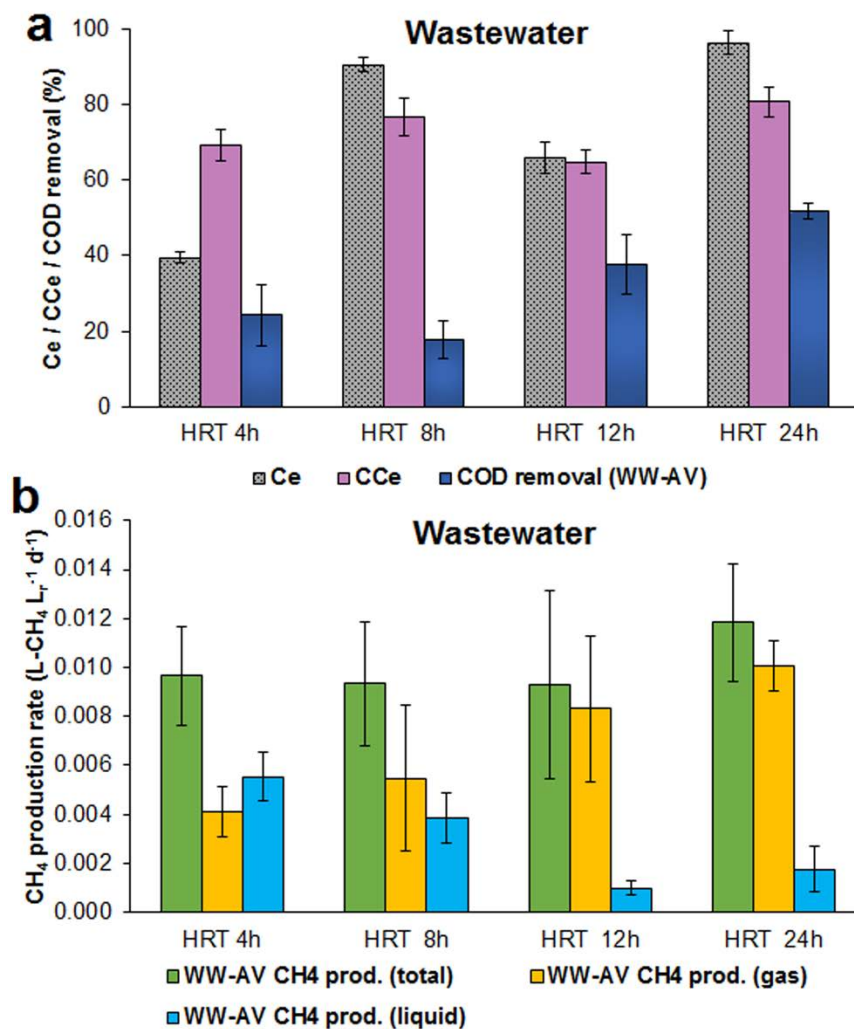
7.4b shows how this ratio increased with HRT as acetate concentration decreased, offering additional evidence of the competitive advantage of ARB over acetoclastic methanogens at low substrate concentrations. Moreover, the improvement of CH<sub>4</sub> production rate in AV conditions is comparable to that of methane yield (data not shown), highlighting the benefits of using an MEC in an anaerobic methane-producing reactor to optimize biogas production.

An important aspect for close consideration when assessing the potential of methane-producing MECs for practical application is how much of the methane produced can be recovered from the off-gas. Indeed, using low-strength WW as a substrate usually results in low methane production, and in this situation methane solubility is not a trivial issue. Figure 7.4b shows that significant amounts of methane left the reactor dissolved in the effluent. Importantly, it also shows that this unrecovered methane can be minimized by increasing the HRT.

When the reactor was fed with WW, CC<sub>e</sub> stabilized at approximately 70% at all HRTs (Figure 7.6a), which contrasts with the relatively high CC<sub>e</sub> observed using SM (over 100% in most cases). This discrepancy can be attributed to the presence of oxidized compounds in the WW that may be reduced at the cathode (as discussed in section 7.3.1), and also to lower activity of acetoclastic methanogens as acetate in the electrolyte becomes scarce. This last could also explain the improvement in C<sub>e</sub> compared to that observed with SM, which could be partially supported by the relatively small gap in the COD balance (undefined COD, Figure 7.5b) that can be attributed to biomass proliferation. The COD balance also showed that large amounts of COD remained untreated even at high HRTs, revealing either the presence of recalcitrant organic matter or a lack of microbial communities able to degrade complex organic matter. As in the previous case, when wastewater was used as the feeding source, an increase in HRTs tested resulted on a lower proportion of dissolved methane in comparison with total CH<sub>4</sub> production (Figure 7.6b).

Finally, energy balance was calculated for both substrates to assess the energy efficiency of the process. In contrast with the energy balance in batch (Table 7.2), there was a positive net energy production in continuous mode when SM was the feeding stock. While net energy production was  $0.308 \pm 0.065$  kWh kg<sup>-1</sup>-COD<sub>rem</sub> for a HRT = 4 h, this parameter was more than double for the other HRTs tested, which presented similar results between them (Table 7.3). On the other hand, with WW as feed, there was a net energy consumption. In any case, both feeding stocks showed a similar pattern, as in both cases longer HRTs resulted in better energetic

efficiencies. The energy use was, for all HRTs tested, below the net energy consumption threshold traditionally associated with aerobic treatment of domestic wastewater (0.7–2 kWh kg<sup>-1</sup>-COD; [35]).



**Figure 7.6.** a) Operational parameters of the MEC at 1V with real domestic WW (WW) under different HRTs. b) Total, gas-phase and dissolved CH<sub>4</sub> production rates under different HRTs.

**Table 7.3.** Net energy balance of the MEC with the synthetic medium (SM) and wastewater (WW) under different HRTs in continuous regime.

|         |    | Net energy balance (kWh kg <sup>-1</sup> -<br>COD <sub>rem</sub> ) |                |
|---------|----|--|----------------|
|         |    | SM   | WW             |
| HRT (h) |    | 0.308 ± 0.065  | -0.134 ± 0.023 |
|         | 4  | 0.689 ± 0.098  | -0.122 ± 0.028 |
|         | 8  | 0.733 ± 0.109  | -0.113 ± 0.039 |
|         | 12 | 0.698 ± 0.105  | -0.100 ± 0.015 |
|         | 24 |  |                |

### 7.3.3. Final considerations and future perspectives

Our results highlight the potential of MECs as a feasible technology for methane recovery from low-strength wastewaters. By applying voltage to an MEC immersed in what could be considered as an anaerobic methanogenic reactor, the rates of acetate consumption and methane production were increased. The simple reactor design, together with the low cost of the building materials, and the relatively low energy consumption of the process, point to an optimistic commercial future. However, MEC technology still presents important deficiencies, such as its inability to remove significant amounts of nutrients from waste streams, which compromise its future use as a stand-alone technology in wastewater treatment facilities. Moreover, our results show that in its present evolution, this is a technology far from techno-economic feasibility, and therefore important improvements are still required. For instance acetate, a substrate that can be readily consumed when fed to the anode of bioelectrochemical reactors, required about 8 days for complete removal in our MEC (Figure 7.2a). This relatively low rate of acetate removal can be explained, at least in part, by inefficient reactor design. An optimized ratio of anode surface area to reactor volume, together with improved hydrodynamics inside the reactor to favor the diffusion of organic matter to the anode, avoid pH gradients that generate over-potentials, and reduce the internal resistance [31] will likely help to improve both COD degradation and methane production rates, and at the same time limit energy consumption.

Overall MEC performance could also be promoted by favoring direct conversion of CO<sub>2</sub> to methane on the cathodic surface, which can be accomplished by controlling the cathode potential [36], and also by using cathode materials with a high specific surface area such as the carbon felt used for the anode, to favor the proliferation of methanogenic microorganisms.

Another issue that threatens the feasibility of MEC for methane production is the loss of methane dissolved in the effluent. Keeping the reactor head at low, or even negative pressure by means of a vacuum pump [37], or degassing membranes [23], can improve the amount of CH<sub>4</sub> recovered in the off-gas. However, this could incur additional costs (both operational and capital) that may not be justified for low methane production rates. Another possible solution might be to optimize the configuration of our reactor by maximizing the ratio between the electrode surface area and the liquid volume. In addition, Figures 7.4b and 7.6b demonstrate that the amount of CH<sub>4</sub> recovered in the off-gas can be improved by increasing the HRT. However, reactor size increases linearly with HRT, with severe impacts on the capital costs of the technology [12].

### **7.4. CONCLUSIONS**

In this study we assessed the feasibility of using a low-cost MEC to treat low-strength WW in parallel to methane production. Batch tests revealed that the use of an MEC in a methane-producing reactor improved organic matter degradation and methane production rate. The results also suggested that homoacetogenic activity (a source of inefficiencies in membrane-less MECs) was ameliorated by promoting hydrogenotrophic methanogenesis. Continuous tests showed that the use of the MEC in a methane-producing reactor was especially favorable at high HRTs, where ARB have a competitive advantage over acetoclastic methanogens. Longer HRTs also favored energy recovery, making the process more cost-effective. Important challenges remain to be addressed, such as the losses of CH<sub>4</sub> in the effluent.

### **Acknowledgements**

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scholarship from the Regional Government of Castilla y León (Orden EDU/828/2014), cofinanced by the European Social Fund. A. Escapa thanks the postdoctoral fellowship of the Regional Government of Castilla y León. The assistance of D. M. García in the laboratory is greatly appreciated. The authors also thank SALEAL (domestic wastewater treatment plant of León, Spain) for providing the wastewater samples.

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# *Chapter 8*

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*Short-term internships  
at European  
research centers*



**8.1. INTERNSHIP AT THE SCHOOL OF CHEMICAL SCIENCE, DUBLIN CITY UNIVERSITY (REP. OF IRELAND), AND AT THE MEDICAL BIOLOGY CENTRE, QUEEN'S UNIVERSITY BELFAST (NORTHERN IRELAND, UNITED KINGDOM)**

**Oct'15 – Jan'16**

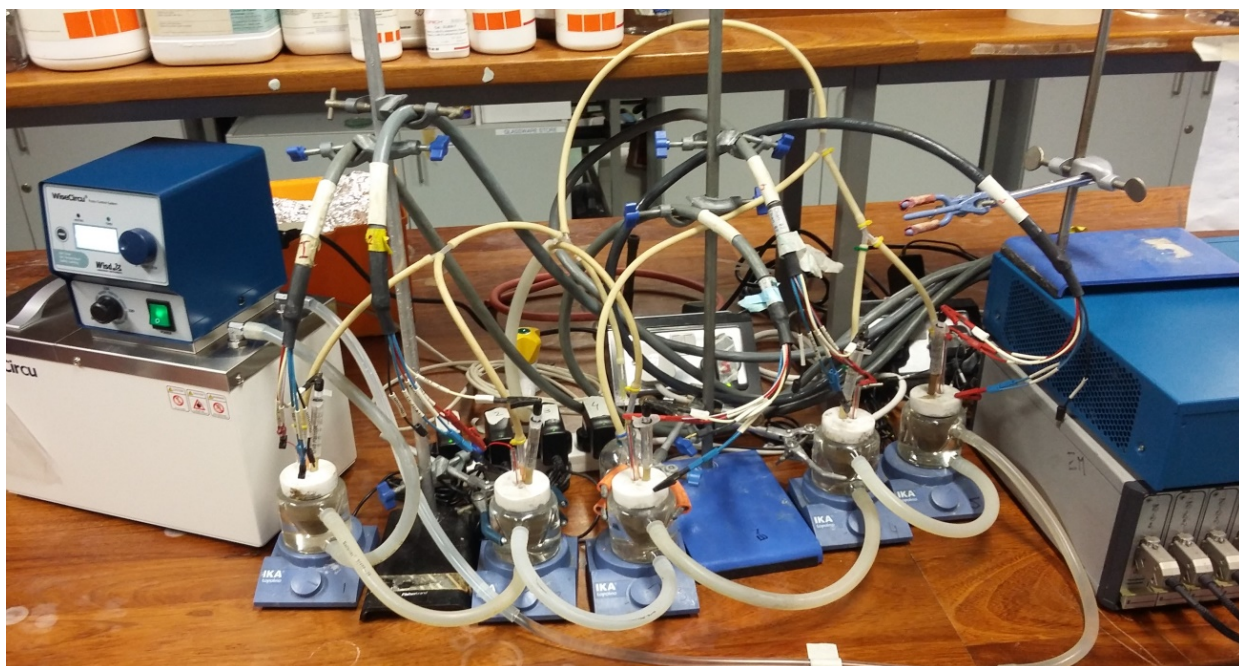
During my stage at Dublin City University (DCU) I was working under the supervision of Dr. Enrico Marsili and Dr. Brian Kelleher. The global aim of this study was the start-up and analysis of results of a bioremediation experiment by means of bioelectrochemical systems, in order to evaluate the selective enrichment of electrogens (as a result of voltage application) and to characterize biofilm development. BESs operation and electrochemical analysis were performed at DCU. Biofilm characterization by PCR and qPCR was carried out at Queen's University Belfast (QUB), where I worked with Dr. Prasanna Pentlavalli.

**8.1.1. Methodology**

**8.1.1.1. Experimental set-up**

This experiment consisted of five potentiostat-controlled electrochemical cells operating in a three electrode configuration under the control of a five channel potentiostat (VSP Bio-Logic, USA). The three electrodes present in each cell were: the working electrodes (WEs), the counter electrode (CE) and the reference electrode (RE). The counter electrode was a 0.25 mm coiled titanium wire approximately 20 cm in length, whereas the working electrode consisted on two 20 x 10 mm carbon felt pieces. The WEs were attached to Ti wire current collector, which was soldered to copper wire in a 6.5mm glass capillary. The resistance of each WE assembly did not exceed 2.5  $\Omega$  in each experiment. The Ag/AgCl reference electrode ( $E=+0.225$  vs. standard hydrogen electrode, SHE) (Als, Japan) was connected to the bioreactor via a salt bridge consistent of 0.1 M Na<sub>2</sub>SO<sub>4</sub> in 1% agar assembled from a 3 mm glass capillary and a 3 mm Vycor frit (BioAnalytical Systems, UK). The CE and WEs were placed approximately 5 mm apart in the electrochemical cells. Both of them and the RE glass capillary were inserted through ports in a custom-made Teflon lid that was sealed with an O-ring gasket. These lids fit onto the single-chambered, water jacketed electrochemical cells with a working volume of 20 mL. The working temperature of the electrochemical cells was controlled at 30 °C by means of an external water bath. To maintain the strict anaerobic conditions required for bacteria, reactors were operated

under a constant flow of sterile humidified N<sub>2</sub>. Each reactor was located above an independent magnetic stirring unit (Figure 8.1).



*Figure 8.1. Experimental set-up of bioelectrochemical systems.*

The potentiostat was used with EC-Lab v10.39 software, in order to run simultaneous multitechnique electrochemistry routines, such as cyclic voltammetry (CV) and chronoamperometry (CA). CA was performed for periods of 48 h between each culture media change. CA was performed at different voltage values for each cell, according to the aim of the experiment. Voltage values tested were +0.4 V and +0.2 V. Besides, another bioreactor was operated in open-circuit voltage as a control test. CV was carried out from  $E_i = -0.8$  V vs. Ag/AgCl to  $E_f = 0.2$  V vs. Ag/AgCl, without shaking. The scan direction was then reversed and the potential was swept back to the original E value of  $-0.8$  V vs. Ag/AgCl. CV was performed at a scan rate of  $1.0 \text{ mV s}^{-1}$ .

#### **8.1.1.2. Substrate**

The substrate was soil slurry, which consisted of a mixture of soil and culture media. The soil was collected from three points next to the nearest pathway of Albert Park, Dublin, to Ballymun Road, in order to get soil samples with a high concentration of poly-hydroxy-alcanoates (PAHs). This was necessary for the lab coworkers, as this experiment was part of a larger PAHs

bioremediation project. Soil samples were taken at 10-20 cm below ground surface. The three samples were then mixed in order to obtain a homogeneous sample, and after sieving it to <2 mm (diameter), soil characterization was performed. Soil pH was 6.53. Soil moisture content was analyzed by the freeze-dry method and it resulted on 29.12% moisture content. Total solids and volatile solids content were 708.8 and 123.0 g kg<sup>-1</sup>, respectively. VS represented 17.4% of TS. Soil slurry was made by mixing soil with culture media (25% w/v). The composition of culture media is detailed in Table 8.1. Sodium acetate was chosen as carbon source due to its good performance with electrogens. The composition of trace metals solution (in g L<sup>-1</sup>) was: FeCl<sub>3</sub>·4H<sub>2</sub>O: 0.2; MgCl<sub>2</sub>·6H<sub>2</sub>O: 0.1; Na<sub>2</sub>WO<sub>4</sub>: 0.02; MnCl<sub>2</sub>·4H<sub>2</sub>O: 0.1; CoCl<sub>2</sub>·4H<sub>2</sub>O: 0.1; CaCl<sub>2</sub>·4H<sub>2</sub>O: 1; ZnCl<sub>2</sub>: 0.05; CuCl<sub>2</sub>·2H<sub>2</sub>O: 0.002; H<sub>3</sub>BO<sub>3</sub>: 0.005; Na<sub>2</sub>MoO<sub>4</sub>: 0.01; NaCl: 1; Na<sub>2</sub>SeO<sub>4</sub>: 0.017.

*Table 8.1. Composition of the culture media used for the bioelectrochemical tests.*

|   | g L <sup>-1</sup>     |
|---|-----------------------|
| <b>CaCl<sub>2</sub>·2H<sub>2</sub>O</b> | 0.08                  |
| <b>NH<sub>4</sub>Cl</b>                 | 1                     |
| <b>MgCl<sub>2</sub>·6H<sub>2</sub>O</b> | 0.2                   |
| <b>NaCl</b>                             | 10                    |
| <b>K<sub>2</sub>HPO<sub>4</sub></b>     | 4.36                  |
| <b>KH<sub>2</sub>PO<sub>4</sub></b>     | 0.58                  |
| <b>Yeast extract</b>                    | 0.5                   |
| <b>Trace metals</b>                     | 10 mL L <sup>-1</sup> |
| <b>AcNa</b>                             | 1.64                  |

### 8.1.1.3. DNA extraction and PCR

As there were two WEs in each of the electrochemical cells, samples from the biofilms of one of the WEs were taken after 180 h of operation, while remaining samples were taken after 360 h. DNA extraction was performed using the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, California, USA). In order to check if DNA extraction had been successful, a PCR was done afterwards. Primers chosen were: forward primer 63f (5'-

CAGGCCTAACACATGCAAGTC-3') and reverse primer 1387r (5'-GGGCGGWGTGTACAAGGC-3'), according to Marchesi et al. (1998), for the amplification of a DNA sequence of approximately 1300 bp of a consensus gene coding for 16S rRNA. The temperature profile for the PCRs was an initial denaturation step of 4 minutes at 95 °C, followed by 35 cycles of 30 seconds at 95 °C, 30 seconds at 52 °C and 90 seconds at 72 °C. A final extension step of 5 minutes at 72 °C was done afterwards. PCR products were visualized by running 5 µL of PCR mixture on 1% agarose gels in 1x TAE (Tris-acetate-EDTA buffer) stained with ethidium bromide (0.00002%). In Figure 8.2 it can be observed that, as expected, a 1300-bp DNA fragment was amplified for each sample.



**Figure 8.2.** Thermocycler used for the PCR, and PCR products in the agarose gel both for 180 and 360 h (PC: positive control).

#### 8.1.1.4. qPCR assays

qPCR was performed by means of a MJ Research Opticon 2 qPCR system (BioRad Laboratories). Two qPCR tests were performed: one for 180h samples, and the other for 360h samples. Primers chosen for qPCR were: primer 1 (5'-CCTACGGGAGGCAGCAG-3') and primer 2 (5'-ATTACCGCGGCTGCTGG-3'), following Muyzer et al. (1993), for the amplification of a DNA sequence of 192bp of the gene coding for 16S rRNA in *E. coli*. qPCR



mixtures contained 1  $\mu\text{L}$  of DNA sample, 5  $\mu\text{L}$  of Thermo Scientific Maxima SYBR-Green/ROX qPCR Master Mix (2x), and 1  $\mu\text{L}$  of each primer. Total reaction volume was 10  $\mu\text{L}$ . In order to generate a standard curve, a DNA vector (a 192 bp fragment inserted in a plasmid) was diluted in series. The concentration of this DNA vector was previously estimated by means of fluorescence, using the double-stranded QuantiFluor® dsDNA System (Promega): DNA concentration of the vector was  $7.40 \cdot 10^{-9}$  and  $8.90 \cdot 10^{-9}$  g  $\mu\text{L}^{-1}$  (for the qPCR for 180 h samples and 360 h samples, respectively). According to the formula:

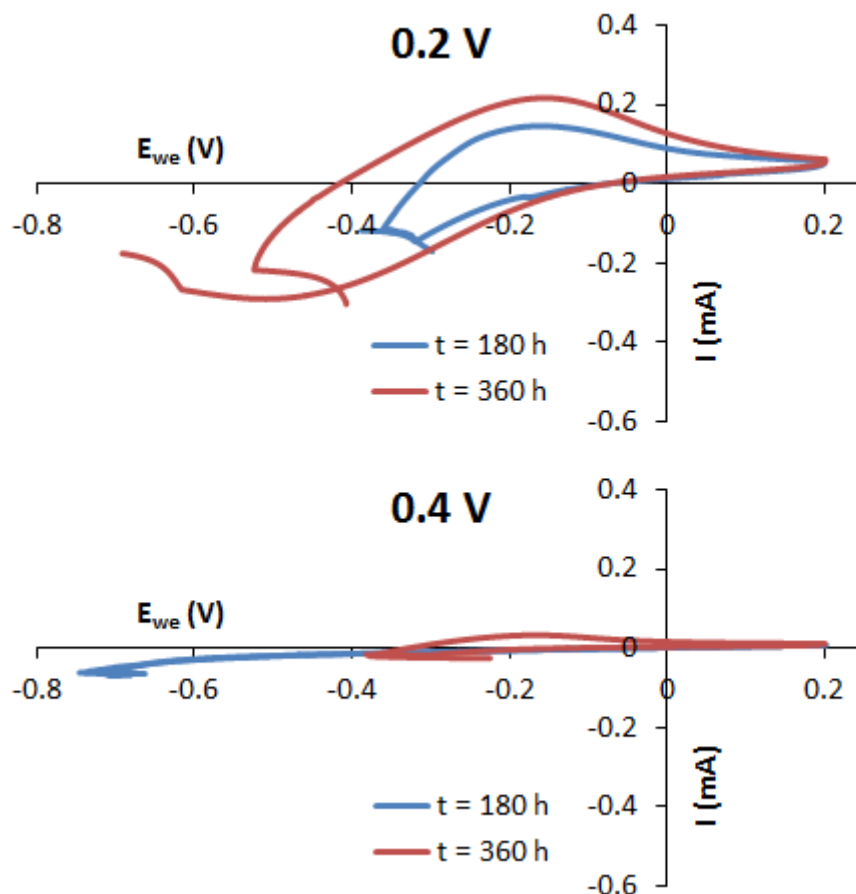
$$\text{DNA copies} / \mu\text{L} = \frac{N_A \cdot [\text{DNA}]}{\text{bp of DNA vector} \cdot 660 \text{ Da}}$$

where  $N_A = 6.022 \cdot 10^{23}$ , and considering that the DNA vector had 3166 bp (192 bp of the standard DNA fragment and 2974 bp of the plasmid where it is inserted), these DNA concentrations resulted on  $2.20 \cdot 10^9$  and  $2.65 \cdot 10^9$  DNA copies  $\mu\text{L}^{-1}$  for the qPCR for 180h samples and 360h samples, respectively.

qPCR protocol consisted on a preliminary incubation at 95 °C for 10 min, and after that, 25 cycles of 30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. After each cycle, the system made a quantification measurement. After cycle 25, melting curves of amplification products were determined from 40 to 100 °C, reading every 5 °C. Data analysis was carried out with the Opticon Monitor Software (BioRad Laboratories).

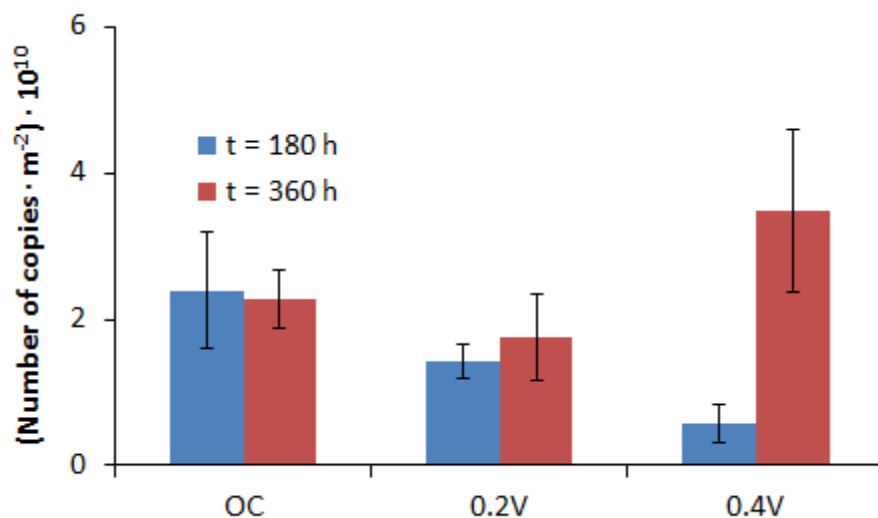
### 8.1.2. General results

Cyclic voltammetry analyses are depicted in Figure 8.3. For biomass grown at +0.4 V, little response can be observed after the first 180 h of operation, suggesting a slow growth rate for electrogenic microbiota. After the second stage of the test (at 360 h), electrogenic performance improves. However, it is still lower than that observed for microbial populations grown at +0.2 V. The improvement in voltammetric behavior at the second stage of the analysis (360 h) in comparison to the first 180 h is enhanced at +0.2 V. Although this may suggest that +0.2 V is the preferred V value for electrogenic selection, further in-depth studies should be done in order to confirm this idea, as this was a superficial approach.



**Figure 8.3.** Cyclic voltammetry analysis for the WEs at different times of the study.

As presented in Figure 8.4, biomass under open-circuit (OC) is quite stable throughout the study, and, in absolute terms, grows more than for reactors at +0.2 V. When voltage is applied a selection step for electrogens is done, which would explain lower growth rates for biomass at +0.2 V when compared to OC. Although growth at +0.2 V seems to be enhanced at the end of the test (t = 360 h) in comparison to OC, growth rates at +0.4 V are much higher than at +0.2 V. As this increase in microbial growth rate does not reflect on an improvement in electrogenic performance, it is suggested that population growth at +0.4 V is not based on electrogenic species.



*Figure 8.4. Quantification of microbial populations in the anodes based on qPCR results.*

### 8.1.3. References

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- Muyzer, G., de Waal, E. C., & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, 59(3), 695-700.

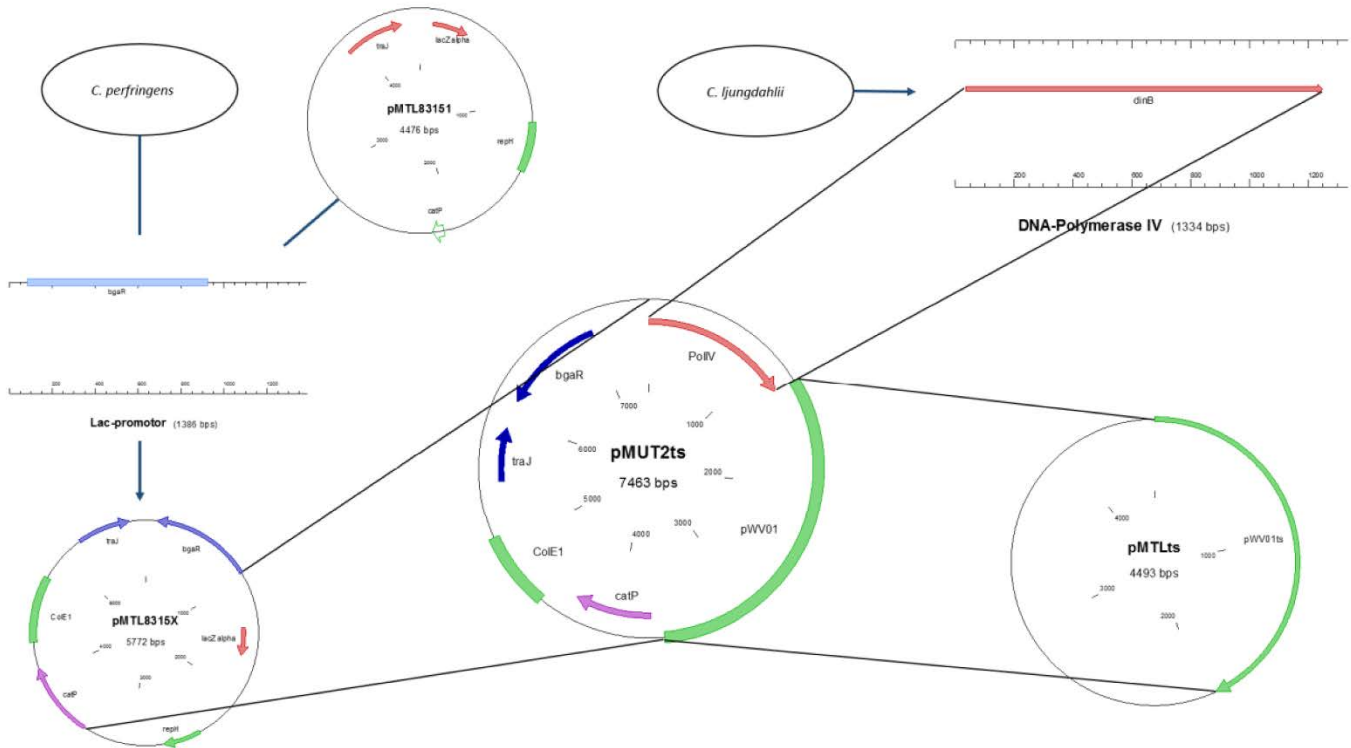
**8.2. INTERNSHIP AT THE INSTITUTE OF APPLIED MICROBIOLOGY,  
RHEINISCH-WESTFÄLISCHE TECHNISCHE HOCHSCHULE AACHEN (GERMANY)**

**Jan'16 – Apr'16**

During my stage in RWTH Aachen I had the opportunity to work under the supervision of Prof. Dr. Miriam Rosenbaum and in collaboration with PhD student Thomas Kirchner. As one of the most important research lines of their group is the characterization of the behavior of bioelectrochemical systems inoculated with pure cultures of *Clostridium ljungdahlii*, the main objective of this internship was to develop a mutagenesis-inducing tool for this microorganism, in order to eventually obtain an improved strain that could optimize reactors performance.

**8.2.1. Methodology**

This experiment consisted on the induction of non-targeted mutagenesis in *Clostridium ljungdahlii*, with the aim of enhancing the growth of auxotrophs for the synthesis of aminoacids (that is, modified *C. ljungdahlii* strains unable to synthesize one particular aminoacid as a result of a mutation in a gene involved in the metabolic pathway), which would play a role as indicators of the increase in mutation rates. In order to do that, a strain of *C. ljungdahlii* was transformed with the plasmid pMUT2. Design of pMUT2 and transformation of *C.ljungdahlii* had been previously done by Thomas Kirchner. The plasmid pMUT2 (Figure 8.5) was based on the commercial plasmid pMTL83151 *C. ljungdahlii-E.coli* shuttle vector (Heap et al., 2009). The *dinB* gene from *C. ljungdahlii* encoding DNA polymerase IV was integrated in the plasmid. DNA polymerase IV is error prone, which means that it usually makes mistakes during DNA replication. This is due to the lack of 3'→5' exonuclease activity in this enzyme, preventing the mistakes to be corrected (Goodman, 2002). In the plasmid, the gene for DNA pol. IV was inserted under the regulation of a lactose-inducible promotor (Banerjee et al., 2014). In order to force the bacteria to retain the plasmid and not reject it when incubated at 30 °C, the plasmid contained a thioamphenicol-resistance gene (*catP*) (Heap et al., 2009). The plasmid was thermosensitive, which means that its origin of replication was only active at 30 °C. If incubated at 37 °C, no replication of the plasmid occurred, while replication of the bacteria did: in other words, if incubated at 37 °C, the plasmid was curated from the bacteria. This was achieved by means of the integration of the temperature sensitive origin of replication pWV01 ori (Framson et al., 1997).



**Figure 8.5.** Schematic representation of plasmid pMUT2 used for the mutagenesis experiment (picture kindly provided by Thomas Kirchner and reproduced here with permission).

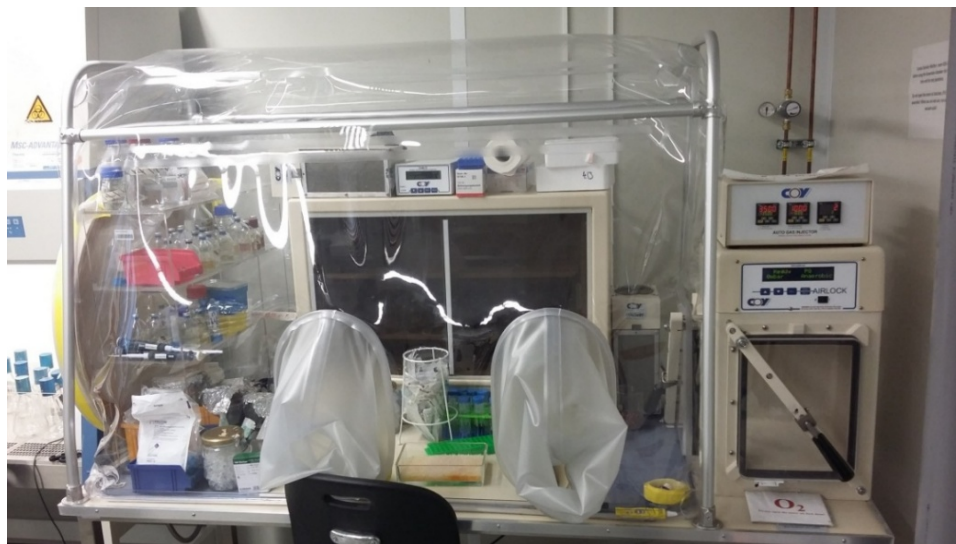
After transformation with pMUT2, the strain was known as *C. ljungdahlii* mut1. *C. ljungdahlii* mut1 was cultivated in PETC medium (the composition is detailed in Table 8.2) supplemented with 5 g L<sup>-1</sup> of fructose as carbon source and 10 mL L<sup>-1</sup> of reducing agent, in Hungate tubes (total culture volume of 5 mL each) into the anaerobic chamber (Figure 8.6). A concentration of 5 mg L<sup>-1</sup> of thioamphenicol was added. pH was adjusted to 5.8 with NaOH 2 M. The composition of trace metals solution (in g L<sup>-1</sup>) was: nitriloacetic acid, 1.50; MgSO<sub>4</sub>·7H<sub>2</sub>O, 3.00; MnSO<sub>4</sub>·H<sub>2</sub>O, 0.50; NaCl, 1.00; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.10; CoSO<sub>4</sub>·7H<sub>2</sub>O, 0.18; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.10; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.18; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.01; KAl(SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O, 0.02; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.01; H<sub>3</sub>BO<sub>3</sub>, 0.01; NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.03; Na<sub>2</sub>SeO<sub>3</sub>·5H<sub>2</sub>O, 0.30·10<sup>-3</sup>; Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O, 0.40·10<sup>-3</sup>; distilled water, 1000 mL. The composition of the Wolfe's vitamin solution (in mg L<sup>-1</sup>) was: biotin, 2; folic acid, 2; pyridoxine-HCl, 10; thiamin-HCl·2H<sub>2</sub>O, 5; riboflavin, 5; nicotinic acid, 5; d-Ca-pantothenate, 5; vitamin B12, 0.1; p-aminobenzoic acid, 5; lipoic acid, 5; bidistilled H<sub>2</sub>O, 1000 mL. The reducing agent solution composition was (in g L<sup>-1</sup>): NaOH, 9; L-Cysteine·HCl, 40; Na<sub>2</sub>S, 40; bidistilled H<sub>2</sub>O, 1000 mL. Gene transcription of the plasmid in the bacteria was induced with

lactose. Three different lactose concentrations were tested: 0, 7.5 and 30 mM. Apart from the blank (0 mM) two controls were used in this experiment: a culture of a wild type of *C. ljungdahlii* (wt) and a culture of this microorganism transformed with the plasmid but lacking the gene for DNA pol. IV, to test if the plasmid itself had some effect. As no differences were found between both controls in a preliminary study using serum bottles, the rest of the experiment had the wt control alone. Two sets of these cultures were incubated at 30 °C, for 5 h or for 24 h.

**Table 8.2.** Composition of *C. ljungdahlii* growth medium.

| <b><i>C. ljungdahlii</i> medium</b>  |         |
|--------------------------------------|---------|
| NH <sub>4</sub> Cl                   | 1.00 g  |
| KCl                                  | 0.10 g  |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O | 0.20 g  |
| NaCl                                 | 0.80 g  |
| KH <sub>2</sub> PO <sub>4</sub>      | 0.10 g  |
| CaCl <sub>2</sub> ·2H <sub>2</sub> O | 0.02 g  |
| Yeast extract                        | 1.00 g  |
| MES buffer                           | 19.54 g |
| Resazurin solution (0.1% w/v)        | 250 µL  |
| Trace element solution               | 10 mL   |
| Wolfe's vitamin solution             | 10 mL   |
| Distilled water                      | 1000 mL |

After this, lactose induction of transcription was stopped. In order to do so, culture medium was centrifuged (10 minutes, 7500 rpm) and the pellet was transferred to new Hungate tubes with fresh PETC medium supplemented with fructose and reducing agent. Neither thioamphenicol nor lactose was present in these new cultures, which were incubated at 37 °C for 24 h, in order to make the bacteria curate the plasmid while promoting bacterial growth. After 24 h, these bacteria were diluted in two different stocks (1:4 and 1:5 dilutions, respectively) and re-cultivated afterwards in Petri plates for single colonies isolation. These plates had the same PETC medium (see Table 8.2), but supplemented with 1.5% (w/v) agar. They were incubated at 37 °C for 72 h.



**Figure 8.6.** Anaerobic chamber for the experimentation stage.

After 72 h of incubation, colonies formed for each lactose concentration were counted from the 1:5 dilutions, and isolated from the 1:4 dilution plates. Single colonies isolation was carried out in duplicate. Isolated single colonies were re-cultivated in new agar PETC plates without yeast extract. In order to do the screening in search of auxotrophies (screening phase), these plates were divided in 16 squares each, so that 16 different single colonies could be cultured in the same plate. As a result, 32 single colonies were isolated for the 5 h lactose-induction period and other 32 colonies were isolated for the 24 h lactose-induction period (in both cases, for each lactose concentration: as three different lactose concentrations were tested, this means 96 colonies were isolated for each period). The medium in these plates was supplemented with different aminoacids solution, so that aminoacids composition was different for each plate. As a first approach, different aminoacids profiles for each plate were based on five aminoacid solutions (A1, A2, A3, A4 and A5). The differences in aminoacids compositions for each of the plates are presented in Table 8.3.

Finally, after having identified potential candidates for auxotrophies, the second part of the screening phase started. As more than one aminoacid was lacking in each of the previous plates, this second part can be considered as a polishing step, so that we could define which particular aminoacid the auxotrophy is for. The potential candidates for auxotrophies were re-cultured in Hungate tubes with the same PETC medium (with fructose, without yeast extract), supplemented with all the aminoacids except one, and grown during 72 h at 37 °C. Their growth was quantified by measuring the optical density at 600 nm.

**Table 8.3.** Aminoacid composition for the agar plating at the first stage of the screening phase.

| Concentration (M) | Total                | Without A1           | Without A2           | Without A3           | Without A4           | Without A5           |
|-------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| <b>Trp</b>        | $1.20 \cdot 10^{-8}$ | 0                    | $1.20 \cdot 10^{-8}$ | $1.20 \cdot 10^{-8}$ | $1.20 \cdot 10^{-8}$ | $1.20 \cdot 10^{-8}$ |
| <b>Phe</b>        | $1.20 \cdot 10^{-8}$ | 0                    | $1.20 \cdot 10^{-8}$ | $1.20 \cdot 10^{-8}$ | $1.20 \cdot 10^{-8}$ | $1.20 \cdot 10^{-8}$ |
| <b>Tyr</b>        | $1.20 \cdot 10^{-8}$ | 0                    | $1.20 \cdot 10^{-8}$ | $1.20 \cdot 10^{-8}$ | $1.20 \cdot 10^{-8}$ | $1.20 \cdot 10^{-8}$ |
| <b>Lys</b>        | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | 0                    | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ |
| <b>Thr</b>        | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | 0                    | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ |
| <b>Met</b>        | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | 0                    | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ |
| <b>Arg</b>        | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | 0                    | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ |
| <b>Ile</b>        | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | 0                    | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ |
| <b>Val</b>        | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | 0                    | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ |
| <b>Leu</b>        | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | 0                    | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ |
| <b>Cys</b>        | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | 0                    | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ |
| <b>His</b>        | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | 0                    | $4.00 \cdot 10^{-8}$ |
| <b>Ser</b>        | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | 0                    | $4.00 \cdot 10^{-8}$ |
| <b>Pro</b>        | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | 0                    | $4.00 \cdot 10^{-8}$ |
| <b>Gly</b>        | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | 0                    | $4.00 \cdot 10^{-8}$ |
| <b>Ala</b>        | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | $4.00 \cdot 10^{-8}$ | 0                    | $4.00 \cdot 10^{-8}$ |
| <b>Glu</b>        | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | 0                    |
| <b>Gln</b>        | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | 0                    |
| <b>Asp</b>        | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | 0                    |
| <b>Asn</b>        | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | $3.20 \cdot 10^{-8}$ | 0                    |

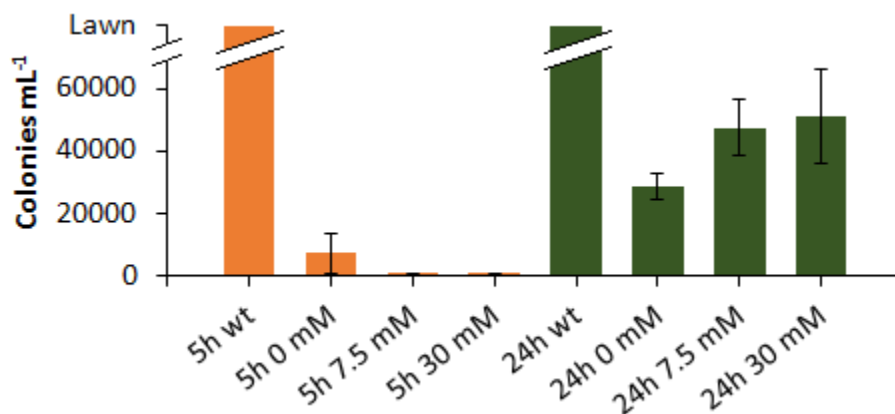
### 8.2.2. General results

The two incubation times (5 and 24 h) were chosen considering that *C. ljungdahlii* mut1 had previously shown a generation time of 7 h, allowing for the analysis of the mutagenic effect of DNA pol. IV after one or three generations, respectively. For both inducing periods, the wild type completely covered the plates (lawn), while the plasmid-containing cultures presented a worse performance (Figure 8.7). A possible explanation for this is the presence of thioamphenicol: wild



type strain is cultivated without antibiotic, while the rest have to activate the *catP* gene for thioamphenicol resistance. This consumes metabolic resources and affects negatively growth performance. Another issue is that differences in growth between 7.5 mM and 30 mM induced cultures were negligible for both inducing periods, suggesting that lactose concentrations higher than 7.5 mM have no significant effect.

The length of the inducing period with lactose seemed to have an important effect on the growth of single colonies in the cultures. As it can be observed in Figure 8.7, strains undergoing the lactose-induction step during 5 h presented a lower number of colonies than those being induced throughout a 24-h period. This might be explained by the longer induction times, which increase the possibility to damage the plasmid itself (as a result of mutagenesis). In that case, cells with a non-functional mutagenesis function could outgrow those with a functional mutagenesis plasmid. In order to confirm this hypothesis, a genome comparison would be necessary.



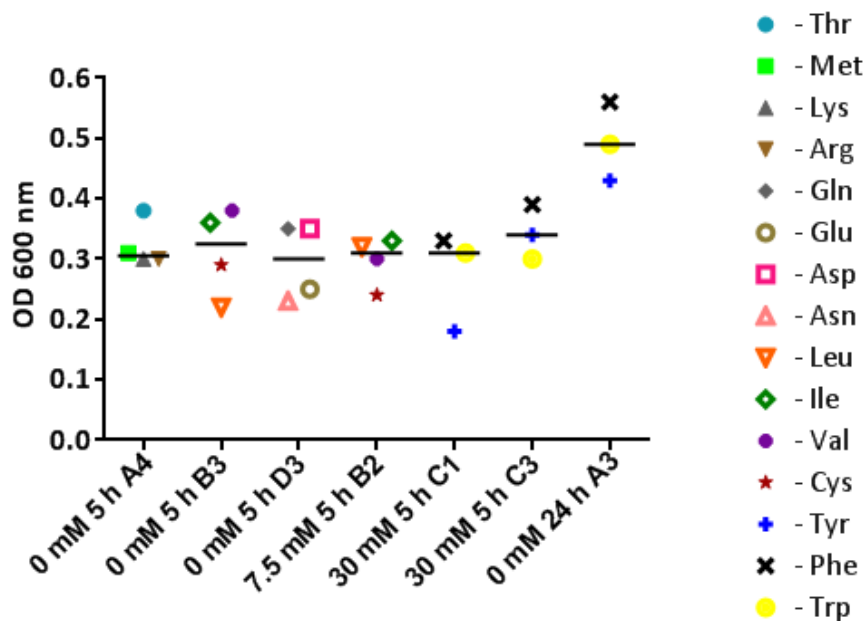
**Figure 8.7.** Growth performance of lactose-induced *C. ljungdahlii mut1* strains in PETC full (including all the aminoacids).

However, this better growth at 24 h did not reflect on a higher performance regarding auxotrophy development. After isolating colonies for each incubation period (and also for each lactose concentration), they were screened in search of auxotrophies for aminoacids synthesis by using plates with different aminoacids compositions. In order to find potential candidates for auxotrophies, it was desirable to find colonies that were able to grow on full PETC plates (containing all the aminoacids) and that did not grow in only one of the other plates. After the screening, 7 potential candidates (colonies) were further evaluated for auxotrophies search. Only one of these colonies had been isolated from the isolated colonies for the 24-h period, while growth

of auxotrophs was more feasible with a 5-h incubating period with lactose. This supports the theory that lactose induction for a period longer than the generation time of the bacteria leads to the prevalence of cells with a non-functional mutagenesis function.

The 7 potential candidates for auxotrophies were recultured in PETC without the aminoacids for which there were options for the development of an auxotrophy. Each screening was done in triplicate. A wild type control was also evaluated for each aminoacid mix, in order to exclude the possibility that detected effects occurred naturally. This wt control showed no natural effects when using any of these aminoacid mixtures. It was found that none of the candidates had actually developed an auxotrophy (Figure 8.8). However, candidates B3, B2 and C1 showed a clear decrease in their growth when a single amino acid was lacking in the culture media (Leu for B3, Cys for B2, Tyr for C1). A possible explanation could be that the corresponding aminoacid synthesis pathway could have been somehow damaged, but not completely knocked out.

Although further analyses should be done with this strain in order to confirm these results, it can be concluded that we could develop a whole cell mutagenesis tool based on DNA polymerase IV for *C. ljungdahlii* mut1 at a lactose concentration of 7.5 mM for periods shorter than *C. ljungdahlii* generation period of 7 h.



**Figure 8.8.** Growth of the 7 potential candidates for auxotrophies from *C. ljungdahlii* mut1 at PETC. Horizontal marks show the median of all aminoacid mixtures tested for each candidate.

### 8.2.3. References

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# *Chapter 9*

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## *General Conclusions*



## 9.1. CONCLUSIONS

As wastewater treatments are considered as energy-intensive processes, new alternatives are being investigated in order to reduce energy needs. Among these, bioelectrochemical systems (BESs) have proved to be a feasible option, since they can recover part of the chemical energy contained in the dissolved organic matter. In this PhD thesis the integration of BESs and other bioprocessing technologies (dark fermentation, DF or anaerobic digestion, AD) was characterized for treating both industrial and domestic wastewater at different configurations. The following conclusions can be drawn from the present study:

1. After the application of response surface methodology (RSM) for optimizing DF of a synthetic feeding, it was found that the best results appear at low values of N and lactose concentrations. An increase in N content negatively affected the H<sub>2</sub> yield, although this effect was attenuated by the increase in substrate concentration.
2. The optimum substrate/inoculum ratio for DF of cheese whey resulted to be 2.5 (VS-substrate:VS-inoculum). When working at this ratio, DF of cheese whey in batch-mode produced 0.7 L H<sub>2</sub> L<sub>r</sub><sup>-1</sup>, and an acidified effluent was obtained.
3. The effect of lactate addition in an acetate-fed microbial electrolysis cell (MEC) was also studied. An acetate:lactate ratio of 70:30 (on a molar basis) was found to optimize the H<sub>2</sub> production rate in the MEC (0.7 ± 0.1 L H<sub>2</sub> L<sub>a</sub><sup>-1</sup> d<sup>-1</sup>).
4. After being processed to fulfil the 70:30 ratio required, the effluent obtained from dark fermentation of cheese whey was fed into the MEC. The H<sub>2</sub> production rate in this second stage amounted to 0.5 L H<sub>2</sub> L<sub>a</sub><sup>-1</sup> d<sup>-1</sup>. As a whole, the global H<sub>2</sub> yield from cheese whey fermentation reached a value of 94.2 L H<sub>2</sub> kg<sub>VS</sub><sup>-1</sup>. This result represents a 20% improvement when compared to CW fermentations yields reported in bibliography.

While the previous conclusions refer to the integration of DF and BESs in a two-stage configuration, the following points are focused on the analysis of an integrative configuration of AD and BESs in the same reactor. It is important to highlight that these

studies were done under extreme conditions (both at high and low organic loads), where AD usually presents inefficiencies. Finally, the last point refers to the different strategies tested for the improvement of exoelectrogenic performance in BESs:

5. With regards to the integration of anaerobic digestion and bioelectrochemical systems in a batch-mode, overloaded anaerobic digester, a delay in VFA build-up, as well as an enhancement of CH<sub>4</sub> production during the first 100 h of the experiment were observed. Interestingly, this amelioration seems to be based on biomass proliferation rather than electrochemical activity, suggesting that the role of the electrode as a support for a better biomass proliferation would be significant.
6. Regarding the integration of MEC electrodes in an underloaded anaerobic reactor (fed with low-strength wastewater) working in batch, the use of MEC electrodes inside the digester improved organic matter removal and CH<sub>4</sub> production rate. Voltage application allowed for an increase of 90% in acetate degradation rate in comparison to open-circuit mode when using an acetate-based synthetic WW. CH<sub>4</sub> production rate was also enhanced by 73% under V application. Besides, hydrogenotrophic methanogenesis was promoted, preventing inefficiencies associated to homoacetogens proliferation.
7. When the same underloaded digester worked in continuous mode, long HRTs (12-24 h) were more favorable, as anode-respiring bacteria have a competitive advantage over acetoclastic methanogens. At an HRT of 24 h, CH<sub>4</sub> production rate in voltage-applied conditions was approximately 40% higher than the obtained in open-circuit mode. Long HRTs also favored energy recovery, making the process more cost-effective.
8. With regards to the improvement of exoelectrogenic response in BESs, a V application of +0.2 V optimized the electrogens selective enrichment from a natural soil substrate; in another strategy, a non-targeted mutagenesis tool based on the overexpression of DNA polymerase IV could be developed. However, in order to confirm these results, further analyses are needed.



## 9.2. FUTURE PERSPECTIVES

Bioelectrochemical systems are a promising technology for biological waste treatments. Moreover, their mutual integration with conventional bioprocesses (anaerobic digestion, dark fermentation) represents an innovative approach that could allow for a better recovery of the energy contained in waste streams, making the processes more profitable. However, there still remain some challenges to be overcome in order to achieve practical implementation of this technology. For instance (and regarding issues contained in this PhD thesis), the optimization of reactor design (particularly concerning the electrode surface/reactor volume ratio) appears as an important point, as this is the cause of many inefficiencies in BESs, such as the great energy loss in the form of dissolved methane. The integration of electrogens-enriched electrodes in an anaerobic digester also presents some issues, such as the need of a better understanding of propionate accumulation and how to prevent it. For a proper characterization of the process, further evaluations of the integrated approach working in continuous-mode at short HRTs are also required.

From a more general perspective, three are the key points that, in the author's opinion, will play a significant role in the future development of BESs technology:

- From the biological point of view, the enhancement of the extracellular electron transfer phenomenon would help to optimize bioelectrochemical activity. This might be done by different approaches, such as genetic engineering, selective enrichment of electroactive species or promotion of biofilm formation by different operational conditions.
- Regarding the feasibility of different processes with BESs, microbial electrosynthesis (MES), a process based on the reduction of carbon dioxide to multiple-carbon organic compounds appears as a promising, environmentally sustainable option for the synthesis of chemical products.
- With regards to the engineering and reactor design aspects, it is known that increasing the size of bioelectrochemical reactors results in a declinment of power density and electrogenic performance. Therefore, if BESs are to be brought into practical implementation, deeper studies at pilot scale for verification of laboratory empirical results must be done.



# *Capítulo 10*

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*Resumen Global*



## 10.1. TECNOLOGÍAS DE TRATAMIENTO BIOLÓGICO PARA LA GESTIÓN DE RESIDUOS

El tratamiento biológico de residuos se ha llevado a cabo tradicionalmente por medio de procesos anaerobios. Entre ellos, el mejor implantado es la digestión anaerobia. La fermentación oscura sería otro ejemplo, aunque no está tan extendida a escala real. En cualquier caso, dado que estos procesos también presentan desventajas, nuevas tecnologías (como los sistemas bioelectroquímicos) se han desarrollado recientemente. La integración de las tecnologías más convencionales y las más innovadoras permitiría un mejor aprovechamiento de sus fortalezas.

### 10.1.1. Tecnologías convencionales de bioprocesamiento: digestión anaerobia y fermentación oscura

La digestión anaerobia (AD\*) es un proceso biológico consistente en la degradación de la materia orgánica en ausencia de oxígeno por parte de microorganismos anaerobios. El principal producto de este proceso es el biogás, que es una mezcla de gases: CH<sub>4</sub> (48-65 %), CO<sub>2</sub> (36-41 %), N<sub>2</sub> (aproximadamente 17%), y proporciones menores de CO, H<sub>2</sub>, H<sub>2</sub>S, etc. (Ward et al., 2008). La digestión anaerobia constituye una alternativa al tratamiento de residuos por métodos aerobios, debido a su menor consumo de energía (Abbasi et al., 2012).

La AD consta de diferentes etapas llevadas a cabo por distintas especies de microorganismos, que trabajan de forma secuencial o simultánea (Massé and Droste, 2000). La primera etapa (hidrólisis) consiste en la solubilización de la materia orgánica más compleja por medio de la acción de enzimas extracelulares secretadas por bacterias hidrolíticas. Al ser esta fase altamente dependiente de la complejidad de la materia orgánica a tratar, suele ser considerada como la etapa limitante del proceso. La segunda fase (acidogénesis) consiste en la metabolización, por parte de bacterias fermentativas, de la materia orgánica soluble producida en la fase anterior, generando ácidos grasos volátiles (volatile fatty acids, VFA), sobre todo acetato, propionato y butirato. En el siguiente paso (acetogénesis), los VFA se transforman en acetato, H<sub>2</sub> y CO<sub>2</sub>. En esta etapa, las bacterias homoacetógenas proliferan y producen acetato a partir del H<sub>2</sub> y CO<sub>2</sub>, compitiendo en parte con los procesos metanogénicos. Estos procesos son la base de la cuarta etapa (metanogénesis), en la que se produce CH<sub>4</sub> por dos posibles mecanismos: metanogénesis acetoclástica (a partir de acetato) y metanogénesis hidrogenotrofa (a partir de H<sub>2</sub> y CO<sub>2</sub>).

\*NOTA: por coherencia entre las distintas abreviaturas, se han mantenido en inglés todas ellas.

El lactosuero (cheese whey, CW) aparece como un sustrato potencial para su tratamiento por AD. Es el principal subproducto de la producción de queso en industrias lácteas, representa aproximadamente el 90% del volumen total de la leche, y retiene aproximadamente el 55% de sus nutrientes (González, 1996). Aunque puede ser usado para la producción de algunos alimentos (por ejemplo el requesón), su producción en exceso lo convierte en un residuo que es necesario gestionar, dado su alto contenido en materia orgánica. Sin embargo, su digestión presenta algunas dificultades: al ser la degradación de VFA por parte de las metanógenas mucho más lenta que la producción de los mismos, la AD de sustratos con alta carga orgánica como el CW suele desembocar en una acumulación excesiva de VFA en el reactor (Prazeres et al., 2012). Así pues, mantener estable el proceso es una tarea compleja (Janczukowicz et al., 2008). Para evitar este problema, se han testado soluciones como son el suplemento de alcalinidad al reactor (Gannoun et al., 2008) o la separación de la acidogénesis y metanogénesis en dos reactores diferentes (Saddoud et al., 2007). Recientemente, procesos de integración de AD con sistemas bioelectroquímicos han sido descritos en bibliografía (ver Sección **10.2**).

Por otra parte, la fermentación oscura (dark fermentation, DF) es una tecnología de tratamiento de residuos cuyo producto principal es el H<sub>2</sub>. Entre los microorganismos productores de H<sub>2</sub>, los más conocidos son *Clostridium* y *Enterobacter*. En ambos casos, el poder reductor obtenido en la glucólisis puede ser utilizado por un complejo enzimático (hidrogenasas) para formar H<sub>2</sub> (Axley et al., 1990; Peters, 1999). Aunque las hidrogenasas de *Clostridium* producen H<sub>2</sub> de forma más eficiente, el hecho de que *Enterobacter* sea anaerobio facultativo (*Clostridium* es estricto) aporta versatilidad a la comunidad microbiana fermentativa, lo que hace que el uso de cultivos mixtos sea recomendable.

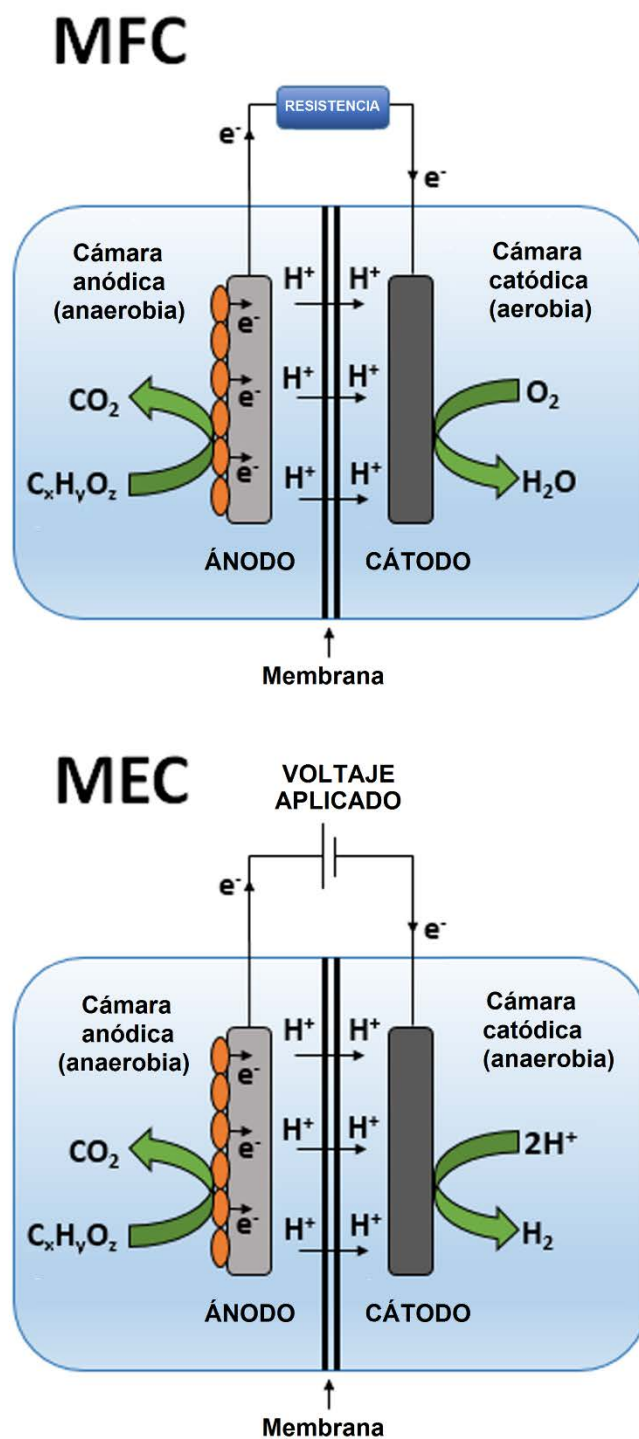
La DF de lactosuero ha sido evaluada en varios estudios. En teoría, este proceso debería conducir a un rendimiento de 8 mol de H<sub>2</sub> por mol de lactosa (Prazeres et al., 2012). Sin embargo, la producción de H<sub>2</sub> es un proceso que sufre inhibición por producto, lo que hace que el rendimiento se vea reducido significativamente (Davila-Vázquez and Razo-Flores, 2007). Dado que el efluente de fermentación oscura de lactosuero requiere de postratamiento (Prazeres et al., 2012) se han buscado alternativas para su gestión, como introducirlo en un reactor de AD (Fernández et al., 2015) o bien usarlo como sustrato para sistemas bioelectroquímicos (ver Sección **10.2**).

### 10.1.2. Sistemas bioelectroquímicos

En términos generales, los sistemas bioelectroquímicos (BESs) son biorreactores en los que se usan los microorganismos adheridos a la superficie de un electrodo para catalizar reacciones de oxidación-reducción (Rabaey et al., 2007). Desde el descubrimiento de microorganismos electroquímicamente activos por Potter (1911), los BESs han aparecido como una tecnología emergente que combina procesos biológicos y electroquímicos para la producción de energía (ya sea en forma de electricidad,  $H_2$ ,  $CH_4$  u otros compuestos), o para el tratamiento de aguas residuales (Bajracharya et al., 2016). En función del proceso requerido, se han desarrollado recientemente diferentes tipos de sistemas bioelectroquímicos, siendo los más importantes las celdas de combustible microbianas (MFCs) para la producción de electricidad (Liu et al., 2005; Logan et al., 2006) y las celdas de electrólisis biocatalítica (MECs), para la síntesis de compuestos químicos, entre los cuales estarían el  $H_2$  y el  $CH_4$  (Rozendal et al., 2006; Escapa et al., 2016).

Una MFC estándar consiste en dos compartimentos diferenciados, conocidos como cámara anódica y cámara catódica, cada uno de los cuales contiene un electrodo (ánodo y cátodo, respectivamente). Ambas cámaras suelen estar separadas por una membrana de intercambio protónico (PEM) (Figura 10.1). En la cámara anódica, la materia orgánica es degradada por bacterias electrógenas, produciéndose  $CO_2$ , electrones y protones. Los electrones son transferidos al ánodo por diferentes mecanismos, y después transportados al cátodo a través de un circuito externo, mientras que los protones alcanzan la cámara catódica atravesando la PEM. En la cámara catódica, los electrones reaccionan con un aceptor terminal de electrones (normalmente el  $O_2$ ), para formar  $H_2O$  (Logan, 2008).

Las celdas de electrólisis biocatalítica (MECs) están dirigidas a la producción de compuestos químicos (por ejemplo,  $H_2$  o  $CH_4$ ), en lugar de a la generación de electricidad. Debido a ello y aunque las MECs no son muy diferentes a las MFCs en cuanto a la arquitectura general del dispositivo, sí se pueden establecer dos diferencias importantes (Figura 10.1): en primer lugar, en las MECs la cámara catódica debe ser anaerobia, para impedir que se produzca la reducción de oxígeno. En segundo lugar, y dado que la reacción de formación de  $H_2$  no es espontánea, en las MECs es necesaria la aplicación de una diferencia de potencial.



*Figura 10.1. Comparación entre las estructuras y las condiciones de operación de una MFC y una MEC. La cámara catódica es aerobia en MFCs y anaerobia en MECs, y una resistencia en el circuito en MFCs es sustituida por la aplicación de voltaje en MECs. Las MECs están dirigidas a la producción de compuestos químicos, mientras las MFCs producen electricidad.*



## 10.2. INTEGRACIÓN DE SISTEMAS BIOELECTROQUÍMICOS CON TECNOLOGÍAS CONVENCIONALES DE BIOPROCESAMIENTO PARA LA GESTIÓN DE RESIDUOS

La habilidad de los BESs para tratar un amplio rango de sustratos diferentes explica la diversidad de usos que estos dispositivos ofrecen. Dada la especial capacidad de los BESs para tratar corrientes residuales ricas en compuestos no fermentables (como los VFA) (Ghimire et al., 2015), la integración de la fermentación oscura y los BESs es particularmente beneficiosa, ya que los efluentes de fermentación oscura son ricos en dichos compuestos (Yang et al., 2013). Debido a ello, la posibilidad de usar MECs como postratamiento para los procesos biológicos ha sido estudiada. Por ejemplo, Liu et al. (2012) usaron el efluente acidificado del proceso de fermentación oscura de lodos activados como sustrato para la producción de  $H_2$  en una MEC, resultando en un rendimiento de  $1.2 \text{ mL } H_2 \text{ mg}_{\text{COD}}^{-1}$  y una tasa de producción de  $H_2$  de  $120 \text{ mL } H_2 \text{ g}_{\text{VSS}}^{-1} \text{ d}^{-1}$ . En otro estudio, Lu et al. (2009) obtuvieron  $700 \text{ mL } H_2 \text{ L}^{-1} \text{ d}^{-1}$  durante la fermentación oscura de melazas y  $1410 \text{ mL } H_2 \text{ L}^{-1} \text{ d}^{-1}$  al tratar el efluente (rico en VFA y etanol) con BESs. En otro estudio, tras conseguir, a partir de la fermentación oscura de restos de tallos de maíz, una producción de  $1.73 \text{ m}^3 \text{ H}_2 \text{ m}^{-3} \text{ d}^{-1}$ , Li et al. (2014) reportaron  $3.43 \text{ m}^3 \text{ H}_2 \text{ m}^{-3} \text{ d}^{-1}$  en la fase bioelectroquímica, mientras Lalaurette et al. (2009) obtuvieron un incremento del 600% en el rendimiento en producción de  $H_2$  al combinar la fermentación oscura de restos de cosecha de maíz con el tratamiento del efluente con BESs.

Además, también existe la opción de integrar la digestión anaerobia con los BESs, lo que abriría la posibilidad a una mejora en el tratamiento de los sólidos separados de las aguas residuales en las estaciones depuradoras. En contraste con el modelo secuencial investigado para la fermentación oscura con BESs, para el caso de integración AD-BES se ha evaluado la combinación de ambas tecnologías en el mismo reactor. Este modelo, que consiste en introducir directamente biomasa electrógena (inoculada en electrodos) en el reactor anaerobio presenta la ventaja de reducir los costes de operación del proceso, así como las pérdidas energéticas, al tiempo que se maximiza la utilización del sustrato (ElMekawy et al., 2014). Además de un incremento en la eliminación de materia orgánica, la integración de AD-BES también contribuiría a la estabilización del proceso de digestión, ya que el consumo del exceso de VFA en el reactor por parte de las electrógenas podría evitar la inhibición del proceso. Este modelo de integración ha sido estudiado

para el tratamiento de diferentes sustratos: remediación de colorantes en aguas contaminadas (Cui et al., 2014), lodos activados (Asztalos et al., 2015) o aguas residuales sintéticas ricas en sacarosa (Zhang et al., 2015).

### 10.3. OBJETIVOS

El objetivo principal de esta tesis es la evaluación y el análisis de la integración de sistemas bioelectroquímicos (BESs) con tecnologías convencionales de tratamiento (como la fermentación oscura y la digestión anaerobia) para el procesamiento de aguas residuales. Este estudio se realizó con aguas residuales de origen industrial (lactosuero) y con aguas residuales urbanas.

Para la consecución de este objetivo principal se determinaron las condiciones de operación que optimizan estos procesos biológicos, y se evaluaron dos configuraciones distintas de integración (en una o en dos etapas). Por tanto, el estudio se centra en cuatro aspectos:

- i) identificar las concentraciones óptimas de nitrógeno y lactosa para fermentación oscura de una alimentación sintética por medio de metodología de superficie de respuesta (RSM), además del ratio sustrato/inóculo óptimo para la fermentación oscura de lactosuero y la relación acetato/lactato más favorable para los procesos bioelectrocatalíticos.
- ii) evaluar la respuesta de un sistema en dos etapas integrado por fermentación oscura y sistemas bioelectroquímicos para el tratamiento de lactosuero.
- iii) estudiar la respuesta de sistemas bioelectroquímicos integrados en un reactor anaerobio bajo condiciones de inhibición por acumulación de VFA.
- iv) analizar los beneficios del uso de procesos de electrometanogénesis con electrodos MEC para asistir la digestión anaerobia en el tratamiento de agua residual urbana de baja carga orgánica.
- v) explorar diferentes estrategias para la mejora del rendimiento bioelectrocatalítico por parte de las bacterias electrógenas: desarrollo de protocolos de enriquecimiento selectivo y de inducción de mutagénesis.

#### 10.4. PRODUCCIÓN DE BIOHIDRÓGENO A PARTIR DE LACTOSA: INFLUENCIA DE LA CONCENTRACIÓN DE SUSTRATO Y NITRÓGENO

Debido al alto contenido en carbohidratos del lactosuero, la producción de biohidrógeno por fermentación oscura de lactosuero aparece como una opción interesante para su valorización (Azbar et al., 2009; Hay et al., 2013). Sin embargo, para un correcto desarrollo de la fermentación, la concentración de materia orgánica y el contenido en nitrógeno del sustrato son factores cruciales. El bajo contenido en nitrógeno del lactosuero hace que este componente deba ser añadido de forma externa. Así pues, en este estudio se evaluó el comportamiento del proceso de fermentación oscura en batch a diferentes contenidos de lactosa (como fuente de C) y a diferentes ratios C/N, por medio de la metodología de superficie de respuesta (RSM). Un segundo estudio usando como sustrato tanto agua residual sintética como lactosuero fue realizado a modo complementario. Se buscaba determinar las condiciones óptimas de operación del proceso de fermentación oscura, de cara a su posterior integración con sistemas bioelectroquímicos.

##### 10.4.1. Determinación de las condiciones iniciales de operación por RSM

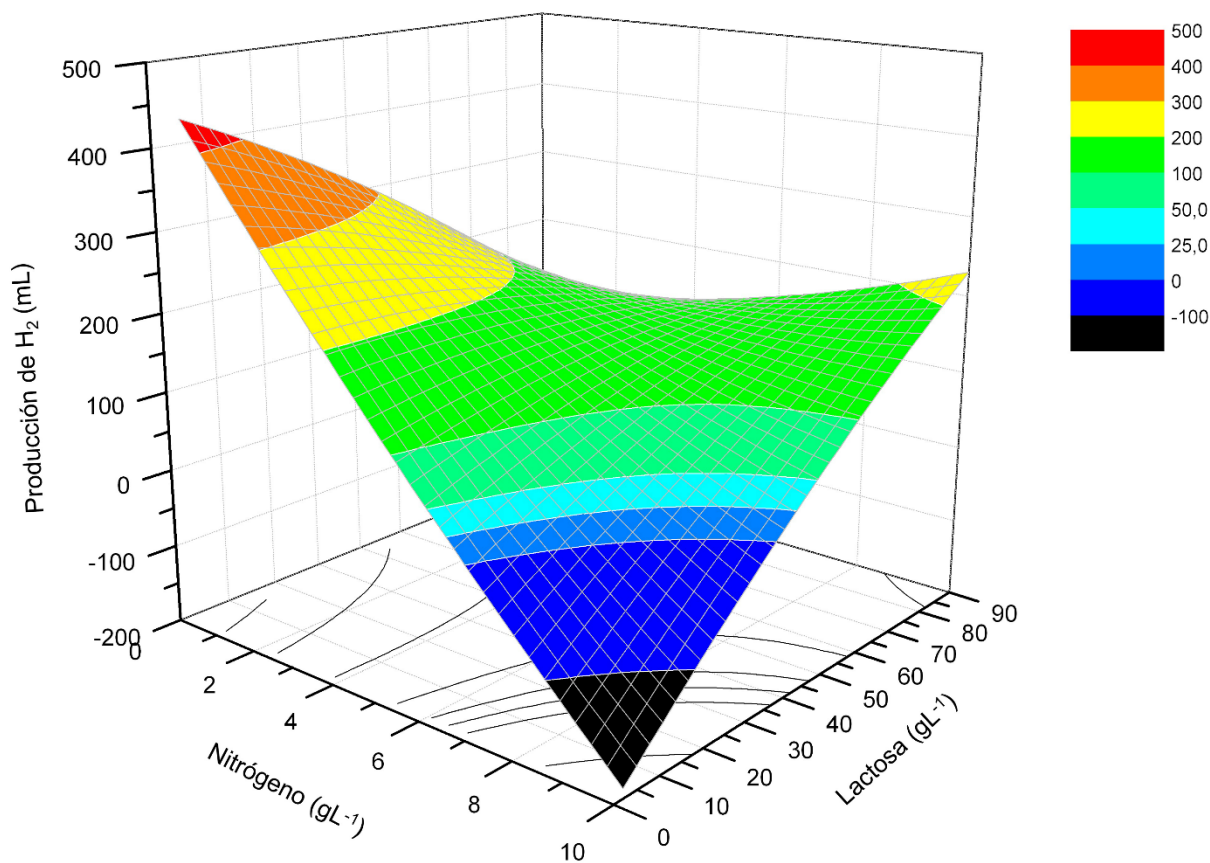
Se diseñó un modelo basado en tres factores (pH, concentración de N y concentración de lactosa, denominados  $X_1$ ,  $X_2$  y  $X_3$ , respectivamente), con el objetivo de ajustar la respuesta a un polinomio de segundo orden. El estudio se realizó en batch, en matraces Erlenmeyer de 250 mL, en rangos de pH entre 4 y 6, concentraciones de N entre 0.5 y 10 g L<sup>-1</sup> y concentraciones de lactosa entre 2 y 90 g L<sup>-1</sup>.

A partir de los datos de producción de gas y del análisis del contenido de los matraces tras la fermentación, se pudo ajustar la respuesta del sistema al siguiente polinomio

$$Y = 305.2 - 128.16X_1 - 78.1X_2 + 29.1X_3 + 0.14X_1X_2 - 5.9X_1X_3 + 0.91X_2X_3 + 29.1X_1^2 + 0.93X_2^2 - 0.012X_3^2$$

donde  $Y$  es la respuesta del sistema (producción de H<sub>2</sub>, en este caso). A partir del análisis de regresión múltiple, se observa que sólo la concentración de lactosa y la interacción entre los factores concentración de N y concentración de lactosa presentaron una influencia significativa sobre la respuesta. Como se puede observar en la Figura 10.2, la producción de H<sub>2</sub> se reduce al aumentar las concentraciones de N y de lactosa. Sin embargo, a altas concentraciones de lactosa,

el efecto del N sobre la respuesta se veía atenuado. En suma, los resultados sugieren que la mejor zona de operación estaría situada a bajos niveles tanto de lactosa como de nitrógeno. Además, a bajas concentraciones de N la producción de ácidos grasos volátiles es máxima. En cuanto al pH, no se observó una influencia significativa de este parámetro sobre el proceso.



**Figura 10.2.** Superficie de respuesta del modelo estudiado para la producción de H<sub>2</sub> a pH=5.5.

#### 10.4.2. Estudios de fermentación en semicontinuo

Posteriormente, se llevaron a cabo, en matraces Erlenmeyer de 250 mL, estudios en semicontinuo con una concentración fija de lactosa de 36 g L<sup>-1</sup> y con cantidades variables de N (0.5, 1.4 y 2.3 g L<sup>-1</sup>) para una alimentación sintética. El tiempo de retención hidráulica (HRT) del sustrato en estos sistemas fue de 2.5 días. A partir de la concentración de N que ofreció mejores resultados, se procedió a un salto de escala a un reactor de 3 L. De nuevo, la concentración de N

se fue reduciendo con el objetivo de comprobar el funcionamiento a concentraciones de N lo más bajas posible (tres períodos de 12 días cada uno, a concentraciones de 1.4, 0.9 y 0.5 g N L<sup>-1</sup>). Finalmente, este mismo reactor fue alimentado con un lactosuero cuya concentración de lactosa fue de 36 g L<sup>-1</sup>, por un período de 20 días. Durante todo el proceso de operación del reactor de 3 L, el HRT fue de 3 días.

Del estudio en matraces Erlenmeyer, se llegó a la conclusión de que la producción de H<sub>2</sub> se veía maximizada a la concentración de 1.4 g L<sup>-1</sup>. Además, al analizar los VFA de los diferentes matraces, se encontró que la máxima producción de butírico se obtenía también a 1.4 g N L<sup>-1</sup>, en comparación con las otras concentraciones estudiadas. Aunque la producción de H<sub>2</sub> es teóricamente mayor con la ruta de fermentación acética en que con la fermentación butírica, esta última es más favorable para el crecimiento de la biomasa fermentativa, lo que hace que, en la práctica, la fermentación butírica sea la ruta de producción de H<sub>2</sub> más efectiva (Hawkes et al., 2007).

A partir, por tanto, de la concentración de 1.4 g N L<sup>-1</sup>, se evaluó el comportamiento de un reactor de 3 L con una alimentación sintética de 36 g L<sup>-1</sup> de lactosa. La concentración de N se fue reduciendo gradualmente, lo que condujo a un aumento en la producción de H<sub>2</sub> del 150% y el 200% en los períodos de 0.9 y 0.5 g N L<sup>-1</sup>, respectivamente. Mientras la concentración de acetato en el efluente se mantuvo constante a lo largo de los tres períodos, se observó que la producción de butirato e isobutirato crecía en aquellos períodos en los que la concentración de N se encontraba en niveles más bajos.

Al pasar a utilizar lactosuero como alimentación, y a pesar de mantener un contenido en lactosa similar (36 g L<sup>-1</sup>), la producción de H<sub>2</sub> aumentó desde 0.14 L H<sub>2</sub> L<sup>-1</sup> d<sup>-1</sup> (en el período anterior) hasta 0.18 L H<sub>2</sub> L<sup>-1</sup> d<sup>-1</sup> con lactosuero, aunque aumentó la inestabilidad del sistema. La producción de butirato e isobutirato también aumentó al usar lactosuero como sustrato en relación a los períodos anteriores. El contenido en N del lactosuero era muy bajo (0.6 g N<sub>Kjeldahl</sub> L<sup>-1</sup>, estando los valores de N en forma de NH<sub>4</sub><sup>+</sup> por debajo de 0.06 g L<sup>-1</sup>), lo que apoyaría la hipótesis de que bajas concentraciones de N fomentan la vía de fermentación butírica por delante de otras rutas metabólicas.

### **10.4.3. Conclusiones**

A partir del estudio del efecto conjunto de la concentración de lactosa y nitrógeno sobre la producción fermentativa de hidrógeno en batch, se obtuvo que el área de operación que optimiza la respuesta se encuentra a bajos niveles tanto de lactosa como de nitrógeno. La evaluación posterior del proceso fermentativo en modo continuo a diferentes concentraciones de N, permitió comprobar que la producción de H<sub>2</sub> aumentaba conforme se iba reduciendo gradualmente la concentración de N. El tratamiento de lactosuero (cuyo contenido en nitrógeno era mínimo) por fermentación oscura en ese mismo reactor resultó en un aumento en la producción de H<sub>2</sub>, aunque el sistema se volvió más inestable.

## **10.5. PRODUCCIÓN DE HIDRÓGENO EN UN PROCESO DE DOS ETAPAS: INTEGRACIÓN DE FERMENTACIÓN OSCURA Y ELECTRÓLISIS BIOCATALÍTICA**

El tratamiento de residuos por fermentación oscura se ha revelado como una opción factible que además permite el aprovechamiento de parte de la energía contenida en el residuo, gracias a la producción de hidrógeno como resultado de este proceso. Sin embargo, debido a limitaciones técnicas, la fermentación oscura sólo permite obtener un rendimiento del 33% del máximo de H<sub>2</sub> que podría obtenerse a partir de la glucosa, según parámetros estequiométricos (Gómez et al., 2011). En este contexto, la electrólisis biocatalítica emerge como una tecnología complementaria a los procesos fermentativos, que permitiría recuperar hasta el 90% de la energía contenida en el sustrato (Cheng and Logan, 2007). Por ello, en este estudio se investigó la capacidad de un proceso integrado fermentación oscura + electrólisis biocatalítica para el tratamiento de un agua residual industrial (lactosuero). Ambos procesos se integran de modo secuencial en dos etapas, de modo que el efluente de la fermentación oscura es tratado en el sistema bioelectrocatalítico.

### **10.5.1. Primera etapa: fermentación oscura**

Se realizaron experimentos en batch en matraces Erlenmeyer de 250 mL usando lodo digerido de depuradora como inóculo (I) y lactosuero (CW) como sustrato. Se hicieron pruebas a diferentes ratios de sólidos volátiles CW:I, con el objetivo de averiguar cuál de ellos optimizaba el proceso fermentativo. Se observó que la producción de H<sub>2</sub> aumentaba al disminuir la proporción

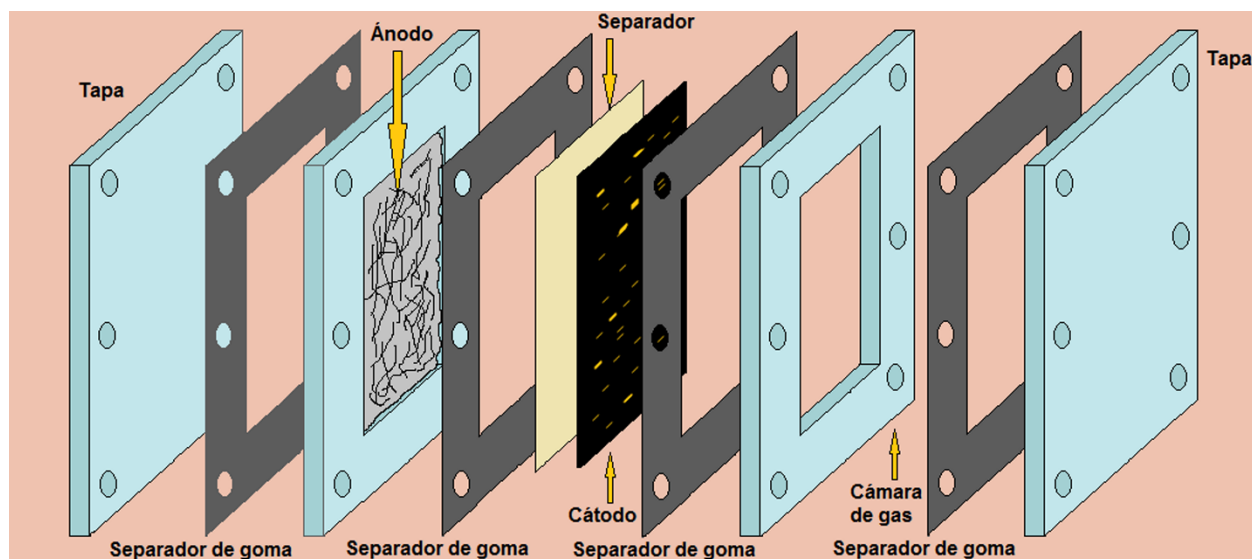
de lactosuero en la alimentación, mientras que la tasa de eliminación de materia orgánica no parecía verse afectada. De este modo, se concluyó que la relación de sólidos volátiles CW:I que optimizaba la respuesta de fermentación oscura resultó ser de 2.5. El análisis de ácidos grasos mostró que, tal y como se esperaba, el ácido láctico fue el producto más abundante en la fermentación oscura de lactosuero (Tabla 10.1). El efluente de fermentación oscura de lactosuero en este ratio fue recogido y procesado para servir de alimentación a la celda de electrólisis biocatalítica en la siguiente etapa.

**Tabla 10.1.** Concentraciones de VFA y ácido láctico durante la producción fermentativa de  $H_2$  con un ratio CW:I de 2.5.

| Hora | Acetato (mM) | Propionato (mM) | Butirato (mM) | Lactato (mM) |
|------|--------------|-----------------|---------------|--------------|
| 0    | 1.9 ± 0.2    | 0.0 ± 0.0       | 0.0 ± 0.0     | 7.5 ± 1.2    |
| 7    | 6.5 ± 0.2    | 0.9 ± 0.0       | 0.7 ± 0.0     | 28.1 ± 3.4   |
| 9    | 9.3 ± 0.3    | 1.6 ± 0.1       | 2.2 ± 0.1     | 36.4 ± 3.2   |
| 11   | 10.5 ± 0.4   | 0.8 ± 0.1       | 3.8 ± 0.1     | 48.6 ± 6.3   |
| 13   | 13.0 ± 0.5   | 1.1 ± 0.0       | 4.7 ± 0.2     | 64.2 ± 8.5   |
| 24   | 12.9 ± 0.4   | 0.9 ± 0.1       | 3.7 ± 0.3     | 99.9 ± 9.6   |

### 10.5.2. Segunda etapa: electrólisis biocatalítica

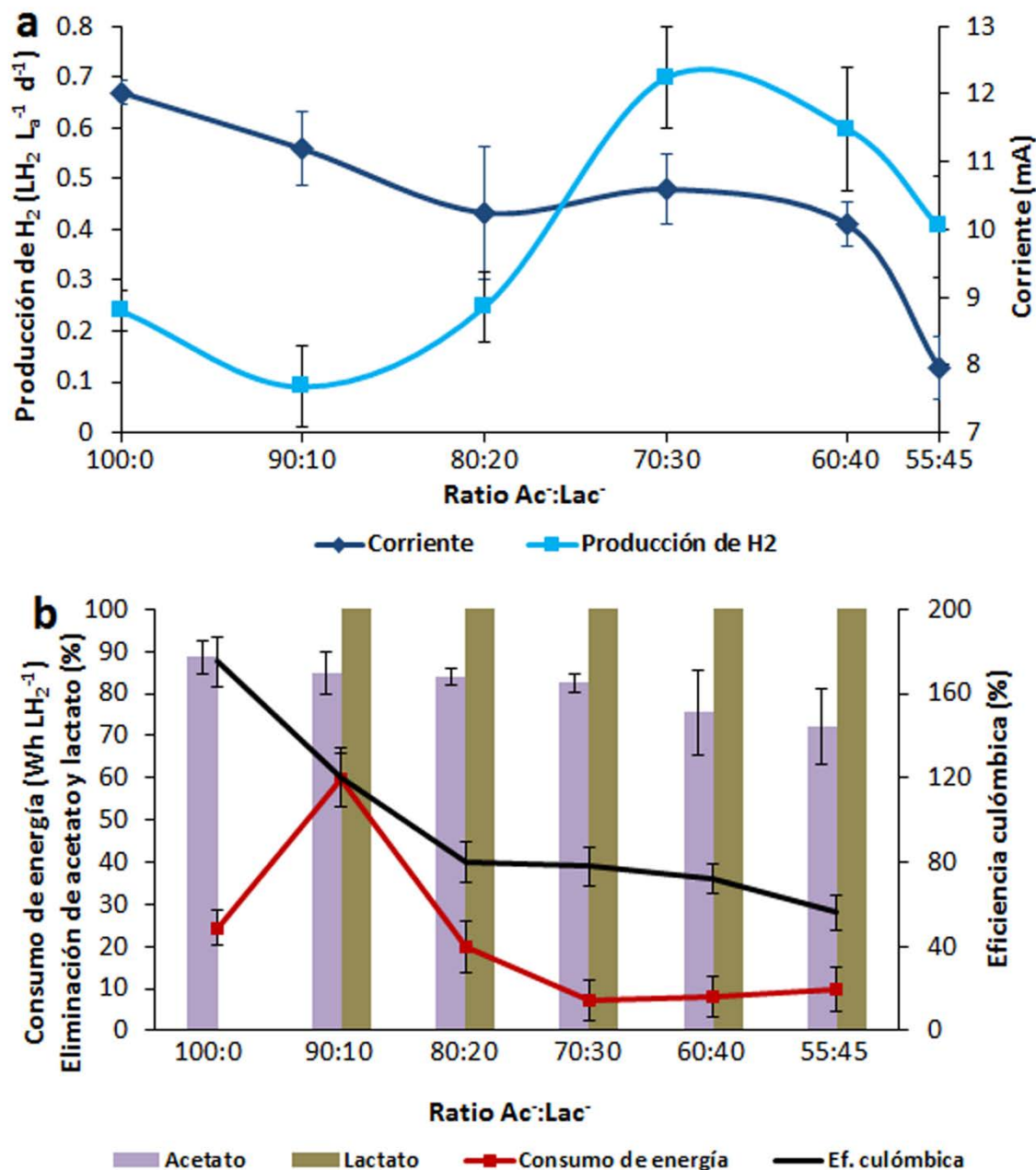
Se utilizó una celda de electrólisis biocatalítica (MEC) construida con una serie de placas de metacrilato (Figura 10.3) para su operación en modo continuo. Esta celda constaba de dos cámaras (anódica y catódica), con un volumen de 50 mL cada una. El ánodo consistió en una capa (5 mm de grosor) de fieltro de carbono, mientras que el cátodo era un electrodo carbonoso de difusión de gas con partículas de Ni electrodepositadas. Una pieza de poliéster evitaba todo tipo de contacto eléctrico entre ambos (cortocircuito). La celda MEC operó a 25 °C y con un voltaje aplicado de 1V.



**Figura 10.3.** Arquitectura de la celda MEC usada en este estudio.

La celda se inoculó con el efluente de otra celda MEC que había operado con agua residual doméstica durante el año anterior. Como alimentación, se fueron probando diferentes proporciones de acetato/lactato hasta encontrar aquel ratio que optimizaba el rendimiento de la celda, con el objetivo de poder adecuar el contenido de lactosuero (rico en lactato) en la siguiente etapa. Se encontró que la adición de lactato a la alimentación provocaba un descenso en la capacidad electrógena de la celda MEC, así como en la producción de  $H_2$ . Sin embargo, con la proporción 70:30 ( $Ac^-:Lac^-$ ) la producción de  $H_2$  se recuperó, mientras que la producción de corriente dejó de reducirse (Figura 10.4a). Además, el bajo pH de la alimentación (debido a la presencia de lactato) contribuyó a la mitigación de un fenómeno indeseable conocido como “hydrogen recycling”, que había sido observado en la celda al principio de la operación (cuando la alimentación constaba únicamente de acetato). Este fenómeno consiste en el consumo, por parte de bacterias homoacetógenas, del  $H_2$  producido para producir acetato, lo que incrementa artificialmente el consumo energético de la celda MEC. La consecuencia de este fenómeno son valores de eficiencia culómbica mayores del 100%. Como se puede observar (Figura 10.4b), la eficiencia culómbica se coloca en niveles menores del 100% a partir de la adición de lactato. Además, el consumo energético de la celda es mínimo para la proporción 70:30 ( $Ac^-:Lac^-$ ), por lo que este ratio es el que se considera óptimo para el rendimiento global del proceso bioelectrocatalítico.





**Figura 10.4.** Evolución de: a) Corriente y producción de H<sub>2</sub>; b) Eliminación de acetato y lactato, eficiencia culómbica y consumo energético para los diferentes ratios Ac<sup>-</sup>:Lac<sup>-</sup>.

Finalmente, se introdujo como alimentación de la celda MEC el efluente acidificado obtenido de la fermentación oscura del lactosuero en la primera etapa. De acuerdo con los resultados recién expuestos, este efluente fue procesado de modo tal que pasó a tener un ratio de 70:30 (Ac<sup>-</sup>:Lac<sup>-</sup>) antes de ser introducido a la celda. Se observó un descenso en la corriente, así

como en la producción de  $H_2$ , al usar este efluente como sustrato (fases CW-DA y CW-D, Figura 10.5). Se achacó este descenso a la falta de sales y oligoelementos en el sustrato. Por ello, tras un período de recuperación de la celda con alimentación sintética 70:30 ( $Ac^-:Lac^-$ ), se reintrodujo el efluente con sales añadidas (fase CW-DAS). Como puede observarse, en este caso el rendimiento de la celda se mantuvo estable. En esta fase se produjo  $H_2$  a un ritmo de  $0.5 \text{ L } H_2 \text{ L}_r^{-1} \text{ d}^{-1}$ , lo que, unido a la producción de  $H_2$  en la fase de fermentación oscura ( $0.7 \text{ L } H_2 \text{ L}_r^{-1}$ ), arroja un rendimiento global de  $94.2 \text{ L } H_2 \text{ kg}_{VS}^{-1}$ , mayor que los obtenidos en otros estudios de tratamiento de lactosuero ( $47.9\text{-}78.2 \text{ L } H_2 \text{ kg}_{COD}^{-1}$ ) (Ferchichi et al., 2005; Venetsaneas et al., 2009). Al retirar posteriormente el suplemento de acetato (fase CW-DS), de nuevo se produjo el colapso de la celda. Así pues, aunque el lactato de la alimentación fue degradado completamente en todas las fases, es importante remarcar el papel crucial que juega el acetato en el mantenimiento de la actividad electrogénica.

### 10.5.3. Conclusiones

Se evaluó un proceso integrado de fermentación oscura y sistemas bioelectroquímicos para el tratamiento de lactosuero y producción de  $H_2$ . Durante la fase de fermentación oscura, se obtuvo una tasa de producción de  $H_2$  de  $0.7 \text{ L } H_2 \text{ L}_r^{-1}$ . El efluente de esta primera etapa fue procesado e introducido como alimentación en una celda MEC, obteniéndose a su vez una producción de  $0.5 \text{ L } H_2 \text{ L}_r^{-1} \text{ d}^{-1}$ . Por tanto, el rendimiento global de producción de  $H_2$  en el proceso corresponde a  $94.2 \text{ L } H_2 \text{ kg}_{VS}^{-1}$ , mayor que otros rendimientos de fermentación de lactosuero hallados en bibliografía. Además, los bajos niveles de pH del efluente de la fermentación oscura ayudaron a controlar los efectos indeseables (“hydrogen recycling”) de la actividad de bacterias homoacetógenas en el reactor bioelectroquímico.

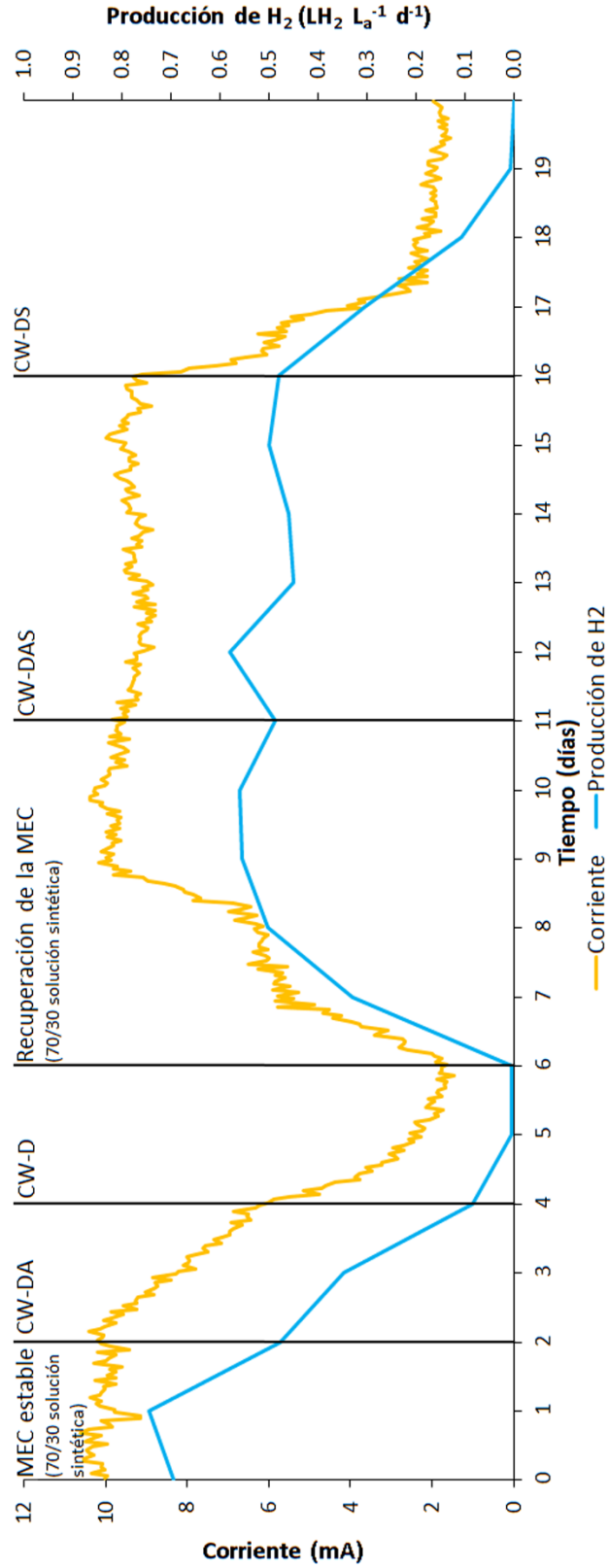


Figura 10.5. Evolución de la corriente y la producción de H<sub>2</sub> en la celda MEC.

## **10.6. PREVENCIÓN DE LA ACUMULACIÓN DE ÁCIDOS GRASOS EN UN DIGESTOR ANAEROBIO MEDIANTE LA INTEGRACIÓN DE ELECTRODOS BIOELECTROQUÍMICOS**

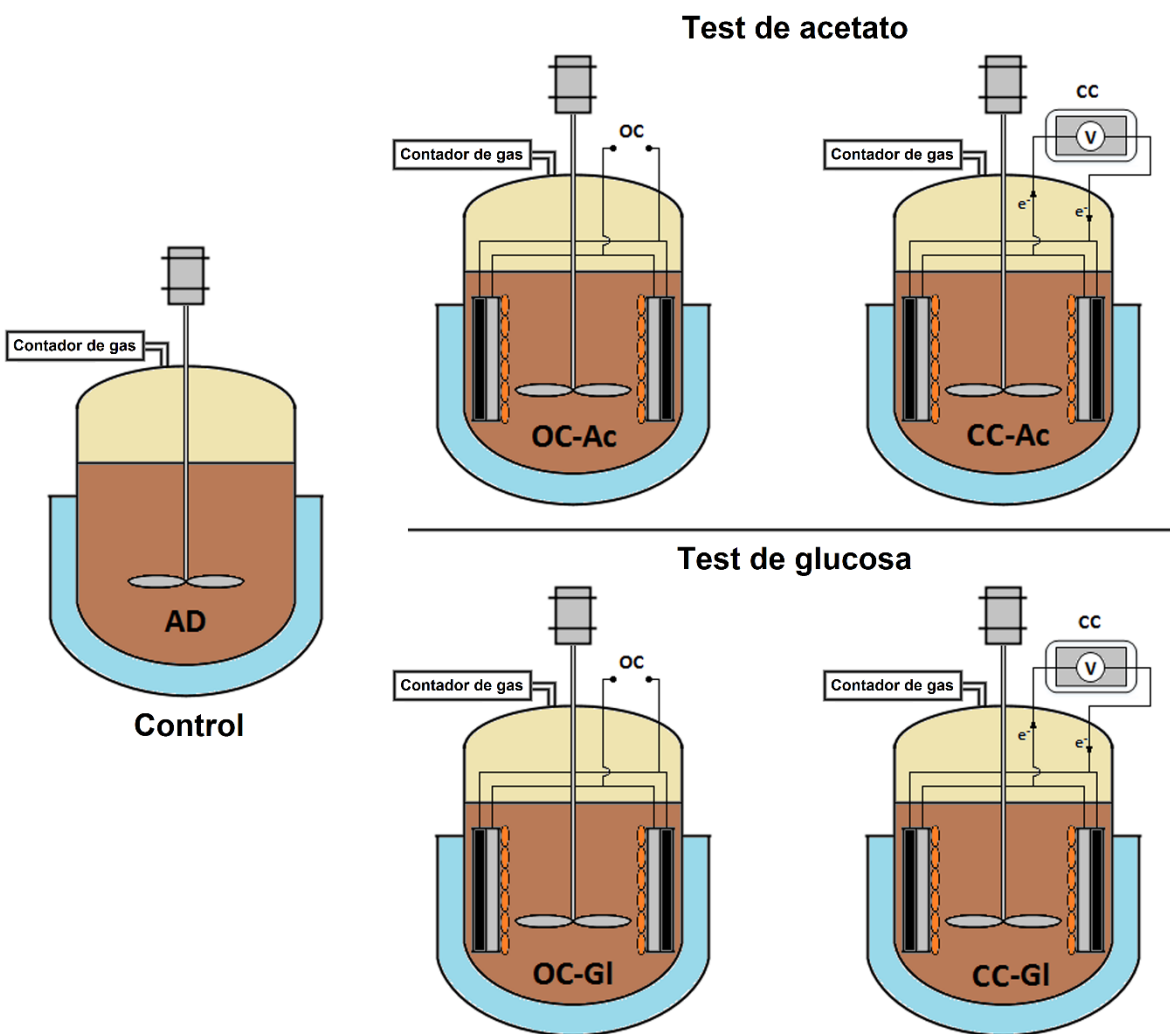
Aunque la digestión anaerobia se ha consolidado como uno de los procesos de valorización de residuos (con producción de  $\text{CH}_4$ ) más robustos, presenta algunos inconvenientes, como su tendencia a la inhibición a altas concentraciones de ácidos grasos en el reactor, que puede acabar desembocando en el colapso del proceso. Dado que los sistemas bioelectroquímicos funcionan muy bien con ácidos grasos como sustrato, la integración de ambos procesos en un mismo reactor aparece como una alternativa viable, capaz de estabilizar el proceso de digestión. Por ello, en este estudio se analizó la capacidad de remediación de la acumulación de VFA por parte de electrodos bioelectroquímicos integrados en el interior de un digestor anaerobio.

Con el objetivo de forzar las condiciones de inhibición por acumulación de VFA en el digestor, se utilizó una alta concentración de sustrato (glucosa). La digestión se realizó en modo batch, a 25 °C y con un volumen total de 2 L. Se realizaron (por duplicado) dos ensayos diferenciados en la fuente de carbono utilizada para la preadaptación de los electrodos a las condiciones del experimento (Figura 10.6): el test de acetato y el test de glucosa. En el test de acetato, a dos de los reactores anaerobios se les introdujo un par de electrodos biocatalíticos previamente adaptados con acetato; en uno de ellos los reactores operaron en circuito abierto (OC), y en el otro, a un voltaje aplicado de 0.9 V (CC). Además, un tercer reactor se dedicó al proceso de digestión anaerobia sin electrodos (control). A continuación se llevó a cabo el test de glucosa, en el que las condiciones eran las mismas con la única salvedad de que los electrodos introducidos habían sido previamente adaptados con glucosa. Se analizó el efecto de la integración con electrodos tanto en términos de producción de  $\text{CH}_4$  como en eliminación (remediación) de VFA en exceso en el reactor.

### **10.6.1. Respuesta en cuanto a la producción de $\text{CH}_4$**

Tal como se observa en la Figura 10.7a, la producción de metano fue mayor en los reactores que contenían electrodos, al menos durante las primeras 100 h del experimento (excepto para el test CC-Gl). De hecho, la producción de  $\text{CH}_4$  aumentó más rápidamente en aquellos reactores que operaron en circuito abierto (OC), lo que sugiere que el mecanismo de transferencia de electrones

más probable fue la transferencia directa interespecífica de electrones (DIET), anteriormente descrito por Rotaru et al. (2014). Así pues, la introducción de electrodos en el reactor anaerobio permitiría un incremento en los procesos metanogénicos como consecuencia de una mayor proliferación de biomasa, y no tanto debido a la aplicación de voltaje, lo que concuerda con observaciones previas (De Vrieze et al., 2014).



**Figura 10.6.** Vista esquemática de los reactores: AD (digestión anaerobia, control), OC-Ac y OC-Gl (electrodos en circuito abierto adaptados con acetato y glucosa, respectivamente) y CC-Ac y CC-Gl (electrodos a  $V_{app} = 0.9$  V, adaptados con acetato y glucosa, respectivamente).

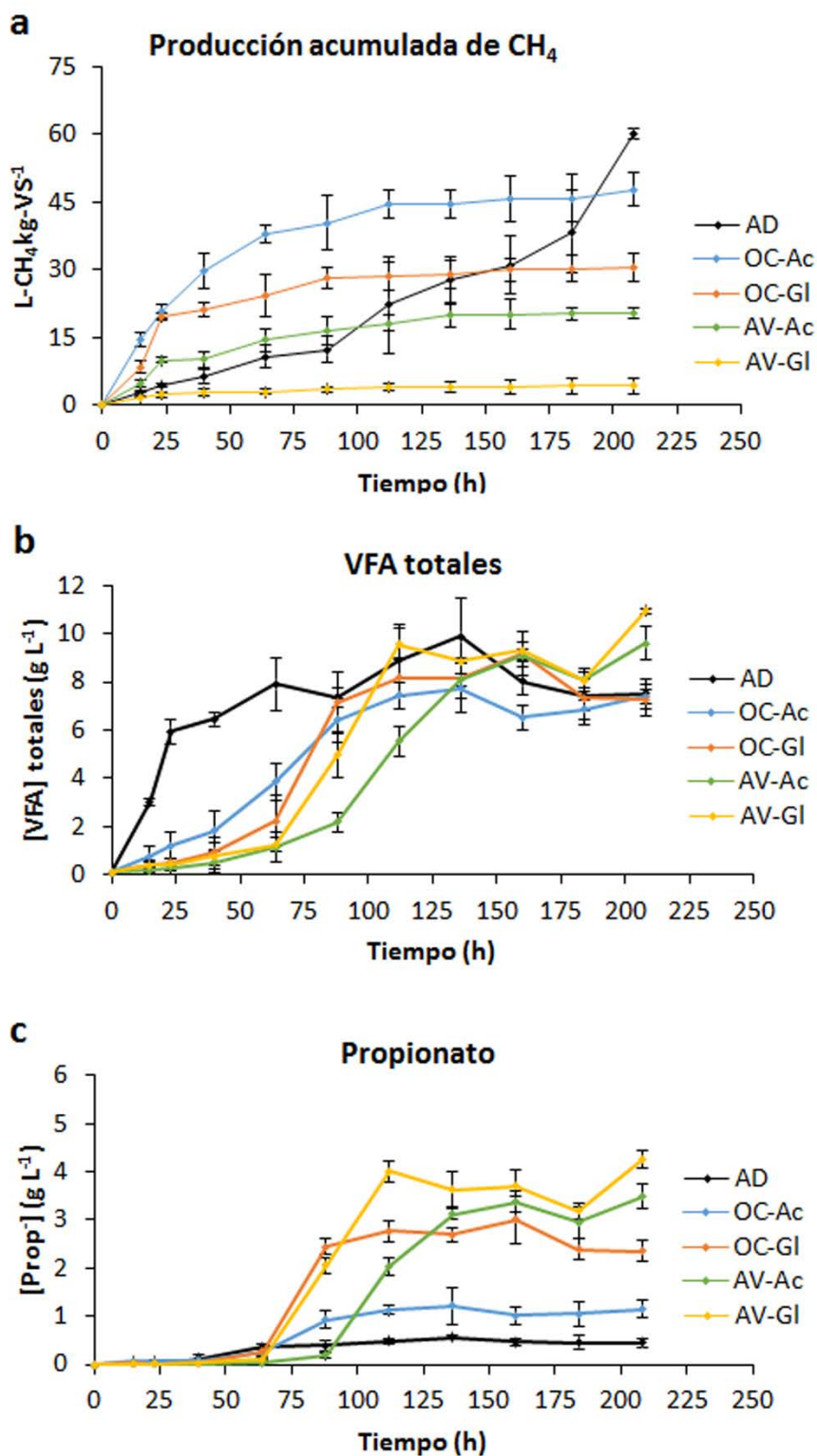
### 10.6.2. Respuesta en cuanto a la remediación de VFA

La Figura 10.7b muestra cómo la presencia de los electrodos permite mantener los niveles de VFA, durante las primeras 100 h del experimento, significativamente más bajos que en el reactor sin electrodos (AD). Este resultado concuerda con los obtenidos en cuanto a los procesos metanógenos, lo cual abre un interesante rango de posibilidades de cara a trabajar en continuo a HRT menores de 100 h para prevenir la acidificación excesiva del reactor.

Por otra parte, se observa que a partir de las 100 h la acumulación de VFA es comparable a la del reactor control. Sin embargo, existe una diferencia en cuanto al tipo de VFA acumulado: la concentración de propionato aumenta en los reactores con electrodos, manteniéndose en valores mínimos en el reactor control (Figura 10.7c). El hecho de que la acumulación de propiónico coincida con el cese de la producción de  $\text{CH}_4$  en los reactores con electrodos parece sugerir una relación entre ambos fenómenos, en concordancia con lo expuesto por Ma et al. (2009). Se observa además que la acumulación de propionato es menor en aquellos reactores con electrodos adaptados con acetato en comparación con los adaptados con glucosa. Sin embargo, la estabilidad de los procesos electrogénicos resultó ser mayor en los electrodos adaptados con glucosa, por lo que nuevas investigaciones deberán realizarse para dilucidar los beneficios e inconvenientes de ambos tipos de adaptación.

### 10.6.3. Conclusiones

A modo de conclusión, podemos decir que la introducción de electrodos bioelectrocatalíticos en un reactor anaerobio proclive a sufrir inhibición por una acidificación excesiva permitió mejorar el proceso de digestión en cuanto a la producción de  $\text{CH}_4$  durante las primeras 100 h de experimento, gracias a la remediación parcial de la acumulación de VFA en las etapas iniciales del experimento. Sin embargo, este efecto parece estar más relacionado con la acumulación de biomasa en el electrodo que con la aplicación de voltaje al mismo.



**Figura 10.7.** Para todas las condiciones de operación testadas: (a) Producción específica de CH<sub>4</sub>. (b) Concentración de VFA totales. (c) Concentración de propionato.

### **10.7. PRODUCCIÓN DE METANO A PARTIR DEL TRATAMIENTO DE AGUAS RESIDUALES EN UNA CELDA DE ELECTRÓLISIS BIOCATALÍTICA**

Aunque la digestión anaerobia es uno de los procesos mejor establecidos para el tratamiento de residuos, la eliminación de materia orgánica puede resultar ineficiente de cara a cumplir los límites legislativos marcados. En este tipo de casos, la electrólisis biocatalítica aparece como la opción más adecuada para maximizar la eliminación de materia orgánica y la recuperación de energía contenida en el sustrato (Villano et al., 2013). Por ello, la electrólisis biocatalítica está indicada para el tratamiento de aguas residuales de baja carga orgánica (como las aguas residuales urbanas). Aunque se han realizado muchos estudios con electrólisis biocatalítica encaminados a la producción de H<sub>2</sub>, existe también la opción de fomentar la producción de CH<sub>4</sub> por medio del proceso conocido como electrometanogénesis, en el que los electrones que llegan al cátodo se utilizan para la reducción de CO<sub>2</sub> a CH<sub>4</sub>. En este estudio se analizó la mejora en el proceso de digestión anaerobia de aguas residuales de baja carga orgánica mediante la introducción de un electrodo biocatalítico en el digestor. Para una mejor caracterización del proceso, el estudio se realizó tanto en modo de operación batch como en continuo.

#### **10.7.1. Respuesta del sistema en batch**

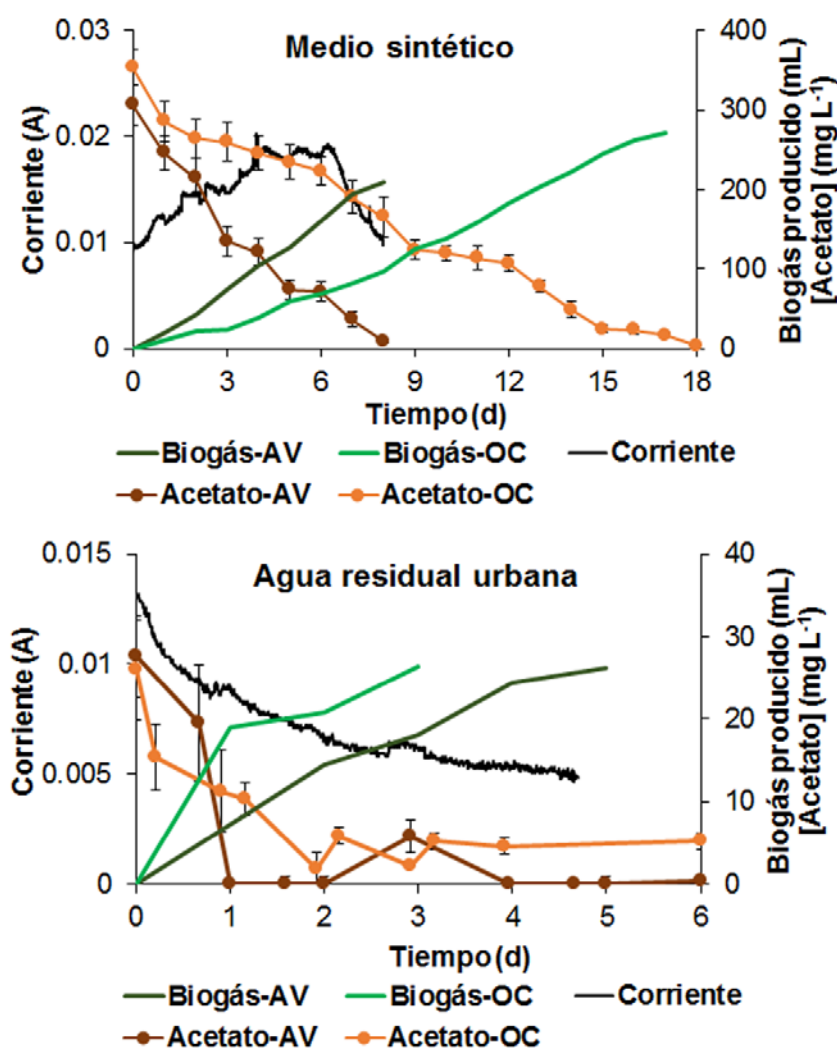
Se utilizó una celda MEC con una sola cámara de 3 L en el que el módulo bioelectrocatalítico (ánodo + cátodo) estuvo sumergido en el medio de alimentación. El ánodo constó de una capa de fieltro de carbono, mientras que el cátodo consistió en una plancha de acero inoxidable. Como en ocasiones anteriores, una pieza de poliéster separaba ambas partes, para evitar cortocircuitos. La celda MEC operó a una temperatura de  $21 \pm 2$  °C, y se introdujeron dos tipos de alimentación: en una primera etapa, alimentación sintética con acetato como fuente de carbono; a continuación, agua residual urbana obtenida de la Estación Depuradora de Aguas Residuales de León. Para ambas alimentaciones, a su vez, hubo un período con voltaje aplicado de 1V y otro en circuito abierto, considerado a modo de control.

Al comparar la operación del sistema en condiciones de voltaje aplicado y en modo circuito abierto con medio sintético como sustrato, se observó que la tasa de producción de CH<sub>4</sub> fue significativamente mayor al aplicar voltaje ( $8.0 \pm 0.2$  mL-CH<sub>4</sub> L<sub>r</sub><sup>-1</sup> d<sup>-1</sup> con voltaje vs.  $4.6 \pm 0.2$  mL-CH<sub>4</sub> L<sub>r</sub><sup>-1</sup> d<sup>-1</sup> en modo circuito abierto). Además, el consumo del  $95 \pm 1\%$  del acetato incluido



en el sustrato requirió de 8 días con voltaje aplicado, mientras que 18 días fueron necesarios en circuito abierto (ver Figura 10.8a). Estas diferencias pueden atribuirse hasta cierto punto a las distintas tasas de crecimiento de biomasa entre microorganismos acetoclásticos y electrogénos.

Con agua residual urbana como sustrato, las diferencias en producción de  $\text{CH}_4$  entre la aplicación y la ausencia de voltaje desaparecen (Figura 10.8b). Sin embargo, y aunque las concentraciones de acetato de partida son mucho menores que en el caso del medio sintético, se sigue observando una tasa de consumo del acetato más rápida durante la operación con voltaje aplicado en contraposición con el circuito abierto.



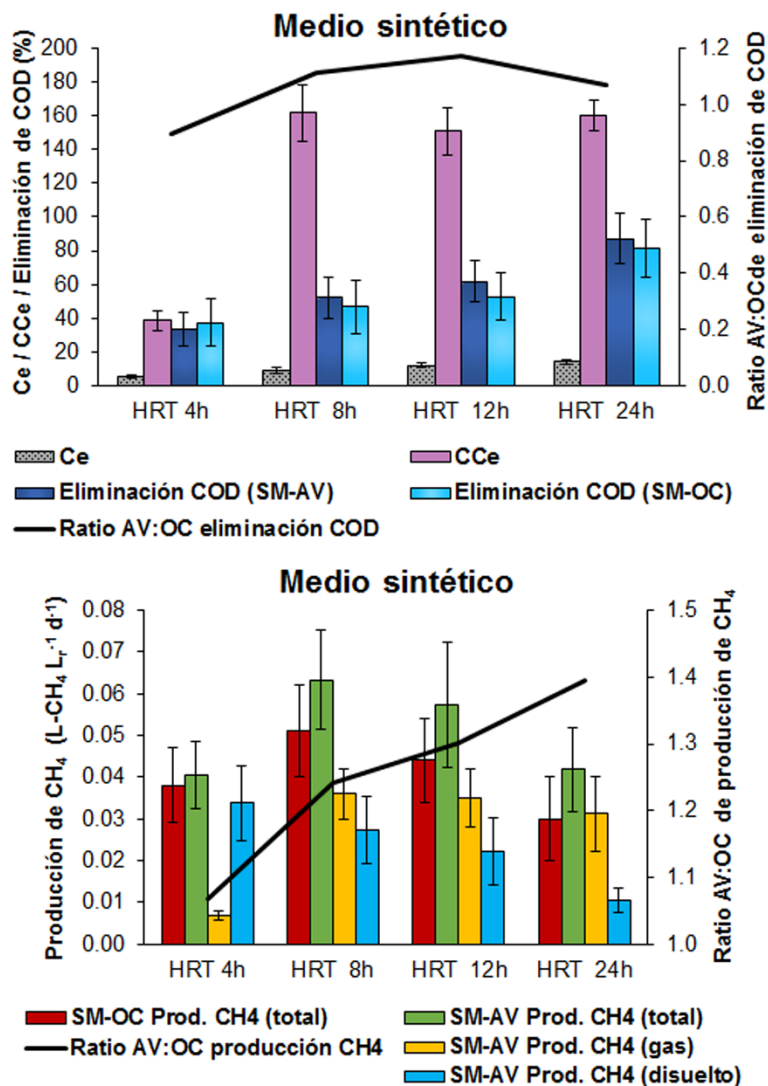
**Figura 10.8.** Perfil de corriente, producción de biogás y concentración de acetato usando: a) medio sintético y b) agua residual urbana aplicando un voltaje de 1V (AV) o en modo circuito abierto (OC), en batch (dos réplicas).

### 10.7.2. Respuesta del sistema en continuo

Posteriormente, la misma celda se alimentó en modo continuo a diferentes tiempos de retención hidráulica (HRT): 4, 8, 12 y 24 h. En esta ocasión, el objetivo del ensayo fue la caracterización y el efecto del HRT sobre la metanogénesis y la degradación de materia orgánica. En primer lugar se alimentó medio sintético (SM) y después agua residual urbana (wastewater, WW).

Al usar SM, se observó que la degradación de materia orgánica en términos de demanda química de oxígeno (COD) aumentó a medida que el HRT fue siendo incrementado (de  $33 \pm 10\%$  a 4 h hasta  $87 \pm 15\%$  a 24 h; Figura 10.9a). Las bajas eficiencias coulombicas observadas sugieren que parte del acetato consumido no derivaba en corriente, sino que se dedicaba a la proliferación de biomasa y, sobre todo, a la producción no electrolítica de metano. Este efecto también se ve en parte paliado a HRT más largos. En la Figura 10.9b se observa que el ratio entre la producción de  $\text{CH}_4$  con voltaje aplicado (1V) frente a circuito abierto aumenta conforme se va incrementado el HRT, aunque la producción de  $\text{CH}_4$  se reduce al aumentar el HRT (debido a la reducción de la carga orgánica alimentada). Esto subraya los beneficios de usar un electrodo MEC para la optimización de la producción de biogás en la digestión anaerobia de sustratos con baja carga orgánica. Es importante remarcar que, a pesar de esta mejora, la baja producción de  $\text{CH}_4$  global hace que el  $\text{CH}_4$  disuelto en el medio suponga un importante porcentaje del total (Figura 10.9b). Por tanto, técnicas baratas de degasificación del medio deberían ser estudiadas en mayor profundidad para minimizar esta desventaja.

Finalmente, se calcularon balances de energía para cuantificar la eficiencia energética del proceso con ambos sustratos. Se encontró que el balance energético neto (es decir, teniendo en cuenta no sólo el consumo de energía de la celda MEC sino también su producción en forma de  $\text{CH}_4$ ) fue positivo para el caso de la alimentación sintética (Tabla 10.2), aunque resultó negativo para el caso de agua residual urbana. Se puede observar una tendencia común en ambos casos: el balance energético neto mejora a medida que el HRT va aumentando.



**Figura 10.9.** a) Parámetros de operación a IV (AV) y en circuito abierto (OC) con medio sintético (SM) a distintos HRTs. b) Producción de CH<sub>4</sub> total, en fase gas y en disolución.

**Tabla 10.2.** Balance energético neto de la celda MEC con SM y WW a diferentes HRT.

|         |    | Balance energético neto (kWh kg <sup>-1</sup> -COD <sub>rem</sub> ) |                |
|---------|----|---|----------------|
|         |    | SM  | WW             |
| HRT (h) |    | 0.308 ± 0.065   | -0.134 ± 0.023 |
|         | 4  | 0.689 ± 0.098   | -0.122 ± 0.028 |
|         | 8  | 0.733 ± 0.109   | -0.113 ± 0.039 |
|         | 12 | 0.698 ± 0.105   | -0.100 ± 0.015 |
|         | 24 |   |                |

### **10.7.3. Conclusiones**

En este estudio se analizaron los beneficios de la integración de los procesos de digestión anaerobia y electrólisis biocatalítica para el tratamiento de sustratos de baja carga orgánica, condiciones bajo las que la digestión anaerobia por si sola presenta muchos problemas. Los tests en batch revelaron que la integración de ambos procesos permitió mejorar la degradación de materia orgánica y la tasa de producción de CH<sub>4</sub>. Los tests en continuo mostraron que el uso de electrodos biocatalíticos como complemento a la digestión fue especialmente favorable a HRT largos, optimizando la recuperación de energía del sustrato y por tanto aumentando la rentabilidad del proceso.

## **10.8. ESTANCIAS CORTAS EN CENTROS DE INVESTIGACIÓN EUROPEOS**

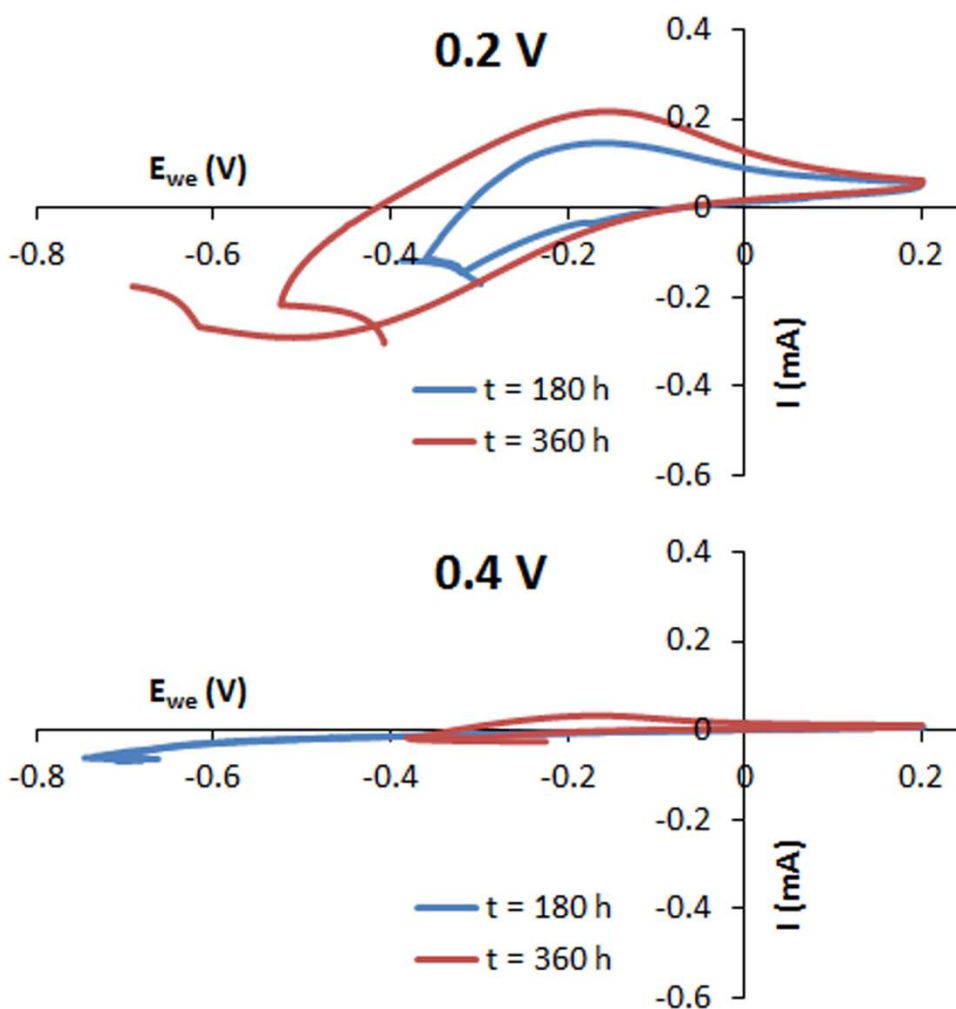
### **10.8.1. Estancia en la Escuela de Ciencias Químicas, Dublin City University (Rep. de Irlanda), y en el Centro de Biología Médica, Queen's University Belfast (Irlanda del Norte, Reino Unido) Oct'15 – Ene'16**

Esta estancia se desarrolló bajo la supervisión del Dr. Enrico Marsili y el Dr. Brian Kelleher (DCU). El objetivo de este trabajo consistió en el arranque y análisis de resultados de un experimento de biorremediación mediante sistemas bioelectroquímicos (BESs), además de la caracterización del desarrollo del biofilm. La operación con los BESs y los análisis electroquímicos se realizaron en la Dublin City University, mientras que la caracterización de la biomasa por PCR y qPCR se llevó a cabo en la Queen's University Belfast, donde mi supervisora fue la Dra. Prasanna Pentlavalli.

Se llevaron a cabo ensayos en cinco celdas bioelectroquímicas bajo la configuración de tres electrodos: electrodo de trabajo (WE), contraelectrodo (CE) y electrodo de referencia (RE). Los electrodos de trabajo consistían en dos pequeñas piezas de fieltro de carbono. Las celdas tenían un volumen de 20 mL y se operó a 30 °C y bajo agitación constante. Se inocularon las celdas con suelo mezclado con medio de cultivo para enriquecer la biomasa electrógena contenida en el suelo. Las celdas operaron a un voltaje (controlado por potencióstato) de +0.2 y +0.4 V. Otra de las celdas operó en circuito abierto (control, OC). A las 180 y 360 h de operación, se realizaron análisis de

voltametría cíclica (desde  $E_i = -0.8$  V vs. Ag/AgCl hasta  $E_f = 0.2$  V vs. Ag/AgCl) sin agitación, y se obtuvieron muestras del WE a esos mismos tiempos para su análisis microbiológico.

Los análisis de voltametría cíclica (Figura 10.10) mostraron un mejor resultado en cuanto a la capacidad electrogénica de la población microbiana para la biomasa crecida a  $+0.2$  V, tanto a las 180 h como a las 360 h. En ambos casos (y de acuerdo a lo esperado) la respuesta mejoró con un mayor período de enriquecimiento: sin embargo, esta mejora es mayor para el caso de  $+0.2$  V. Del análisis de cuantificación poblacional llevado a cabo por qPCR se obtuvo que la población crecida en ausencia de voltaje es algo mayor que con el voltaje de  $+0.2$  V. Esto es previsible, puesto que al aplicar voltaje estamos llevando a cabo un proceso de selección que nos reduce la población de biomasa no electrogénica, lo que redunda en la cuantificación de la biomasa total.



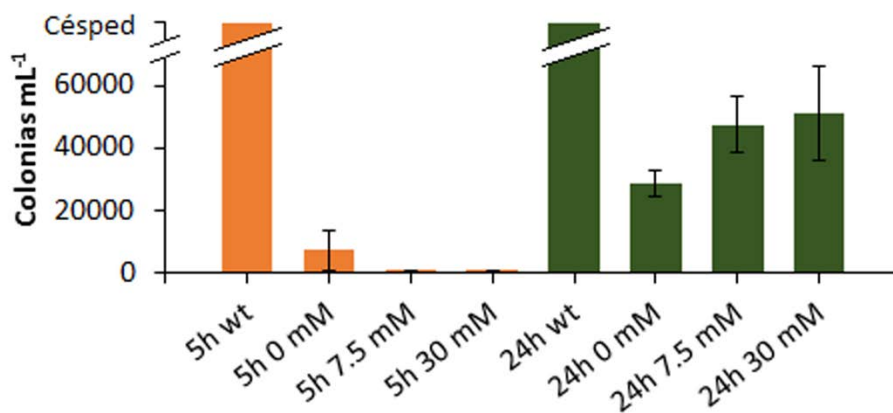
**Figura 10.10.** Análisis de voltametría cíclica para los WEs a diferentes tiempos.

### **10.8.2. Estancia en el Instituto de Microbiología Aplicada, Universidad Técnica de Renania del Norte-Westfalia en Aquisgrán (Alemania) Ene'16 – Abr'16**

Durante mi estancia en la RWTH Aachen (Aquisgrán) tuve la oportunidad de trabajar bajo la supervisión de la Prof. Dra. Miriam Rosenbaum, y en colaboración con su estudiante de doctorado Thomas Kirchner. Dado que una de las líneas de investigación de su grupo consistía en la caracterización de sistemas BESs inoculados con cultivos puros de *Clostridium ljungdahlii*, el objetivo principal de la estancia fue el desarrollo de una herramienta de inducción de mutagénesis para este microorganismo, para poder obtener una cepa mejorada del mismo que pudiera optimizar el comportamiento de los BESs.

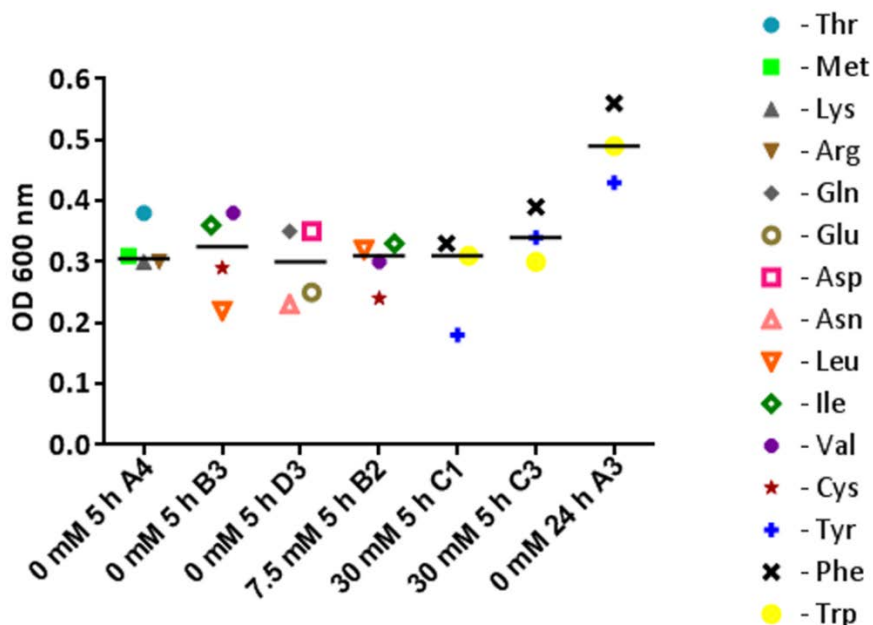
El protocolo de mutagénesis no dirigida consistió en la sobreexpresión del gen para la DNA polimerasa IV, enzima que carece de actividad exonucleasa 3' → 5', lo que redundó en una alta tasa de error en la replicación del ADN (Goodman, 2002). El gen para esta polimerasa se introdujo en un plásmido bajo la regulación de un promotor inducible por lactosa (Banerjee et al., 2014). Con posterioridad a la transformación de *C. ljungdahlii* mut1 con este plásmido, se indujo la expresión del gen mediante diferentes concentraciones de lactosa (0 mM, 7.5 mM y 30 mM). Se probaron dos duraciones del período de inducción: 5 h y 24 h. Al ser el tiempo de generación de *C. ljungdahlii* de 7 h, el probar estos dos tiempos nos permite comprobar el efecto de mutagénesis de la DNA pol. IV tras una o tres generaciones, respectivamente. Al finalizar el período correspondiente, se detuvo la inducción de la expresión de la DNA pol. IV, y se recultivaron las bacterias en agar, para hacer contaje y aislamiento de colonias.

Como puede observarse (Figura 10.11), la longitud del período de inducción parece tener un efecto importante, ya que el número de colonias es mayor en las inducidas durante 24 h. Una posible explicación podría ser que los períodos de inducción largos incrementan la opción de dañar el plásmido mismo en su replicación con la DNA pol. IV, por lo que resultaría no funcional. Por otra parte, es importante remarcar que, tanto para las inducidas a 5 h como a 24 h, no existen diferencias significativas entre la inducción a 7.5 y a 30 mM de lactosa, lo que sugiere la existencia de un umbral de saturación de inductor.



**Figura 10.11.** Crecimiento de *C. ljungdahlii mut1* bajo inducción por lactosa.

Para comprobar la tasa de mutagénesis, se realizó un recultivo de colonias aisladas para buscar desarrollo de auxotrofías (incapacidad para la síntesis de algún aminoácido) como resultado de una mutación. Para ello, el recultivo de cada colonia se realizó en diferentes placas Petri en las que el medio de cultivo carecía de un grupo de aminoácidos concreto (como paso preliminar); las que presentaban un crecimiento deficiente se consideraron candidatas a haber desarrollado una auxotrofia para alguno de los aminoácidos ausentes en esa placa Petri, y fueron recultivadas nuevamente para constatar si esa posible auxotrofia se mantenía para un único aminoácido concreto. Aunque se encontró que ninguna de las candidatas había desarrollado una auxotrofia de forma concluyente (Figura 10.12), las candidatas B3, B2 y C1 sí desarrollaron un descenso claro en su crecimiento en ausencia de un aminoácido determinado (Leu para B3, Cys para B2 y Tyr para C1), lo que sugiere que la ruta de síntesis de estos aminoácidos puede haberse visto dañada como resultado de la mutagénesis no dirigida, aunque sólo de forma parcial. Así pues, aunque son necesarias más investigaciones con esta cepa para confirmar estos resultados, se puede afirmar que se pudo desarrollar un protocolo de mutagénesis no dirigida basado en la DNA pol. IV a concentraciones de inductor (lactosa) de 7.5 mM y con un período de inducción menor que el período de generación de *C. ljungdahlii* (7 h).



**Figura 10.12.** Crecimiento de las cepas candidatas a desarrollo de auxotrofías de *C. ljungdahlii mut1*. Las marcas horizontales muestran la mediana para todos los aa testados para cada cepa candidata.

## 10.9. CONCLUSIONES GENERALES

Dado que los tratamientos de aguas residuales presentan altos requerimientos energéticos, se están investigando nuevas alternativas para reducir el consumo de energía. Entre ellas, los sistemas bioelectroquímicos han demostrado ser una opción factible, ya que permiten recuperar parte de la energía química contenida en la materia orgánica disuelta. En esta tesis doctoral, se caracterizó, en distintas configuraciones, la integración de BESs y tecnologías convencionales de tratamiento (DF o AD) para el tratamiento de aguas residuales (tanto de origen industrial como urbano). Del presente estudio se pueden extraer las siguientes conclusiones:

1. Al probar diferentes concentraciones de nitrógeno y de sustrato para optimizar la DF de una alimentación sintética (con lactosa como fuente de C) por medio de la metodología de superficie de respuesta (RSM), se encontró que el área de operación que optimiza la respuesta se corresponde con bajos niveles tanto de lactosa como de



nitrógeno. Un incremento en el contenido de N afectó negativamente al rendimiento en producción de  $H_2$ , aunque este efecto se vio atenuado al aumentar la concentración de sustrato.

2. La proporción óptima sustrato/inóculo para la DF de lactosuero resultó ser de 2.5 (VS-substrate:VS-inoculum). Al trabajar con este ratio, la producción de  $H_2$  en batch fue de  $0.7 \text{ L } H_2 \text{ L}_r^{-1}$ , obteniéndose también un efluente de naturaleza ácida.
3. También se estudió el efecto de la adición de lactato en una celda de electrólisis microbiana (MEC) alimentada inicialmente con acetato. Se estableció el ratio de acetato:lactato de 70:30 (en base molar) para optimizar la tasa de producción de  $H_2$  en la MEC ( $0.7 \pm 0.1 \text{ L } H_2 \text{ L}_a^{-1} \text{ d}^{-1}$ ).
4. Tras ser procesado hasta cumplir el ratio obtenido de 70:30, el efluente obtenido de la fermentación oscura de lactosuero fue introducido en la celda MEC. La tasa de producción de  $H_2$  en esta segunda etapa fue de  $0.5 \text{ L } H_2 \text{ L}_a^{-1} \text{ d}^{-1}$ . En conjunto, el rendimiento global de producción de  $H_2$  a partir de lactosuero alcanzó un valor de  $94.2 \text{ L } H_2 \text{ kg}_{\text{VS}}^{-1}$ . Esto representa un 20% de mejora en comparación con otros estudios descritos en bibliografía.

Las conclusiones anteriores se refieren a la integración de fermentación oscura y BESs en una configuración de dos etapas. Los siguientes puntos, por el contrario, se enfocan en el análisis de integración de digestión anaerobia y BESs en el mismo reactor. Además, es importante subrayar que estos estudios fueron realizados bajo condiciones extremas (a alta y baja carga orgánica), donde la AD convencional suele presentar ineficiencias. Finalmente, el último punto hace referencia a diferentes estrategias estudiadas para la mejora de la respuesta exoelectrógena en BESs:

5. Con respecto a la integración de digestión anaerobia y sistemas bioelectroquímicos en un reactor anaerobio propenso a sufrir inhibición por acidificación debido a altas cargas orgánicas, se observó un retraso en la acumulación de VFA, así como un incremento

en la producción de  $\text{CH}_4$  durante las primeras 100 h del experimento. Esta mejora, sin embargo, parece estar más basada en la proliferación de biomasa que en la actividad electroquímica, lo que sugiere que el papel del electrodo como soporte para un mayor crecimiento de biomasa sería relevante.

6. En relación a la integración de electrodos MEC en un reactor anaerobio alimentado en batch con sustrato de baja carga orgánica, el uso de los electrodos dentro del reactor incrementó la degradación de la materia orgánica y la tasa de producción de metano. La aplicación de voltaje condujo a un incremento del 90% en la tasa de eliminación de acetato (en comparación con la operación en circuito abierto) al trabajar con agua residual sintética como sustrato. La tasa de producción de  $\text{CH}_4$  también se vio incrementada en un 73%. Además, los procesos de metanogénesis hidrogenotrofa se vieron aumentados, evitando así las ineficiencias asociadas a la proliferación de homoacetógenas.
7. Finalmente, cuando el mismo reactor alimentado a bajas cargas orgánicas trabajó en modo continuo, se encontró que HRTs largos (12-24 h) resultaron ser más favorables, al permitir a la biomasa electrógena tener una ventaja competitiva frente a las metanógenas acetoclásticas. A un HRT de 24 h, la tasa de producción de  $\text{CH}_4$  (con voltaje aplicado) sobrepasó en aproximadamente un 40% el mismo parámetro en condiciones de circuito abierto. HRTs largos también son favorables para la recuperación de energía, aumentando así la rentabilidad del proceso.
8. Con respecto a la mejora de la respuesta bioelectrocatalítica en los BESs por parte de las bacterias electrógenas, la aplicación de un voltaje de +0.2 V parece optimizar el enriquecimiento selectivo de electrógenas a partir de suelo como sustrato; en el marco de otra estrategia, se pudo desarrollar una herramienta de inducción de mutagénesis no dirigida basada en la sobreexpresión de la DNA polimerasa IV. Sin embargo, para poder confirmar estos resultados serán necesarias investigaciones posteriores.

**10.10. BIBLIOGRAFÍA**

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