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Novel bioelectrochemical approaches for exploring extracellular electron transfer in *Geobacter sulfurreducens*

Marta R. Estévez Canales

2016

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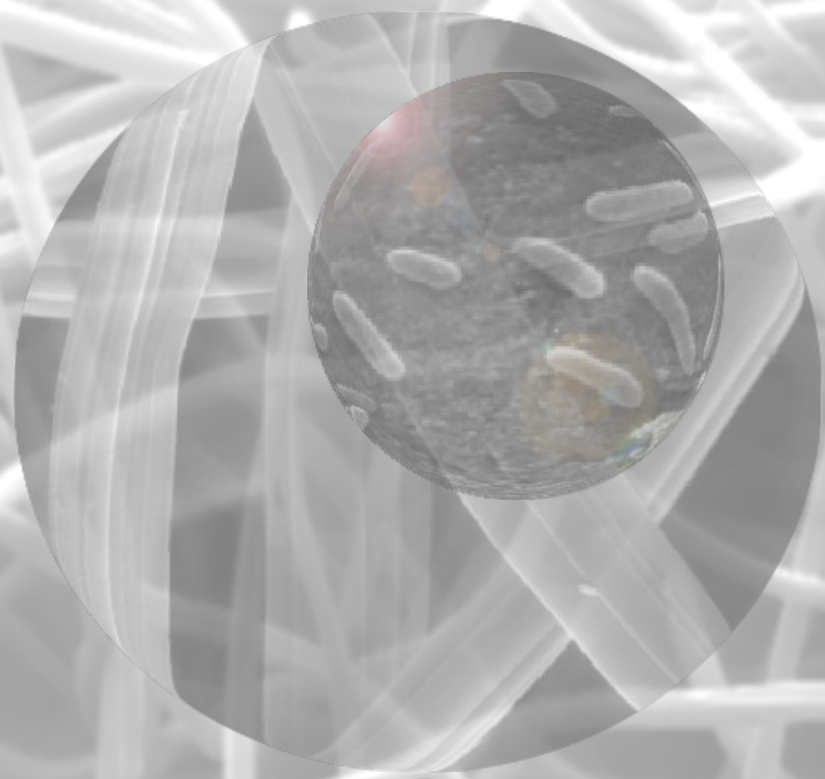

Ph.D. Thesis

MARTA ROSA ESTÉVEZ CANALES

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CERTIFICA:

Que el trabajo descrito en la presente memoria, titulado "**Novel bioelectrochemical approaches for exploring extracellular electron transfer in *Geobacter sulfurreducens***" ha sido realizado bajo su dirección por Dña. Marta Rosa Estévez Canales en el Área de Ingeniería Química del Departamento de Química Analítica, Química Física e Ingeniería Química de la Universidad de Alcalá. Asimismo, autorizo su presentación para que sea defendido como Tesis Doctoral.

Y para que conste y surta los efectos oportunos, firma el presente en Alcalá de Henares a 14 de Abril de 2016

Abraham Esteve Núñez

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Dicha tesis reúne los requisitos necesarios para su presentación y defensa.

Y para que conste y surta los efectos oportunos, firma el presente en Alcalá de Henares a 14 de Abril de 2016

Jesús Alberto Escarpa Miguel

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Novel bioelectrochemical approaches for exploring extracellular electron transfer in *Geobacter sulfurreducens*

A mi familia

y a mis profesores

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SUMMARY

Microbial electrochemical systems deal with electrochemical systems in which living cells like electroactive microorganisms serve as catalysts for either oxidation or reduction reactions. These devices enable to transform chemical energy into electricity by the action of some bacteria (e.g. *Geobacter sulfurreducens*) that are able to oxidize organic compounds coupled to the reduction of an extracellular electron acceptor (e.g. electrode). The merging of two fields as different as microbiology and electrochemistry have resulted in a very powerful and versatile discipline that requires the synergy of both.

This PhD thesis consists of 5 chapters, 3 of them experimental, that explore different aspects of microbial electrochemical systems, based on the model electroactive bacteria *G. sulfurreducens.* The research aims to develop novel approaches for further understanding Extracellular Electron Transfer (EET) process as well as for providing cutting-edge bioelectrochemical platforms.

In view of the foregoing, **Chapter 1** provides a literature review and contextualization of the use of *G. sulfurreducens* in microbial electrochemical systems. The mechanisms that allow *G. sulfurreducens* to establish redox contact with extracellular acceptors are based on the presence of a vast network of *c*-type cytochromes that facilitates electron transport from central metabolism. In order to verify the role of *c*-type cytochromes in EET, some deletion studies have attempted to obtain entirely EET-free cells. Nonetheless, knock-out cells did not display the expected phenotype, therefore we plan to follow an alternative approach as one of task of this thesis.

From a microbiology point of view, classical systems used in Microbial Electrochemical Technologies (METs) involve complex experimental set-ups, long conditioning periods, and substantial costs. So thus, miniaturization of bioelectrochemical devices may bring considerable advantages, such as rapid electrical responses or cost reduction. These features would be especially desirable in systems with a potential use in electroanalysis.

Another requirement for microbial electrochemical systems is the formation of a mature microbial biofilm. However, the long process needed for its development could be overcome by artificially assembling a bacteria-electrode system (bioelectrode). Ready-to-use artificial bioelectrodes may represent a versatile time and cost saving strategy for microbial electrochemical technologies. They could be customized in terms of size, bacterial concentration or conductive support materials, among others aspects.

According to the identified challenges, **Chapter 1** ends describing the three main research goals of this thesis:

i) investigating the role of *Geobacter´*s cytochromes in EET,

ii) exploring SPEs as miniaturized tools for rapid assessing microbial electrochemistry, and

iii) constructing ready-to-use *Geobacter*-based artificial bioelectrodes.

Chapter 2 shows a straightforward method for growing cytochrome-depleted cells of *G. sulfurreducens* with the aim to confirm the role of *c*-type cytochromes in EET. Heme-staining based protocols together with other tecniques like inductively coupled plasma mass spectrometry, and *in situ* nanoparticle enhanced raman spectroscopy analysis, revealed that the cytochrome content could be severely decreased by limiting iron availability in growth media. The cytochrome-depleted cells were viable since they

could reduced fumarate to succinate at inner-membrane level using a cytochrome-free reaction. However, cytochrome-free cells were unable to reduce Fe (III) citrate or to exchange electrons with a graphite electrode. These results confirm that *c*-type cytochromes are essential for extracellular electron transfer in *G. sulfurreducens.*

Simplifying the electrochemical aspects for culturing electroactive bacteria is a must in this new discipline. So thus, **Chapter 3** investigates the use of Screen-Printed Electrodes (SPEs) as a novel low-cost platform for assessing microbial electrochemical activity at the microscale level. SPEs proved to be robust for identifying the bioelectrochemical response, while avoiding complex electrochemical set-ups. The system was successfully validated for characterizing the response of *G. sulfurreducens* under diverse physiological states revealing different electron transfer responses. Moreover, a combination of SPE and *G. sulfurreducens* resulted to be a promising biosensor for quantifying the levels of acetate and for using samples as complex as urban wastewater. In addition, the potential of the technology for identifying electroactive consortia was also successfully tested.

As a final scientific contribution, **Chapter 4** explores a new strategy for constructing ready-to-use artificial bioelectrodes of *G. sulfurreducens* by means of effective immobilization of cells inside silica gel and carbon felt electrodes. Viability test confirmed that the vast majority of bacteria survived the encapsulation process and cell density did not change, at least, for a 96 h period. This double entrapment prevents bacterial release from the electrode but allows solute diffusion, making possible the electrochemical characterization of the system. Artificial bioelectrodes were evaluated in 3-electrodes reactors for several days, using several electron donors. Cyclic voltammetry of acetate-fed bioelectrodes revealed a sigmoidal catalytic

oxidation wave from the very beginning, typical of more aged biofilms. Furthermore, the presence of *G. sulfurreducens* within the fibers and silica gel could be verified by scanning electron microscopy and the transcriptomic response of cells encapsulated was analyzed as well.

Based on our results, a general discussion, conclusions and future outlook are presented in **Chapter 5**. This chapter was structured in a question-answer mode. We believe this format could help the reader to understand the reasons beyond the experimental activities together with those supporting the research interpretation. The main value of this work is the straightforward, fast and economical nature of the tools provided that can help other researchers in microbial electrochemistry field.

RESUMEN

Los sistemas electroquímicos microbianos son aquellos en los que los microorganismos actúan, en combinación con electrodos, como catalizadores de reacciones redox. Estos sistemas permiten transformar la energía química en energía eléctrica mediante la acción de ciertos microorganismos (e.g. *Geobacter sulfurreducens*) que tienen la capacidad de oxidar compuestos orgánicos, acoplando dicha reacción a la reducción de un aceptor de electrones extracelular (e.g. electrodos). La fusión de dos campos tan diferentes como la microbiología y la electroquímica ha dado lugar a una disciplina nueva y versátil que requiere la sinergia de ambos.

Esta tesis doctoral se compone de 5 capítulos, 3 de ellos experimentales, que exploran diferentes aspectos de los sistemas electroquímicos microbianos, utilizando como modelo de bacteria electroactiva a *G. sulfurreducens.* El objetivo principal de la investigación fue desarrollar nuevas estrategias para profundizar en la comprensión del proceso de transferencia de electrones extracelular (TEE), así como para explorar nuevas plataformas bioelectroquímicas.

En vista de lo anterior, el **Capítulo 1** explora el estado del arte sobre el uso de *G. sulfurreducens* en sistemas electroquímicos microbianos. Dicha bacteria posee la capacidad de establecer contacto electroquímico con aceptores extracelulares mediante a la presencia de una extensa red de citocromos de tipo c que conecta el citoplasma con el exterior celular. Con el fin de confirmar el papel de los citocromos de tipo c en la TEE se han realizado estudios de deleción génica para intentar obtener células incapaces de producir determinados citocromos tipo c. Sin embargo, las construcciones genética resultantes no mostraron el comportamiento

(fenotipo) esperado por lo que, en esta tesis, nos planteamos un enfoque alternativo basado en el metabolismo asimilatorio del hierro y su efecto en la síntesis de grupos hemo.

Desde un punto de vista microbiológico los sistemas clásicos que se emplean en el desarrollo de la electroquímica microbiana implican complejos montajes experimentales, largos períodos de acondicionamiento y costes sustanciales. En este sentido, la miniaturización de los dispositivos bioelectroquímicos puede suponer ventajas considerables, tales como respuestas en producción de corriente o la reducción de costes. Estas características serían especialmente deseables en sistemas electroanalíticos. Otro requisito para los sistemas electroquímicos microbianos es la formación de un biofilm bacteriano maduro, lo cual suele implicar largos períodos de tiempo. Este proceso podría simplificarse mediante la construcción artificial de un sistema bacteria-electrodo.

De acuerdo con los retos identificados, el **Capítulo 1** concluye describiendo los tres objetivos específicos que conforman esta tesis doctoral:

i) Investigar el papel de los citocromos de *Geobacter* en la TEE,

ii) Explorar los electrodos serigrafiados como instrumentos miniaturizados para una rápida evaluación electroquímica de microorganismos electroactivos,

iii) Construir bioelectrodos artificiales basados en *Geobacter*

Con el objetivo de confirmar el papel de los citocromos de tipo c en la TEE, el **Capítulo 2** describe un método para el cultivo de células de *G. sulfurreducens* carentes de citocromos. Los análisis bioquímicos basados en la tinción específica del grupo hemo, junto a otros basados en espectrometría de masas y en espectroscopía Raman, demostraron que es factible modificar el contenido de citocromos en *G. sulfurreducens* limitando

la disponibilidad de hierro en el medio de cultivo. Las células carentes de citocromos eran viables ya que pudieron reducir fumarato a succinato, una reacción a nivel de la membrana interna que no requiere citocromos, pero no fueron capaces de reducir hierro citrato (III) o de intercambiar electrones con un electrodo. Estos resultados confirmaron que los citocromos de tipo c son esenciales para la TEE en *G. sulfurreducens*.

Teniendo en cuenta la necesidad de simplificar los aspectos electroquímicos, el **Capítulo 3** investiga el uso de electrodos serigrafiados (SPEs) como una nueva plataforma de bajo coste para evaluar la actividad electroquímica microbiana a un nivel de microescala. Los SPEs resultaron ser válidos para identificar respuestas bioelectroquímicas, evitando complejos montajes. El sistema se validó mediante el estudio de la respuesta de *G. sulfurreducens* bajo diversos estados fisiológicos. Además, el uso de SPE y *G. sulfurreducens* reveló su potencial como biosensor para la cuantificación de acetato y para explorar muestras de naturaleza tan compleja como las aguas residuales urbanas. La identificación de consorcios electroactivos resultó ser otra de las aplicaciones de esta tecnología.

Como aporte científico final, el **Capítulo 4** explora una nueva estrategia para utilizar *G. sulfurreducens* en la de construcción bioelectrodos artificiales, mediante la inmovilización de las células en gel de sílice sobre electrodos de fieltro de carbono. El análisis de viabilidad confirmó que la mayoría de las células sobrevivieron al proceso de encapsulación y que la densidad celular no evolucionó, al menos, durante un período de 96 h. Esta doble encapsulación impide la liberación de bacterias del electrodo, pero permite la difusión de solutos, haciendo posible la caracterización electroquímica del sistema. Los bioelectrodos artificiales se evaluaron en sistemas de 3 electrodos durante varios días,

utilizando diversos donadores de electrones. El análisis voltamétrico de los bioelectrodos alimentados con acetato mostró una onda de oxidación catalítica sigmoidal desde el inicio, característica de biofilms más maduros. Además, se confirmó la presencia de *G. sulfurreducens* dentro de las fibras y del gel de sílice por microscopía electrónica de barrido y también se realizó un análisis transcriptómico para estudiar el efecto de la encapsulación a nivel intracelular.

A partir de los resultados obtenidos, el **Capítulo 5** presenta una discusión general, conclusiones y perspectivas de futuro. Este capítulo fue concebido con una estructura de preguntas y respuestas que confío ayude al lector a entender las razones que sustentaron tanto nuestra experimentación como la interpretación que hicimos de la misma. El valor fundamental de este trabajo es la naturaleza sencilla, rápida y económica de las herramientas proporcionadas que pueden ayudar a otros investigadores en el campo de la electroquímica microbiana.

Chapter 1

General introduction & Objectives

GENERAL INTRODUCTION

1. MICROBIAL ELECTROCHEMISTRY: AN EMERGING FIELD OF STUDY

Microbial electrochemistry deals with the interactions between microorganisms and electronic devices. At the beginning of the 20th century it was first conceived the idea of using microbial cultures in an attempt to produce electricity (Potter, 1911), in the first approach of what it is known nowadays as a microbial fuel cell (MFC). Generally speaking, a MFC is a system in which microorganisms catalyze the conversion of chemical energy into electrical energy. The process can occur in two directions, microorganisms can oxidize organic matter while an insoluble acceptor (e.g. electrode) is subsequently reduced or either, they can accept electrons from the electrode (Rosenbaum *et al*., 2011). Even though the concept emerged long time ago, it has been in the recent decades when it has really started to take off. Such is the case that, in addition to MFCs, a vast number of derivatives technologies have appeared recently for several purposes, for instance: microbial electrolysis cells (MECs) (Verea *et al*., 2014), microbial desalination cells (MDCs) (Cao *et al*., 2009), microbial electrosynthesis (MES) (Rabaey and Rozendal, 2010), or microbial electroremediating cells (MERCs) (Rodrigo *et al*., 2016), among others. All these diverse technology are included in what is named as microbial electrochemical technologies (METs) (Schröder *et al*., 2015).

Several type of microorganisms can exchange electrons with an electrode, by donating or accepting them (Bond, 2010; Franks and Nevin, 2010; Logan, 2009; Rosenbaum *et al*., 2011). Nevertheless, the mechanisms for such outstanding interaction, have been thoroughly studied

primarily in two bacteria *Shewanella Oneidensis* and *Geobacter sulfurreducens* (Carmona-Martínez *et al*., 2013; Marsili *et al*., 2008a; Lovley *et al*., 2011; Lovley, 2011; Schrott *et al*., 2011). The interest in *G. sulfurreducens* has exponentially grown, due to its easy culturing conditions, metabolism, its broad environmental relevance and because of its intrinsic capacity for establishing direct contact with electrodes. Hence it is not surprising that it has become the model organisms for the study of METs, and the focus of this work as well.

2. THE ELECTROACTIVE BACTERIA: *GEOBACTER SULFURREDUCENS*

General features

Geobacter species can be classified in the family *Geobacteraceae*, belonging to the class *Deltaproteobacteria* and phylum *Proteobacteria.* They represent a wide genus of Gram-negative, metal-reducing microorganisms which have an important role in natural environments, where dissimilation of iron and manganese are predominant processes (Lovley *et al*., 2011). Since the discovery of *G. metallireducens* in 1987, it has been described more than a dozen subgenera, being *G. sulfurreducens* the one of the most extensively studied (Lovley *et al*., 2011). *G. sulfurreducens* strain PCA was isolated in 1994, and it was the first *Geobacter* strain able to grow with elemental sulfur as an electron acceptor (Caccavo *et al*., 1994). Moreover it was the first for which methods for genetic manipulation were developed (Coppi *et al*., 2001). Over time, it was shown the most relevant feature of these bacteria: its ability for respiring electrodes (Bond and Lovley, 2003). That implies the coupling of the intracellular oxidation of an electron donor, with the extracellular reduction of an electron acceptor. This milestone marked the beginning of METs and *G. sulfurreducens* became the model electroactive bacteria.

Electron donors and acceptors

G. sulfurreducens has a broader range of electron donors than originally reported by Caccavo *et al* in 1994.

Electron donors:

Acetate is the preferred electron donor for *G. sulfurreducens.* It can be completely oxidized in the tricarboxylic acid (TCA), and electrons generated in the process can be accepted by intracellular (e.g fumarate) or extracellular electron acceptors (e.g Fe (III) or electrodes) (Lovley *et al*., 2011). In addition, acetate provide carbon to the cell, via its conversion to pyruvate, for gluconeogenesis, biomass synthesis, and other anabolic reactions (Galushko and Schink, 2000; Segura *et al*., 2008; Yang *et al*., 2010). Acetate is the central intermediate in the anaerobic degradation of organic matter in sedimentary environments, as well as the end-product of the acetogenic phase in the anaerobic wastewater treatments, and its presence is directly related with the formation of biogas in anaerobic digestion (Henze, 2008).

Lactate is oxidized as well, producing piruvate and acetate coupled with the reduction of Fe (III), fumarate or an electrode (Call and Logan, 2011). However, lactate-fed systems produce lower power density than acetate ones (Speers and Reguera, 2012). Formate and hydrogen, are also oxididized by *G. sulfurreducens*, with Fe (III) or electrode as terminal electron acceptors (TEAs), although in the case of the second, a carbon source (e.g. lactate) is required (Speers and Reguera, 2012).

Reduced humic substances or the analog anthrahydroquinone-2,6-disulfonate (AHQDS), can also act as electron donor for *G. sulfurreducens.* Nonetheless, due to its large size, they are extracellularly oxidized (Smith *et*

al., 2015). Finally, *G. sulfurreducens* have been reported to utilize graphite electrodes as electron donor for the reduction of fumarate (Gregory *et al*., 2004).

Electron acceptors:

Fe (III) (in soluble and insoluble forms) is the natural TEA of *G. sulfurreducens* when coupled with the oxidation of acetate, hydrogen or lactate (Caccavo *et al*., 1994; Call and Logan, 2011). Fe (III) is the most abundant acceptor available in soils and sediments, the natural habitat of *G. sulfurreducens* (Lovley., 2011). Is interesting to notice that the reduction Fe (III) is preferred over the fumarate, despite the lower growth yield showed (Esteve-Núñez *et al*., 2004).

Another relevant acceptor in natural environments are the humic substances, that are the most abundance source of organic matter (Lovley., 2011). In addition, *G. sulfurreducens* can use the following TEAs: elemental sulfur, Mn(IV), uranium, malate, even other microorganisms, what is named as microbial direct interspecies electron transfer (DIET) (Caccavo *et al*., 1994; Mehta *et al*., 2005a; Lovley *et al*., 2011; Lovley, 2011).

With the isolation of *G. sulfurreducens* it was described that fumarate can be reduced coupled to the oxidation of acetate (Caccavo *et al*., 1994). A few years later, it was demonstrated that fumarate can be reduced using lactate (Call and Logan, 2011) or electrodes (Gregory *et al*., 2004) as electron donors. Fumarate reduction in *G. sulfurreducens* is in fact an artificial process, based on the succinate deshydrogenase (Sdh) activity. The natural rol of Sdh is to oxidize succinate as part of the TCA cycle, but it can operate backwards in presence of high concentration of fumarate. Considering that in sedimentary environments the exogenous fumarate is not abundant, it is likely that the primary function of the Sdh is the catalysis of succinate oxidation rather than fumarate reduction (Butler *et al*., 2006; Zaunmüller *et*

al., 2006). However, fumarate is the intracellular electron acceptor of choice for the culturing of *G. sulfurreducens* due to the high growth yield obtained in comparison with the natural acceptor Fe (III) (Esteve-Núñez *et al*., 2004, 2005).

Surprisingly, several years after its isolation, *G. sulfurreducens* was reported not to be a strict anaerobe, and it can indeed grow with oxygen at the sole electron acceptor at low concentrations (Lin *et al*., 2004). Finally, the utilization of electrodes as TEAs was demonstrated by coupling the electron transfer to acetate, lactate, formate, and hydrogen oxidation (Bond and Lovley, 2003; Speers and Reguera, 2012).

Why *G. sulfurreducens* interact with electrodes?

The fact that a microorganism can interact with electrodes is even more surprising taking into account that such conditions are not part of the natural environment. An initial hypothesis was simply that this type of microorganisms adapted their molecular machinery, over millions of years, to extracellular reduction of insoluble minerals. Apparently, these evolutionary mechanisms resulted to be effective for reducing electrodes as well (Lovley, 2012). However, when *Geobacter* species are grown on Fe (III) oxides, they express flagella that need for its continuously search of the next source of Fe (III) (Childers *et al*., 2002). A different situation is observed when *Geobacter* is cultured in an electrochemical system, where bacteria are permanently attached to electrodes (Bond and Lovley, 2003). So the origin of this evolutionary adaptation is likely to be different.

Another possible explanation are the so-called natural geobatteries. Geobbateries are formed when graphite deposits in the subsurface are able to transfer electrons between anaerobic an oxic zones (Bigalke and Grabner, 1997), which constitute a long-term electron acceptor for the

surrounding microbes and have more in common with the electrodes acting as anodes in a MFC (Leung and Xuan, 2015) **(Figure 1).**

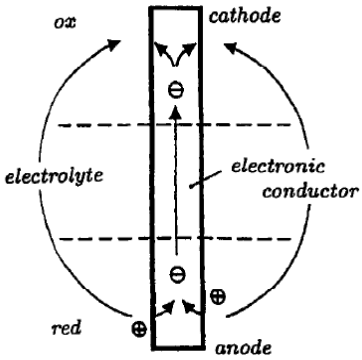

Figure 1: Schematic representation of a geobattery. Ox: oxidizing zone. Red: reducing zone. Extracted from (Bigalke and Grabner, 1997).

Types of mechanisms for electron transfer

There is a great diversity of electroactive microorganisms which are able to exchange electrons with an electrode, hence they use different strategies for making extracellular electron transfer (EET). Three EET mechanisms between microorganisms and electrode surfaces can be distinguished **(Figure 2),** namely, i) indirect electron transfer (IET), ii) mediated electron transfer (MET), and iii) direct electron transfer (DET).

i) IET occurs when a fermentative microorganism convert glucose into reduced metabolic products (hydrogen, formic acid, alcohol...) which can be secreted and react with electrons to an electrode (Karube *et al*., 1977, Sydow *et al*., 2014).

ii) MET involves the use of mediators or electron shuttles which are soluble molecules that can accept electrons from microorganisms and subsequently, donate them to the electrode. In contrast to IET, mediators can be reversibly oxidized and reduced, so they have become a widely used

strategy in microbial electrochemistry field. Electron shuttles can be artificially added, like quinones (Adachi *et al*., 2008), however, its addition could be toxic and expensive for large-scale applications (Thrash and Coates, 2008). Some microorganisms are able to secrete their own electron shuttles to establish electric contact with the electrode, such is the case of *Shewanella oneidensis*, which produce flavins (Marsili *et al*., 2008a; Kotloski and Gralnick, 2013) or *Pseudomonas aeruginosa* that secrete phenazines (Pham *et al*., 2008).

iii) Regarding DET, microorganisms such *G. sulfurreducens* establish direct electric contact with the electrode via an outer-surface redox network, which is formed basically by *c*-type cytochromes (Lovley, 2011; Lovley *et al*., 2011; Lovley, 2012).

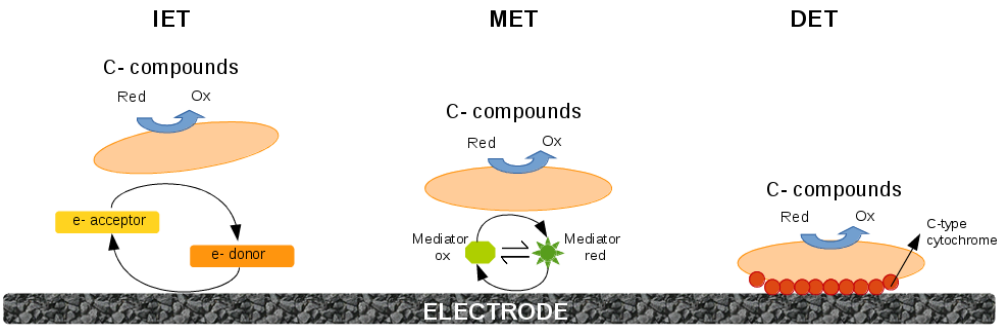

Figure 2: Representation of bacterial mechanisms for: indirect electron transfer (IET), mediated electron transfer (MET) and direct electron transfer (DET)

Direct extracellular electron transfer (DEET) in *G. sulfurreducens:* the role of the cytochromes

The unique ability of *Geobacter* to establish a direct contact with an insoluble electron acceptor is due to the presence of a vast network of

cytochromes C that connects the internal cytoplasm with the outermost environment of the cell (Morgado *et al*., 2012; Aklujkar *et al*., 2013; Estévez-Canales 2015b). There are about 100 putative *c*-type cytochrome genes encoded in *G. sulfurreducens* genome (Methé *et al*., 2003), which contain heme groups that can act as electron transfer groups. Many of these *c*-type cytochromes are located in the periplasmic space (Morgado *et al*., 2010), (e.g. PpcA) exposed on the outermost membrane of the cell, (e.g. OmcB), (Mehta *et al*., 2005; Ding *et al*., 2006; Qian *et al*., 2007), aligned along the pili structure, (e.g. OmcS) (Leang *et al*., 2010), while others, are found well beyond the cell membrane, embedded in the extracellular matrix (e.g. OmcZ) **(Figure 3)** (Inoue *et al*., 2011; Rollefson *et al*., 2011). Knock-out studies suggest that these *c*-type cytochromes transfer electrons *in vivo* to a diversity of natural extracellular electron acceptors, such as metals and humic substances (Leang *et al*., 2003; Leang *et al*., 2005; Mehta *et al*., 2005b; Shelobolina *et al*., 2007; Voordeckers *et al*., 2010; Orellana *et al*., 2013). Furthermore, numerous authors have demonstrated that *c*-type cytochromes directly participate in the electrochemical communication with the anode (Holmes *et al*., 2006; Nevin *et al*., 2009; Busalmen *et al*., 2010; Esteve-Núñez *et al*., 2011; Millo *et al*., 2011; Kuzume *et al*., 2013; Kuzume *et al*., 2014).

The network of cytochromes in *Geobacter* can also function as a capacitor accepting electrons from the acetate metabolism (iron lungs) (Esteve-Núñez *et al*., 2008) when extracellular electron acceptors are not available (Esteve-Núñez *et al*., 2008; Lovley, 2008). Indeed, the abundant *c*-type cytochromes in current-producing biofilms provide a capacitance comparable to that of synthetic supercapacitors with low self-discharge rates (Schrott *et al*., 2011; Liu *et al*., 2011; Malvankar *et al*., 2012).

Although the exact pathway of each cytochrome of the network remains unknown, some elements seem to have a different role in the

respiration of electrodes or Fe (III) oxides **(Figure 3)**. That is the case of the pili structure or nanowire (Reguera *et al*., 2005; Malvankar *et al*., 2011). Deletion studies proved that pili are crucial for reduction of Fe (III) oxides, but not for other extracellular acceptor such an electrode (Reguera *et al*., 2005). However, in current producing biofilm growing on electrodes, pili are proposed to provide a conductive network through the internal structure of the biofilm, which allows to perform long-range electron transport (Lovley, 2012).

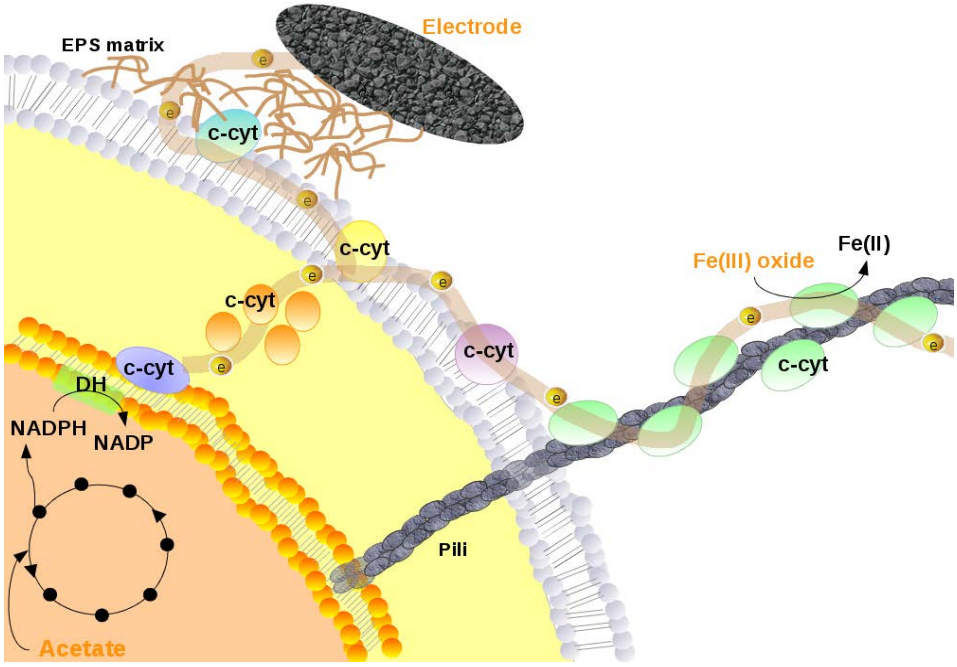

Figure 3: Representation of different pathways of the cytochromes network (c-cyt) proposed for short-range DEET to electrodes and Fe (III) oxides in *G. sulfurreducens*.

The deeper understanding about the mechanism for the long-range electron transport through the inside of the biofilms, has led to two different school of thoughts: electron hopping and metallic-like conductivity.

Metallic-like conductivity model, proposes that the pili structure posses

conductivity itself, and the overlapping π-orbitals of aromatic amino acids (forming the protein structure), are thought to be responsible for this model based on electron delocalization (Malvankar *et al*., 2011; Malvankar *et al*., 2015). Conversely, the electron hopping model is based on the classical biological thought for electron transfer, and electrons are proposed to hop from one cytochrome to another. In this way electrons are always localized in discrete biomolecules, and they move through multi-step hopping (Snider *et al*., 2012; Bond *et al*., 2012).

Some authors has pointed that the combination of both models, is the more plausible hypothesis (Bonanni *et al*., 2013).

Despite the great research work in recent years, the complete understanding of the interaction bacteria-electrode represents a challenge and it will be key for establishing new frontiers and practical applications in the field of electromicrobiology.

Influence of cell culturing methods

G. sulfurreducens can be cultured using different approaches depending upon the application (e.g. fundamental studies, laboratory scale, actual implementations...) or physiology desired (e.g. over-expression of *c*-type cytochromes, change of extracellular polymeric substances (EPS) profile...). In this thesis, batch and chemostat mode were used **(Figure 4)**.

Batch culture:

It can be defined as a closed system where culture medium is inoculated and confined into a culture vessel. Then, it is incubated at a suitable temperature and gaseous environment for an appropriate period of time (Lee, 2006). The composition of the medium, the biomass and the metabolite concentration change as a result of cell metabolism. Four typical growth phases can be distinguished: lag, exponential, stationary and death

phase. Batch culture is common for fundamental studies (Kuzume *et al*., 2013; Aklujkar *et al*., 2010).

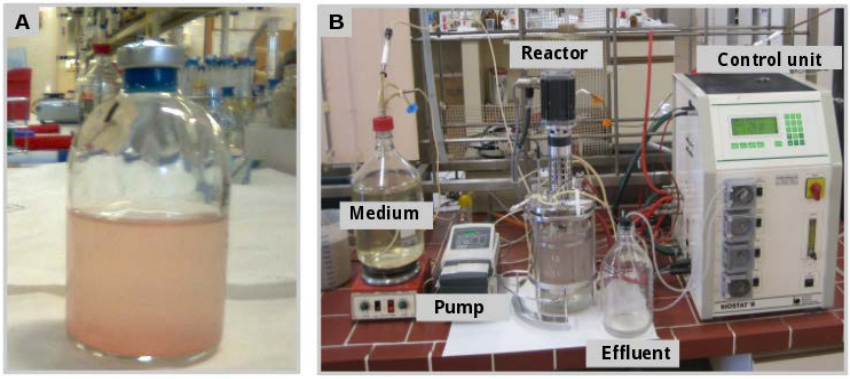

Figure 4: Photos of batch culture bottle (A) and chemostat culture (B) of *G. sulfurreducens*.

Chemostat culture:

It consists of an open system where sterile nutrient medium is added to the bioreactor continuously and an equivalent volume of used medium with microorganisms is simultaneously removed. The physiological steady state is controlled by means of the availability of a limiting substrate. A key parameter that must be fixed in a chemostat is the dilution rate, which is defined as the flow of medium per time over the volume of culture. At steady state, the specific growth rate of the microorganism is equal to the dilution rate, thus the biomass remains constant (Lee, 2006). The chemostat provides a great opportunity to study microbial physiology under specific and reproducible conditions, (Esteve-Núñez *et al*., 2004; Esteve-Núñez *et al*., 2005a). In particularly, for *G. sulfurreducens,* it has been showed that electron acceptor limitation growth condition in a chemostat, enhances extracellular electron transfer rates due to the overproduction of cytochromes at the cell surface (Esteve-Núñez *et al*., 2011), minimizing considerably the start-up process of METs (Borjas *et al*., 2015).

Others conditions:

Recent unexpected findings have shown an alternative way for culturing highly electroactive cells by adding NaCl into batch cultures. These conditions modify the EPS profile so cells displayed 3-fold higher DEET capacity in comparison with standard batch cells (Borjas *et al.* personal communication).

Biofilm vs planktonic growing

The typical approach to study the electroactivity is *G. sulfurreducens* is to grow a biofilm on the electrode surface. Operating with a mature biofilm, allows to obtain steady and reproducible conditions (Harnisch and Freguia, 2012; Schrott *et al*., 2011; Beyenal and Babauta, 2015; Marsili *et al*., 2008b). A biofilm can be defined as an assemblage of surface-associated microbial cells. In *G. sulfurreducens*, the biofilm thickness has been reported to be of several tens of micrometers, although the physiological state is not homogeneous. As matter of fact, once biofilms are thicker than 60-70 μm, the accumulation of cells does not contribute to current production (Schrott *et al*., 2014).

Alternatively, planktonic cells (bacterial suspension) have been also utilized for short time studies, in where the main goal is a rapid characterization, electrochemical or metabolic, of the system instead large current production (Esteve-Núñez *et al*., 2011; Estevez-Canales *et al*., 2015a; Shelobolina *et al*., 2007). Moreover, the growth of planktonic electroactive cells of *G. sulfurreducens* could be supported by a Microbial Electrochemical-Fluidized Bed Reactor (ME-FBR), obtaining electron transfer rates similar to those reported for electroactive biofilms (Tejedor *et al.* personal communication).

Despite the divergent growth conditions, both approaches provide an

opportunity to deepen understanding of *G. sulfurreducens*.

Regulation of metabolism

The expression of genes in an organism can be influenced by the environment, which may impose significant stress on cultures. In order to understand how *G. sulfurreducens* is likely to change its expression and metabolism in response to these variations, it is important to know how gene expression is regulated (Mahadevan *et al*., 2008). Among the mechanisms for regulating gene expression, the sigma and transcription factors are the most important (Methé *et al*., 2003; Lovley *et al*., 2011; Qiu *et al*., 2013).

Sigma factors:

Sigma factors play a key role in the regulation of gene expression in response to changing environments. σ-Factors are proteins needed for initiation of RNA synthesis that recognize specific promoter elements of a certain set of genes and initiate their transcription. The genome of *G. sulfurreducens* encodes homologs of RpoD, RpoS, RpoH, RpoN, RpoE and FliA found in *E. coli* and many other bacteria; indeed they are reported to be the major regulator of energy metabolism (Methé *et al*., 2003; Núñez *et al*., 2004; Yan *et al*., 2006; Krushkal *et al*., 2007; Ueki and Lovley, 2007; Leang *et al*., 2009; Qiu *et al*., 2013).

Transcription factors:

Transcription factors are typically related with more specific cellular function than sigma factor in response to environmental and physiologic changes. Its operating mechanism includes a repressor and an activator which inhibits or promotes transcription, although some transcription factors can act in both ways. *G. sulfurreducens* genome encodes for 151 putative transcription factors (Methé *et al*., 2003).

One example of a transcription factor is the system for regulating the iron metabolism, the ferric-uptake regulator (Fur). Fur is a protein that acts as a transcriptional repressor. In response to the intracellular iron availability, Fur blocks the access of RNA polymerase to the DNA and controls many genes related to iron acquisition as well as redox-stress resistance and central metabolism (Embree *et al*., 2014; Wan *et al*., 2004).

Bacterial immobilization

With the aim of simplifying the start-up process and stability of the biofilm, recent studies have pointed at the possibility of artificially improving electroactive biofilms performance. Some examples reported in the literature using *Shewanella*, have introduced the concept of making artificial colonization of the biofilm, where bacteria are encapsulated in a material to conform a bioelectrode (Luckarift *et al*., 2012). Others bet on doping the biofilm, adding conductive compounds, in order to improve the electrical conductivity through it (Yu *et al*., 2011). Unlike traditional encapsulation, in order to build an artificial electroactive biofilm, aside from bacterial viability, it is key to ensuring electrical communication with the electrode. Among the matrices where cells can be immobilized, agar, pectine, alginate (organic polymers) and silica gels (inorganic polymer) are the most common choices (Bjerketorp *et al*., 2006). On one hand, organic polymers have the advantage to be always biocompatible as well as allows solute diffusion, (Srikanth *et al*., 2008). On the other hand, inorganic polymers, like silica gels, also allow solute diffusion but they offer better optical and mechanical properties, making the gel more robust and easier to control (Depagne *et al*., 2012; Ouay *et al*., 2013).

Another possibility for bacterial immobilization on the electrode

surface is the union by using artificial linkers. For *G. sulfurreducens* it has been demonstrated that carboxyl-terminated alkanethiols linkers, such as 6-mercaptohexanoic acid, effectively bind to gold electrodes and outermost cytochromes providing biocompatible conditions (Kuzume *et al*., 2013; Füeg *et al*., Submitted). The alkanethiol group binds to gold surface and the negatively charged carboxyl groups binds electrostatically to a positively charged pocket of the cytochromes.

Although the cellular and enzymatic entrapment have been studied for several decades (Calabretta *et al*., 2012; Datta *et al*., 2013), the interest in this methodology for electroactive bacteria is very recent, and it could be a helpful and versatile strategy both for bacterial conservation and for its implementation in METs, especially for biosensing purpose (Luckarift *et al*., 2012; Yang *et al*., 2015).

3. ELECTROCHEMICAL ASPECTS: TOOLS FOR STUDYING DEET IN *G. SULFURREDUCENS*

The merging of two fields as different as microbiology and electrochemistry, have resulted in a very powerful and versatile tool for both, basic and applied research leading to a newborn field: METs.

Microbial fuel cell (MFC) vs microbial electrolysis cell (MEC)

Traditionally, the typical configuration of MFC consists of two chambers (anode and cathode) separated by a proton exchange membrane **(Figure 5)**. In the anode, microorganisms oxidize organic matter, producing CO_2, electrons and protons. Electrons are transferred to the cathode compartment through an external electric circuit, while protons are transferred to the cathode compartment through the membrane. Electrons and protons are consumed in the cathode, combining with the catholyte and

reducing it. The combination of the electrical current production (*I*) and the voltage difference (*V*) between anode and cathode determine the amount of power produced:

$$P = I\,V$$

However, when it comes to the study of biological process in METs, it is key to maintain constant the anode potential, in order to obtain reproducible results. These conditions can be achieved by using a potentiostat which set a specific potential to the working electrode (anode) versus a reference electrode, in a MEC **(Figure 5)** (Beyenal and Babauta, 2015).

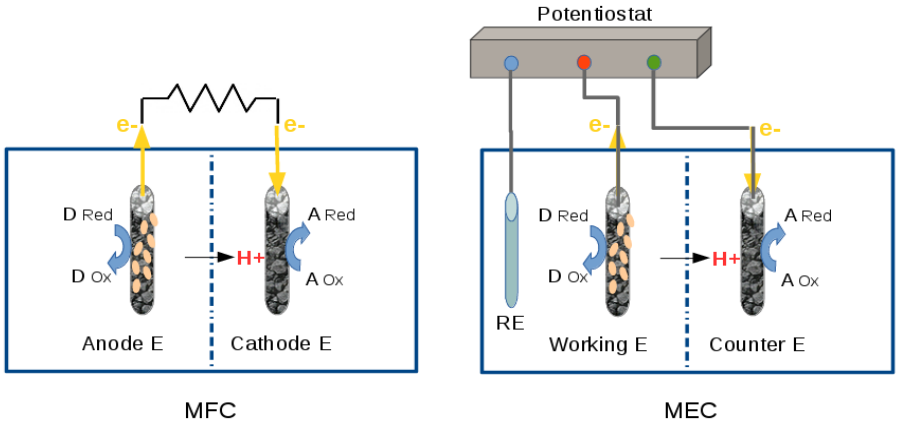

Figure 5: Scheme of a two-chambered MFC and MEC, both having an electron donor (D red/ox), an electron acceptor (A red/ox) and a proton exchange membrane. In the MEC, a potentiostat fix the working electrode potential to a specific value.

Although traditionally, many MEC-related works are performed using a two-chamber configuration, it is also possible to operate in a single-chamber, where there is not membrane, and working and counter electrodes share the same electrolyte. In a MEC, at equal number of electrodes, this simplified architecture allows to make the same electrochemical tests, and is especially useful in controlled systems where

the reactions than can take place are limited, for instance, working with pure cultures.

Electrochemical methods for the study of the microbial electroactivity in *G. sulfurreducens*

The use of a potentiostat allows real-time measurement of the microbial electroactivity by different techniques, such as chronoamperometry, cyclic voltammetry, open circuit potential or spectroelectrochemical techniques.

Chronoamperometry (CA):

This technique fixes a given potential between the working and the reference electrode, and the current produced or consumed is monitored as a function of time, as a result of the oxidation or reduction of a compound on the surface of the working electrode.

In *G. sulfurreducens* there is a wide variety of examples in where CA has been used. On one hand, current produced serve as an status check of the system as well as to screening various levels of electroactivity (Harnisch and Rabaey, 2012; Borjas *et al*., 2015; Esteve-Núñez *et al*., 2011). On the other hand potentials applied, have great influence in the adaptation of exocellular electron transfer elements as well as in the microbial diversity of a biofilm community (Busalmen *et al*., 2008b; Torres *et al*., 2009). In addition, CA can be used in biosensors, as the current produced can be directly related with the concentration of electron donor that it is being oxidized (Li *et al*., 2011; Tront *et al*., 2008).

There is no consensus about the optimal potential that should be applied, because it is dependant of many factors, but there are a few considerations. If the goal is promoting oxidation process, the potential applied must be more positive than the open circuit potential (OCP) of the

working electrode. Conversely, a negative potential accelerates reduction at the working electrode (Babauta *et al*., 2012).

Cyclic voltammetry (CV):

This electrochemical procedure consists of a cyclic potential sweep, at a fixed scan rate, during which the current of the working electrode is registered. CV is a powerful technique that provides valuable information about the redox behaviour of the reactions that *G. sulfurreducens* is able to catalyze (Harnisch and Freguia, 2012; Fricke *et al*., 2008). Understanding this process, from a strictly electrochemical point of view, might be confusing, since the oxidation or reduction mediated by a whole cell is far more complex that enzymatic catalysis or inorganic electrocatalysis. During a whole cell catalysis, there are a considerable number of enzymatic sub-steps in the redox cascade, hence, peaks responses overlap, making difficult assigning them to specific redox couples (Fricke *et al*., 2008; Babauta *et al*., 2012;). In addition, voltammograms differ as function of the physiologial state, cellular arrangement and experimental conditions (Marsili *et al*., 2008b; 2012; Busalmen *et al*., 2008b; Esteve-Núñez *et al*., 2011;Strycharz-Glaven and Tender). However, this fact can be considered an advantage as well. For instance, the voltammogram shape allows to determine whether the system is under turn-over (excess of substrate) or non turn-over conditions, and therefore, to obtain the maximum and limiting current value **(Figure 6)** (Harnisch and Freguia, 2012). Furthermore, through CV it is possible to determine the working electrode potential window in which DEET can occur for a given electron donor and experimental conditions (Fricke *et al*., 2008). CV can also serve a biosensor detection technique, since maximum current display is related with the concentration of electron donor, in a similar way that occurs in chronoamperometry. Moreover, the peak potential of catalytic curves ensures the proper performance of the system (Marsili *et al*., 2008b).

Regarding some technical aspect, it is worth noting that the scan rate selected is crucial, considering that capacitive currents can significantly mask the faradaic response (LaBelle and Bond, 2009; Harnisch and Freguia, 2012).

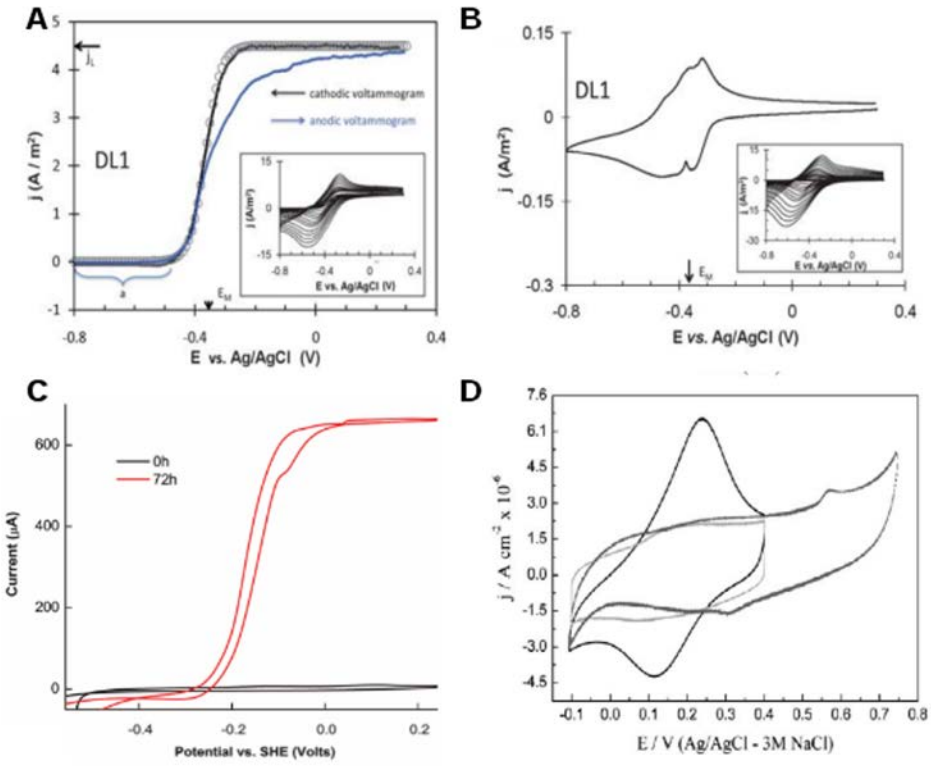

Figure 6: Voltammetric characterization of the catalytic activity of *G. sulfurreducens* in A) Mature biofilm at turn-over conditions, B) Same biofilm after acetate depletion, C) At differents time of biofilm formation and D) Comparing different physiological state. (A) y B) extracted from (Strycharz *et al*., 2011), C) from (Marsili *et al*., 2008b), and D) from (Esteve-Núñez *et al*., 2011).

Open circuit potential (OCP):

This parameter is defined as the potential of an electrode (working or counter) measured versus the reference electrode. In long term

experiments, OCP value can be used as a diagnosis, of the microbial colonization of the working electrode. So thus, the more negative the value, the higher level of bacterial colonization; until reach a steady-state OCP, due to complete coverage of electrode (Babauta *et al*., 2012).

Spectroelectrochemical techniques:

Spectroelectrochemistry combines electrochemistry with spectroscopy, and it relates the changes in the spectral signature with electrochemically active compounds above an electrode set at a certain polarization potential (Bard and Faulkner, 2000).

Spectroelectrochemical techniques can provide *in vivo* understanding of electron transfer mechanisms as well as molecular structures (Scott and Yu, 2015). This approach has been used for studying the interface between *G. sulfurreducens* and gold electrodes using Attenuated Total Reflection-Surface Enhanced Infrared Absorption Spectroscopy (ATR-SEIRAS), showing oxidized/reduced states in *c*-type cytochromes, and evidencing that *c*-type cytochromes are responsible for DEET (Busalmen *et al*., 2008a; Busalmen *et al*., 2010; Esteve-Núñez *et al*., 2011) .

Another spectroelectrochemical technique for the characterization of *G.sufurreducens* is the *in situ* Nanoparticle Enhanced Raman Spectroscopy (NERS), a powerful technique that can detect and further provide structure information of hemes groups of *c*-type cytochromes (Kuzume *et al*., 2013; Kuzume *et al*., 2014; Estevez-Canales *et al*., 2015b).

From microbial metabolisms to electrical current

Unlike other techniques to convert organic material to electricity, METs are unique because it allows direct electricity generation (Rabaey and Verstraete, 2005). In order to confirm that the current generated is due the

consumption of a given nutrient, the total charge transferred (Q) must be correlated with the electron donor consumption, by integrating current over time, following Faraday's law:

$$Q = \int Idt = n_e N_{mol} F$$

The difference between the electrons released by the bacterial metabolism and the electrons recovered by the electrode, it is known as coulombic efficiency. In *G. sulfurreducens,* for each mol of acetate oxidized, eight electrons are harvested (n_e), what corresponds with a coulombic efficiency of ca. 95 %, indicating that acetate was completely oxidized to CO_2 (Bond and Lovley, 2003). Besides, coulombic efficiency, this relation can be used for comparing metabolic rates of oxidation under several conditions (Esteve-Núñez *et al*., 2005b).

Screen-printed electrodes

Screen-printing is an established technique to fabricate micro-scale electrochemical devices. A standard Screen Printed Electrode (SPE) comprise a variable number of electrodes and all the compounds necessary for completed the electric circuit placed over a supporting material (Hayat and Marty, 2014). Its inherent advantages include miniaturization, versatility, low cost and the possibility of mass production. SPEs can be manufacture using several materials as electrodes, including carbon inks that have very similar properties than conventional electrodes, such as low background signal and a wide range of working potentials. SPEs are accurate and rapid analysing devices (Taleat *et al*., 2014) that can be also combined with functionalization (through electrochemical techniques) of biomolecules (Gómez-Mingot *et al*., 2011) or bacteria (Chang *et al*., 2012). Moreover, they require small volumes of the analyte (microlitres), they are suitable for off-the-bench assays.

All these benefits turns SPEs into a natural choice that could serve as biosensor, as well as a fast screening of physiology, among others potential

application, from electroactive microorganisms (Estevez-Canales *et al*., 2015a) .

4. APPLICATIONS OF MINIATURIZED METs

Among the classical applications known as METs, simultaneous electricity generation with wastewater treatment (Kim *et al*., 2015; Borjas *et al*., 2015; Malaeb *et al*., 2013) or bioremediation (Zhang *et al*., 2010; Rodrigo *et al*., 2014); have been the topics most widely studied. However, one of the major handicaps for practical implementation of METs is the scale-up, due to the lack of linearity between current density production and the active surface area (Babauta *et al*., 2012).

For this reason, an alternative research line is receiving more and more attention: the down-scaled METs (Choi *et al*., 2015; Jiang *et al*., 2015; Yoon *et al*., 2014). Such platforms offer considerably advantages starting by the process of microfabrication itself: mass production and costs reduction (Choi, 2015), making them more applicable and potentially realizable than macro-scale METs, in terms of profitability. Second, micro-scale METs exhibit a higher surface area to volume ratio which enhance the efficiency usage of substrates per unit volume and the mass transport (Ren *et al*., 2014). These features allow to obtain significantly shorter start-up time and rapid electrical response, compared with classical MFCs (Estevez-Canales *et al*., 2015a; Wang *et al*., 2011; Jiang *et al*., 2015; Li *et al*., 2011).

From such appealing characteristics, the future applications of miniaturized METs, seem to point at: a) small portable electronic elements in remote locations, b) biosensing, and c) fundamental studies and screening of microorganisms.

a) Small portable electronic devices in remote location

Power supply for small devices in remote location is one of the scenarios that can take more advantage from miniaturized METs in the future (Ren *et al*., 2012). Normally, disposable batteries are used with this purpose, with the inconvenient that they must be replaced because of the limited service life.

In recent years some researchers (Choi and Chae, 2013; Ren *et al*., 2014) have achieved power densities in the range of microwatts, that should be enough for feeding small devices, functioning as permanent power source in hard-to-access conditions (Wei and Liu, 2008). This technology would be especially useful for medical devices, such as intraspinal microelectrodes for treating paralysis that could operate consuming the glucose as fuel in the bloodstream (Siu and Chiao, 2008). However, further studies about toxicity, implantation rejection or bacterial leakage, are needed to investigate its suitability for *in vivo* applications. As a matter of fact, even for environmental applications, there are others aspects to overcome in micro-sized METs. For instance, high internal resistances that limit their power output, which makes fast responding analyses, the more promising applications in the foreseeable future (Wang *et al*., 2011; Choi, 2015).

b) Biosensing

One of the most feasible trends for micro-scale METs is the biosensing based on electrochemical detection, as they can perform *in situ* analysis at short time with high sensitivity and stability (Yang *et al*., 2015). In wastewater technologies, researchers have successfully developed miniaturized sensors based on MFCs for measuring the biological oxygen demand (BOD) (Peixoto *et al*., 2011), acetate (Li *et al*., 2011), pH (Uria *et al*., 2016) as well as toxic compounds (Davila *et al*., 2011; Liu *et al*., 2014;

Lee *et al*., 2015).

Moreover, scaling down the architecture of the biosensor allows multiple assays on a single platform (lab-on-chip). For instance it has been reported a multianod MFC-based biosensor that could serve as water diagnostic (Fraiwan *et al*., 2014), and a microfluidic MFC-based biosensor capable of screening several analytes simultaneously related to water toxicity **(Figure 7)** (Ben-Yoav *et al*., 2009).

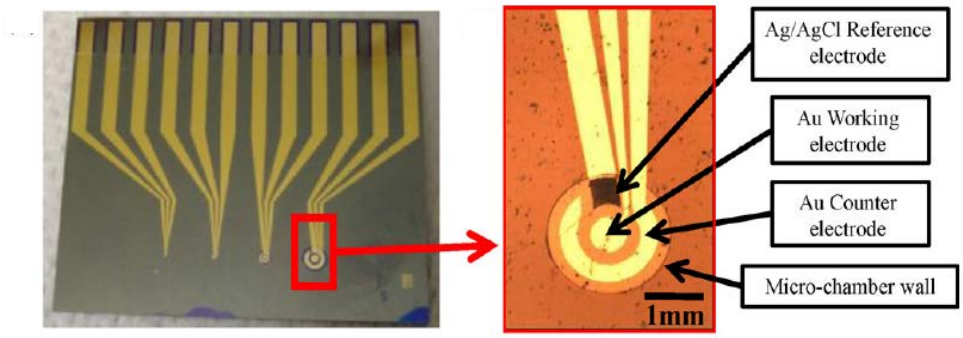

Figure 7: Silicon-based micro-chip comprising four electrochemical micro-chambers, and inside view of a single three-electrode electrochemical micro-chamber. (Extracted from Ben-Yoav *et al*., 2009).

Microfluidics is considered a keystone technique for lab-on-chip development. It is defined as a science and a technique that deals with the flow of liquids inside micrometer-sized channels. Micro-scale fluids behaviour is substantially different than macro-scale, mainly due to the flow regime which become laminar instead of turbulent (Wang *et al*., 2011; Squires and Quake, 2005). This feature makes that viscous and surface forces start to dominate the fluid behaviour, resulting in precisely control of the electrolytes inside the channels, among others benefits of microfluidics like low consumption of costly reagents, short reaction time, multiple

analysis in parallel, portability, and versatility in design (Li *et al*., 2011; Choi, 2015; Luka *et al*., 2015).

Another interesting branch in order to make whole cell biosensors with a higher robustness, sensitivity and specificity, is the synthetic biology approach (Bereza-Malcolm *et al*., 2015). Generally speaking synthetic biology consist of designing, constructing and assembling biological components for useful purpose, such as industry, science or even space exploration (Menezes *et al*., 2015). For instance, it has been described a genetic engineered modified *E.coli* (a non eletrogenic bacteria), which has acquired a portion of the extracellular electron transfer chain of *Shewanella oneidensis* MR-1, making it able to perform a low but detectable EET (Jensen *et al*., 2010).

c) Fundamental studies and screening of microorganisms

Another field that can benefit from miniaturized METs is the fundamental studies and screening of microorganisms. Despite the continued advances of the field, the precise mechanisms of electron transfer in electroactive bacteria is still poorly understood. The kind of assays required for the study of this outstanding process, usually involve rather complex experimental setups and limited access to microbial tools (Harnisch and Rabaey, 2012; Schröder, 2012). Hence, there is a clear niche for the development of appropriate, simpler and faster devices that supply reproducible and reliable data for the characterization and selection of microorganisms.

Screen-printed electrodes and microfluidic devices provide a great opportunity for the study, understanding and rapid screening of electroactivity or metabolic features of microorganisms in a single or multi-plate analysis **(Figure 8)** (Wang and Su, 2013; Estevez-Canales *et al*.,

2015a; Choi *et al*., 2015). Some researchers went further, and they have developed a device that enables simultaneous electrical and optical measurements without fluorescent markers, which allow observation of live bacteria (Dai *et al*., 2013).

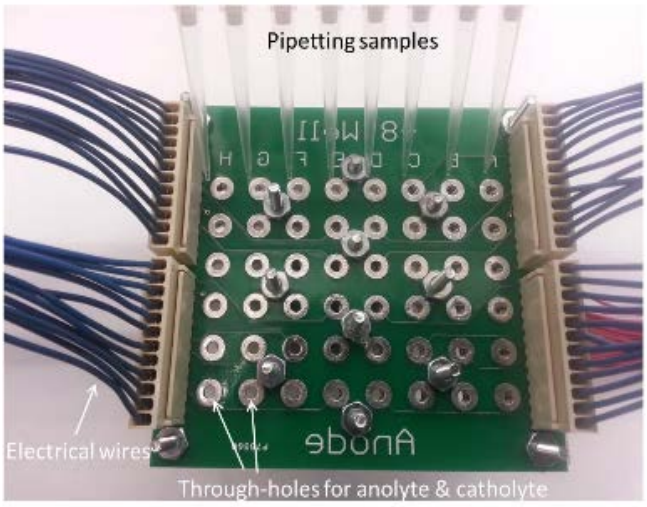

Figure 8: Image of the assembled sensor array for high-throughput screening of electrochemically (Extracted from Choi *et al*., 2015)

There is still a long way to go for the real implementation of miniaturized METs in our daily life. In any case, the way to improve such devices is, for sure, through the integration of a variety of fields of knowledge: engineering, material science, chemistry, biochemistry, physics, nanotechnology, and biotechnology.

OBJECTIVES AND OUTLINE

The present PhD thesis aims to explore novel approaches for a further understanding of EET process in *G. sulfurreducens,* as well as for providing advantageous electrochemical platforms for assisting basic and applied studies on METs. So thus, the following specific objectives were proposed:

- To study the role of c-type cytochromes in EET from *G. sulfurreducens* by developing a method for obtaining cytochrome-depleted cells.

- To explore Screen Printed Electrodes as miniaturized tools for rapid assessing microbial electrochemistry, avoiding complex electrochemical set-ups. More specifically, to implement a suitable platform for screening different physiologies of *G. sulfurreducens*, as well as desirable metabolic features from a microbial consortium. Moreover, to provide a system robust enough for performing studies in real wastewater and for biosensing of acetate.

- To develop an artificial bioelectrode based on silica encapsulated *G. sulfurreducens* that allows a fast electrochemical characterization while also ensuring cell viability.

The mentioned objectives are developed through the following chapters, each of which corresponds to a self-standing unit organized around the topics proposed. Except for **Chapter 1** which is introductory

and **Chapter 5** which presents a general discussion, conclusions and future outlook; the remaining chapters correspond to articles published or submitted to peer-review journals. A brief description of the following chapters is presented:

Chapter 2 shows a straightforward method for growing *G. sulfurreducens* in a low iron medium that included a chelator, resulting in cytochrome-depleted cells (heme$^-$ cells), which were characterized in detail by electrochemical, molecular, spectroscopic and microscopic techniques.

Chapter 3 investigates the use of SPEs as a novel low-cost platform for assessing microbial electrochemical activity at the microscale level, using either pure cultures of *G. sulfurreducens* or a microbial consortium. The study also included electrochemical analysis of different microbial physiological states, together with several materials for working electrodes

Chapter 4 explores a new strategy for manufacturing ready-to-use artificial bioelectrodes of *G. sulfurreducens* by means of effective immobilization of cells inside silica gel and carbon felt fibers electrodes. Such bioelectrodes were electrochemically characterized using several electron donors. In addition, viability assays and an analysis of the transcriptomic response for the encapsulated cells were performed.

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Chapter 2

The role of c-type cytochromes in extracellular electron transfer

Contents

Estevez-Canales, M., Kuzume, A., Borjas, Z., Füeg, M., Lovley, D., Wandlowski, T., Esteve-Núñez, A., 2015.
"A severe reduction in the cytochrome C content of Geobacter sulfurreducens eliminates its capacity for extracellular electron transfer".
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The role of c-type cytochromes in extracellular electron transfer

ABSTRACT

The ability of *Geobacter* species to transfer electrons outside the cell enables them to play an important role in a number of biogeochemical and bioenergy process. Gene deletion studies have implicated periplasmic and outer-surface *c*-type cytochromes in this extracellular electron transfer. However, even when as many as five *c*-type cytochrome genes have been deleted, some capacity for extracellular electron transfer remains. In order to evaluate the role of *c*-type cytochromes in extracellular electron transfer, *Geobacter sulfurreducens* was grown in a low iron medium that included the iron chelator (*2,2'-bipyridine)* to further sequester iron. Heme-staining revealed that the cytochrome content of cells grown in this manner was 15-fold lower than in cells exposed to a standard iron-containing medium. The low cytochrome concentration was confirmed by *in situ* Nanoparticle Enhanced Raman Spectroscopy (NERS). The cytochrome-depleted cells reduced fumarate to succinate as well as the cytochrome-replete cells grown, but were unable to reduce Fe (III) citrate or to exchange electrons with a graphite electrode. These results demonstrate that *c*-type cytochromes are essential for extracellular electron transfer by *G. sulfurreducens*. The strategy for growing cytochrome-depleted *G. sulfurreducens* will also greatly aid future physiological studies of *Geobacter* species and other microorganisms capable of extracellular electron transfer.

INTRODUCTION

G. sulfurreducens is an intensively studied microorganism that serves as a model system to investigate extracellular electron transfer (EET) in bacteria (Lovley *et al*, 2011). EET is the ability that certain bacteria have for coupling the oxidation of cytoplasmic electron donors with the reduction of insoluble electron acceptors located outside the cell. EET is responsible of biogeochemical processes such as the reduction of Fe-oxides and other metals in soils and sediments (Lovley *et al.* 2004) and for syntrophic electron transfer to methanogens (Rotaru *et al*., 2013). EET is also behind practical applications in the emergent field of electromicrobiology (Lovley, 2011), where bacteria are directly involved in redox processes with conductive materials (electrodes), which serve as electron acceptors. Microbial electrochemical technologies for harvesting energy from waste (Logan and Rabaey, 2012) or from soil enviroments (Dominguez-Garay *et al*., 2013), bioremediating polluted sediments (Lovley 2011, Rodrigo *et al.,* 2013) or biosensing **(**Dávila *et al.,* 2011**)** are all based on effective EET.

The unique ability of *Geobacter* to establish a direct contact with an insoluble electron acceptor is due to the presence of a vast network of cytochromes C that connects the internal cytoplasm with the outermost environment of the cell (Aklujkar *et al*., 2013; Morgado *et al*., 2012). There are about 100 putative c-type cytochrome genes encoded in *G. sulfurreducens* genome (Methé *et al.*, 2003), most of which contain multiple heme groups that can act as electron transfer mediators. Many of these *c*-type cytochromes are exposed on the outermost membrane of the cell (Metha *et al*., 2005; Ding *et al*., 2006; Qian *et al.*, 2007; Leang *et al.,* 2010; Inoue et al., 2011). Knock-out studies suggest that these c-type

cytochromes transfer electrons *in vivo* to a diversity of natural extracelular electron acceptors, such as metals and humic substances (Leang *et al*., 2003; Leang *et al*., 2005; Mehta *et al.*, 2005; Shelobolina *et al*., 2007; Voordeckers *et al*., 2010; Orellana *et al.* 2013). Furthermore, numerous studies have demonstrated that *c*-type cytochromes directly participate in the electrochemical communication with the anode (Holmes *et al.*, 2006,; Nevin *et al*., 2009; Busalmen *et al*., 2010; Esteve-Nuñez *et al*., 2011; Millo *et al*., 2011; Jain *et al*., 2011; Liu *et al.*, 2011; Richter *et al*., 2009; Strycharz *et al*., 2011).

The network of cytochromes in *Geobacter* can also function as a capacitor accepting electrons from the acetate metabolism (Esteve-Núñez *et al*., 2008) when extracellular electron acceptors are not available (Esteve-Núñez *et al*., 2008; Lovley., 2008). Indeed, the abundant c-type cytochromes in current-producing biofilms (Schrott *et al*., 2011; Liu *et al*., 2011) provide a capacitance comparable to that of synthetic supercapacitors with low self-discharge rates (Malvankar *et al*., 2012).

The synthesis of c-type cytochromes constitutes a complex process in which iron must be incorporated to the protoporphyrin ring to conform each heme group that subsequently will be attached (Stevens *et al*., 2004). A recent study has explored the iron stimulon, reporting how twenty-four different c -type cytochromes were slightly downregulated with decreasing iron levels (Embree *et al*., 2014). Interestingly, strategies for promoting transposon insertions in the cytochrome c maturation genes ccmC and ccmF1 led to *Shewanella* oneidensis strains unable to perform any kind of anaerobic respiration including the donation of electrons to extracellular electron acceptors like iron, or manganese or intracellular molecules like fumarate or nitrate (Bouhenni *et al*., 2005).

Although iron is an abundant element in nature, its low solubility forces microorganisms to develop regulatory and transport mechanisms with the purpose of maintaining the iron homeostasis. In *G. sulfurreducens*, two systems belonging to the Feo family have been identified to facilitate the transport of Fe (II) (Cartron *et al*., 2006). All Feo genes as well as eleven genes encoding components for heavy metal efflux pumps were found to be most downregulated during iron-excess conditions (Embree *et al*., 2014).

The most important system for regulating the iron metabolism is the ferric-uptake regulator (Fur). Fur acts as a transcriptional repressor which in response of the iron availability, controls many genes related to iron acquisition as well as redox-stress resistance, central metabolism, and energy production in *Geobacter sulfurreducens* (O'Neil *et al.,* 2008; Embree *et al*., 2014). Along with Fur, an additional transcriptional regulator called IdeR has been recently suggested to have a role in iron homeostasis for *G. sulfurreducens* (Embree *et al.*, 2014).

In some bacteria, such as the *Rhizobium* genus (Johnston *et al*., 2007), the Fur-like iron response regulatory protein (Irr) regulates the heme biosynthetic pathway according to the iron availability. Under iron limitation conditions, Irr reduces the heme synthesis in order to avoid porphyrins accumulation that can be highly toxic (Qi *et al.,* 1999; Ishikawa *et al*., 2011). Although Irr has not yet been found in *Geobacter* species, it is likely that *G. sulfurreducens* has developed a system to limit the synthesis of cytochromes under iron-limiting conditions based on either Fur or IdeR regulators (Embree *et al*., 2014).

In the present study, we demonstrate that limiting the availability of iron to *G. sulfurreducens* resulted in a decreased cytochrome abundance

and a concomitant loss of its capacity for extracellular electron transfer while keeping the cell viability.

MATERIALS AND METHODS

Media and growth conditions

Geobacter sulfurreducens (strain DSM 12127; ATCC 51573) was grown at 30ºC in batch cultures. The freshwater medium contained the following mineral salts (per liter): 2.5 g of $NaHCO_3$, 0.25 of NH_4Cl, 0.06 g of $NaH_2PO_4H_2O$ and 0.1 g of KCl, 0.024 g of $C_6H_5FeO_7$ (ferric citrate), 10 mL of a vitamins mix and 10 mL of a trace mineral mix (for details see Lovley *et al.,* 1988). For batch cultures, acetate (20 mM) was supplied as the sole carbon source and electron donor, and fumarate (40 mM) as electron acceptor. Anaerobic conditions were achieved by flushing the culture media with N_2-CO_2 (80:20) to remove oxygen and to keep the pH of the bicarbonate buffer at pH 7.

Geobacter sulfurreducens was also cultured in a chemostat under acetate-limiting conditions (Esteve-Núñez *et al.,* 2005). Fe(II) for supplementing iron in the culture media was supplied in form of $(NH_4)_2Fe(SO_4)_2 \cdot 6H2O$.

The bacteria were grown according to the following two-step protocol to obtain heme$^-$ cells with a reduced content of cytochromes. First, the cells were cultured in a freshwater medium prepared in the absence of Fe sources. Next, this Fe-free culture was used to inoculate (10%) of a Fe-free medium containing 30 µM of the iron chelator 2,2'-bipyridine. The resulting cells are named heme$^-$ cells in this work.

Analytical methods

The cells were disrupted by boiling the samples for 10 minutes in a lysis Tris-HCl buffer (100 mM, pH 6.8, 8% SDS) before the cytochrome analysis. To confirm the absence of cytochromes, the cellular proteins were

analyzed by electrophoresis on 10 % acrylamide tris-tricine SDS gels. The reagent 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) was used for staining the heme-bands following published protocols (Francis *et al*., 1984). The gel was stained with Coomassie Brillant Blue (Bio Rad) for the determination of the total protein content. The Fe (III) reduction was measured employing a ferrozine assay, which was described previously (Lovley and Phillips, 1986).Organic acids were determined using an HP series 1100 high-pressure liquid chromatograph coupled with a UV detector (210 nm). The compounds were separated using a Supelco C-610H column with 0.1 % H_3PO_4 as mobile phase with a flow rate of 0.5 mL min^{-1}.

Electrochemical measurements

Cyclic voltammetry was performed in a 3-electrodes configuration DRP-110 (Dropsens) using a potentiostat (Nanoelectra) connected to a PC and controlled by specific software. The potential was scanned between -0.8 and 0.8 V, with a scan starting in the positive direction at 0.0 V. The scan rate was fixed at 0.005 V s^{-1}. All potentials are reported versus a saturated Ag/AgCl electrode. All experiments were performed under anaerobic conditions (anoxic chamber Coy) guaranteeing the presence of a negligible amount of oxygen. An equal number of cells were adjusted at OD_{600} = 2 for the heme-free solution and the standard reference culture of *G. sulfurreducens*. Subsequently, both samples were resuspended in 30 mM phosphate buffer solution (pH = 6.8) containing 30 mM KCl and 20 mM acetate as electron donor.

RAMAN

In-situ NERS measurements were performed with a LabRAM HR800 confocal Raman microscope (Horiba Jobin Yvon). The excitation wavelength was 532 nm from a Nd:YAG laser. The power of the laser on the sample was 1 mW, and a 50 times magnification long-distance (8 mm) objective

with a numerical aperture of 0.1 was used to focus the laser onto the sample for collecting the signal in a backscattering geometry. Citrate-stabilized Ag nanoparticles with an average diameter of 80 nm were synthesized by reducing 200 mL of a boiling 0.018 wt% $AgNO_3$ solution with 4 mL of 1 wt% sodium citrate solution. A color change from yellow to pale green took place in about 20 sec. The solution was kept boiling for 1 h and was then stirred until cooling down to room temperature (Lee and Meisei.,1982). 1.5 mL of the Ag nanoparticle solutions was subsequently cleaned and concentrated by a factor of 100 via centrifugation (5500 rpm for 15 min, 3 times), which led to 10-20 µl of a dark green sediment. This sediment was mixed with concentrated *G. sulfurreducens*, casted and dried on a flat Ag electrode in an Ar atmosphere and subsequently transferred into a home-made Raman cell to perform the NERS experiments.

SEM

The cells were harvested in the late exponential growth phase by centrifugation for 10 minutes at 9000 g. The cell pellets were harvested and fixed by immersion for one hour at room temperature in Cacodylate buffer (0.2 M, pH 7.2) containing 5 % glutaraldehyde. The samples were rinsed two times in 0.2 M Cacodylate buffer, pH 7.2 for 10 minutes, and subsequently dehydrated by a graded ethanol series (25, 50, 70, 90, 100 and 100 %; 10 minutes each stage). Finally, the samples were rinsed two times in acetone for 10 minutes and immersed in anhydrous acetone at 4 ºC overnight. Finally, dehydrated cells were dried in CO_2 at the critical point and processed using a scanning electron microscope DSM-950 (Zeiss).

ICP-MS

Aliquots (20 mL) of cultures at equal cell density were harvested by centrifugation (8000 g, 10 min). The cells were then washed with 20 mL of phosphate buffer (20 mM, pH = 7). The washed pellets were treated with 5

mL of 1 M NaOH for 3 hours to dissolve the intracellular content. The total iron content was analyzed by inductively coupled plasma mass spectrometry (ICP-MS). The samples were ionized and quantified using the method Icp/ms 7700x (Agilent). The iron concentrations were normalized with a control culture of *Pseudomonas putida* strain ATCC 12633 treated under the same conditions.

RESULTS AND DISCUSSION

High iron requirement for the optimal growth of *Geobacter sulfurreducens*.

The standard freshwater medium for *Geobacter* growth contains ca. 2 µM Fe as part of its trace element cocktail (Lovley and Phillips, 1986). This concentration has been reported to be sufficient to satisfy the Fe bacterial requirement (Fukushima *et al*., 2012). However, it might be expected that the synthesis of the abundant cytochromes in *Geobacter* might impose a need for additional iron. In order to evaluate this, *G. sulfurreducens* was grown in chemostats under continuous culture conditions. Iron was supplied in the ferrous form because the presence of ferric iron results in transcriptional repression of the fumarate respiration (Esteve-Núñez *et al*., 2004).

With 2 µM ferrous iron, typically used in *G. sulfurreducens* medium, the steady state acetate concentration and the biomass concentrations were adjusted to 1.5 mM and 42.6 mgprot/l, respectively. Increasing the ferrous iron concentration to 150 µM led to a reduction of the residual concentration of acetate by a factor of 10 (150 µM). The biomass concentration increased to 51.7 mgprot/l culture. Adding a pulse of ferrous iron had a similar impact **(Figure 1)**.

These results suggest that the iron availability limits the growth in typical *G. sulfurreducens* growth medium. The higher assimilation of acetate in the presence of iron could be explained by the lower Ks obtained in chemostats with Fe (III) rather than with fumarate as TEA, which leads to a higher affinity for acetate (Esteve-Nuñez *et al*., 2005) when the iron supply is abundant.

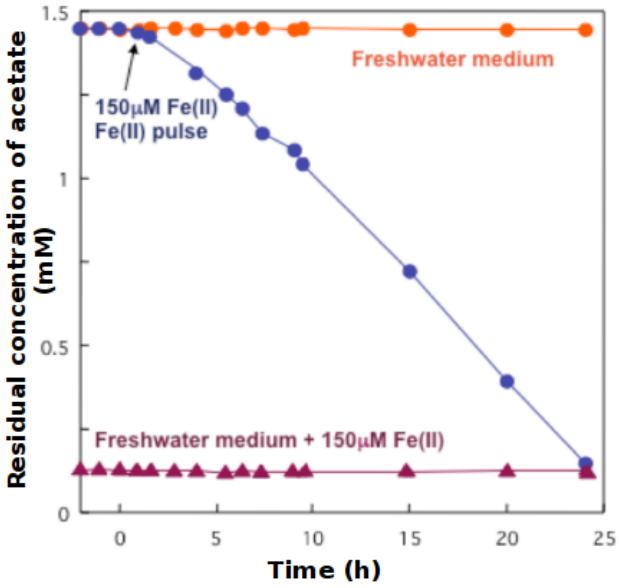

Figure 1: Residual acetate concentration under acetate-limiting conditions with a) a culture growing in a standard freshwater medium (orange line), b) growing in Fe (II)-supplemented freshwater medium (blue line), and c) growing in a standard freshwater medium, but spiked with Fe(II) as indicated by the arrow (purple line).

When cultured with 150 µM ferrous iron *G. sulfurreducens* cells contain 1.9 x 10^{-6} ng iron/cell. This is orders of magnitude higher than the average iron content of other bacteria such as *E. coli* (10^{-8} – 10^{-7} ng/cell, as derived by Andrews *et al.* 2003) **(Figure 2A).** One reason for the difference in iron content between *G. sulfurreducens* and *E. coli* is that the *G. sulfurreducens* genome encodes more than 100 *c*-type cytochromes, whereas only 5 genes encoding cytochromes are present in *E. coli* (Grove *et al.,* 1996*;* Reid *et al*., 2003). Many of the *G. sulfurreducens* cytochromes are constitutively expressed, regardless of the culture conditions (Ding *et al.,* 2006), including during growth in the absence of extracellular electron acceptor, e.g. under fumarate-reducing conditions (Holmes *et al.*, 2006; Esteve-Nuñez *et al.,* 2008). There is remarkably little conservation of c-type cytochromes genes across the six *Geobacter* species whose genomes have been sequenced.

This suggests that there has not been evolutionary pressure to maintain specific structures that might promote interactions of the cytochromes with the electron acceptors (Lovley, 2008). However, there has been evolutionary pressure for the *Geobacter* species to maintain an abundance of hemes. The energetic investment that *Geobacter* species make in the c-type cytochrome production could be very adaptive in providing an 'iron lung' that permits electron transfer in the temporary absence of Fe (III) oxides (Esteve-Núñez *et al.,* 2008; Lovley, 2008). The hypothesis of the cytochrome network acting as capacitor, where multi-heme could store charge (Esteve-Núñez *et al.,* 2008; Schroot *et al.,* 2011; Robuschi *et al.,* 2013), may be the key to understand this biosynthetic pathway. The electron-accepting capacity of the cytochromes network would be useful in the absence of an electron acceptor while conferring *Geobacter* the ability to satisfy maintenance energy requirements to develop motility and search for the nearest available electron acceptor (Childers *et al*., 2002).

Heme$^-$ *Geobacter* cells

The high requirement of *G. sulfurreducens* for iron suggests that it might be possible to limit the cytochrome production by limiting the iron availability. In order to further lower the iron availability, the iron-non-supplemented medium was amended with bypiridine, an iron chelator. The iron content of cells grown in this manner was 15-fold less (1.2 ng x 10^{-7}/ cell) than in cells grown in a typical iron-containing medium **(Figure 2A)**. Heme staining of whole-cell lysate proteins separated with SDS-PAGE demonstrated that the cytochrome content of cells grown in the low-iron medium was much lower than in cells grown in standard iron-containing medium **(Figure 1B-D)**. The cultures grown in the low-iron medium were much less red than cells grown in typical iron-containing medium **(Figure 2B).**

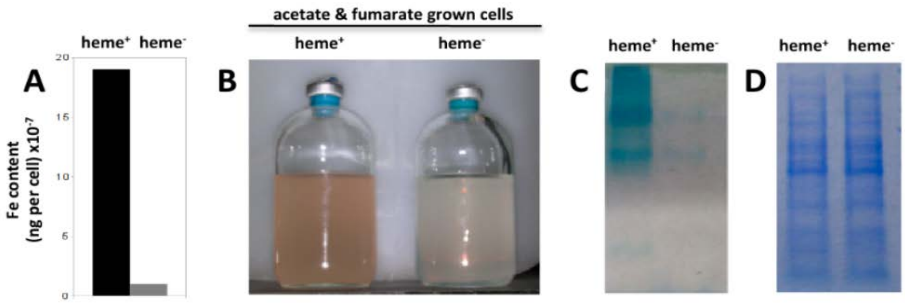

Figure 2: (A) Total cellular iron content analyzed by ICP-MS; heme$^+$ (black) and heme$^-$ (grey) cells of *G. sulfurreducens.* (B) Photo of the heme$^+$ and *the* heme$^-$ batch cultures of *Geobacter sulfurreducens.* The nalysis of the SDS-PAGE for the protein fraction of both the heme$^+$ and *the* heme$^-$ *Geobacter cells* followed by heme staining (C) and Coomassie staining (D).

Scanning electron microscopy revealed no difference in cell morphology between cells grown with limited iron concentration versus the standard culture medium, indicating that the cells do not suffer from any major morphological damage due to the absence of the cytochromes network **(Figure 3)**.

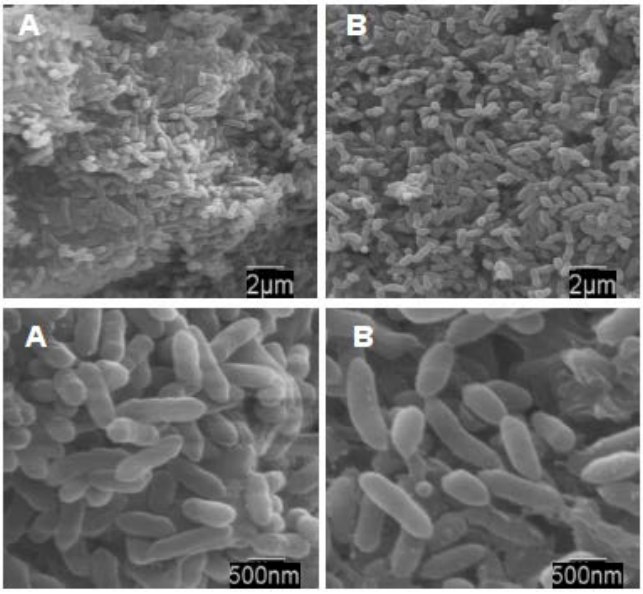

Figure 3: SEM images of heme$^+$ *G. sulfurreducens* (A) and heme$^-$ *G. sulfurreducens* (B).

The growth rate of *G. sulfurreducens* reduces from 0.050 h^{-1} to 0.035 h^{1} when iron limits the growth. In contrast, the **r**ates of fumarate reduction per cell in $heme^{+}$ (2.0 x 10^{-10} mmol/h cell) and $heme^{-}$ (1.9 x 10^{-10} mmol/h cell) growing cells were similar demonstrating that this key central metabolism reaction was not affected by the absence of cytochromes. This is consistent with the fact that fumarate is reduced at the inner membrane by a membrane-bound fumarate reductase/succinate dehydrogenase that does not involve cytochromes (Butler *et al.,* 2006). These results demonstrate that the low-iron culture conditions provide enough iron for cells to perform central metabolism reactions and assure viable cells

Cytochromes *c* were shown for first time to release electron *in vivo* on electrodes in spectroelectrochemical studies of the outermost membrane of *Geobacter* cells upon reduction on a gold electrode (Busalmen *et al.*, 2008a). Since then, a number of techniques involving infrared (Busalmen *et al*., 2010; Esteve-Núñez *et al*., 2011) and Raman (Millo *et al.,* 2011; Virdis *et al*., 2013; Kuzume *et al*., 2013; Robuschi, *et al*., 2013) were applied successfully to explore the surface of the bacteria.

In order to analyze the outermost membrane of $heme^{-}$ cells, we used in this study Nanoparticle Enhanced Raman Spectroscopy (NERS), a powerful technique that can detect and further provide structure information of hemes, which are vicinal to the coinage metal nanoparticle surface. For the NERS measurement in this work, Ag nanoparticles, which act as optical antennas to enhance the Raman response, were deposited onto a submonolayer of bacteria. The Raman scattering is enhanced by the plasmonic Ag nanoparticles by several orders of magnitude (10^{3}-10^{6}) (Hildebrandt *et al.,* 1986) allowing the selective probing of the vibrational signature of adsorbates adjacent to the nanoparticles. A SEM/EDX analysis

revealed that the Ag nanoparticles located vicinal to the bacterial cells are sufficiently close to enhance the Raman signals of the outermost domains. (Kuzume *et al.,* 2013).

Figure 4 displays a NER spectrum of *G. sulfurreducens* cells mixed with Ag nanoparticles in an Ar atmosphere. It shows a typical NER spectrum of the heme^{+} *G. sulfurreducens* cells, which correspond with key heme-related bands described for 532 nm laser excitation line (Biju *et al.,* 2007; Oellerich *et al.,* 2002; Eng *et al.,* 1996; Yeo *et al.,* 2008). No specific Raman signals from heme-related domains were found in heme^{-} sample prepared in this work, which represents a direct proof of the absence of heme-groups. The four signals between 1400 and 1600 cm^{-1} can be assigned to the amino acid adenine (Papadopoulou *et al.*, 2010), and to citrate-stabilized Ag NP (Kuzume *et al.,* 2013). These bands are located on the surface of the cells and are not related to heme-domains.

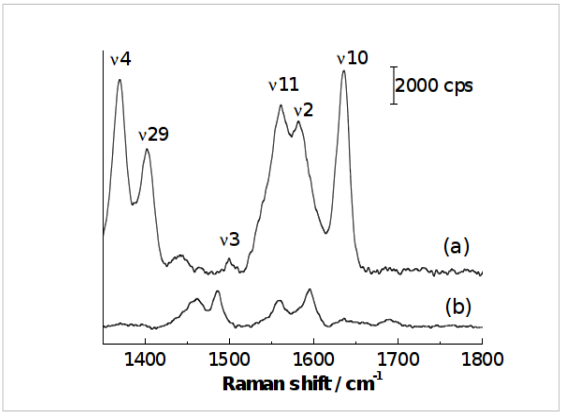

Figure 4: Nanoparticle enhanced Raman spectra of heme^{+} *G. sulfurreducens* (a) and heme^{-} *G. sulfurreducens* (b) mixed with Ag nanoparticles.

Extracelullar Electron Transfer (EET) assays

Gene deletion studies have implicated a number of *c*-type cytochromes in extracellular electron transfer, but even when multiple cytochrome genes are deleted in the same strain, some extracellular

electron transfer capability remains (Voordecker *et al*., 2010; Orellana *et al*., 2013). However, the number of cytochrome genes that can be deleted in a single strain is limited.

To determine if the lack of cytochromes associated with the growth in a low iron concentration medium could completely remove the capacity for extracellular electron transfer, cells growing with fumarate as electron acceptor were pulsed with 10 mM Fe (III) citrate. No Fe (III) was reduced **(Figure 5)**, and the rate of fumarate reduction to succinate (1.9 x 10^{-10} mmol/h per cell) was unaltered. In contrast, when Fe (III) was added to cells growing in a medium with the standard iron content, Fe (III) was rapidly reduced (5 x 10^{-10} mmol/h per cell) and the fumarate reduction was inhibited.

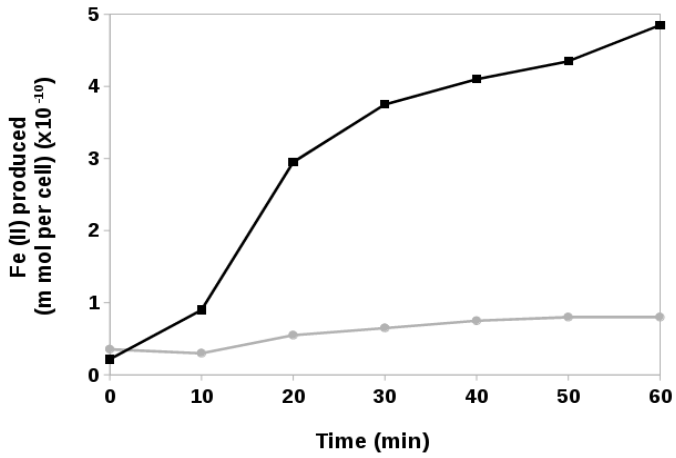

Figure 5: Fe (III) reduction after addition of 10 mM ferric citrate to heme^{+} *G. sulfurreducens* (black line) and to heme^{-} *G. sulfurreducens* (grey line).

Another EET process, where cytochromes have been reported to participate, is the electrode reduction in microbial electrochemical

technologies. By using electrochemical approaches, such as cyclic voltammetry (Busalmen *et al*., 2008), the bioelectrochemical response for the extracellular electron transport was monitored. *G. sulfurreducens* was resuspended in phosphate buffer in the presence of an electron donor but in absence of a soluble electron acceptor.

Consequently, when *G. sulfurreducens* cells were incubated in a three-electrode cell, just the electrode could act as TEA. A typical voltammogram shows two redox peaks with current maxima at 0.2 and -0.2 V vs. Ag/AgCl (Busalment *et al.*, 2008; Richter *et al*., 2008; Fricke *et al*., 2008), which represent the corresponding oxidation and reduction processes, respectively. In contrast to the wild type, *G. sulfurreducens* heme$^-$ cells did not display any redox peak demonstrating that the presence of cytochromes is required for performing a sufficient redox communications with an exocellular electron acceptor, such as a polarized electrode **(Figure 6)**.

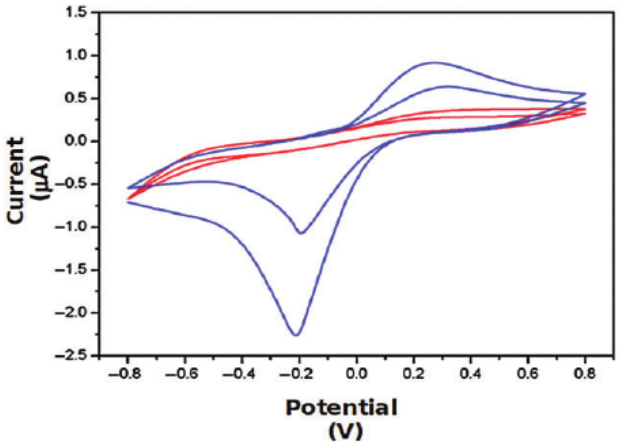

Figure 6: Cyclic voltammograms of *G. sulfurreducens* cells deposited on a carbon electrode. Blue line: heme$^+$ *G. sulfurreducens.* Red line: heme$^-$ *G.sulfurreducens.* The potential refers to an Ag/AgCl reference electrod.

The absence of additional current peaks confirms that the

cytochrome-related redox reactions comprise the major active compound in *Geobacter* redox-activity on polarized electrodes in BES. This conclusion is confirmed by recent findings of several groups (Busalmen *et al*., 2008a; Millo D *et al.,* 2011; Kuzume *et al*., 2013).

CONCLUSIONS

These results demonstrate dramatic impact of available iron on the growth and activity of *G. sulfurreducens*. Adjusting laboratory media to provide a higher iron concentration than that *Geobacte*r species experience in a natural environment may promote important applications, such as METs that rely on optimized extracellular electron exchange.

Alternatively, making iron less available yielded cells unable to produce heme groups and studies with these cells confirmed the key role of the vast cytochrome network in EET. Our bioelectrochemical results confirm that cytochromes are essential for direct electron transfer to electrodes. Although we have focused on getting heme$^-$ cells, our methodology allows controlling the level of cytochrome production by varying the doses of the chelator. In consequence, we could generate *Geobacter* cells with different levels of heme content in contrast with previous strategies performed in bacteria for erasing all c-type cytochromes through transposon insertions that led to unviable cells under anaerobic conditions (Bouhenni *et al.*, 2005). Furthermore, we believe that heme$^-$ cells reported in this work will also be relevant for other researchers targeting investigations on the physiology of *Geobacter* under EET-free background conditions.

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Chapter 3

Screen-printed electrodes : new tools for developing microbialelectrochemistry at microscale level

Contents

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Screen-printed electrodes: new tools for developing microbial electrochemistry at microscale level

ABSTRACT

Microbial electrochemical technologies (METs) have a number of potential technological applications. In this work, we report the use of screen-printed electrodes (SPEs) as a tool to analyze the microbial electroactivity by using *Geobacter sulfurreducens* as a model microorganism. We took advantage of the small volume required for the assays (75 µL) and the disposable nature of the manufactured strips to explore short-term responses of microbial extracellular electron transfer to conductive materials under different scenarios. The system proved to be robust for identifying the bioelectrochemical response, while avoiding complex electrochemical setups, not available in standard biotechnology laboratories. We successfully validated the system for characterizing the response of *G. sulfurreducens* in different physiological states (exponential phase, stationary phase, and steady state under continuous culture conditions) revealing different electron transfer responses. Moreover, a combination of SPE and *G. sulfurreducens* resulted to be a promising biosensor for quantifying the levels of acetate, as well as for performing studies in real wastewater. In addition, the potential of the technology for identifying electroactive consortia was tested, as an example, with a mixed population with nitrate-reducing capacity. We therefore present SPEs as a novel low-cost platform for assessing microbial electrochemical activity at the microscale level.

INTRODUCTION

Since the discovery in the last decade of electrode-respiring microbes, such as *Geobacter sulfurreducens* and some species of *Shewanella* (Bond and Lovley, 2003; Kim *et al*, 2002) there has been an increasing interest in their potential applications for designing several types of microbial electrochemical technologies (METs), like electricity production from wastewater in microbial fuel cells (MFC), bioremediation in microbial electroremediating cells (MERCs) (Rodrigo *et al*, 2014), electrosynthesis, or biosensing (Schröder *et al*, 2015).

Many attempts to optimize METs have been focused on reactor design and operational aspects (Rozendal *et al*, 2008; Clauwaert *et al*, 2008). Different electrochemical approaches, such as chronoamperometric assays, cyclic voltammetry (CV), sometimes coupled to spectroscopic techniques as infrared (IR) or Raman (Busalmen *et al*, 2008a; Kuzume *et al*, 2013, 2014), have been applied for the study of this fascinating phenomenon. All of these have helped to investigate and analyze electroactive microorganisms and learn valuable information about how microbial physiology at different hierarchical levels affects the capability of electron transfer to solid electrodes at the whole biofilm, single cell and sub-cell (molecular) level (Schrott *et al*, 2011; Carmona-Martínez *et al*, 2013; Busalmen *et al*, 2008b).

Nevertheless, much remains unknown regarding the electrochemical properties of electroactive bacteria and their interaction with electrodes. This is partially due to difficulties in monitoring METs or the requirement of rather complex experimental setups for growing bacteria in electrochemical

environments with limited access to microbial tools (Harnisch and Rabaey, 2012; Schröder, 2012).

Although considerable efforts have been made to scale up METs for industrial applications, there is also a great interest in miniaturizing bioelectrochemical devices for research purposes where large current production is not the main goal. Those devices are typically designed to work under fixed potential conditions with a three-electrode setup. Some examples include flow cells, microfluidic devices or micro-sized microbial fuel cell (Davila *et al*, 2011; Li *et al*, 2011, Fraiwan *et al*, 2014)

Screen-printing is an established technique to fabricate electrochemical devices. A standard screen-printed electrode (SPE) comprises a variable number of electrodes and all the compounds necessary for completed the electric circuit placed over a supporting material (Hayat and Marty, 2014). Moreover, for carbon SPEs, the inks employed are based on graphite and have very similar properties to conventional electrodes, such as low background signal and a wide range of working potentials. With inherent advantages including miniaturization, versatility, low cost and the possibility of mass production. SPEs have been proved to be disposable, yet highly accurate and rapid devices (Taleat *et al*, 2014), with a promising future as a tool for bioelectrochemical systems. SPE can be also combined with functionalization (through electrochemical techniques) of biomolecules (Gómez-Mingot *et al*, 2011) or bacteria (Chang *et al*, 2012). Moreover, they require small volumes of the analyte (microlitres), they are suitable for off-the-bench assays and they are marketed by several companies. All these benefits make SPEs a natural choice that can serve as biosensors, as well as for a fast screening of the physiology and/or metabolism of electroactive microorganisms, among other potential applications, in kinds of assays that

performed with conventional electrochemical devices involve more time and complexity (Asturias-Arribas *et al*, 2014, Esteve-Núñez *et al*, 2011).

Considering that *G. sulfurreducens* is a well-accepted model system in METs, it could be the ideal candidate for exploring its activity and its potential applications on SPEs, for which, to our knowledge, there is no precedent using *Geobacter* species. There is indeed one interesting study where SPEs are tested with *Shewanella* sp. (Sudhakara Prasad *et al*, 2009), but *Shewanella* species are reported to release electron shuttles as the primary mechanism to make extracellular electrons (Marsili *et al*, 2008; Kotloski *et al*, 2013), in contrast to *Geobacter* species that perform direct extracellular electron transfer (DEET) (Lovley 2011).

Herein, we report the use of SPEs as a tool to analyze the electrochemical behavior of *Geobacter* species and mixed cultures, under several physiological conditions. Our analysis was performed with planktonic cells in 75 µL drop-assays, at short term, using working electrodes made from SPEs either as electron acceptor and electron donor. SPEs provide us with the possibility to develop quick drop-assays and rapidly assess the electrochemical properties of a bacterial culture, avoiding complicated experimental setups. We, therefore, present SPEs as a novel, low-cost platform for studying microbial electrochemical activity at the microscale level.

MATERIALS AND METHODS

Bacterial Culture

Geobacter sulfurreducens (strain DSM 12127; ATCC 51573) was grown at 30 °C in freshwater medium containing the following mineral salts (per liter): 2.5 g of $NaHCO_3$, 0.25 g of NH_4Cl, 0.06 g of $NaH_2PO_4 \cdot H_2O$ and 0.1 g of KCl, 0.024 g of $C_6H_5FeO_7$ (ferric citrate), 10 mL of a vitamins mix and 10 mL of a trace mineral mix (Lovley and Phillips, 1988). Anaerobic conditions were achieved by flushing the culture media with N_2:CO_2 (80:20) to remove oxygen and to keep the pH of the bicarbonate buffer at pH = 7. For batch cultures, acetate (20 mM) was supplied as the sole carbon source and electron donor, and fumarate (40 mM) as electron acceptor. Mid-log cells and stationary cells were harvested 40 h and 72 h, respectively, after the inoculation procedure.

G. sulfurreducens was also cultured in continuous mode using a chemostat. This growth method allowed to achieve fumarate-limiting conditions, a situation that led to a better EET, as previously described (Esteve-Núñez *et al*, 2005; (Esteve-Núñez *et al*, 2011). The nitrate reducing microbial consortium was directly harvested from an anaerobic reactor operating under nitrate reducing conditions.

Electrochemical Analysis

Bacterial cultures were harvested by centrifugation at 6000 rpm during 10 min. Subsequently, bacterial pellet were resuspended (OD_{600} = 2) in filtered wastewater or 30 mM phosphate buffer solution (pH = 6.8) containing 30 mM KCl. Then, 75 µL of a *G. sulfurreducens* cell suspension was added to the SPE and, immediately, the electrochemical assays were performed without any preconditioning period. All electrochemical assays

were performed in a screen-printed three electrode configuration using electrodes made of carbon (DRP-110, Dropsens, Asturias, Spain) or graphene (DRP-110GPH, Dropsens).

These electrodes consist of a working electrode of carbon ink (surface = 0.12 cm^2), a carbon counter electrode and a silver reference electrode; assembled on a ceramic platform (3.4 cm × 1.0 cm × 0.05 cm), connected to a potentiostat and controlled by specific software (Nanoelectra, Madrid, Spain). All potentials were reported versus an Ag/AgCl electrode. For chronoamperommetric assays the current was registered every 0.5 s and two electrode potentials were explored: current producing assays by supplying acetate as electron donor to the cell suspension and fixing the potential at 0.2 V; and current consuming assays, by supplying nitrate as electron acceptor and fixing the potential at −0.5 V. For CV, the potential window was scanned between −0.8 V and 0.8 V at 0.005 V s^{-1}. All experiments were performed under anaerobic conditions by using an anoxic chamber (Coy, Grass Lake, MI, USA) or, alternatively, a sealed cap-tube. The sealed cap-tube was assembled by gluing the upper section of a cut-tube (1.5 mL, Eppendorf, Hamburg, Germany) to the ceramic surface of the SPE. Anoxic conditions inside the tube were achieved by flushing the headspace with N_2 **(Figure 1)**.

Perfomance of the Acetate Biosensor Assay

Early stationary bacterial cultures were harvested by centrifugation at 6000 rpm during 10 min. Then, samples were resuspended (OD_{600} = 5) in 30 mM phosphate buffer solution (pH = 6.8) containing 30 mM KCl. Subsequently, a drop of 75 µL of the cell suspension was placed on the electrode and cells were fixed by cycling the working electrode between 0.1 V and −0.1 V at 100 mV s^{-1} during 100 scans (Gómez-Mingot, 2011). Then,

the electrode was washed with phosphate buffer in order to remove the unattached cells. Finally, a 75 µL drop of acetate solutions (1–20 mM) in phosphate buffer were added, and the electrode was polarized at 0.2 V during 15 min. For each acetate concentration a new SPE was used.

Scanning Electron Microscopy (SEM)

The microbial attachment of the electrode surface was observed by SEM. Microbial SPEs were fixed by immersion for one hour at room temperature in cacodylate buffer (0.2 M, pH = 7.2) containing 5% glutaraldehyde. The samples were rinsed two times in 0.2 M cacodylate buffer, pH = 7.2 for 10 min, and subsequently dehydrated by a graded ethanol series (25%, 50%, 70%, 90%, 100% and 100%; 10 min each stage). Then, the samples were rinsed two times in acetone for 10 min and immersed in anhydrous acetone at 4 °C overnight. Finally, dehydrated cells were dried in CO_2 at the critical point and processed using a scanning electron microscope DSM-950 (Zeiss, Oberkochen, Germany).

RESULTS AND DISCUSSION

Testing Electroactivity in Geobacter

It is well known that the central metabolism of Geobacter can fully oxidized acetate to CO_2 by coupling it with the extracellular electron transfer to electrodes of different nature (Bond and Lovley, 2003; Kuzume *et al*, 2014; Maestro *et al*, 2014). The purpose of this work was to test if SPEs could be suitable tools for assaying such a reaction.

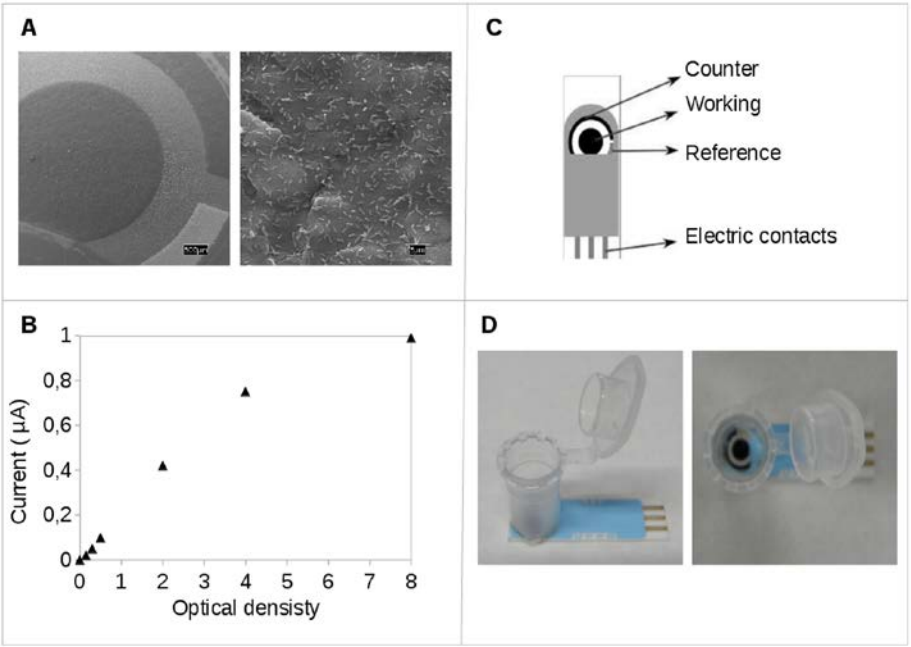

Figure 1. (A) Scanning electron microscopy (SEM) image of *G. sulfurreducens* attached to a carbon screen-printed electrode (SPE); (B) steady current production of *G. sufurreducens* at different cell density; (C) scheme of a SPE; and (D) detail of a lid-tube sealed to the SPE ceramic surface.

With the aim of standardize our methodology, a preliminary study was performed by increasing the cell concentration of *G. sulfurreducens* and measuring the current production using carbon SPEs. Due to electrode sensitivity, we were able to detect changes in the current values by varying

the amount of cells in contact with the electrode. We concluded that a cell suspension of bacteria with a value of 2 units of optical density (1.6×10^9 cells/mL) can generate a current value in the mid-linear phase of the response **(Figure 1B)**.

The electrogenic response of *G. sulfurreducens* under electron acceptor limitation has been previously studied by electrochemical and spectroelectrochemical techniques (Busalmen *et al*, 2008a,b). Bacteria were grown in continuous culture with acetate as electron donor and fumarate as sole electron acceptor. Attenuated Total Reflection-Surface Enhanced Infrared Reflection Absorption Spectroscopy (ATR-SEIRAS) and CV, in a classical three-electrode chamber setup, showed that terminal electron acceptor (TEA) limited cells, exhibited a constitutive capacity for extracellular electron transfer (EET) (Esteve-Núñez *et al*, 2011). The capability of cells in fumarate-limiting conditions to respire electrodes was analyzed on disposable miniaturized carbon SPEs, in order to compare the results to those previously obtained with a classical electrochemical cell configuration.

The fumarate-limited cells response showed that current production from metabolic acetate oxidation is predominant from the very beginning, displaying an electron transfer rate of 32.7 pmol electrons/s per cm^2, which indicates the constitutive capacity for electron transfer to the electrode. In contrast, mid-log cells exhibit a lag phase, lower current production and ca. 2-fold lower electron transfer rate (15.6 pmol electrons/s per cm^2), when they were tested with a SPE polarized at 0.2 V **(Figure 2A)**. The electrochemical properties of both physiologies were also then tested by CV in SPEs. Once again, fumarate-limited cells presented a well-defined redox process showing the two characteristic redox peaks **(Figure 2B)**, while mid-log cells exhibit a remarkably lower peak current under identical analysis

conditions. This result is consistent with previous data that suggest a different bacterial electroactive response for each physiological condition (Esteve-Núñez *et al*, 2011).

Moreover, it is possible to use these SPEs for testing the electrochemical response corresponding to the growth phases of *G. sulfurreducens*, since it will be a key factor in the electrochemical performance. The availability of TEAs in microorganisms entails physiological changes in the cell, especially in those adapted to oligotrophic environments, such as *G. sulfurreducens*. As previous studies showed, the electron acceptor limitation triggers a metabolic adaptation that includes the overexpression of redox-active proteins, such c-type cytochromes (Esteve-Núñez *et al*, 2011).

Following this strategy, electrons generated from acetate metabolism can be stored in a c-type cytochrome network which act as capacitor-like element (Esteve-Núñez *et al*, 2008), that would discharge them as soon as a TEA is available. Interestingly, the current generated and the electron transfer rate (30.2 pmol electrons/s per cm^2) by cells in stationary phase, was similar to the one found on fumarate-limited cells, suggesting that cells in stationary phase are also well adapted to EET **(Figure 2).**

This behavior could be justified with the proteomic profile, which reveals the increase of several proteins at stationary phase, including cytochromes an oxidoreductase involved in electron transport (Bansal *et al*, 2013; Ding *et al*, 2006. In contrast, the significant lower electron transfer rate in mid-log phase cells, suggests that they still have not synthesized all the redox players for performing an efficient electron transfer to the electrode, so analyzing the electroactivity of a bacterial culture may help to predict the growth phase of the cells.

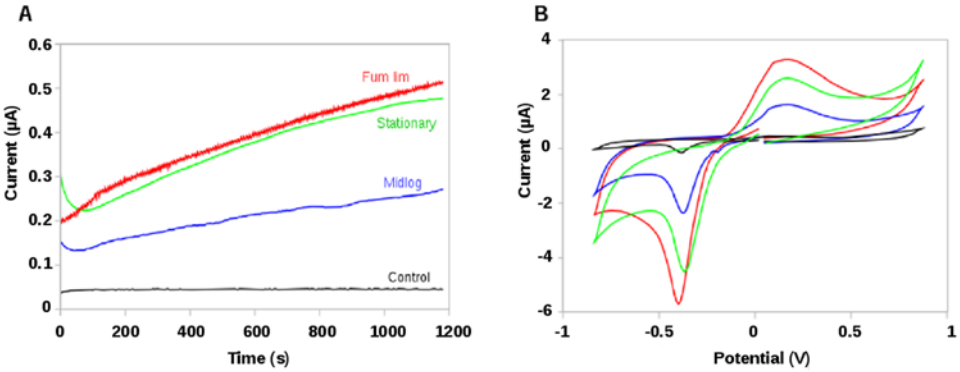

Figure 2. (A) Current production by *G. sulfurreducens* cells under different growth conditions: chemostat cells under fumarate-limiting conditions (red), early stationary cells (green), midlog cells (blue) and buffer control (black), polarized a 0.2 V; (B) Cyclic voltammetry (CV) of *G. sulfurreducens* cells under different growth conditions: chemostat cells under fumarate-limiting conditions (red), early stationary cells (green), mid-log cells (blue) and buffer control (black).

It is interesting to mention that cells are still able to produce current when adsorbed onto a polarized electrode, even if acetate is not present in the electrolyte solution **(Figure 3A)**. This can be explained by the presence of some intracellular acetate that remains as a source of electrons. Using this internal organic fuel, cells are able to produce current for ca. 3 min, after then intracellular acetate is not enough to produce a stable current value, and the current decreased to zero, presumably because the electron donor was fully consumed. From the gathered data, we calculated using the Faraday equation that the intracellular acetate content is about 1.51×10^{-6} pmol per cell. Regarding the acetate oxidation rate, it is worth noting that the remaining stored acetate is consumed ca. 4-fold slower (1×10^{-9} pmol/s per cell) than when it is at high concentration as 20 mM in the extracellular medium (4×10^{-9} pmols/s per cell).

During the last years, *G. sulfurreducens's* electroactivity was tested on several materials serving as TEAs, such as graphite, gold, silver and

platinum (Kuzume *et al*, 2014; Maestro *et al*, 2014). Among all the conductive materials that can be tested nowadays, graphene is for sure one of the most attractive ones. Its single atomic layer of graphite allows it to have higher electric conductivity than conventional carbon materials (Castro Neto *et al*, 2009). Despite the excellent features of graphene, there are few examples of its use in METs (Guo *et all*, 2014; Kumar *et all*, 2014).

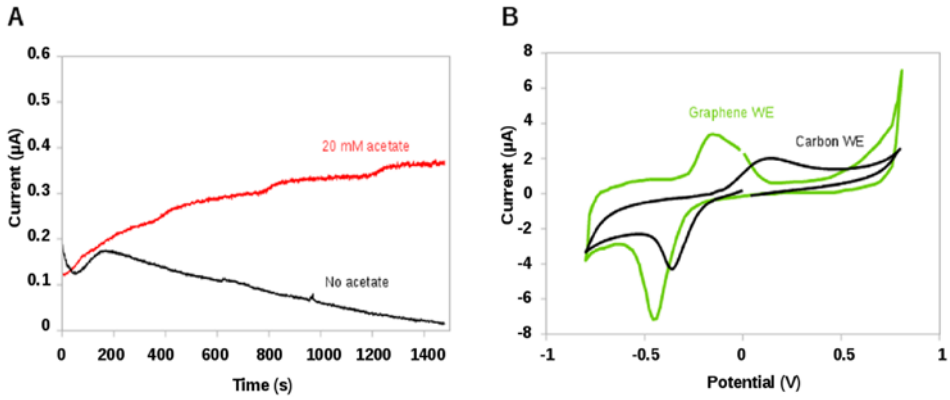

Figure 3. (A) Current production (at 0.2 V vs. Ag/AgCl) by *G. sulfurreducens* under acetate-excess conditions (red), and under acetate-limiting conditions just using intracellular acetate content (black); (B) CV of *G. sulfurreducens* on a graphene SPE (green) and a carbon SPE (black).

The electroactivity in stationary cells of *G.sulfurreducens* using graphene SPEs was also tested in this work, which resulted in a considerably increase of the signal intensity in contrast to carbon SPEs **(Figure 3B).** Moreover the redox signal appeared now at more negative values of potential (ca. 0 V) in contrast with the typical value of ca. 0.2 V detected on standard graphite electrodes (Busalmen *et al*, 2008a; Estevez-Canales, 2015). The shift in the oxidation potential might be related with graphene's better electric conductivity, which is several orders of magnitude higher than that of carbon (Castro Neto *et al*, 2009). In addition, it is well

known that the working electrode material can influence the microbial response at METs. Even using the same material, a mere change in the active surface could lead to different electroactive microorganism behavior (Maestro *et al*, 2014) so the improved response could be a combination of both electric conductivity and a different bacteria-electrode interaction. Graphene is by far the best up-and-coming material which is being implemented in several fields, so further studies will be required to take advantage of its unique conductive properties.

Practical Applications

a) Screening of metabolic features from a microbial consortium: SPEs acting as electron donor

Another desirable property for a microorganism employed in METs, is the ability of using an electrode as electron donor source, conforming a biocathode. In comparison with an abiotic cathode, promoting the redox reaction at the cathode by microorganisms, increases the operational sustainability as well as reduces the cost of construction and operation of METs (Rabaey and Keller, 2008). This trait could be exploited with the aim of screening predominant metabolic pathways from a mixed culture, by monitoring the electroactive response in the presence of several oxidizing substrates.

As a proof of concept, a consortium well adapted to nitrate reduction obtained from an anaerobic reactor was tested. In contrast, a culture of *G. sulfurreducens* was used as a negative control for electroactive bacteria unable to reduce nitrate. The current consumption of both types of cells was evaluated in the presence of the selected electron acceptor nitrate (2 mM) after polarizing the SPE-working electrode at −0.5 V. After a brief conditioning period of 5 min, we observed that the current consumption was

20-fold higher in the microbial consortium assay in comparison with the pure culture of *G. sulfurreducens.* **(Figure 4)**.

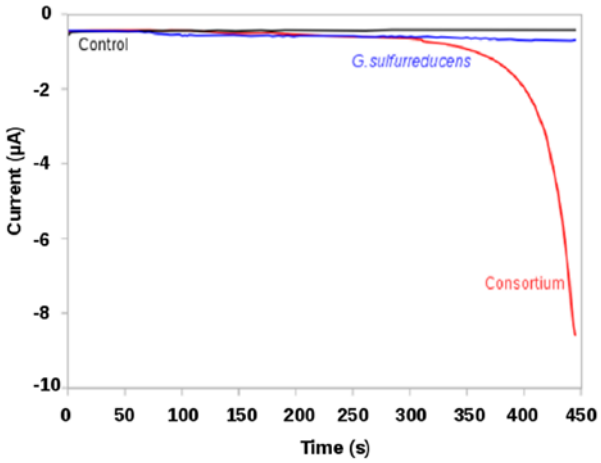

Figure 4. Electricity consumption by a microbial consortium (red) and *G. sulfurreducens* (blue) and buffer control (black) using nitrate (2 mM) as TEA, polarized at −0.5 V.

The results are not unexpected, considering that the consortium was fully adapted to nitrate-reducing conditions, while *G. sulfurreducens* is very well known for its ability to interchange electrons with electrodes, but not with nitrate as sole electron acceptor (Caccavo *et al*, 1994).

Aside from this example, the use of SPEs appears as an easy and quick method to identify potential microbial cultures showing activity in biocathodes. The microbial metabolism in biocathodes, indeed provides an excellent opportunity to find candidates for produce useful products performing bioelectrosynthesis (Nevin *et al*, 2010) or for removing unwanted compounds from water like sulfate (Coma *et al*, 2013), nitrate (Clauwaert *et al*, 2007), uranium (Gregory and Lovley, 2005, chloroaromatic compounds (Alulenta *et al*, 2005), which could be exploited in METs.

b) Bioelectrochemical Sensing of Acetate

Among the METs, there are a few examples of whole-cell biosensors as the electric signal transducer for generating and quantifying soluble organic matter (Tront *et al*, 2008; Li *et al*, 2011), or for detecting the presence of a toxic compound like formaldehyde (Davila *et al*, 2011). In contrast to those studies, we combine the use of SPEs with *G. sulfurreducens* to follow a CV-based strategy and construct a prototype microbial electrochemical sensor for quantifying acetate. Our assays revealed that the microbial current displayed by the CV at a potential of 0 V seems to be proportional to the concentration of acetate **(Figure 5)**. The fact of using voltammograms for extrapolating current produced at 0 V, allows us to take advantage of both electrochemical methods. Amperometry makes it possible to detect down to picoamperes, while performing CV offers a low noise signal on top of verifying the proper electrode-bacteria interaction (Su *et al*, 2010). Indeed, the current response to acetate concentration at this conditions, exhibited a coefficient of determination (R^2) of 0.98 with a detection limit between 20 mM and 1 mM.

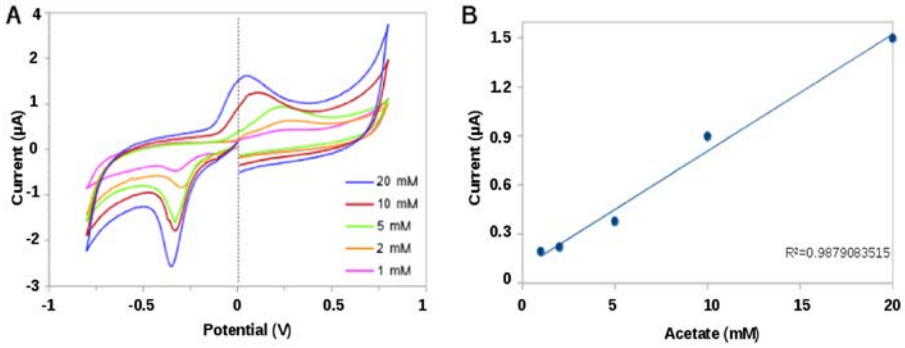

Figure 5. (A) Voltammograms corresponding to SPE-monolayer of *G. sulfurreducens* exposed to solutions with different acetate concentration; (B) Calibration curve for acetate concentration between 1 mM and 20 mM, calculated from current display at 0 V according to the CV.

Similar results were previously obtained by other authors, although they used more sophisticated microfluidic devices (Li *et al*, 2011). Although further investigation for optimization of the sensor will be required, nevertheless, to our knowledge, this is the first approach to an amperometric acetate biosensor using a pure culture of *G. sulfurreducens* in combination with a SPE.

c) Effects of a Background of Real Urban Wastewater

In contrast with the fascinating profile of electron acceptors (Fe-oxides, uranium, humic acids, graphite or gold electrode) that *G. sulfurreducens* can use (Lovely *et al*, 2011), its ability for oxidizing organic electron donors is very limited. Actually just acetate (Bond *et al*, 2003) and lactate (Speers and Reguera, 2012) can be converted into electrical current by this strain. This apparent limitation can nevertheless be a positive feature because just acetate will be converted into electrical current regardless of the complexity of mixture. This is especially relevant if we consider that acetate is the end-product of the acetogenic phase in the anaerobic wastewater treatments, and its presence is directly related with the formation of biogas in anaerobic digestion (Henze, 2008).

Therefore, as a first approach for future applications, we tested the current production of our microbial-SPE-based in a background sample of real urban wastewater, using acetate as electron donor. According to the electrical current production, there was no substance in the sample of non-buffered wastewater able to significantly compete with the electrode as TEA. Moreover, current production was doubled when the acetate concentration was artificially increased from 5 mM to 10 mM demonstrating

that the system was robust enough for performing assays in any kind of medium **(Figure 6)**.

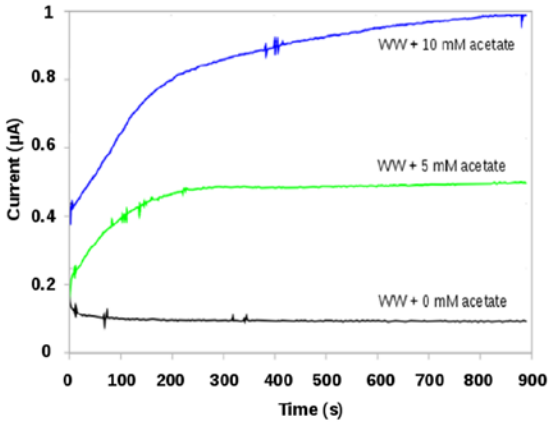

Figure 6. Current production of *G. sulfurreducens* from a real urban wastewater supplemented with 0 (black), 5 (green) and 10 mM acetate (blue). Working electrode was polarized a 0 V (vs. Ag/AgCl).

CONCLUSIONS

The results presented in this work demonstrate that screen-printed disposable electrodes can be used as a novel platform to assess within minutes the electron transfer capacities of electroactive microorganisms in quick drop assays that only require microlitres of culture samples. SPEs have shown to be sensitive to different physiological conditions in *Geobacter sulfurreducens* and excellent working electrode materials. Furthermore, SPEs could be exploited for fast screening methods to select tailor made biocathodes. SPEs were designed for electroanalytical applications, and we have demonstrated that they are ready to accept whole-living cells and explored biosensor development for uses such as acetate detection in wastewater. Finally, we understand this work as a proof of concept for exploring new scenarios to investigate microbial electrochemistry without setting-up large electrochemical devices.

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Chapter 4

Ready-to-use artificial bioelectrodes: a promising strategy for Microbial Electrochemical Technologies

Contents

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Ready-to-use artificial bioelectrodes: a promising strategy for Microbial Electrochemical Technologies

ABSTRACT

Microbial Electrochemical Technologies (METs) deal with the interactions between microorganisms and electronic devices, enabling to transform chemical energy into electricity. We report a new approach to construct artificial *Geobacter sulfurreducens* bioelectrodes by immobilizing cells in silica gel and carbon felt fibers. Viability test confirmed that the majority of bacteria (ca. 70 ± 5 %) survived the encapsulation process and cell density did not increase in 96 h. This double entrapment prevents bacterial release from the electrode but allows a suitable mass transport conditions (ca. 5 min after electron donor pulse), making the electrochemical characterization of the system possible. The artificial bioelectrodes were evaluated in 3-electrodes reactors, using different electron donors. The maximum current displayed was ca. 220 and 150 μA/cm^3 for acetate and lactate respectively. Cyclic voltammetry of acetate-fed bioelectrodes revealed a sigmoidal catalytic oxidation wave, typical of more advanced stage biofilms. The presence of *G. sulfurreducens* within the fibers and silica gel was verified by SEM analysis. Moreover, the transcriptomic response of encapsulated *G. sulfurreducens* was analyzed. Therefore, ready-to-use artificial bioelectrodes, represent a versatile time and cost saving strategy for microbial electrochemical systems.

INTRODUCTION

The advent of Microbial Fuel Cells (MFC) marked the beginning of a new research area, between biology and electrochemistry (Schröder and Harnisch., 2015). These electrochemical devices enable the transformation of chemical energy into electricity by means of electrode-respiring microorganisms. They are able to oxidize organic compounds (electron donors) to reduce an electrode as an extracellular electron acceptor (Kim *et al*., 2002; Bond and Lovley, 2003; Logan *et al*., 2006; Lovley *et al*., 2011). Advances in Microbial Electrochemical Technologies (METs) have led to a wide range of systems, like microbial desalination cells (Ping *et al*., 2016), microbial electroremediating cells (MERCs) (Rodrigo *et al*., 2016), and microbial electrosynthesis cells (MES) (Rabaey and Rozendal, 2010).

Among electroactive bacteria, *G. sulfurreducens* has become a model system for the study of all kind of METs (Yates *et al*., 2015; Yu *et al*., 2015; Dantas *et al*., 2015; Borjas *et al*., 2015; Estevez-Canales *et al*., 2015) in order to achieve a deeper understanding of the biological mechanisms involved in direct extracellular electron transfer (DEET) (Lovley, 2011; Bond *et al*., 2012; Bonanni *et al*., 2013). In long term experiments, with no terminal electron acceptor available apart from the electrode, *G. sulfurreducens* forms a biofilm on the electrode, reaching a thickness of tens of microns (Snider *et al*., 2012; Stephen *et al*., 2014; Schrott *et al*., 2014). The natural formation of an electrogenic biofilm, with stable current production, entails prolonged conditioning periods, as long as days or weeks, depending on the architecture of the electrochemical system, and bacterial physiology (Vargas *et al*., 2013; Borjas *et al*., 2015).

Recent studies have pointed out the possibility of artificially improving

electroactive biofilms with the aim of simplifying this process. Some examples are based on doping the electrode in order to improve the electrical conductivity through the biofilm (Adachi *et al*., 2008; Liang *et al*., 2011; Katuri *et al*., 2011; Nguyen *et al*., 2013). Others have introduced the concept of making an artificial biofilm, where electroactive bacteria are encapsulated in a material to constitute a bioelectrode (Srikanth *et al*., 2008; Yu *et al*., 2011; Luckarift *et al*., 2012; Sizemore *et al*., 2013).

Biomolecule encapsulation has been studied for several decades due to its benefits in both, longevity and practical applications, especially for biosensing purposes. (Bjerketorp *et al*., 2006; Balcão and Vila, 2015; Nimse *et al*., 2014; Liu *et al*., 2015). Nevertheless, the interest in this methodology for electroactive bacteria, such as *Shewanella* (Yu *et al*., 2011; Luckarift *et al*., 2012; Sizemore *et al*., 2013) or *Geobacter* (Srikanth *et al*., 2008) is very recent.

In contrast to traditional encapsulation, in order to build an artificial electroactive biofilm, aside from bacterial viability, it is vital to ensure electrical contact with the electrode. There is a wide variety of matrices used for biomolecules immobilization, including agar, pectin, alginate (organic polymers) and silica gels (inorganic polymer) (Bjerketorp *et al*., 2006). Organic polymers have the advantage of being biocompatible, allowing solute diffusion and electron exchange (Srikanth *et al*., 2008). On the other hand, inorganic polymers, like silica gels, allow both solute diffusion and electrons exchange, but offer better optical and mechanical properties, making the gel more robust and easier to control than organic polymers (Depagne *et al*., 2012; Ouay *et al*., 2013; Wang *et al*., 2015).

To the best of our knowledge, silica gel encapsulation remains unexplored for the whole-cell immobilization of the model bacteria *G.*

sulfurreducens, though its use for immobilizing *Shewanella* in artificial bioelectrodes has been reported (Yu *et al*., 2011; Luckarift *et al*., 2012; Sizemore *et al*., 2013). However, *Shewanella* species are reported to release electron shuttles as the primary mechanism of extracellular electron transfer (Marsili *et al*., 2008a; Kotloski and Gralnick, 2013). This may be a drawback in case of serial fed-batch operation. In contrast, *G. sulfurreducens* establishes DEET so its performance is not affected when the media is refreshed (Lovley, 2011).

In this work, we report a new approach to construct ready-to-use artificial bioelectrodes of *G. sulfurreducens* by means of immobilizing cells in silica gel and carbon felt fibers electrodes.

MATERIALS AND METHODS

Bacterial culture

Geobacter sulfurreducens (strain DSM 12127; ATCC 51573) was grown at 30ºC in freshwater medium containing the following mineral salts (per liter): 2.5 g of $NaHCO_3$, 0.25 of NH_4Cl, 0.06 g of $NaH_2PO_4H_2O$,0.1 g of KCl, 0.024 g of $C_6H_5FeO_7$ (ferric citrate), 10 mL of a vitamins mix and 10 mL of a trace mineral solution (Lovley and Phillips, 1988). Acetate (20 mM) was supplied as the sole carbon source and electron donor, and fumarate (40 mM) as the sole electron acceptor. Anaerobic conditions were achieved by flushing the media with N_2-CO_2 (80:20) to remove oxygen and to keep the pH of the bicarbonate buffer at pH 6.8.

Bioelectrode construction

First, the electrode was treated in order to make it more hydrophilic (Ouay *et al*., 2013). Carbon felt (Mersen) was immersed in nitric acid (65%, Sigma Aldrich) for 48 h. Then the felt was rinsed with bicarbonate buffer (pH 6.8) and stored in the same solution before use.

Prior to the gel encapsulation, early stationary bacterial cultures were harvested by centrifugation at 8000 rpm for 10 minutes. These samples were resuspended (OD_{600} = 5) in 90 mM phosphate buffer (pH = 6.8).

For the encapsulation of *G. sulfurreducens* in silica gel, 0.52 mL of sodium silicate (2 M, Sigma Aldrich), 0.1 mL of Ludox HS-40 (Sigma Aldrich) and 1.5 mL of bicarbonate buffer (90 mM), were mixed, deoxygenated and neutralized with 310 µl of HCl (3 M). Subsequently, 2 mL of the concentrated bacterial suspension was added to the mixture, followed by the immersion of the pretreated carbon felt, which lead to a final bacteria concentration of 2 OD_{600} units. Gelation process ends after ca. 20 minutes, with continuously bubbling with a flux of N_2-CO_2 (80:20) to maintain anoxic

conditions inside the bioelectrode.

Electrochemical analysis and calculations

All electrochemical assays were performed in a 3-electrodes configuration reactor. This system consisted of a hermetically sealed glass vessel, where electrodes were assembled. The finished silica-carbon biolectrode was used as a working electrode, a carbon plate (5 x 2 x 0.5 cm) as counter electrode and an Ag/AgCl 3M reference electrode (BASI). The system was controlled by a PC connected-potentiostat (Bio-Logic Science Instruments, SP-150). The reactor was filled with 125 mL of 90 mM bicarbonate buffer amended, when specified, with sodium acetate (20 mM) or lactate (20 mM).

All potentials were quoted versus an Ag/AgCl electrode. For chronoamperometric assays the current was recorded every 10 seconds and the potential was fixed at 0.25 V.

For cyclic voltammetry, the initial potential was 0 V and the potential window was scanned between -0.6 and 0.6 V at 0.005 V s^{-1}. All experiments were performed under a continuous bubbling of N_2-CO_2 (80:20) to maintain an anoxic enviroment and a pH of 7.

Faradays's law ($Q = \int Idt = n_e N_{mol} F$) was used for the oxidation rate estimations.

Viability test

The viability of silica gel encapsulated *G. sulfurreducens* was tested at 24, 72 and 96 hours in the presence of an electron donor and an acceptor. An aliquot of each sample was removed from the bulk of the gel and was fluorescently stained with the LIVE/DEAD Baclight bacterial viability kit (Invitrogen). For staining mixture, 3 µL of SYTO 9 and 3 µL of propidium iodide were mixed in 1 mL of bicarbonate buffer (90 mM). The samples of encapsulated bacteria were stained for 1 h at room temperature in the dark. Post staining samples were washed twice with bicarbonate buffer, in order

to remove the excess of staining, which might cause background noise at the observation. Confocal images were captured using a confocal and multiphoton fluorescence microscope (Leica TCS SP5). Images were analyzed by the ImageJ software for the semi-quantitative analysis.

SEM

A functional bioelectrode was extracted from the reactor, after 72 h of polarization (steady current producing) using acetate as electron donor. Samples were immersed at room temperature for one hour in cacodylate buffer (0.2 M, pH 7.2) containing 5 % glutaraldehyde for the cellular fixation. Samples were rinsed twice in 0.2 M cacodylate buffer, pH 7.2 for 10 minutes.Samples were then dehydrated with a series of ethanol solutions (25, 50, 70, 90, and 100 %) for 10 minutes at each stage. Finally, ethanol was removed by evaporation at room temperature, before the samples were cut in half and imaged with a scanning electron microscope DSM-950 (Zeiss).

RNA extraction and transcriptomic analysis

Encapsulated and free *G. sulfurreducens* cells were incubated at 30ºC for 96 h in the presence of acetate (20 mM) and fumarate (40 mM) prior to RNA extraction. To harvest RNA, 500 µL aliquots were scraped (encapsulated sample) or pipetted (free culture sample) into 1.5 mL tubes. Samples were homogenized by the addition of 500 µL of Purezol (BioRad, USA) and vortexed in the presence of glass beads (1 mm diameter, Sigma Aldrich) for 2 min. Samples were then incubated at 65ºC for 10 min with occasional mixing. 100 µL of chloroform (Sigma Aldrich) was added into each tube, vortexed for 15 s, placed on ice for 10 min and centrifuged for 15 s at 15,000*xg*. The aqueous phase of each tube (approximately 200 µL) was mixed with 500 µL of 2-Propanol (Sigma Aldrich). Then, samples were transfer to RNeasy spin columns (RNeasy kit, Qiagen) and centrifuged for

15 s at 8000 *x g*, discarding the eluate. RNA was purified and concentrated in Rnase-free water, following the protocol provided in the RNeasy kit (Qiagen). RNA yield was measured by absorbance at 260 nm (NanoDrop ND-100, Thermo Fisher Scientific). RNA purity was assessed by examining A260/280 ratio with both samples exceeding a 1.8 value. RNA extracts were sent to FPCM (Campus Cantoblanco, Madrid) to perform the RNA sequencing (RNA-seq). Bioinformatics analysis was performed by Era7 Bioinformatics (Granada, Spain).

RESULTS AND DISCUSSION

Survival of *G.sulfurreducens* after encapsulation

Cell viability after silica encapsulation, was monitored by confocal and multiphoton fluorescence microscopy in the presence of soluble electron donor and acceptor, using Live/Dead staining taking advantage of the good optical properties of the silica gel. This kit allows simultaneous observation of living (green) and dead bacteria (red) based on two different fluorescent dyes. **Figure 1** shows that the viability after 24 h of encapsulation was reasonably good with the majority of bacteria (ca. 70 ± 5 %) surviving the process. This result is corroborated by similar studies conducted on *Escherichia coli* (Ouay *et al*., 2013) and *Shewanella* (Yu *et al*., 2011). Moreover, 96 h after encapsulation, viability appeared to remain constant, demonstrating that encapsulation in silica gel did not significantly affect to *G. sulfurreducens*.

It is also worth noting that cell density did not appear to change over time **(Figure 1)**. Silicate cages are reported to prevent for both escape and proliferation of immobilized bacteria, depending on the selected porosity of the three-dimensional network of the gel. This is feasible because the silica network is not degradable by most living organisms (Bjerketorp *et al*., 2006; Wang *et al*., 2015). Nevertheless it has been recently showed that silica-encapsulated *E. coli* may divide if the inorganic network is flexible enough (Depagne *et al*., 2012; Eleftheriou *et al*., 2013). It would be interesting to study this aspect further, considering that for certain application, such as biosensing, maintaining the number of cells it would be crucial.

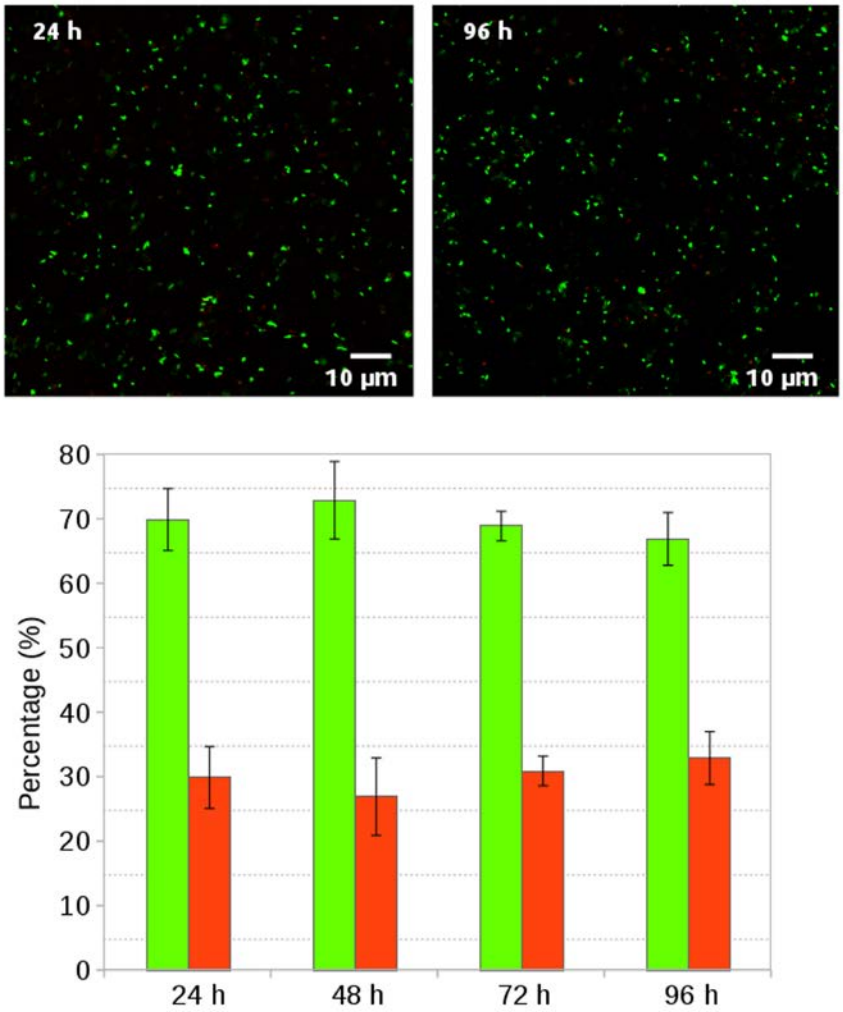

Figure 1: Viability of encapsulated *G. sulfurreducens* within silica gel by Live (green)/Dead (red) staining, up to 96 h.

Bioelectrodes running test

Artificial bioelectrodes were assembled by encapsulating a cell suspension in silica gel within the carbon felt. Once the bioelectrode was assembled, it was immersed in a 3-electrodes reactor containing acetate as sole electron donor and polarized at 0.25 V. Likewise, a bioelectrode was incubated in a free-acetate reactor, and an abiotic electrode containing silica and electron donor were used as a controls. After 60 min of polarization at

0.25 V, a cyclic voltammetry analysis was performed in order to explore the catalytic activity of the three conditions. In the presence of acetate and encapsulated cells the voltammogram displayed a sigmoidal shape profile, typical of the catalytic activity of an advanced-stage biofilm of *G. sulfurreducens* (Marsili *et al*., 2010; Strycharz *et al*., 2011) **(Figure 2A).** However, in the absence of electron donor the catalytic activity displayed was not significant and there is no catalytic activity in the abiotic control, as expected.

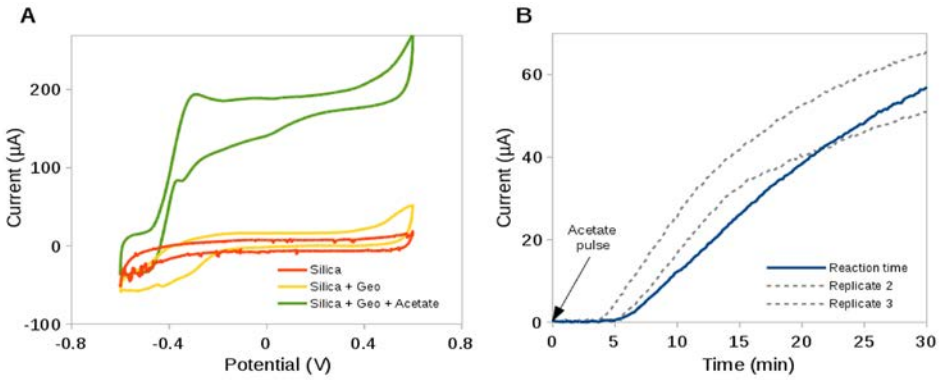

Figure 2: A) Cyclic voltammetries of *G. sulfurreducens* bioelectrodes in the presence of electron donor (green), without electron donor (yellow) and abiotic control with electron donor (red). B) Diffusion of acetate with time.

These results confirm that silica encapsulation does not hinder the electric contact bacteria/electrode, while making possible the acetate to diffuse through the gel.

On these bases, acetate diffusion from the solution to the microbial conversion to electricity was monitored over time. An assembled bioelectrode was placed in a 3-electrodes reactor with no electron donor. It was polarized at 0.25 V until the complete depletion of intracellular acetate, leading to null current production. Then, a pulse of acetate was added to the reactor to produce a final concentration of 20 mM. As shows **Figure 2B**, the current production was restablished ca. 5 minutes after the pulse, revealing

that the diffusion of acetate through the silica gel occurred in a short time. Indeed, our results are consistent with alternative studies conducted without immobilized cells (Bond and Lovley, 2003; Tront *et al*., 2008). So thus, acetate diffusion is unlikely to be a limiting step for characterization of the bioelectrode.

Electrochemical behaviour of the bioelectrode using different electrons donors

In order to explore the behaviour of the system, the electrochemical activity of the bioelectrode was monitored over time using two different electron donors: acetate and lactate.

Acetate is the preferred electron donor for *G. sulfurreducens* and the end-product of the acetogenic phase in anaerobic wastewater treatments, so it is widely used in MET studies (Wang and Ren, 2013; Scott and Yu, 2015). The artificial bioelectrode was polarized in a 3-electrodes reactor for 5 days. During this time cyclic voltammetry (CV) and open circuit potential (OCP) measurements were performed every 24 h. Chronoamperometric results showed a brief initial phase where the current increased, while cells were adapting to the system. Maximum current production (ca. 220 $\mu A/cm^3$) was reached after 24 h of polarization and it kept stable for 96 h. After that period it started to drop **(Figure 3A)**. Assuming that cell density remained constant during the experiment, the average oxidation rate of acetate was estimated in ca. 3.5 x 10^{-10} pmol/s per cell, which is an order of magnitude lower than values for non-encapsulated cells (Estevez-Canales *et al*., 2015). This lower oxidation rate of acetate in comparison with non-encapsulated cells could be explained by the limited access of *G. sulfurreducens* to the electrode. This is likely considering that not every cell immobilized in silica matrix may be in contact with the electrode or other cells close to it.

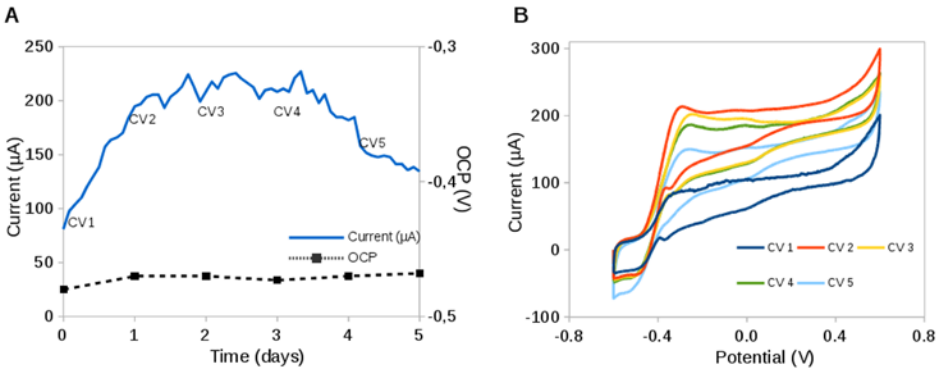

Figure 3: A) Current production at 0.25 V vs Ag/AgCl and open circuit potential vs time of acetate-fed artificial bioelectrode. B) Cyclic voltammetries performed at different time of acetate-fed artificial bioelectrode.

Accordingly with amperometric results, the voltammograms exhibit a sigmoidal catalytic wave from the very beginning, with an onset potential near -0.4 V. Moreover, they achieved similar limiting current values (ca. 200 µA) as in chronoamperometric assays, which is commonly reported for *G. sulfurreducens* anodic biofilms under turn-over conditions **(Figure 3B)** (Katuri *et al*., 2010; Marsili *et al*., 2010). Once maximum limiting current was reached after 24 h, it remained quite steady for the next 72 h. Again, from 96 h onwards, limiting current in the CV also decreased, although the onset oxidation potential as well as the OCP remained constant (- 0.48 V) during the whole experiment. Similar OCP values have been commonly reported for acetate-fed biofilms, demonstrating the sturdiness of our bioelectrode (Babauta *et al*., 2012).

Since acetate concentration in the reactor is not limited, biofilm acidification might be a possible interpretation for the decrease of current production at the final phase. During the reduction of an external electron acceptor, there is a remarkably accumulation of protons that typically limits the performance of MFCs (Franks *et al*., 2008). Opposite, when the terminal

electron acceptor is intracellular, like fumarate, those protons are consumed in the process, so this explains the lack in decrease of cell viability seen earlier.

The behaviour using lactate (20 mM) was also explored. Lactate is metabolized by producing pyruvate and acetate coupled with the reduction of an electrode in *G. sulfurreducens* (Call and Logan, 2011; Speers and Reguera, 2012). Regarding chronoamperometric results, the overall behaviour allows to distinguish as well an initial, steady and drop phase; however the time scale is quite different. The initial phase was considerably longer when lactate was the sole electron donor, and it took a week to reach the stable current production **(Figure 4A)**. Lower steady current (ca. 150 $\mu A/cm^3$) and oxidation rate (ca. 1 x 10^{-10} pmol/s per cell) were observed, which is consistent with others studies that attribute this fact to the lactate diversion to anabolic activities rather than electricity production (Speers and Reguera, 2012).

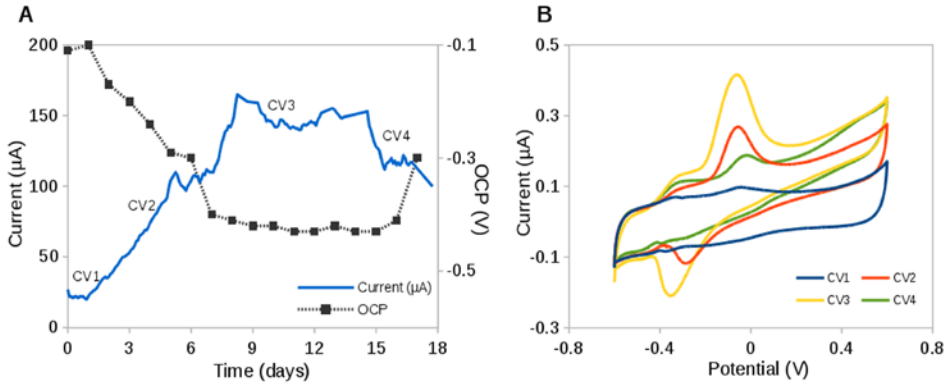

Figure 4: A) Current production and open circuit potential evolution of lactate-fed artificial bioelectrode, polarized at 0.25 V. B) Cyclic voltammetries at different times of lactate-fed artificial bioelectrode.

In contrast to the results obtained with acetate, lactate-fed cyclic voltammetry assay displays two well defined peaks characteristic of the

catalytic activity in non-turnover conditions, when the electron donor is limited in a biofilm (LaBelle and Bond, 2009; Strycharz *et al*., 2011) **(Figure 4B)**. In non-encapsulated mature biofilms, acetate concentrations lower than 3 mM showed indeed a great influence in oxidation waves on voltammograms (Marsili *et al*., 2008b). It is not surprising that in lactate-fed systems, electron donor was limited regarding its interaction with the electrode due to metabolic constrains. *G. sulfurreducens* encodes for glycolate oxidase (GO), an enzyme homologous to lactate deshydrogenase in *S. oneidensis* that poorly catalyze the oxidation of lactate (Speers and Reguera, 2012). Moreover, the pyruvate released is described as a poor electron donor for *G. sulfurreducens*, thus current contribution in lactate-fed system is mainly due to its partial oxidation to acetate (Segura *et al*., 2008; Speers and Reguera, 2012). These studies could explain the prolonged lag phase required to achieve maximum current values. Moreover, the voltammetric profile obtained in our work may correspond with a gradual generation of acetate and a low concentration of it.

In agreement with these results, OCP acted analogously to current production toward more negative values until entering in the steady phase, where the potential reaches ca. -0.45 V. This is similar to previously experiment, using acetate as the sole electron donor. Higher initial and final values of OCP, could be accounted for a lower concentration of acetate. Likewise, biofilm acidification could be the limiting factor for the bioelectrode performance, resulting in current decrease.

Metabolic constraints in lactate oxidation coupled with electrode reduction need further study. For instance, they might be partially overcome by adding other carbon sources that displace lactate for anabolic activities (Speers and Reguera, 2012).

In both acetate and lactate fed bioelectrodes there was no increase in turbidity of the media (data not shown), demonstrating that the double entrapment (silica gel and carbon fibers) prevented bacterial release from the electrode.

Cross-section morphology of the artificial bioelectrode

Furthermore, the inner structure of an acetate-fed artificial bioelectrode in steady state current production (72 h) was analyzed by SEM. Images obtained reveal a heterogeneous coverage, yet the presence of *G. sulfurreducens* cells within carbon felt fibers and silica gel was confirmed **(Figure 5)**.

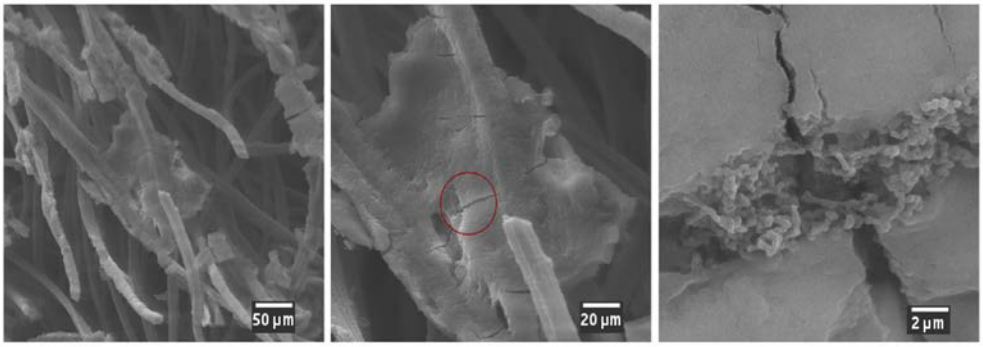

Figure 5: Characterization of the cross-section morphology and structure of the artificial bioelectrodes by SEM.

Such irregular distribution suggests that no every cell was contacting with the electrode, this fact would explain the lower acetate oxidation rate estimated in the preceding section when compared to other studies. It would be interesting to explore other techniques of immobilization in the future, to attain a more homogeneous coverage improving the performance of the bioelectrode.

Transcriptome analysis

To further understand the cell's response to immobilization, the preliminary transcriptomic response of encapsulated *G. sulfurreducens* was analyzed. Transcript abundance of free cells and encapsulated cells were analyzed. Silica encapsulation resulted in differentially expression of 86 genes, 69 overexpressed and 17 underexpressed, considering a *P* value<0.01. The 30 most differentially expressed genes are listed in Table 1.

Table 1: The 30 most strongly overexpressed and underexpressed genes in response to 96 h of silica gel encapsulation compared with a control in standard culture conditions of *G. sulfurreducens*.

Gene	Annotation	Fold change	Name
Overexpressed			
GSU0837	Phosphorelay signal transduction system	8,19	-
GSU2373	WHy domain-containing lipoprotein. Response to desiccation	6,01	-
GSU2374	Lytic transglycosylase lipoprotein. Cellular component	5,1	-
GSU3125	Mannitol dehydrogenase	4,88	*mdt*
GSU1142	Scaffold protein CheW associated with MCPs. Signal transducer activity	4,74	*cheW34H-1*
GSU0663	Peptidyl-tRNA hydrolase. Aa translation	4,57	*pth*
GSU1336	TerC family integral protein membrane	4,51	-
GSU0966	hypothetical protein	4,36	-
GSU0832	Lipoprotein. Molecular funtion lipoprotein	4,31	-
GSU0842	Phosphorelay signal transduction system	4,3	-
GSU2863	DNA-directed RNA polymerase subunit beta. Transcription	4,27	*rpoB*
GSU1141	Methyl-accepting sensory transducer. Signal transducer activity	4,19	*mcp34H-10*
GSU1603	3-oxoacyl-ACP reductase. Fatty acid biosynthetic process	3,92	*FabG-2*
GSU1140	Methyl-accepting sensory transducer class 34H. Signal transducer activity	3,83	*mcp34H-3*
GSU0151	Acetylornithine aminotransferase. Arginine biosynthetic process	3,73	*argD*
GSU0841	Sigma-54-dependent transcriptional response regulator	3,68	-
GSU1607	Serine hydroxymethyltransferase. Glycine biosynthetic process	3,63	*glyA*
GSU1602	Malonyl-CoA carrier protein transacylase. Fatty acid biosynthetic process	3,6	*FabD-2*
Underexpressed			
GSU3493	Hypothetical protein	-7,13	-
GSU0766	Methyl-accepting sensory transducer. Signal transducer activity	-5,42	*Mcp64H-8*
GSU3410	Hypothetical protein	-4,87	-
GSU2529	Elongation factor G. Translation	-4,62	*FusA-2*
GSU0767	Outer membrane channel protein	-4,4	-
GSU0593	Cytochrome b	-4,37	-
GSU3560	Hypothetical protein	-4,01	-
GSU2470	Hypothetical protein	-4,01	-
GSU0768	Amino acid transmembrane transporter activity	-3,85	-
GSU2469	Hypothetical protein	-3,71	-
GSU1639	Rrf2 family winged helix-turn-helix transcriptional regulator	-3,2	-
GSU2590	Hypothetical protein	-3,2	-

Genes encoding proteins related to osmotic stress are the most strongly overexpressed when *G. sulfurreducens* was encapsulated **(Figure 6)**. These include genes involved in the synthesis and metabolism of compatible solutes such as aminoacids (GSU0151, GSU1607, GSU2371, GSU0153, GSU2874) and sugar alcohol (GSU3125), which were overexpressed between 4.8 and 3-fold. Another bacterial common response to environmental changes, including osmotic stress, is the modification of the membrane composition (Zhang and Rock, 2008). Silica encapsulation of *G. sulfurreducens* triggered the overexpression (between 6 and 2.9-fold) of several genes involved in the synthesis and transport of membrane components such as fatty acids (GSU1603, GSU1602, GSU1601), lipoproteins (GSU2373, GSU2374, GSU0832), proteins (GSU1336, GSU2267) and lipopolysaccharides (GSU2085, GSU2490) **(Figure 6)**.

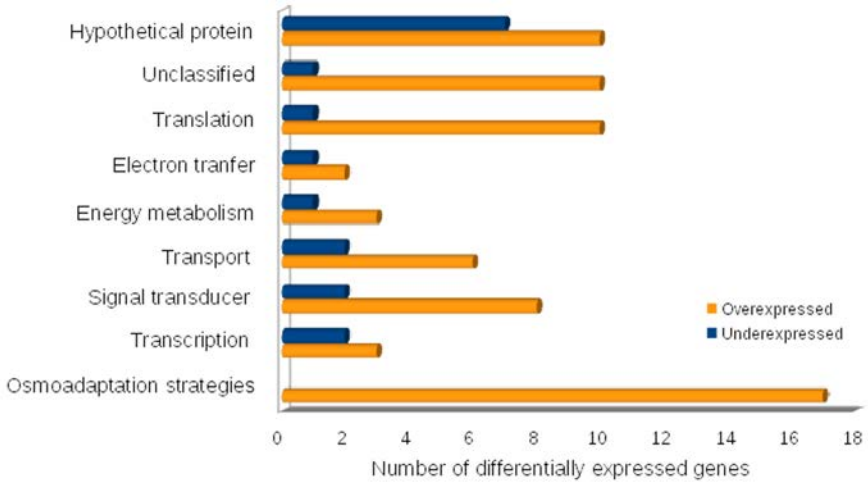

Figure 6: Differentially expression of silica encapsulated *G.sulfurreducens* according to their annotation function in the genome (NCBI database).

Moreover, bacteria are equipped with a broad number of mechanisms in order to sense information caused by external stimuli into the interior of the cell, and to initiate an according response (Krämer *et al*., 2010).

Therefore it is not surprising that several genes involved in signal transduction, translation, transport and transcription, exhibited changes in their expression when cells are silica encapsulated, in response to osmotic upshift among other stimuli **(Figure 6)**.

The transcriptomic results are consistent with previous work that has identified high salinity as the predominant stress for aqueous alkaline silicates precursors of silica gels, like sodium silicate (Dickson *et al*., 2012; Wang *et al*., 2015). Furthermore, similar changes in expression patterns have been recently observed in our research group when *G. sulfurreducens* is exposed to NaCl over several generations, although cell adaptation had surprising effects like higher electroactivity (Borjas *et al* 2016, Submitted). These saline-adapted *G. sulfurreducens* could be used to improve silica gel encapsulation, by avoiding the need for artificial osmoprotectants, and perhaps enhancing the electroactivity of artificial bioelectrodes. Nonetheless, additional analysis of target genes by qRT-PCR would be required to obtain more conclusive results.

CONCLUSIONS

These results show an effective method for the immobilization of *G. sulfurreducens* within silica gel and carbon felt fibers to produce a ready-to-use artificial bioelectrode. Viability test of silica encapsulation confirmed that the majority of bacteria survived the process and the apparently cell density did not change over time. This double entrapment prevented bacterial release from the electrode, so the system could be electrochemically characterized under suitable mass transport conditions. *Geobacter*-based artificial bioelectrodes were tested using acetate and lactate as electron donors. In both cases biofilm acidification provided a plausible explanation for the limitation of long term performance. Furthermore the cross-section of this artificial bioelectrode was interrogated by SEM, and the presence of a heterogeneous coverage of *G. sulfurreducens* within the fibers and silica gel was confirmed. Transcriptomic analysis suggested that osmotic pressure was the predominant stress for silica gel encapsulation of *G. sulfurreducens*. Our methodology will allow the use of different fibrous conductive materials, silica gel porosity and cell densities, for a wide range of applications. Herein, ready-to-use artificial bioelectrodes represent a versatile time and cost saving strategy that could be relevant for short and medium term experiments in microbial electrochemical systems.

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Chapter 5

General discussion, Conclusions & Future outlook

DISCUSSION, CONCLUSIONS & FUTURE OUTLOOK

What is the role of iron in G. sulfurreducens for performing DEET?

Iron is crucial for synthesizing c-type cytochromes as it must be incorporated to the protoporphyrin ring to conform the heme groups. Making iron less available, leads to cells unable to produce enough heme groups for reducing extracellular electron acceptors (Fe(III) or electrodes). Therefore, iron is essential for synthesizing the functional cytochromes network that makes DEET a feasible process. Moreover, supplying enough concentration of iron in the culture media promotes an optimal production of this redox network what ensures maximal electron transport to electrodes.

What could be the molecular mechanisms acting in response to low-iron availability in G. sulfurreducens?

We hypothesize that low-iron availability triggers the expression of certain transcription factors, such as Fur or IdeR, present in G. sulfurreducens as well as in many others bacteria. Once the regulator protein is expressed, it blocks the access of RNA polymerase to those genes related to iron acquisition as well as redox-stress resistance. The so-called Fur-like iron response regulatory protein (Irr) has been described in many bacteria genus and it regulates specifically the heme biosynthetic pathway according to the iron availability. Although Irr has not been found in Geobacter species yet, it is likely that *G. sulfurreducens* has developed a similar system to limit the synthesis of cytochromes under iron-limiting conditions.

What are the advantages of using a low-iron culture methodology for avoiding cytochromes synthesis?

This iron chelator-based methodology for eliminating cytochromes is straightforward, fast and economical. Furthermore, heme$^-$ cells obtained by this culture approach led to viable cells able to perform a functional central metabolism (eg. Fumarate respiration). So thus, heme$^-$ cells reported in this thesis will be extremely useful for other researchers targeting investigations on the physiology of *G. sulfurreducens* under EET-free background conditions. Moreover, it would be possible to generate cells with different levels of heme groups by varying the doses of the chelator.

What are the main advantages and drawbacks of using SPEs for testing microbial electrochemistry?

Incorporating SPEs as regular tools for METs could be very helpful due to their reduced sized together with a low volume requirement (microliters range). Moreover, the larger surface area to volume ratio enhances the efficiency usage of substrates per unit volume. Such a feature allows to significantly reduce the start-up operation time while obtaining a rapid electrical response compared with classical systems. In addition, they are commercially marketed by several brands and they are available in various configurations and materials at low cost. SPEs are indeed more applicable and potentially realizable than macro-scale METs.
Nonetheless, they are probably not suitable for long term experiments due to its disposable nature.

What is the framework for Geobacter-based SPEs in a basic research context?

SPEs were successfully validated for characterizing the response of *G. sulfurreducens* in different physiological states (exponential phase, stationary phase, and steady state under continuous culture conditions) revealing different electron transfer responses. The results are consistent with previous data that used classical electrochemical systems.

Nonetheless SPEs offer a much faster electrical response, which can be used for identifying a certain physiology.

What are the practical applications of SPEs in microbial electrochemical systems?

The results obtained demonstrated that SPEs could be exploited for fast screening methods to select tailor made biocathodes, but SPEs could be used for the detection electroactive microorganisms in bioanodes as well. Moreover, it was displayed a promising biosensor for quantifying the levels of acetate, based on the combination of SPE and *G. sulfurreducens.* In addition, it was shown that the system was robust enough for performing assays in real urban wastewater.

How is G. sulfurreducens affected by the silica-based encapsulation?

Viability test results confirmed that the vast majority of bacteria (ca. 70 ± 5 %) survived the process, demonstrating that encapsulation in silica gel did not significantly affect to *G. sulfurreducens.* Moreover, the apparently cell density did not change over time. Nevertheless it has been recently showed that silica-encapsulated *E. coli* might even divide if the inorganic network is flexible enough. Under this scenario we found interesting to further study this aspect in encapsulated *G. sulfurreducens.* This is especially key in applications like biosensing where maintaining the number of cells can be crucial.

Encapsulation must impose some kind of stress in the cells, and eventually such a stress will generate changes in gene transcription to cope with the environmental shift. So thus, we decided to interrogate the cellular gene expression in order to identify what cellular processes are affected by encapsulation. Nonetheless, an RNAseq-based transcriptomic analysis suggested that high salinity was the predominant stress for silica gel

encapsulations of *G. sulfurreducens*. Saline-adapted *G. sulfurreducens* could be further investigated avoiding the use of artificial osmoprotectants during silica gels encapsulation.

Do the architecture of artificial bioelectrodes enable electrical conductivity and solute diffusion?

The results demonstrate that the double entrapment within silica gel and carbon felt fibers did not hinder the electric contact bacteria/electrode and the system could be electrochemical characterized. Moreover the catalytic activity displayed few minutes after encapsulation was comparable to a more non-encapsulated advanced-stage biofilm. Furthermore, the architecture of these artificial bioelectrodes also allows well mass transport conditions.

What could be the limiting factors in acetate-fed artificial bioelectrodes?

The restricted contact between bacteria and the electrode might be one limiting factor due to the heterogeneous coverage observed in SEM analysis. That could explain the lower acetate oxidation rates estimated in comparison with non-encapsulated cells. Hence, in the future it would be interesting to explore other techniques for silica immobilization, in order to improve the performance of the system.

Moreover, biofilm acidification might be a possible reason for the decrease of current production at the final phase. During the reduction of an external electron acceptor, protons are not consumed at the same rate that EET takes place. As a result, a remarkably accumulation of protons released by the cells inhibit the cell activity, and this is considered one of the main limitations for the performance in microbial electrochemical systems. So thus, promoting the proton flux out of the bioelectrode, by modifying the

porous structure, could be an interesting strategy that should be explored in the future.

What are the main advantages of artificial bioelectrodes?

Ready-to-use artificial bioelectrodes of *G. sulfurreducen*s allow a fast electrochemical characterization of cell response, avoiding long periods typically required for electroactive biofilm formation. This feature could be useful for conducting basic research as well as for electroanalytical purposes such as biosensors.

The fabrication methodology would also allow to explore different fibrous conductive materials and to select silica gel with different porosity, as well as to use different cell density, depending on the desired end use.
Herein artificial ready-to-use bioelectrodes represent a versatile time and cost saving strategy that will be key for short and medium term experiments in microbial electrochemical systems.

Future outlook

This thesis offers several bioelectrochemical approaches to explore EET in *G. sulfurreducens* although further research is still needed. The main value of this work is the straightforward, fast and economical nature of the tools provided. An upcoming tendency to scale-down the electrochemical platforms is not exclusive for SPEs but also for microfluidics, the cornerstone of lab-on-chip biosensing tools. Another future trend is the use of synthetic biology leading to make robust and reliable microorganisms able to assist on long-term and *in situ* analysis. Hard-to-reach natural locations such as sea and river bottoms, or more applied situations like wastewater treatment plants will be benefit of such a technology. There is still a long way to go for the optimization of this fascinating discipline where biology meets electrochemistry and engineering in a manner not expected just 15 years ago.

Abbreviations

ABBREVIATIONS

ATR- SEIRAS	Attenuated Total Reflection-Surface Enhanced Infrared Absorption Spectroscopy
CA	Chronoamperometry
CV	Cyclic Voltammetry
DET	Direct Electron Transfer
DEET	Direct Extracellular Electron Transfer
DIET	Direct Interspecies Electron Transfer
EET	Extracellular Electron Transfer
EPS	Extracellular Polymeric Substances
Fur	Ferric-uptake regulator
IET	Indirect Electron Transferred
MDC	Microbial Desalination Cell
MEC	Microbial Electrolysis Cell
ME-FBR	Microbial Electrochemical-Fluidized Bed Reactor
MERC	Microbial Electroremediating Cell
MES	Microbial Electrosynthesis
MET	Mediated Electron Transfer
METs	Microbial Electrochemical Technologies
MFC	Microbial Fuel Cell

NERS	Nanoparticle Enhanced Raman Spectroscopy
OCP	Open Circuit Potential
RNA-seq	RNA- sequencing
SPEs	Screen Printed Electrodes
TEA	Terminal Electron Acceptor
TCA:	Tricarboxylic acid cycle or citric acid cycle

Curriculum vitae

CURRICULUM VITAE

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ACADEMIC BACKGROUND

From March 2011 to present

PhD student at *Bioelectrogenesis* research group at University of Alcalá

Thesis: *Novel bioelectrochemical approaches for exploring electron transfer in G. sulfurreducens*

Participation in European projects:

Bacwire: Bacterial Wiring for Energy Conversion and Bioremediation (7^{th} FP, Nanosciences, Nanotechnologies, Materials and New Production Technologies)

Visiting researcher (2 months) at Laboratoire Chimie de la Matière Condensée de Paris (LCMCP).

From September 2010 – to February 2011

Postgraduated researcher grant- Molecular Biology Severo Ochoa Centre (CBMSO)

Project: Purification and characterization of yeast enzymes with prebiotic activity

From September 2009 – to September 2010

Master degree on Biotechnology – Autónoma University of Madrid
MSc thesis: Studies on β- Fructofuranosidase from *Xanthophyllomyces dendrorhous* toward its crystallization and heterologous expression" at Molecular Biology Severo Ochoa Centre (CBMSO).

From September 2007 – to June 2009

Degree in Food Science and Technology – Autónoma University of Madrid
Internship at Molecular Biology Severo Ochoa Centre (CBMSO)

From September 2004 – to June 2007

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OTHER RELEVANT EXPERIENCE

- Teaching experience on theoretical and practical classes in "Bioengineering and new sources of energies " (Biology Degree of University of Alcalá)
- Teaching experience on practical sessions in " Chemical engineering " (Chemistry Degree of University of Alcalá)
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- Scientific dissemination in Researchers´ Night in Madrid 2012, 2013, 2014, 2015

SCIENTIFIC CONTRIBUTION

Publications

- **Estevez-Canales M,** Pinto D, Corandin T, Laverty-Robert C, and Esteve-Núñez A. Ready-to-use artificial bioelectrodes: a promising strategy for

Microbial Electrochemical Technologies. Submitted.

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Participations in workshops & congress

- Poster at European Workshop ISMET 2014 (Alcalá de Henares, Spain). Authors: **Marta Estévez**, Akiyoshi Kuzume, Zulema Borjas, Michael Fueg, Derek Lovley and Abraham Esteve-Núñez. Tittle: "A severe reduction in the cytochrome C content of *Geobacter sulfurreducens* eliminates its capacity for extracellular electron transfer".

- Poster at VI Workshop of Nanoscience and Analytical Nanotechnology 2013 (Alcalá de Henares, Spain). Authors: **Marta Estévez**, Zulema Borjas, Antonio Berná and Abraham Esteve-Núñez. Tittle: "Microbial electrogenic biosensors: acetatee into electricity by bacteria of the genus *Geobacter*".
- Oral communication at Electron transfer at the microbe-mineral interface International Congress 2012 (Norwich, UK). Authors: Cristina Gutierrez-Garrán, Zulema Borjas, **Marta Estévez**, Tristano Bascchetti, Derek Lovley and Abraham Esteve-Núñez. Tittle: "Electron-acceptor limitation triggers exocellular electron transfer in planktonic culture of *Geobacter sulfurreducens*".
- Poster at 7th European Workshop on Bacterial Respiratory Chains: Biochemistry, Genetics, Assembly and their Regulation 2011 (Lund, Sweden). Authors: Cristina Gutierrez-Garrán, Zulema Borjas, **Marta Estévez**, Abraham Esteve-Nuñez. Tittle:"Electron acceptor limitation controls extracellular respiration mechanisms in *Geobacter Sulfurreducens*.
- Oral communication at 3rd International Congress Smallwat 2011 (Sevilla, Spain). Authors: Alejandro Reija, Sara Tejedor, Antonio Berna, Karina Boltes, CristinaGutierrez, Zulema Borjas, **Marta Estevez**, Jose Carpatos, Miriam Biel, Eduardo Montero, Belén Barroeta and Abraham Esteve-Núñez. Tittle: Microbial electrogenesis: wastewater as an energy resource.
- Poster at BIOTECH 2010, (Pamplona, Spain). Authors: Patricia Gutierrez, Dolores Linde, LucíA Fernández-Arrojo, Bárbara Rodríguez-Colinas, Miguel Alvaro-Benito, Miguel de Abreu, David Gónzalez-Pérez, **Marta Estevez**, Francisco J Plou, María Fernández Lobato.Tittle: "Production of prebiotic oligosaccharides using extracellular glycosyl- hydrolases from non conventional yeasts".

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