Universidade do Minho
Escola de Engenharia

Luciana de Jesus dos Santos Peixoto

Microbial Fuel Cells for autonomous systems: kinetics and technological advances in wastewater treatment and sensor applications

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Microbial Fuel Cells for autonomous systems: kinetics and technological advances in wastewater treatment and sensor applications

Doctoral Dissertation for PhD degree in Chemical and Biological Engineering

Supervisors of the thesis:
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"*Tira a mão do queixo, não penses mais nisso*
O que lá vai já deu o que tinha a dar
Quem ganhou, ganhou e usou-se disso
Quem perdeu há-de ter mais cartas para dar
E enquanto alguns fazem figura
Outros sucumbem à batota
Chega aonde tu quiseres
Mas goza bem a tua rota

Enquanto houver estrada para andar
A gente vai continuar
Enquanto houver estrada para andar
Enquanto houver ventos e mar
A gente não vai parar
Enquanto houver ventos e mar"

Jorge Palma

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O tempo é fugaz, os planos são guiões e dificilmente têm um término, a aprendizagem é uma busca incessante pelo desconhecido que, quando é conhecido despoleta mais uma dúzia de questões, dando a sensação que temos sempre mais "estrada para andar"... É assim que revejo estes meus últimos 4 anos, sendo esta dissertação apenas o termo de uma etapa e não o fim de uma caminhada. Contudo, não poderia deixar de agradecer àqueles que direta ou indiretamente, me acompanharam ou a tornaram mais fácil. Assim, agradeço:

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Microbial Fuel Cells for autonomous systems: kinetics and technological advances in wastewater treatment and sensor applications

Abstract

Microbial Fuel Cell (MFC) technology is a novel approach for the production of bioelectricity. In MFC, electroactive bacteria oxidize organic matter and have the particularity to transfer the electrons, directly to external electron acceptors producing electricity. *Geobacter* species are one of the most effective microorganisms known to use electrodes as the sole electron acceptor. In that regard, the present work was focused on the study of the parameters which influence the bacterial electron transfer process to electrodes and on the advancement of wastewater valorization and monitoring technologies based on the MFC concept.

The electrochemical behavior of *Geobacter sulfurreducens* in different stages of the biofilm formation in MFC anodes was assessed by cyclic voltammetry in a three-electrode glass cells with two compartments separated by an ion exchange membrane at room temperature and 35 °C. Cyclic voltammograms showed that the presence of *G. sulfurreducens* biofilm results in an increase of current intensities between - 0.5 V and 0.89 V *vs.* SCE with three well defined oxidation peaks at 0.16 V, 0.6 V and 0.8 V *vs.* SCE respectively. In mature biofilm the current intensity were higher. The current intensities for both cases were dependent on operating temperature (≈ 22 °C and 35°C). In every stage of the biofilm formation, the oxidation reaction rate, for the peaks presenting higher current intensities, was limited by the diffusion step with one electron at a time transferred.

Besides, the influence of the growth temperature in the induction of pili production and consequence on the expression of the outer membrane proteins of *G. sulfurreducens* and its effect on the electrochemical behavior as evaluated. Proteins from *G. sulfurreducens,* cultured at 25 °C and 37 °C, were analysed by two-dimensional gel electrophoresis. The results obtained at both temperatures showed that 13 proteins were differentially expressed. The identified proteins were related to membrane permeability, structural integrity, protein's synthesis and energy generation. From the identified proteins, only the LamB porin family was overexpressed at 37 °C. Cyclic voltammetry was used to compare the electrochemical

behaviour of *G. sulfurreducens* grown at both temperatures. The results showed a dependency of the electrochemical behaviour of the bacteria, on its growth temperature. Three well-defined oxidation peaks were observed at 0 V, 0.69 V and 0.82 V *vs*. SCE in voltammograms of bacteria grown at 25 °C while only two peaks were observed at 37 °C at 0.08 V and 0.74 V *vs*. SCE. The oxidation processes characteristic of *G. sulfurreducens* grown at 25 °C presented a mixed kinetic control and showed an irreversible behaviour in most sweep rates. At 37 °C, two distinct irreversible processes were observed, one controlled by diffusion and another by adsorption.

A filter-press MFC was adapted for testing electricity production and wastewater treatment. The cell, with 64 cm^2 of electrode area (carbon Toray) and 1 cm^3 of electrolyte volume, was operated with domestic wastewater in a batch mode (load 622 ± 10 mgL^{-1} COD) with recirculation in the anodic chamber. In the cathode compartment phosphate buffer with hexacyanoferrate was used. The results showed that the maximum current density increased to 823 Wm^{-3}, resulting in a carbon removal of 84 %. Interruptions in the supply of fresh wastewater slightly decreased power density, while the increase/decrease of the recirculation flow rate did not influence power generation.

Finally, a sensor based on the MFC principle was tested for online and in-situ monitoring of Biodegradable Oxygen demand (BOD), a well-known parameter in environmental monitoring in natural waters and wastewater treatment processes. A stable current density of 282 ± 23 mAm^2 was obtained with a wastewater containing a BOD_5 of 317 ± 15 mgL^{-1} O_2 at 22 ± 2 °C, 1.53 ± 0.04 $mScm^{-1}$ and pH 6.9 ± 0.1. The current density showed a linear relationship with BOD_5 concentration ranging from 17 ± 0.5 mgL^{-1} O_2 to 78 ± 7.6 mgL^{-1} O_2. The current generation from the BOD biosensor was dependent on the measurement conditions such as temperature, conductivity, and pH. These results provide precise knowledge for the development of a biosensor for real-time in situ monitoring of environmental quality.

Células de Combustível Microbianas para sistemas autónomos: cinéticas e avanços tecnológicos para aplicação a tratamento de águas residuais e sensores

Sumário

A Tecnologia de Células de Combustível Microbianas (MFC) é uma nova abordagem para a produção de bioeletricidade. Em MFC, as bactérias eletroativas oxidam matéria orgânica transferindo eletrões diretamente para aceitadores de eletrões externos, produzindo eletricidade. Espécies do género *Geobacter* são conhecidas pela capacidade de usar elétrodos como único aceitador final de eletrões. Neste sentido, o presente trabalho foi focado no estudo dos parâmetros que influenciam a transferência de eletrões de bactérias para elétrodos e nos avanços das tecnologias de valorização de águas residuais e de monitorização baseadas no conceito de MFC.

O comportamento eletroquímico de *Geobacter sulfurreducens* em diferentes fases da formação de biofilme em ânodos de MFC foi avaliado por voltametria cíclica em célula eletroquímica de vidro com três elétrodos e dois compartimentos separados por uma membrana de troca iónica, à temperatura ambiente (≈ 25 °C) e a 35 °C. Os voltamogramas cíclicos na presença de biofilme de *G. sulfurreducens* mostraram um aumento da intensidade de corrente entre – 0.5 V e 0.89 V *vs.* SCE, com três picos de oxidação bem definidos a 0.16 V, 0.6 V e 0.8 V *vs.* SCE, respectivamente. No biofilme maduro as intensidades da corrente foram maiores. As intensidades de corrente para os dois casos eram dependentes da temperatura de funcionamento da MFC (≈ 25 °C e a 35 °C). Em cada fase da formação de biofilme, a etapa limitante da reação de oxidação, para os picos que apresentam mais elevadas intensidades de corrente, foi a difusão, sendo que um eletrão de cada vez foi transferido nesta reação.

Além disso, foi avaliada a influência da temperatura de crescimento na indução da formação de pili e na expressão das proteínas da membrana externa de *G. sulfurreducens* e por conseguinte foi observado o consequente efeito no comportamento eletroquímico. Proteínas de *G. sulfurreducens*, cultivadas a 25 °C e 37 °C, foram analisadas em géis de eletroforese bidimensional. Os resultados obtidos em ambas as temperaturas mostraram que 13 proteínas foram expressas diferencialmente. As proteínas identificadas foram relacionadas

com a permeabilidade da membrana, a sua integridade estrutural, a síntese proteica e geração de energia. A partir das proteínas identificadas, só a família LamB porin foi superexpressa a 37 °C. A voltametria cíclica foi usada para comparar o comportamento eletroquímico de *G. sulfurreducens* cultivada em ambas as temperaturas. Os resultados mostraram que o comportamento eletroquímico das bactérias dependeu da sua temperatura de crescimento. Três picos bem definidos foram observados em 0 V, 0.69 V e 0.82 V v*s*. SCE em voltamogramas de bactérias cultivadas a 25 °C, enquanto apenas dois picos foram observados a 37 °C a 0.08 V e 0.74 V *vs.* SCE. O processo de oxidação de *G. sulfurreducens* aumentou a 25 °C, apresentou um controle misto da cinética e mostrou um comportamento irreversível na maioria das velocidades de varrimento. A 37 °C, foram observados dois processos irreversíveis distintos, um controlado por difusão e outro por adsorção.

Uma MFC *filter-press* foi adaptada para testar a produção de eletricidade e tratamento de águas residuais. A célula, com 64 cm^2 de área do elétrodo (carbono Toray) e 1 cm^3 de volume de eletrólito, foi operada com água residual doméstica em recirculação na câmara anódica num modo *batch* (carga 622 ± 10 mgL^{-1} CQO). No compartimento do cátodo foi usado tampão fosfato com hexacianoferrato. Os resultados mostraram que a densidade de corrente instantânea aumentou para 407 Wm^{-3}, resultando numa remoção de 84 % do carbono. Interrupções no fornecimento de água residual fresca diminuiu ligeiramente a densidade de potência, enquanto o aumento/diminuição do caudal de recirculação não influenciou a produção de energia.

Finalmente, um sensor com base no princípio de MFC foi testado para a monitorização *in situ* da carência bioquímica de oxigénio (CBO), um parâmetro bem conhecido na monitorização ambiental em águas naturais e processos de tratamento de águas residuais. Densidades de corrente estáveis (282 ± 23 mAm^{-2}) foram obtidas com uma água residual contendo CBO_5 de 317 ± 15 mgL^{-1} O_2 a 22 ± 2 °C, 1.53 ± 0.04 $mScm^{-1}$ e pH 6.9 ± 0.1. A densidade de corrente mostrou uma relação linear para concentrações de CBO_5 variando de 17 ± 0.5 mgL^{-1} O_2 para 78 ± 7.6 mgL^{-1} O_2. A corrente eléctrica gerada é dependente das condições de medição, tais como temperatura, pH e condutividade. Estes resultados forneceram conhecimento preciso para o desenvolvimento de um biossensor para controlo em tempo real e *in situ* da qualidade ambiental.

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Abbreviations

A – Ampere

AEM – Anion exchange membrane

Ao – Surface Area

ATP – Adenosine triphosphate

BMFC – Benthic microbial fuel cell

BOD – Biological oxygen demand

BOD_5 – Biological oxygen demand after five days

BUG – Benthic unatted generator

CEM – Cation exchange membrane

COD – Chemical oxygen demand

CV – Cycliv voltammetry

d – days

DET – Direct electron transfer

DGGE – Denaturing gradient gel electrophoresis

DNA – Deoxyribonucleic acid

dNTP - deoxynucleoside triphosphate

DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen

EET – Extracellular electron transport

Ep – Peak potential

EPS – Extracellular polymeric substance

F – Farady constant

G – *Geobacter*

h – Hour

i – Current intensity

IEF – isoelectric focusing buffer

j – Current density

L – Liter

Log – Logarithm

MDS – Microbial desalination cell

MEC – Microbial electrolysis cell

MET – Mediated electron transfer

MFC – Microbial fuel cell

min – Minute

°C – degree Celsius

OCV – Open circuit voltage

Omc – Outer membrane cytochrome

OMP – Outer membrande proteins

P – Power density

PCR – Polimerase chain reaction

PEM – Proton Exchange membrane

PI – Isoelectric point

R – Resistance

R_{int} – Internal resistance

RNA – Ribonucleic acid

SCE – Saturated Calomel Electrode

SDS-PAGE

SEM – Scanning electron microscopy

SMFC – Submersible microbial fuel cell

T – Temperature

UM – University of Minho

V – Voltage

W – Watt

σ – Conductivity

Ω – Ohm

2-DE – Two-dimensional gel electrophoresis

Preface

This thesis is based on research for a PhD project undertaken at the IBB - Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, University of Minho, from October 2007 to February 2012. The thesis is composed of a summary, an introduction and 4 publications in scientific journals.

(I) L. Peixoto, A.G. Brito, R. Nogueira, P. Parpot (2012) "Kinetic study of the redox processes at electrodes coated with *Geobacter sulfurreducens* biofilm in a microbial fuel cell" (Submitted to Bioelectrochemistry)

(II) L. Peixoto, I. Machado, A.F. Santos, A.G. Brito, T. Jouenne, P. Parpot, M.O. Pereira, R. Nogueira (2012) "Influence of the temperature in the outer membrane proteins expression and electrochemical behavior of *Geobacter sulfurreducens*" (Submitted to Biochimica et Biophysica Acta).

(III) L. Peixoto, A.L. Rodrigues, M.M. Silva, A. Nicolau, A.G. Brito, P. Parpot, R. Nogueira (2012) "Optimization of a filter-press type Microbial Fuel Cell for electricity production and wastewater treatment" (Submitted to Energy and Environmental Science).

(IV) L. Peixoto, B. Min, G. Martins, A.G. Brito, P. Kroff, P. Parpot, I. Angelidaki, R. Nogueira (2011) "In situ microbial fuel cell-based biosensor for organic carbon". Bioelectrochemistry 81 (2), 99-103.

Publications co-authored and closely related to the topic of the thesis, but not explicitly comprised here are listed below.

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G. Martins, **L. Peixoto**, A.G. Brito, R. Nogueira, (2012) "Exploring the microbial diversity of eutrophic lake sediments in order to simultaneously immobilize phosphorus and generate electricity" (submitted to FEMS Microbiology Letters).

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Poster Presentations:

L. Peixoto, A.F.S. Santos, I. Machado, A.M. Sousa, A.G. Brito, P. Parpot, M.O. Pereira, R. Nogueira, "Electronic transference assessment of the redox processes at carbon electrodes

coated with *Geobacter sulfurreducens* that grown at different temperatures", Semana da Escola de Engenharia, Guimarães, Portugal, 20 to 26 October 2011.

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Chapter 1

Introduction and objectives

As energy is the basis of all life, bioelectricity can be the basis of a better life for all. In a changing world seems impossible that there are still huge areas on the Globe, where electricity, water supply and public sanitation have not yet arrived. Electricity gives personal comfort and is essential for the generation of industrial, commercial and public wealth. On the other hand, electricity production places considerable pressure on the environment, because is produced with nuclear reactions, burning of fossil fuels and in dams. These pressures include the emission of greenhouse gases and air pollutants, land-use, waste generation, contributing to climate change, damage natural ecosystems and cause adverse effects on human health. Nowadays, the renewable energies obtained from the wind, the waves, the biomass, the bio-fuels, the geothermic and the solar energy can satisfy part of the modern energetic necessities, nevertheless, the electricity remains essential.

Microbial Fuel Cells (MFC), a novel approach for the production of bioelectricity using organic compounds (e.g. wastewater), presenting considerable potential for small on-site applications in autonomous systems. In MFC, Electroactive bacteria degrade/oxidize organic matter and have the particularity to transfer the electrons, resulted by the respiratory process (where electrons are transferred gradually in respiratory chain to store energy in the form of ATP), directly to external acceptors [1].

The generation of an electrical current by bacteria during the decomposition of organic compounds was observed for the first time in 1911 by Potter from the University of Durham [2]. After a long period of latency, bioelectricity was again referred in 1966 by Lewis, naming this process as biochemical fuel cells [3]. However, only after 90s years, more advances were obtained in this field. In 1993, Allen and Bennetto, added chemical mediators to assist the microorganisms in shuttling electrons to anodes [4] and the efficiency of extracellular electron transfer in the anode became high enough to warrant sustained academic research. In the early 2000s, several research groups, including Derek Lovley's at the University of Massachusettes, discovered that certain species of bacteria could transfer electron (during respiration) directly to the anode of MFC, namely from the genera *Geobacter* [1, 5, 6] and *Shewanella* [7, 8], resolving the problem of the cost and toxicity of artificial mediators. Thus was born the research around the MFC.

After these discoveries, Bruce Logan from the Penn State University recognizes the commercial potential of MFC, investigating the use of MFC as a wastewater treatment method with production of electricity [9]. The organic waste present in the water, whether sewage or industrial byproducts, is consumed in MFC as fuels, producing electricity [10]. In this way, a MFC can serve two important environmental functions: generating electricity while simultaneously treating wastewater from a renewable energy source [10].

Even after 100 years of exoelectrogenesis discovery, the electron transfer, from the bacteria to anodes, is the main reason that limits the electricity production in MFC. High current densities were obtained in MFC inoculated with *G. sulfurreducens* when compared with other pure bacterial cultures [11, 12]. *Geobacter* species are important in the reduction of metals (e.g. Fe, Mn, U) in soils and sediments and one of the most effective microorganisms know to use electrodes as the sole electron acceptor in MFC to generate electricity [4-6]. *G. sulfurreducens* does not require exogenous mediators to transfer electrons to extracellular electron acceptors, although two main mechanisms for electron transfer have been proposed: i) direct extracellular electron transfer via redox-active proteins on the outer membrane surface [13-16], and ii) long-range electron transfer via pili [17, 18]. The understanding of the electron transfer mechanisms between the electroactive bacteria and the anode surface will certainly allow the optimization of this new and promising biotechnology.

The ability of certain microorganisms transfer electron to electrodes, has been recently recognized for new bioelectrochemical applications to produce fuels such as hydrogen and methane gases in microbial electrolysis cells (MEC) and desalinate water, partially or fully, in microbial desalination cells (MDC)[19, 20]. In cathode chambers of MEC, the electron acceptor is absent and additional voltage (≥ 0.2 V) is added to the circuit, allowing hydrogen gas production [19]. In MFC or MEC, membranes are included to create specialized compartments where desalinate water [20].

Currently, MFC projects tend to be small, and scale-up the MFC reaction to industrial operation will likely involve significant technical challenges and a deeper understanding of electron transfer to electrodes.

In that regard, the present work was focused on the study of the parameters which influence the bacterial electron transfer process to electrodes and on the advancement of wastewater

valorization and monitoring technologies based on the MFC concept. The specific aims of the work were the following:

- to asses the electrochemical behaviour of *G. sulfurreducens* in different stages of the biofilm formation in microbial fuel cell anodes, to assess the kinetic parameters of the redox reactions;
- to evaluate the influence of the growth temperature in the induction of pili production and consequence on the expression of the outer membrane proteins of *G. sulfurreducens* and its effect in the electrochemical behaviour;
- to optimize and adapted a filter-press type MFC for electricity production and wastewater treatment in autonomous systems;
- to evaluate the correlation between electrical current of a submersible MFC and the biodegradable organic content of domestic wastewater, hypotheses it as BOD biosensor.

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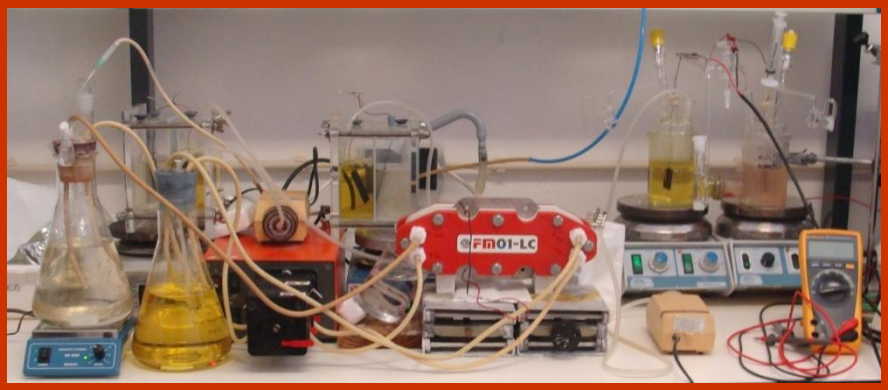


Chapter 2

Outline of the thesis

This thesis is organized in seven Chapters. Firstly, in **Chapter 1** is presented the context and motivation of the work, as well as its main objectives.

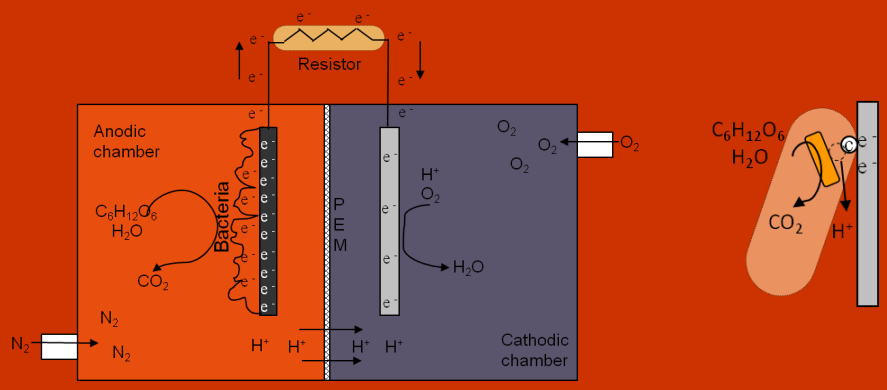
Chapter 3, gives an overview of the literature related to this thesis. In a first part, the principles and fundaments of microbial fuel cells (MFC) were clarified. The electroactive bacteria and the mechanisms that these ones can use to transfer electrons to electrodes were presented. A second part handles about MFC to show the flexibility of its design, resulting in a versatility of applications.

Chapter 4, presents the results of electrochemical studies of *Geobacter sulfurreducens* in carbon electrodes. In **chapter 4.1**, it is presented the results of the electrochemical behaviour of *G. sulfurreducens* in different stages of the biofilm formation on MFC anodes and the kinetic parameters of the redox reactions. **Chapter 4.2**, presents the study of the influence of growth temperature on the expression of the outer membrane proteins of *G. sulfurreducens* and this reflex in the electrochemical behaviour.

Chapter 5, describes the results obtained during the adaptation of a filter-press FM01 reactor to a filter-press type MFC. This MFC was tested to simultaneously produce electricity and treat domestic wastewater. Several effects on MFC operation conditions were tested in order to allow the use in autonomous systems.

Chapter 6, presents the application of the MFC principals in the development of an in situ BOD biosensor. In this study, a submersible MFC was adapted and tested to respond with different current signals at different carbon concentrations in domestic wastewater.

Chapter 7, summarizes the results obtained during this thesis and provides future perspectives.



Chapter 3

Theoretical Background

3.1. Principles and fundaments of microbial fuel cells

Microbial fuel cell (MFC) technology is a novel approach for the production of bioelectricity using organic compounds (e.g. glucose, acetate and wastewater), thus showing considerable potential for small on-site applications. The MFC technology is based on the capacity of some carbon oxidizer microorganisms to transfer electrons directly to the anode, thus generating electricity [1, 2]. This conversion is done through catalytic reactions operated by electroactive microorganisms under anaerobic conditions [3, 4]. The Figure 3.1 shows a general scheme of a MFC for bioelectricity generation.

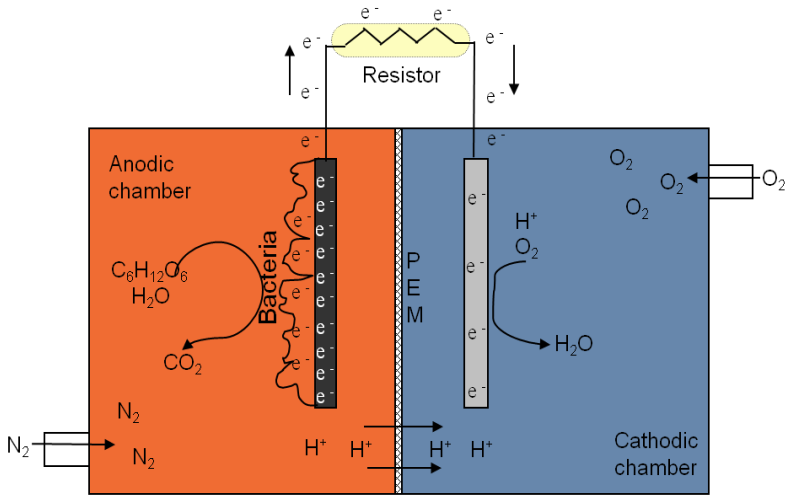


Figure 3.1. Scheme of a MFC for bioelectricity generation.

In the anodic chamber, electroactive bacteria, under anaerobic conditions and in absence of terminal electron acceptors, as example oxygen, nitrate or sulfate, oxidize substrates and generate electrons resulted by the respiratory process (where electrons are transferred gradually in respiratory chain to store energy in the form of ATP) [5-8] as well as protons. The electrons are transferred to the anode and transported to the cathode through an external circuit, the protons are transferred to the cathode chamber by the proton exchange membrane (PEM) where they combine with oxygen to form water [9]. Electricity generation is promoted forcing the electrons to pass through an external resistance [10]. A simplified representation of the anode reaction involved in the oxidation of glucose in a MFC would be as follows:

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \quad (3.1)$$

For the cathode, the reaction can be demonstrated by:

$$6O_2 + 24H^+ + 24e^- \rightarrow 12H_2O \quad (3.2)$$

3.2. Bacterial communities of microbial fuel cells

The electrodes can be colonized by several groups of bacteria [10]. These bacteria could growth using soluble electron acceptors, or growth via fermentation, or from exoelectrogenic activity (known as electroactive bacteria) [10]. Electroactive bacteria can transfer electrons directly from the carbon source to an anode, without the need of redox mediators [10]. Among the electrochemically active microorganisms the most well-known are *Shewanella putrefaciens*, a *Gamma-proteobacterium* [11, 12], *Geobacter sulfurreducens*, *Geobacter metallireducens* and *Desulfuromonas acetoxidans*, all *Deltaproteobacteria* [3, 4, 11] and *Rhodoferax ferrireducens*, a *Betaproteobacterium* [11, 13].

The electrode community, can vary attending the initial inoculums, the MFC design and in particular the final electron acceptor. An overview of the different taxonomic classes in MFC is present in Figure 3.2. For MFC, using oxygen at the cathode, river sediment as inoculums and glucose with glutamic acid as substrate, 65 % of the identified anode bacteria were from *Alphaproteobacteria* [14]. In the same conditions, but with river water as substrate, the *Betaproteobacteria* (related to *Leptothrix spp.*) predominated with 46 % [14]. Using marine sediments as inoculums and cysteine as substrate, the *Gammaproteobacteria* predominated, namely 40 % of *Shewanella affinis* [15]. For MFC inoculated with wastewater, and acetate as substrate, *Alpha-*, *Beta* and *Gammaproteobacteria* were identified in the same ranges of concentrations [16], however with starch as substrate, an unidentified bacteria was predominant

[17]. For sediment MFC, *Deltaproteobacteria* (related to *Geobacteraceae* family and *Desulfuromonas acetoxidans*) dominated the anode communities [1,3]. The identified sequences in MFC with ferrycianide at the cathode and glucose as substrate were derived from *Firmicutes*, *Gamma-*, *Beta-* and *Alphaproteobacteria* [18, 19]. The *Firmicutes* clones were 99% similar to *Brevibacillus agri* [19]. The dominant members of anode communities did

not revealed any specific trend and the mechanisms of electron transfer from bacteria to electrodes are not clearly understood in most of the bacterial groups.

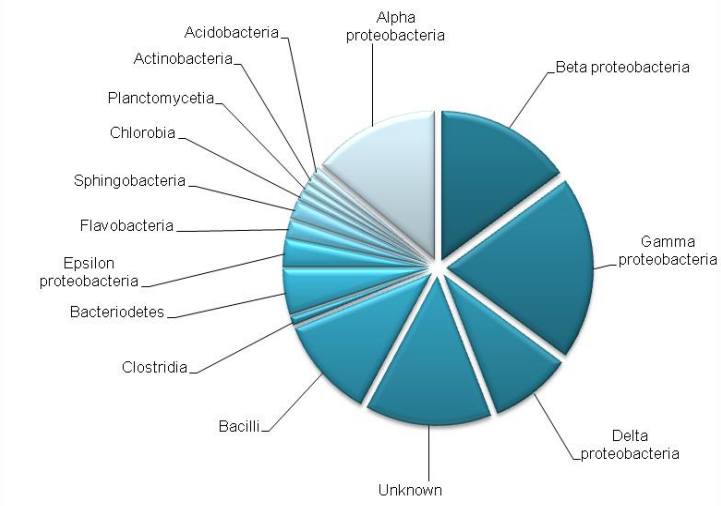


Figure 3.2. Overview of the different taxonomic classes in MFC [20].

Geobacter spp. and *Shewanella spp*. are the bacterial groups where the exoelectrogenic activity was well understood and are recognized as models [21, 10]. *Geobacteraceae* family have the ability to metabolise two common fermentation by-products, acetate and hydrogen and have been found to use dissimilatory iron reduction as the terminal electron accepting process [22]. *Geobacter* species are important in the bioremediation of subsurface environments contaminated with organic and/or metals [23]. *Geobacter sulfurreducens* does not require exogenous mediators to transfer electrons to extracellular electron acceptors, although two main mechanisms for electron transfer have been proposed: i) direct extracellular electron transfer via redox-active proteins on the outer membrane surface [24-27], and ii) long-range electron transfer via pili [21, 28]. Several genetic studies suggested that outer membrane c-type cytochromes are involved in electron transfer to extracellular iron (III) oxides showing that iron (III) reduction is inhibited when the genes for these cytochromes are deleted [29, 30]. According to the literature, the electricity produced by *G. sulfurreducens* biofilms increased with biofilm thickness, which means that most of the cells contributed to the production of electricity without being in direct contact with the anode surface [25, 31]. It was suggested that the long range electron transfer through anode biofilms takes place via conductive pili. The deletion of the gene PilA (responsible for the pili expression) caused only a decrease in power production without a complete inhibition due to the continuous transfer of

the electrons from the cytochromes. Contrarily, the deletion of some outer membrane *c*-type cytochrome genes completely inhibits power production [25].

3.3. Mechanisms of electron transfer from bacteria to electrodes

Bacteria which can transfer electrons to outside of the cell have great importance in the natural environment, mainly in metal oxidation and reduction and associated effects on mineral dissolution, carbon cycle, sorption and complexation of phosphorus and heavy metals. The electron transfer to electrodes as final electron acceptors allow the generation of electricity. The electron transfer from bacteria to electrodes during the decomposition of organic compounds was observed for the first time in 1911 by Potter from the University of Durham [32]. However only in 1993, with Allen and Bennetto [33] the efficiency of extracellular electron transfer in the anode became high enough to warrant sustained academic research. The bacteria can transfer electrons directly to electrodes via membrane bound redox proteins (without the involvement of dissolved species) or with dissolved redox species as mediators [34]. An overview of the electron transfer mechanisms were presented in the following organogram (Figure 3.3).

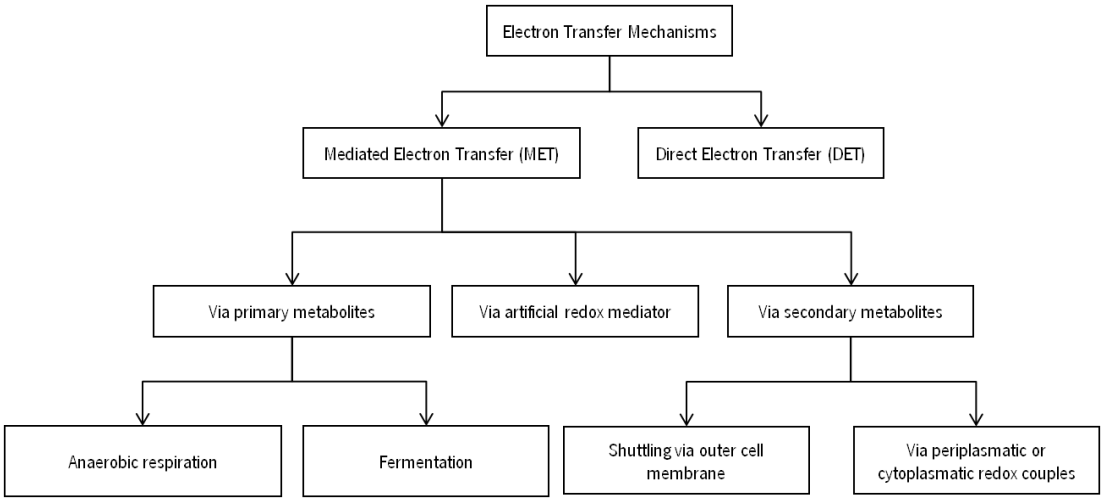


Figure 3.3. Overview of the electron transfer mechanisms involving bacteria [34].

3.3.1. Direct Electron Transfer

In direct electron transfer (DET) mechanisms, the bacterial cells membrane are in physical contact with the anode of MFC, with no diffusional redox species involved. The electron transfer to extracellular electron acceptor, namely anodes, occurs from the membrane bound electron transport proteins existing inside the membrane via outer membrane redox proteins [34]. Outer membrane proteins recognized to establish a direct connection are c-type cytochromes (Figure 3.4), that are multi-heme proteins especially involved in the reduction of iron (III) oxides in sea or lacustrine sediments by *Geobacter* [35, 36], *Rhodoferax* [13] and Shewanella [12] species. This mechanism requires the adherence of the cytochromes to the anode and only a monolayer of bacteria can transfer the electrons to the electrode [34].

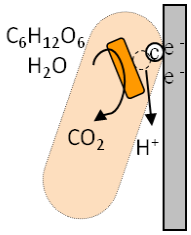


Figure 3.4. Illustration of DET via membrane bound cytochromes.

Geobacter and *Shewanella* strains can develop a conductive pili, also named nanowire [37, 38]. The pili are connected to the membrane-bound cytochromes, making the electron transfer to the outside of the cell more efficient due to the more distant solid electron acceptors in this case. The developed electroactive biofilms are thicker and consequently higher power densities are reached [34]. Direct electron transfer is considering by several authors as the first and only choice for an efficient current generation in MFC [39].

3.3.2. Mediated Electron Transfer

The power densities generated in MFC with mixed biofilms suggests that, not only DET can be considered. The mediated electron transfer (MET) represents an effective mean to transfer electrons from bacteria to the outside during its metabolism (Figure 3.5). The MET can be classified by the nature of the mediator [34].

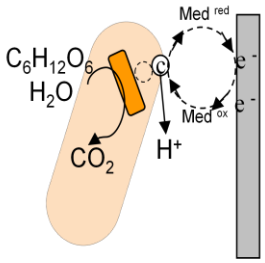


Figure 3.5. Illustration of MET via secondary metabolites.

MET via artificial redox mediators

Mediated electron transfer using artificial mediators correspond to the use of inorganic or organic substances like potassium ferricyanide or benzoquinone to facilitate the electron transfer ("electromotively active oxidation-reduction products" [40]) from bacterial cultures to electrodes. A large number of artificial compounds were investigated, the majority based on phenazines, phenothiazines, phenoxazines and quinones, considering their suitability and behavior as MFC exogenous mediators [11, 34, 41-49].

There are various disadvantage of the use of exogenous redox mediators, beside the usually low current densities, their toxicity and chemical instability are limiting effects and the necessity of a regular addition of the exogenous compound is technologically unfeasible and environmentally questionable and have motivated the abandonment of this approach [34].

MET via primary metabolites

The production of primary metabolites is closely associated with oxidative substrate degradation; the total amount of reduction equivalents produced matches the amount of oxidized metabolites. In order to be utilizable as a reductant for anodic oxidation the metabolite has to accomplish certain requirements:

- Its redox potential should be as negative as possible, but within the limit imposed by the oxidation potential of the substrate [34];
- It must be accessible for electrochemical oxidation under MFC conditions [34].

Anaerobic respiration and fermentation are theoretically the two major pathways that can lead to the formation of reduced metabolites suitable for MFC utilization [34].

Anaerobic respiration

In MFC, the use of primary metabolites as mediators for electron transfer formed during anaerobic respiration is not usual, but is possible [34].

The sulfate/sulfite redox couple is thermodynamically the most suitable mediator, due to its biological standard potential (- 0.22 V) (reaction 3.3).

$$SO_4^{2-} + 8H^+ + 8e^- \leftrightarrow S^{2-} + 4H_2O \quad (3.3)$$

Sulfate reduction is a frequent respiratory path in anaerobic bacteria [50], and is a possible electron transfer mechanism in MFC inoculated with wastewater [51] and benthic MFC [52]. However, the sulfate reducing bacteria are unable to metabolize carbohydrates [34]. They depend on a symbiotic relation with fermenting bacteria that provide low-molecular organic acids and alcohols [50]. Furthermore, the electrochemical re-oxidation of sulfide to sulfate is difficult, since metallic electrodes are easily poisoned by the adsorption of sulfide. Additionally, the electrochemical oxidation is usually hindered by the formation of solid sulfur that inhibits further oxidation [34, 53],

Fermentation

A large variety of fermentative and photo-heterotrophic process results in the production of energy rich reduced metabolites such as hydrogen (e.g., reactions 3.4 and 3.5) ethanol or formate. The substrate (e.g., glucose) can be oxidized directly in the microbial medium. The assisted electrocatalytic oxidation is implemented to prevent the use of the metabolites by other biological processes [34].

Fermentation of glucose to butyrate:

$$C_6H_{12}O_6 \rightarrow C_3H_7COOH + 2CO_2 + 2H_2 \quad (3.4)$$

Fermentation of glucose to acetate:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (3.5)$$

Fermentation for MFC operation is limited by lower energy yield and Coulombic efficiency. Different strategies have been proposed to increase the Coulombic and energy efficiency. An electrochemical approach is the electrocatalytic oxidation of additional energy rich reduced

organic fermentation side products [54]. A biological approach focuses on a combination of dark fermentation with photofermentation in order to increase hydrogen yield and thus the Coulombic and energy efficiency [55]. An additional approach may represent the combination of fermentation with other electron transfer mechanisms (e.g. DET) in MFC [34].

MET via secondary metabolites

Where neither soluble electron acceptors (e.g. like O_2) nor solid electron acceptors are in direct contact, microorganisms may either use externally available (exogenous) electron shuttling compounds like humic acids or metal chelates, or can itself produce low- molecular electron shuttling compounds via secondary metabolic pathways [34, 56, 57], for example, bacterial phenazines like pyocyanine and Phenazine-1-carboxamide and, 2-amino-3-carboxy-1,4-naphthoquinone(ACNQ). The thick biofilms are an example where oxygen diffusion into the depth of the film is limited and the cells are not in direct contact with a solid electron acceptor [34].

For MFC applications, the secondary metabolites (endogenous redox mediators) are especially of great interest, as their synthesis makes the electron transfer independent of the presence of exogenous redox shuttles [34]. The mediator serves as a reversible terminal electron accepter, transferring electrons from the bacterial cell either to a solid oxidant (MFC anode) or into aerobic layer of the biofilm, where it becomes re-oxidized and is again available for subsequent redox processes [34]. One molecule can thus serve for thousands of redox cycles. Therefore, the production, directly in the anodic biofilm, of small amounts of these compounds enables the organism to dispose of electrons at sufficiently high rates [34]. Particularly in batch culture, reports [18, 58] show that these redox mediators effectively facilitate the electron transfer and increase the efficiency of current generation. Some authors [35, 39] argued that this high efficiency may be limited to batch systems, whereas in continuous flow systems a steady lost of mediators may occur, leading to a decreasing value of electrons transferred and thus to a decreasing Coulombic and energetic efficiency. Recent studies however suggest that such losses may be due to a confinement of redox shuttles within the biofilm via electrostatic forces [34]. The production of these electron shuttling compounds is, however, probably energetically expensive, leading to additional biological losses [34].

3.4. Microbial fuel cells design

The construction and analysis of MFC requires knowledge of several scientific areas, which vary from microbiology, electrochemistry, materials, environmental and biological engineering. MFC are being constructed using a variety of materials and in an ever increasing diversity of configurations [2]. These systems are operated under a range of conditions that include differences in temperature, pH, electron acceptor, electrode surface area, reactor size and operation time [2]. Conventional MFC consist of an electrochemical reactor with anodic and cathodic compartments separated by a proton exchange membrane. Anode and cathode are connected to each other using an external resistance. With this principle, several new configurations can be more efficient and adapted to different applications.

3.4.1. Electrode materials

The electrodes can be constructed with a large variety of material (Figure 3.6). The most versatile material is carbon, available like compact material, as graphite, presented in the forms of plates, tube or granules, like fibrous material (filters, cloth, paper, fibers and foams), like brushes and glassy carbon [2].

Figure 3.6. Different electrode materials tested in MFC [59].

A proportion between the current generated and the geometrical electrode area has been demonstrated according to the order: carbon brush > carbon filters > carbon foam> graphite [13]. Higher surface areas are achieved by the use of compact materials like reticulated vitreous carbon that is available in different porosities, and can be used in layers [10]. The high porosity of the materials is important to prevent fouling phenomena [2]. The effect, in the long term stability of the biofilm, in any one of the materials, above stated, was not studied appropriately [2].

A smaller brush anode in a cube-type MFC produced the highest power density yet achieved for an air-cathode MFC, 2400 mWm^{-2} (73 Wm^{-3}) [60].

Carbon fibers can be obtained, between other precursors, from several sources of tar, but the most common sources are PVC, asphalt oil, coal tar [61]. These materials are attractive sources due to the fact of being inexpensive and allow obtaining elevated profits, in carbon fibers [61].

Different chemical and physical strategies can be followed to increase the performance of electrodes. The integration of Mn (IV) and Fe (III) in to the electrode structure [62] and the use of artificial exogenous mediators allow to obtain a superior electron transfer rate to anode [2].

The use of conductive polymers coatings and metal coatings have also been demonstrated, but the performance of these systems has erratic and inconsistent, indicating both maximum power densities and stability of these materials will be a concern, however additional work is needed in this area [10].

In synthesis, the anode materials require [2, 10]:

- To be electrically conductive, preferentially with high conductivity;
- To be biocompatible;
- To have chemical stability in the reactor solution;
- Non-fouling;
- Non-corrosive;
- High specific surface area (area per volume) adapted for the growth of the biofilme, which will be responsible for most of the electron transfer;
- Capacity to promote sufficient turbulence for an appropriate diffusion of protons for the membrane and cathode;
- Inexpensive and easy made and scaled to larger sizes.

The same materials that have been described above for use as the anode have also been used as cathodes. Studies have reported the use of graphite, graphite felt, carbon paper, carbon-cloth, Pt, Pt black and reticulated vitreous carbon [2]. The main difference is that a catalyst is usually need (e.g. Pt for oxygen reduction or potassium ferricyanide - $K_3[Fe(CN)_6]$ in this case).

Potassium ferricyanide - $K_3[Fe(CN)_6]$] is very popular as liquid catalyst-electron acceptor mainly due to the lower overpotential of ferricyanide when associated with carbon, allowing cathode working potential close to its open circuit potential [2]. The great disadvantage of potassium ferricyanide is that the solution cannot be reoxidized totally with oxygen, making the regular substitution of the cathodic solution a necessity [63]. Additionally, the performance of the system can be affected by the diffusion of the ferricyanide through the PEM and consequently inside the anodic chamber [10].

Oxygen is the electron acceptor more adapted for the MFC systems due to its elevated oxidation potential, accessibility, low cost and because the resultant product water is harmless [2]. To increase the rate of oxygen reduction, Pt catalysts are usually used for dissolved oxygen [52] or open-air (gas diffusion) cathodes [2, 64, 65].

3.4.2. Membrane materials

Membranes are primarily used in two chambers MFC for keeping the anolyte and catholyte separated. Ferricyanide, water or other solution present in the cathode chamber, containing dissolved oxygen cannot be mixed with the anolyte. These membranes need to be permeable to protons produced at the anode. Membranes also serve as barriers to unwanted substrate flux from the anode to the cathode (fuel cross over) and oxygen from the cathode to the anode, improving Coulombic and power efficiency [10]. The disadvantages of membranes in MFC are their high cost (Nafion 117 can cost around 2800 € per m^2 and the performance of the system decrease by increasing internal resistance and thus reduce power generation [10].

The most commonly Cation exchange membrane (CEM) is Nafion (Dupont Co. USA) available in different thickness being 117 the more usual [10]. Ultrex CMI-7000 (Membranes International Inc.) is a CEM alternative [66] with a superior relation cost/effectiveness when compared with Nafion [10]. The Anion exchange membrane (AEM) AMI-7001 (Membranes International Inc.) was tested by Kim *et al.* [67] with higher power densities. Bipolar membranes, anion and cation membrane can be used for specific applications [68]

Most of the configurations of MFC require a physical separation between the anodic and cathodic compartments, sediment MFC [52] and single-chamber MFC [69] being exceptions.

3.4.3. MFC configurations

The MFC materials can affect the power density, as well as the MFC configuration, decreasing the internal resistance. From the simpler to the more complex, for example the submersible MFC with one chamber (air-cathode) (Figure 3.7), the MFC configurations are divided in 2 types: two-chamber MFC and single-chamber MFC. In each type of MFC is possible to use oxygen or artificial electron acceptors as final electron acceptor.

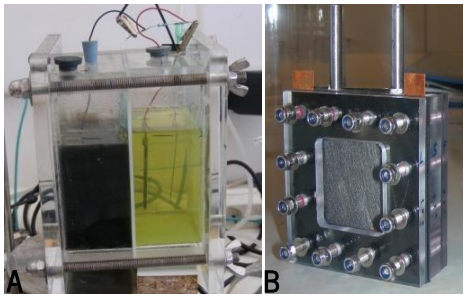


Figure 3.7. Two distinct MFC configurations: Two-chamber MFC (A) and Submersible air cathode single-chamber MFC (B).

3.4.3.1. Two-chamber MFC

A standard MFC is a two-chamber MFC composed by anodic and cathodic chambers separated by a proton exchange membrane or a salt bridge that allows the protons to flow from the anode to the cathode and at the same time obstructs the oxygen diffusion from the cathodic chamber to the anodic chamber. Two-chamber MFC are normally operated in batch mode with diverse carbon sources (e.g. glucose and acetate solutions or wastewater) to generate bioelectricity [20]. The compartments can assume several designs: H-type, tubular upflow and flat plate [2, 10].

The H-type consists in two bottles connected by a tube containing a cation exchange membrane (CEM) or a plain salt bridge [70]. In the H-configuration, the membrane is clamped in the middle of the tubes connecting the bottle (Figure 3.8) [2]. A glass tube that is heated and bent into a U-shape can be used and filled with agar and salt to do the connection between the chambers [70]. H-shape systems are used only in research parameters, such as examining power production using new materials, or types of microbial communities that arise

during the degradation of specific compounds, but they typically produce low power densities [2]. The two compartments of a MFC can be connected by membrane and held together by bolts (Figure 3.7A) in an assembled MFC [58].

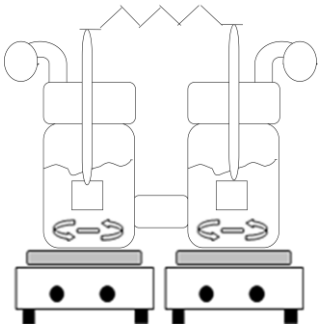


Figure 3.8. H-Type MFC scheme.

A Flat Plate MFC was developed by Min and Logan in 2004 [71], with only a single electrode/PEM assembly (Figure 3.9). A carbon-cloth cathode was hot pressed in a Nafion PEM and placed in direct contact with a single sheet of carbon paper (the anode), to form an electrode/PEM set. This configuration is composed by two non-conductive plates of polycarbonate attached together. The PEM separates the cathodic and the anodic compartments (Figure 3.9 B). The anodic chamber can be fed with residual waters or another organic matter and dry air can be pumped from side to side in to the cathodic chamber without the necessity of a catalytic solution [71].

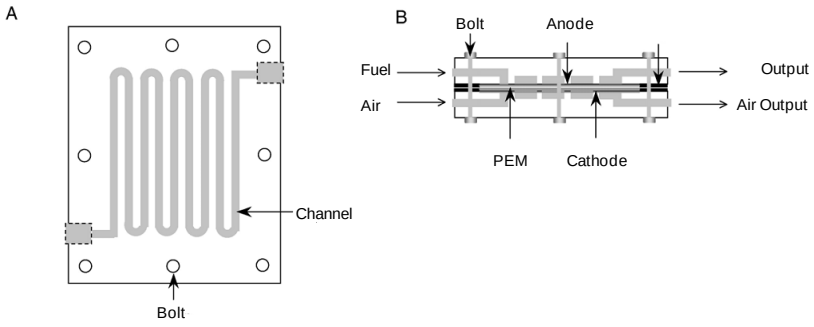


Figure 3.9. Schematic of a Flat Plate MFC: top view (A) and side view (B) [71].

The tubular upflow configuration [66, 72], present more adapted system for wastewater treatment applications due to the facility of scale-up [20]. On the other hand upflow models need energy for pumping the recycled water and consequently the energy output of these systems are much lower than the single-chamber MFC. This configuration offers low internal

resistance (around 4 Ω) due to the proximity of the anode and cathode with a large PEM with high surface area [20].

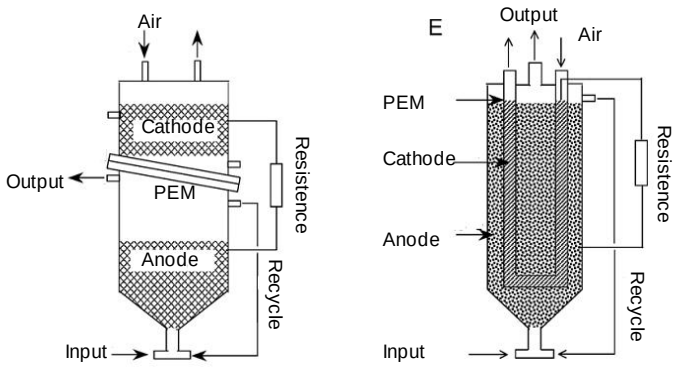


Figure 3.10. Two different tubular upflow configurations [20, 66, 72].

A tubular upflow MFC consist in a Plexiglas cylinder divided in two sections (anodic and cathodic chambers) using the glass beads and glass wool layers [66]. The graphite felt anode and the cathode were situated in the inferior and superior part of the reactor, respectively (Figure 3.10 A). Inspired in the same concepts another arrangement of this configuration can be observed in Figure 3.10 B. In this MFC there is no separation between the anode and cathode. The substrate is supplied from the inferior part of the reactor and the effluent passes through the cathodic chamber in a continuously mode [72].

4.4.3.2 Single-chamber MFC

The Two-chamber MFC are complex and difficult to scale-up [20]. Single-chamber MFC offers a simple design and less investment costs, and can operate in continuous or batch mode [2, 20]. Typically, in this configuration both anode and cathode are introduced in the same compartment, without the necessity of the aeration system [2, 20]. In the simplest configuration, the anode and cathode are placed on either side of a tube, with the anode sealed against a flat plate and the cathode exposed to air on one side, and water on the other [69]. The anode was made of carbon paper with the characteristic of not being water proof [69]. The cathode was either, an assembly of carbon electrode/PEM, manufactured by joining a PEM directly to a flexible carbon cloth electrode, or a system without PEM formed by a rigid

carbon paper [69]. The utilization of oxygen by bacteria in the anode chamber can result in a lower Coulombic efficiency [69].

Another arrangement of single-chamber MFC consists of a tubular MFC with exterior cathode and an inner anode composed by graphite granules [69]. In the absence of a cathodic chamber, the cathode of woven graphite in in direct contact to the air, the catholyte [69].The application of these open-air cathodes is seen as critical to practical implementation of MFC [69].

Another type of single-chamber MFC is a cylindrical MFC, which use in the same chamber the anode and the cathode. It consists of a Plexiglas chamber with eight graphite tubes (as anode), in a concentric display, around a single cathode [64, 73]. A PEM/catalyst composed by a carbon/platinum exchange membrane layer was combined with a plastic support tube to form an air-porous cathode [64].

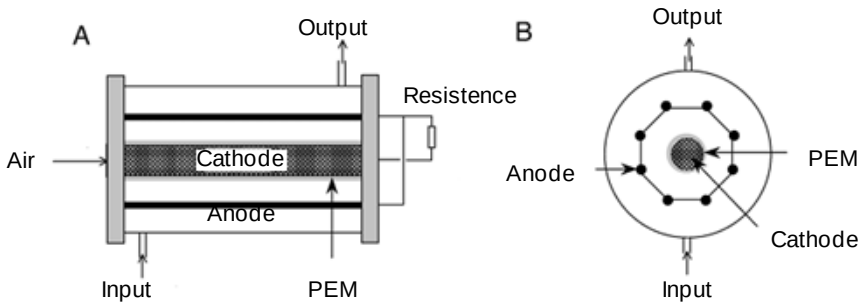


Figure 3.11. Schematic of cylindrical single-chamber MFC containing eight graphite tubes which serve as anode in a concentric display surrounding a single cathode: side view (A), top view (B) [20, 64].

3.4.3.3 Stacked MFC systems

The association of MFC is useful to investigate the performance of MFC coupled in series and in parallel configurations [19]. An increase of voltage and current intensities can be achieved by linking several MFC in series or in parallel mode, improving consequently the Coulombic efficiency and the maximum power output per unit of MFC [10, 20]. The Coulombic efficiency describes the part of the electrons which can be subtracted by the electrode from the total obtained for oxidation of the organic complex matter. The associations of MFC in parallel mode obtain a Coulombic efficiency of around six times higher than those obtained for the

association of the same units in series for the same volumetric flow rate [10, 20]. This means that the connection of MFC in parallel achieves higher maximum bioelectrochemical reaction rate than in serial configuration [10, 20]. Therefore to maximize the substrate consumption rate and consequently accomplish higher values of Coulombic efficiency (higher voltage, current and power generation standards) a parallel connection is preferred if MFC units are not independently operated [19].

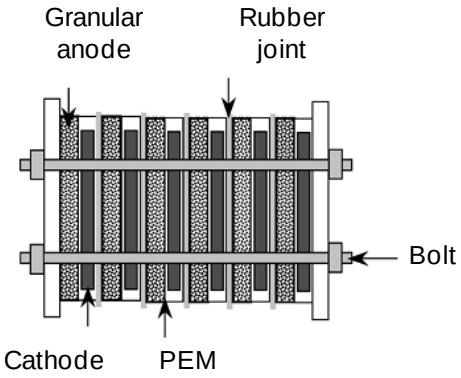


Figure 3.12. MFC association of six individual units in parallel with granular graphite anodes [19, 20].

3.4.3.4. Benthic MFC

Previous studies have shown that electrical energy can be harvested from organic-rich aquatic sediments by electrochemically active microorganisms and a new application of the microbial fuel cell technology has emerged: the Benthic Microbial Fuel Cell (BMFC) or sediment MFC. A BMFC consists of an anode embedded in the anoxic sediment and a cathode suspended in the aerobic water column (Figure 3.13) [52]. Bacteria in a BMFC mediate the transfer of electrons from carbon sources to the anode thus generating an electric current. The first BMFC described in the literature were associated to marine sediments due to the better ion conductivity between electrodes in saline environments [1, 74]. Recently, sediments from rivers and lakes were also used to operate BMFC [75, 76]. The main application of BMFC described in the literature is long-term power sources for autonomous sensors and communication devices [3, 52, 74, 77].

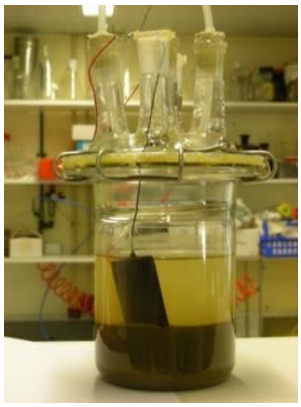


Figure 3.13. Illustration of a microcosm that can represent a BMFC [76].

3.5. Current applications of MFC.

3.5.1. Wastewater treatment

The MFC can be an alternative treatment method for low concentrated wastewater, removing carbon or another biodegradable compounds under anaerobic conditions and generating electricity [64, 78]. The observation that the use of mediators (chemicals that shuttle electrons) is not essential for MFC stimulated the development of these devices for wastewater treatment [69]. The substrates which can be degraded by MFC include acetate [36, 79], glucose [80], starch [81], cellulose [82], wheat straw [83], pyridine [84], phenol [85], *p*-nitrophenol [86] and complex solutions such as domestic waste water [69, 87], brewery waste [88], land file leachate [89], chocolate industry waste [90], mixed fatty acids [91] and petroleum contaminants [92]. The MFC can produce less biomass then the equivalent aerobic wastewater treatment and doesn't need an energy intensive aeration process [93]. MFC for the large scale treatment of wastewaters still face problems related to the scale up from laboratory experiments and to the slow rates of substrate degradation [94]. In a large single-chamber MFC it was possible to produce power up to 26 mWm^{-2} from continuous treatment of domestic wastewater. In this process besides the electricity generation up to 80 % of BOD was removed. Power generation was related to the wastewater strength (COD), and was linearly dependent on the range examined [93]. Thus, it can be expected that high power densities should be achieved in this system with more concentrated wastewater [93]. Removal of BOD was also proportional to residence time [93].

The first large-scale test of MFC for wastewater treatment was developed by the Advanced Water Management Center at the University of Queensland, under the supervision of Jurg Keller and

Korneel Rabaey (www.microbialfuelcell.org) and tested at Foster's brewery in Yatala, Queensland (Australia). The reactor consisted of 12 modules, each 3 m high, with a total volume of approximately 1 m^3 (Figure 3.14) [96]. The reactor contained carbon fiber brush anodes inside tubular reactors, with flow up through the tubes the outside of the reactor was covered with graphite fiber brush cathodes [96]. This design was similar to one tested in the laboratory with a ferricyanide catholyte [63]. The purpose of the pilot plant was to remove at least 5 kg of organic matter per m^3 of reactor volume per day. Depending on the removal, a power output of 500 W could be reached continuously [10].

Figure 3.14. Tubular MFC tested for power production using wastewater produced at Foster's brewery in Yatala, Australia (Photo author: Jeremy Patten from the University of Queensland).

Another pilot-scale of MFC was tested by researchers from the University of Connecticut and their coworkers (Fuss & O'Neill, and Hydroqual Inc.) at a site in the USA (Baikun Li, personnel communication). The reactors contain granular graphite as the anode, and Pt-catalyzed carbon cloth cathodes, based on a design published by Jiang and Li [95]. The systems are treating wastewater, removing up to 80% of the chemical oxygen demand present at 300–600 mgL^{-1} [96].

The MFC technology can be a novel approach for the production of bioelectricity using organic compounds, presenting considerable potential for small on-site applications in isolated and autonomous systems and can also be useful for individual applications in residences.

3.5.2. Batteries and environmental sensors

Data about environmental parameters can be useful for the understanding and modeling of the answers of the ecosystems, but the sensors in order to operate in the environmental placement need energy. Meteorological buoys capable of measuring air temperature, pressure, relative humidity, water temperature, are operated and the data are transmitted at real-time mode using sight radio frequency telemetry by benthic MFC [9, 94].

One of the most exciting discoveries in the last years, concerning the technology of the microbial fuel cells, was developed by Reimers and Tender and it consists of a MFC which produce electricity from organic matter present in aquatic sediments [52, 74, 97]. These systems are known like Benthic Unatted Generators or BUGs [35]. The systems BUGs were projected to supply electric energy to electronic devices placed in remote locations, like the bottom of the ocean, where it is technically difficult and expensive the maintenance of an energy system for these electronic sensors [35]. The BUGs consist of Benthic MFC as was described earlier. In this context two separate benthic MFC have been tested, the first, a prototype with a mass of 230 kg and a volume of 1.3 m^{-2}, which could provide 24 mW or the equivalent of 16 alkaline D-cell batteries per year [97]. A second design was developed with a mass reduced to 16 kg, a volume of 0.03 m^{-2} and furnished 36 mW or the equivalent of 26 alkaline D-cell batteries per year [97] (Figure 3.15).

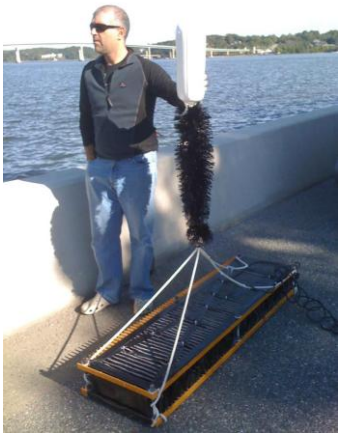


Figure 3.15. Benthic MFC with Leonard Tender. Graphite plates (in the yellow casing) are deployed in the marine sediment with a graphite brush cathode in the overlaying water column [94, 97].

Several configurations based on MFC have been tested and optimized to be used as a biological oxygen demand (BOD) biosensor [98]. The usual configuration was a mediator-less MFC, with two chambers separated by a cation exchange membrane with a continuous wastewater flow in the anode chamber [98-104]. The main drawback of this configuration is the complexity of the setup that is not suitable for in situ applications. Therefore, a very compact MFC configuration, known as submersible microbial fuel cell (SMFC), was developed by Min and Angelidaki [105]. This configuration uses an air-cathode which simplifies considerably the MFC configuration [106] and was adapted and tested as an in situ BOD biosensor, that can be submerged in anaerobic wastewater [107].

Figure 3.16. Submersible MFC tested as in situ BOD biosensor [107].

3.5.3. Biorremediation

The ability of the MFC microbial communities to degrade a wide range of environmental pollutants can be used to clean up the environmental samples *in situ* [94]. *Geobacter* species have been used for the anaerobic degradation of petroleum components and landfill leachate contaminants in ground water [108]. *Geobacter metallireducens* are able to accelerate the degradation rate of toluene, benzene and naphthalene in hydrocarbon-contaminated soils using an electrode as the final electron acceptor [109]. In this setting the current intensities produced by the bacteria are irrelevant when compared to the high rates of bioremediation [94].

3.5.4. Another applications of the MFC principal

The ability of certain microorganisms to transfer electrons to the electrodes, has been recently associated to the new bioelectrochemical applications such as the production of hydrogen and methane gases in microbial electrolysis cells (MEC) and the desalinization of water, partially or fully, in microbial desalination cells (MDC)[110, 111]. The MEC is a modified MFC in which the cathode is completely anoxic, besides the voltage produced by the cell and additional voltage is applied to allow the hydrogen evolution. At the cathode, electrons combine with protons to form hydrogen via the hydrogen evolution reaction:

$$2H^+ + 2e^- \rightarrow H_2 \quad (3.6)$$

Bacteria at the anode consume organic matter and produce a voltage of approximately 0.3 V, while the hydrogen evolution reaction requires 0.41 V, demanding a theoretical input of 0.11 V. In practice, additional voltage (≥ 0.2 V) is added to the circuit, allowing hydrogen gas production [110].

In the anode chamber of two-compartment MFC, the H^+ accumulation promotes the transport of Na^+ and K^+ through the CEM. A device with three chambers, with an AEM next to the anode and a CEM next to the cathode, and a middle chamber between the membranes can be used in water desalination. When current is generated by bacteria on the anode, and protons are released into solution, positively charged species are prevented from leaving the anode by the AEM and therefore negatively charged species move from the middle chamber to the anode. In the cathode chamber protons are consumed, resulting in the transport of the positively charged species from the middle chamber to the cathode chamber. This loss of ionic species from the middle chamber results in water desalination without request of energy or water pressure [111].

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Chapter 4

Electron transfer: from bacteria to electrodes

4.1. Kinetic study of the redox processes at electrodes coated with *Geobacter sulfurreducens* biofilm in a microbial fuel cell

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Geobacter species are one of the most effective microorganisms known to use electrodes as the sole electron acceptor in microbial fuel cells to generate electricity, and the electronic transfer mechanisms have been largely studied. The knowledge of the mechanisms involved in redox processes between electrodes and bacteria allows engineering biofilms with a higher power production. In that regard, the main goal of the present work is to go deeper in understanding of the kinetics of the redox processes at electrodes in a microbial fuel cell during the biofilm formation of *Geobacter sulfurreducens*. *G. sulfurreducens* strain PCA (DSMZ 12127) was inoculated in three-electrode glass cells with two compartments separated by an ion exchange membrane at room temperature and 35 °C. Cyclic voltammetry was used to characterize the oxidation-reduction reactions and for each oxidation peak the reversibility of the process and the limiting step was assessed. Cyclic voltammograms showed that the presence of G. sulfurreducens, immediately after inoculation and in a mature biofilm results in an increase of current intensities with three well defined oxidation peaks. The current intensities, corresponding to the mature biofilm were higher than those observed for the bacteria after inoculation due probably to the increases of the active surface. The current intensities for both cases were dependent on operating temperature. In every stage of the biofilm formation, the oxidation reaction rate, for the peaks presenting higher current intensities, was limited by the diffusion step. One electron at a time was transferred with diffusing electron transfer mediators. In conclusion, this study pointed out that in order to optimize current generation mass transfer resistance concerning the biofilm should be minimized.

Keywords: *Geobacter sulfurreducens*, Microbial Fuel Cell, Cyclic voltammetry, Electronic transfer, Electricity.

4.1.1. Introduction

Iron (III)-reducing bacteria belonging to the *Geobacteraceae* family within the delta-subdivision of the Proteobacteria are among the most well studied electrochemical active microorganisms [1]. The *Geobacteraceae* family have the ability to metabolise two common fermentation by-products, acetate and hydrogen, and have been found to use dissimilatory iron reduction as the terminal electron accepting process [2]. *Geobacter* species are important in the bioremediation of subsurface environments contaminated with organic and/or metals [3]. They can also convert organic compounds to electricity in microbial fuel cells (MFC), using the electrode as the sole electron acceptor [4]. MFC is one of the most interesting applications of these electroactive bacteria.

Present understanding of electron transfer mechanisms in biofilms and the specific interactions between biofilm matrix and the electron-accepting electrode are far from to be completed. However, it is known that direct electron transfer (DET) via membrane bound redox proteins does not involve dissolved species, which should exclude the need of the presence of bacterial mediators. Mediated electron transfer (MET) is based on dissolved species and can be classified by the nature of the mediating redox species; they can be secondary metabolites shuttled via the outer cell membrane cytochromes and via periplasmatic/cytoplasmatic redox couples, or primary metabolites via reduced terminal electron acceptors (anaerobic respiration) and oxidation of reduced fermentation products [5]. The reversibility of MET via exogenous or artificial redox mediators depend on the nature of the mediator itself.

Recent research suggested that, in the most efficient microbial fuel cells (MFC) electron transfer proceeds through biofilms, a phenomenon for which there is little prior guidance in the literature; however, the mechanisms of electron transfer involved in an anode biofilm are yet not well understood. Data from literature suggested that, in *G. sulfurreducens* the electronic transfer to extracellular electron acceptors do not require exogenous mediators (DET) [4]. For low current production (e.g. 0.012 $mAcm^{-2}$), some authors support the idea that outer membrane *c*-type cytochromes are involved in electron transfer through an anode

biofilm [13, 14], while for high current outputs (e.g. 0.2 $mAcm^{-2}$) the presence of conductive pili (also known as bacterial nanowires) are required [15].

According to the literature, the electricity produced by *G. sulfurreducens* biofilms increases with the thickness of the biofilm, which means that most of the cells are not in direct contact with the anode surface but contribute to the production of electricity [13, 16]. It was suggested that long range electron transfer through anode biofilm takes place via conductive pili; however, the deletion of the gene PilA (responsible for the pili expression) cause just a decrease in power production without a complete inhibition it because of the continuous transfer of the electrons from the cytochromes. Contrarily, the deletion of some outer-membrane *c*-type cytochrome genes completely inhibited power production. [13]. In the most recent studies, the deletion of the gene OmcZ greatly inhibited current production [14]; this fact corroborates the idea that the cytochromes *c*-type are required to facilitate electron transfer between the biofilm and the anode surface [16] either directly or via pili in long distances.

The cyclic voltammetry (CV) is an electrochemical technique that can be used to assess the redox reactions between planktonic microorganisms or microbial biofilms and electrodes as was performed by several authors [12, 17-22]. The application of a continuously time-varying potential to the working electrode results in the occurrence of redox reactions of electroactive species in the solution (faradaic reactions). The electroactive species might adsorb to the electrode, according to the potential, originating a capacitive current due to double layer charging [23]. In previous electrochemical studies, CVs performed with and without bacteria confirmed that G. *sulfurreducens* was fully responsible for the catalysis of acetate oxidation. Biofilms of *G. sulfurreducens* were able to catalyze the oxidation of acetate transferring the electrons directly to graphite anodes [10]. A recent study reported that the oxidation potential of the electrode influences the expression of those cytochrome proteins for which an energetically favourable interaction between electrode-biofilm can be established [24].

The present work aims to study the electrochemical behaviour of *G. sulfurreducens* in different stages of the biofilm formation and to assess the kinetic parameters of the redox reactions.

4.1.2. Materials and methods

4.1.2.1. Geobacter sulfurreducens growth

Geobacter sulfurreducens (DSM 12127) was obtained from DSMZ (Braunschweig, Germany) and cultivated as was suggested by Caccavo *et al.* [2]. The growth of *G. sulfurreducens* was done in 100 mL bottles sealed with leak proof sealings, to ensure anaerobic sterilized microenvironment, at 35 °C with sodium acetate as electron donor and sodium fumarate as electron acceptor. The growth medium had the following composition per litre (ultra-pure water): 1.5 g of NH_4Cl, 0.6 g of Na_2HPO_4, 1.5 g of NaH_2PO_4, 0.1 g of KCl, 0.8 g of Na-acetate, 8.0 g of Na_2-fumarate, 2.5 of $NaHCO_3$, 10 mL of vitamins solution (in a 1 L solution: 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyridoxine - HCl, 5.0 mg thiamine HCl . 2 H_2O, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg d-Ca-pantothenate, 0.1 mg vitamin B_{12}, 5.0 mg p-aminobenzoic acid, 5.0 mg lipoic acid), 10 mL of trace metals solution (in a 1 L solution: 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4 \cdot 7\ H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1.0 g NaCl , 0.1 g $FeSO_4 \cdot 7\ H_2O$, 0.18 g $CoSO_4 \cdot 7\ H_2O$, 0.1 g $CaCl_2 \cdot 2\ H_2O$, 0.18 g $ZnSO_4 \cdot 7\ H_2O$, 0.01 g $CuSO_4 \cdot 5\ H_2O$, 0.02 g $KAl(SO_4)_2 \cdot 12\ H_2O$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4 \cdot 2\ H_2O$, 0.025 g $NiCl_2 \cdot 6\ H_2O$, 0.3 mg $Na_2SeO_3 \cdot 5\ H_2O$) and 1 mL of a selenio-tungstate solution (in a 1 L solution: 0.5 g NaOH, 3 mg $Na_2SeO_3 \cdot 5\ H_2O$, 4.0 mg $Na_2WO_4 \cdot 2\ H_2O$). All components (except $NaHCO_3$ and Na_2-fumarate) were dissolved by heating the medium until boiling, cooling down to room temperature with ice, and simultaneously, bubbling a gas mixture N_2-CO_2 (80:20) from PraXair® in the solution to assure anaerobic conditions. Then, $NaHCO_3$ was added and the pH was equilibrated at 6.8 with the gas mixture. The growth medium without fumarate was autoclaved for 20 min at 121 °C. Afterwards, the electron acceptor (50 mM of sodium fumarate) was added from a stock solution using a sterilized filter (0.22 μm) (medium 826 adapted from DSMZ method).

4.1.2.2. Electrochemical set up

A thermostated three-electrode glass cell with two compartments separated by an ion exchange membrane (Nafion 117, DuPont Co., USA) was used as a Microbial Fuel Cell (MFC)

for the voltammetric study and for the biofilm formation. For the voltammetric study, a saturated calomel electrode (SCE) was used as reference electrode and a carbon Toray sheet (E-TEK division, USA) was used as both working and counter electrodes. The carbon Toray sheet (3 cm x 3 cm) was glued to a platinum (Alfa Aesor, Germany) wire using conductive carbon cement (Fluka, Sigma-Aldrich) and was dried at room temperature during 24 h. Before each experiment, the anodic and cathodic chambers were filled with the supporting electrolyte (KCl 0.1 M) and deaerated with pure argon (U Quality from L'Air Liquid). Then, the cleanness of the surfaces and solutions were tested prior to each experiment by recording voltammograms in the supporting electrolyte and in medium alone. The electrochemical instrumentation consisted of a potentiostat/galvanostat from Amel Instruments coupled to a microcomputer through a AD/DA converter. The Labview software (National Instruments) and a PCI-MIO-16E-4 I/O module were used for generating and applying the potential program as well as acquiring data such as current intensities. Cyclic voltammetric studies were carried out with *G. sulfurreducens* biofilm modified anodes at different stages of biofilm formation.

For biofilm formation on the anode surface in the MFC, an enriched culture of *G. sulfurreducens* (150 mgL^{-1} of protein) in the logarithmic growth phase was inoculated in growth medium containing sodium acetate as electron donor, without any electron acceptor but the anode. The MFC was operated in batch mode at room temperature (≈ 22 °C), this temperature was chosen because it corresponds to a situation that occurs frequently in operation of biological technical systems, and at 35 °C, as proposed by DSMZ for *G. sulfurreducens* cultivation based in Caccavo *et al.* [2]. The temperature was maintained constant using a thermostat bath. The cathodic chamber was filled with a solution of KCl (0.1 M) containing potassium hexacyanoferrate (50 mM), as electron acceptor, and was aerated continuously using an air pump. The anodic chamber was flushed with the gas mixture N_2-CO_2 (80:20) from PraXair® and sealed to maintain anaerobic condition. The anode and cathode, both of carbon Toray, were connected via a 500 Ω resistor. The voltage (V) difference between anode and cathode electrodes was recorded every 30 minutes. A concentrated solution of sodium acetate was added regularly (periods of 4 days) in order to maintain a concentration of 20 mM in anode compartment.

4.1.2.3. Analytical methods and calculations

The current intensity (i) was calculated according to the Ohm´s law, i=V/R, where V is the voltage and R the resistance. Current density (j) was calculated as j=i/Ao, where Ao is the projected surface area of the anode. Power density (P) was calculated as the product of the current intensity and the voltage divided by the projected surface area (Ao) of the anode (P=iV/Ao).

To follow the cellular concentration, the protein was measured according to the Lowry method [25], modified by Peterson [26], using the Sigma protein assays kit (Sigma Diagnostics, St Louis, MO, USA).

4.1.2.4. Scanning electron microscopy (SEM)

A sample of *G. sulfurreducens* in suspension, which was used to inoculate the MFC, and two samples of anodes with biofilm, corresponding to the 4th day of growth at 22 °C and 35 °C, were prepared to be analysed by SEM. The samples were dehydrated using different ethanol solutions with increasing concentrations (10 %, 25 %, 50 %, 75 %, 90 % and 100 % (V/V)) two times for each solution during 15 minutes. After the final concentration, the samples were dried in a desiccator for 2 d. To increase their conductivity, the samples were mounted on aluminium stubs with carbon tape and sputtered with gold. The electronic microscopic images were obtained with a Leica Cambridge S360 microscope (UM – Electron Microscopy lab).

4.1.3. Results and Discussion

4.1.3.1. Scanning electronic microscopy analysis

SEM analysis of the *G. sulfurreducens* suspension used as inoculum showed rod shaped bacteria with 1.2 ± 0.1 μm of length (Figure 4.1A). The biofilms observed on the surface of the anodes at both temperatures and after 4 days of growth contained cells covered by a layer of extracellular polymeric substances (EPS) (Figure 4.1B, C). It can be observed that the amount EPS was considerably higher in the biofilm grown at 35°C.

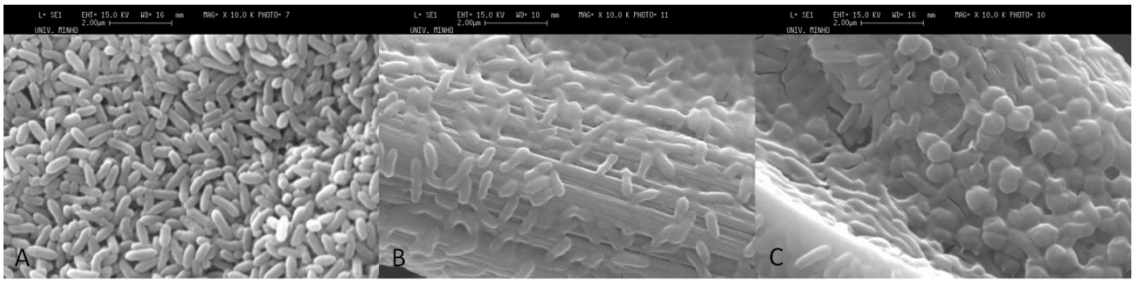

Figure 4.1. *G. sulfurreducens* observed by SEM. Bacteria used as inoculum (A) and biofilm with 4 days in the carbon Toray electrode at 22 °C (B) and 35 °C (C).

4.1.3.2. Electrochemical behaviour of *G. sulfurreducens* after inoculation and during the biofilm formation

4.1.3.2.1. *G. sulfurreducens* after inoculation

The voltammograms of carbon Toray at 50 mVs^{-1}, in growth medium with and without *G. sulfurreducens*, at both temperatures 22 °C and 35 °C, are given in Figure 4.2. The voltammogram in growth medium without *G. sulfurreducens* at 22 °C exhibited an oxidation peak at 0.57 V *vs*. SCE with a moderate current intensity (0.273 mA) and a reduction peak at 0.22 V *vs*. SCE. At 35 °C, an oxidation peak was observed at 0.55 V *vs*. SCE with a current intensity of 0.544 mA. In the reverse scan, a reduction peak was observed at 0.33 V *vs*. SCE. Higher current intensities, observed for this last temperature in comparison with those obtained for 22° C, show the influence of the temperature on reaction rate. The redox process observed in culture medium without *G. sulfurreducens* was probably due to the presence of trace minerals existing in this medium. This fact was confirmed by the voltammetric study of the individual components of the medium (data not shown).

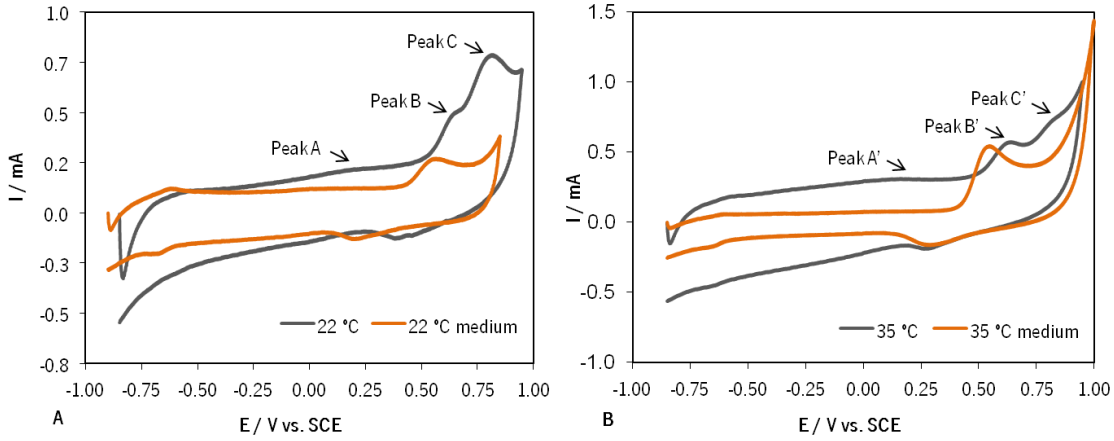

Figure 4.2. The voltammograms of carbon Toray in growth medium without and with *G. sulfurreducens* (immediately after inoculation) at 22 °C (A) and 35 °C (B) (50 mVs^{-1}; 150 mgL^{-1} of bacteria; pH 7; 20 mM acetate).

After the addition of *G. sulfurreducens* (Figure 4.2), an increase of the overall current intensities was noticed for the potential region between - 0.5 V and 0.89 V *vs*. SCE. At 22 °C, three peaks of oxidation during the positive sweep and one peak of reduction during the negative sweep were observed at 0.16 V (peak A), 0.60 V (peak B), 0.80 V (peak C) and 0.39 V (peak D) *vs*. SCE, respectively. The corresponding current intensities for the peaks A, B, C and D were respectively 0.22 mA, 0.42 mA, 0.79 mA and -0.13 mA (Figure 4.2A). The same potentials were observed for the oxidation peaks A', B' and C' at 35 °C with maximum current intensities of respectively 0.31 mA, 0.54 mA and 0.70 mA (Figure 4.2B). The reduction peak D', with a current intensity of -0.18 mA, was noticed at 0.30 V *vs*. SCE. These results are consistent with those presented by Liu *et al.* [27] reporting a positive influence of temperature in the electroactivity of mixed cultures. The three distinct oxidation peaks can be attributed to independent or successive oxidation processes, which reveal the existence of three distinct redox couples, suggesting that different redox species present in the biofilm contribute to the current intensities. This fact was previously described by several authors as for example Katuri *et al.* [19] and Strycharz *et al.* [28]. Comparing the peak potentials of this study with the ones present in literature for graphite at 10 mVs^{-1} [7], the results were quite similar. The observed difference in the potential peaks might be due to the electrode material. Analysing the results of the present study, carbon Toray appears to be more sensitive detecting bacterial oxidation since it was observed a higher oxidation potential intervals, resulting in the detection of other

peaks, for example peak A. The oxidation currents observed in a large potential region are in agreement with the existing data on the availability of different cytochromes groups in bacteria which can be oxidized at different potentials [12]. The increase of temperature from 22 °C to 35 °C increased the maximum current intensity of peak B while a slight decrease was noticed for peak C. It can be concluded from these results that at 35 °C the process that occurs at lower potentials (peak B'), with a higher reaction facility, was privileged.

Figure 4.3 shows the voltammograms corresponding to the successive cycles at 22 °C and 35 °C, with *G. sulfurreducens*. A fast decrease of the current intensity was noticed for peak B, showing an important deactivation of the active sites. Considering the relation between successive cycles and the accumulation of the irreversibly adsorbed species on the electrode surface, it can be concluded that peak C can be more dependent on surface phenomena than peak B.

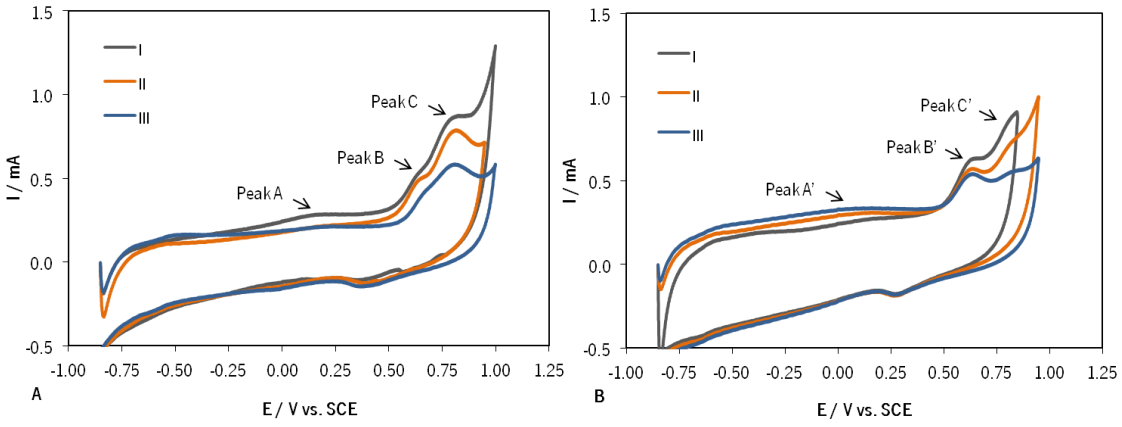

Figure 4.3. Behaviour of the oxidation peaks of *G. sulfurreducens* immediately after inoculation in carbon Toray when subjected to successive voltammetric experiments at 22 °C (A) and 35 °C (B) (50 mVs^{-1}; 150 mgL^{-1} of bacteria; pH 7; 20 mM acetate).

4.1.3.2.2. Biofilm of *G. sulfurreducens*

Two MFC containing a *G. sulfurreducens* suspension (≈ 150 mgL^{-1} of protein) were used simultaneously to study the mechanisms involved in electron transfer during biofilm formation at different temperatures, 22 °C and 35 °C. Sodium acetate was added regularly (once after every 4 days) to avoid the decrease of current density. The catholyte was also renewed regularly to minimize limitations concerning the cathodic process. The current density of the

cells versus time was registered at 22 °C and 35 °C during biofilm formation (Figure 4.4). After around 2 days latency, the current density of the MFC operated at both temperatures started to increase gradually to reach 17.8 mAm^{-2} at 22 °C and 80 mAm^{-2} at 35 °C after 29 days (Figure 4.4). The renewal of the catholyte after this period did not cause any change in current densities, at 35 °C, while an increase of current densities with a maximum of 45.5 mA m^{-2} was noticed at 22 °C, suggesting an electron acceptor limitation in this last case. The maximum current densities for both temperatures were maintained constant during two weeks and a half. No further work was carried out to optimize current densities in order to reach those mentioned in the literature considering that the principal objective was the investigation of kinetic parameters of the redox reaction in different stages of biofilm formation. A higher output current density was noticed at 35 °C compared to that at 22 °C. These results are in agreement with those presented by Fricke *et al.* [29]. After the stabilization of the current densities, the measured open circuit voltage (OCV) was 197 mV at 22 °C and 240 mV at 35 °C.

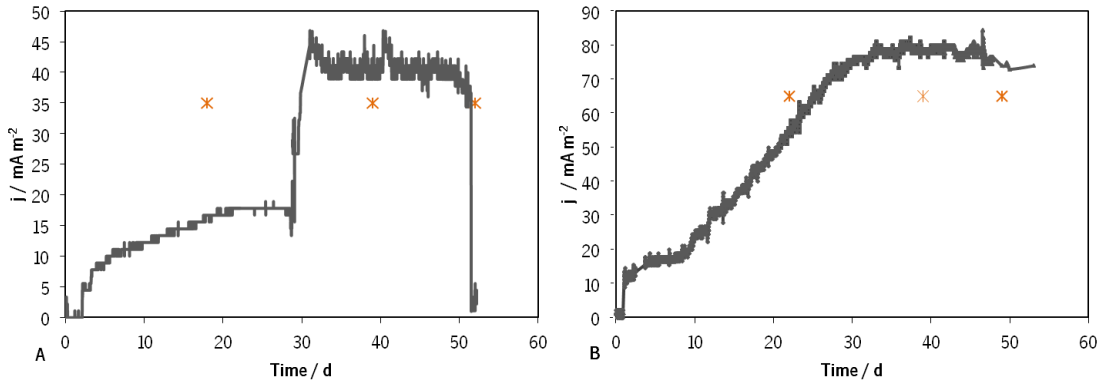

Figure 4.4. Current densities over time of a MFC with *G. sulfurreducens* at 22 °C (A) and 35 °C (B) (20 mM acetate, external resistance of 500 Ω). Cyclic voltammetry experiments were done at defined moments presented in the figure by an asterisk.

Cyclic voltammetry (5n) was carried out regularly to determine peak potentials and current intensities of the oxidation process during the biofilm growth. Figure 4.5 shows the evolution of the maximum current intensities of the voltammetric peaks versus time. During biofilm formation, the current intensities of the oxidation peaks increased significantly in comparison with those obtained for bacteria immediately after inoculation. No significant changes were noticed for the peak potentials at 22 °C, the current intensities increased regularly up to the

39th day of operation and reached 22.4 ± 2.4 mA at 0.73 ± 0.05 V. At 35 °C, the current intensities stabilised after the same period, at 11.7 ± 2.8 mA with a peak potential of 0.78 ± 0.08 V.

The voltammograms registered at 22 °C show a large peak (peak E) at 0.75 V *vs.* SCE which probably includes three oxidation processes attributed to peaks A, B and C, observed immediately after inoculation with *G. sulfurreducens*, being peak C predominant in this case. Contrarily to the previous case, two peaks can be observed on the voltammograms registered at 35 °C (Figure 4.6). These results show that the increase of temperature enhanced the current density of peak A' in comparison with those corresponding to peaks B' and C'. The reduction peaks may be due to the reduction of mediators existing in their oxidized forms due to acetate oxidation and/or, according to Schröder [5], to the reduction of the metabolites produced during the biofilm growth. Comparing our current densities of the oxidation peaks with the ones of Strycharz *et al.* [28] and Richter *et al.* [12], we concluded that both current density data are similar.

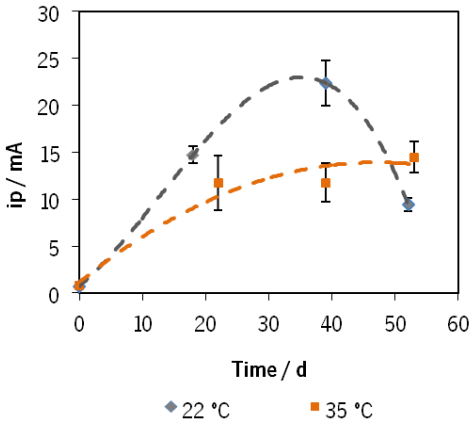

Figure 4.5. Maximum current intensities (5n) of the oxidation peaks obtained during biofilm formation.

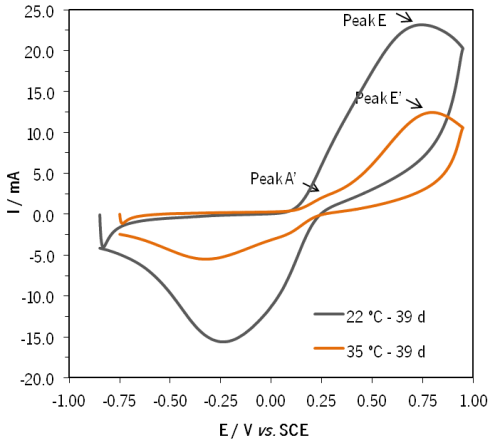

Figure 4.6. Voltammograms of carbon Toray with a *G. sulfurreducens* biofilm in the 39th day of growth at 22 °C and 35 °C (50 mVs^{-1}).

These results allow to conclude that more than one type of cytochromes were involved in the electron transfer process. Each cytochrome may be associated with a range of electrical potentials, being some cytochromes energetically more favourable than others. Different growth conditions of the biofilm, such as temperature, may influence the prevalence of certain cytochromes in the external membrane.

4.1.3.3. Determination of the kinetic parameters

The kinetic parameters of the reactions were determined by cyclic voltammetry. A study was carried out with different sweep rates (from 5 to 1000 mVs^{-1}) to investigate the rate limiting step of the oxidation reactions during biofilm formation. The current intensities resulting from the electroreactivity of *G. sulfurreducens* were calculated after the peaks deconvolution to minimize the distortion of the current waves. Peak deconvolution was performed using OriginPro 7.5 (OriginLab corporation). The slope of the straight lines of the log i *vs.* log v, i being the peak current intensity expressed in mA and v the potential sweep rate in mV s^{-1}, gave information about the limiting step. Slope values close to 0.5 indicate that the reaction is mainly diffusion controlled, while a slope value of 1.0 shows that adsorption is the limiting step of the reaction [30]. To determine the reversibility of the charge transfer, the dependence of potential (E) on the log (v) was evaluated; the peak potential being dependent on scan rate for irreversible processes and independent in the case of reversible processes [30]. The curves representing the log i *vs.* log v and E *vs.* log (v) are depicted in Figures 4.7 and 4.9.

Considering the kinetics of the electrode processes with coupled heterogeneous catalytic reactions, two kinetic regions can be defined assuming a sufficiently fast heterogeneous electron transfer (Figure 4.7B and 4.7E). In the first region, corresponding to the kinetic control of the catalytic process, the current intensities are associated to the catalytic reaction rate and independent of the scan rate [31]. Assuming the model of catalytic reactions on coated electrodes, the current intensity can be expressed as:

$I = nFA\, k_s \Gamma C_s$

where k_s is the second-order rate constant, Γ the active sites of catalyst (mol cm^{-2}) homogenously distributed throughout a layer of uniform thickness, d ($C_{cat} = \Gamma/d$), C_s the concentration of substrate, n the number of electrons involved in the electrode reaction of the catalyst, A the electrode area, and F the faraday constant. The second region, which is dependent of the sweep rate, relates to the kinetics of a charge transfer without a chemical reaction step. Taking into account both soluble and bounded electron transfer mediators in the biofilm, the current can be limited by the diffusion of soluble mediators or by the diffusion of charge compensating ions in the case of adjacent bounded mediators.

4.1.3.3.1. *Geobacter sulfurreducens* after inoculation

Figure 4.7 (A, B and C) shows log i *vs*. log v curves for carbon Toray electrode immediately after inoculation with *G. sulfurreducens* at 22 °C. A summary of the kinetic parameters of the redox reactions during biofilm formation at 22 °C and at 35 °C is presented in Table 4.1. At 22 °C, the slope value of the log i *vs*. log v curve in the entire sweep rate region for peak A was 0.89, suggesting that this process was mostly limited by adsorption. For peak B (Figure 4.7B), the calculated slope value was 0.25 for the lower sweep rates (≤ 0.010 Vs^{-1}), suggesting that the reaction kinetic was controlled by the catalytic reaction. For higher sweep rates (≥ 0.25 Vs^{-1}), it can be concluded from the slope value corresponding to 0.5 that the reaction kinetic was controlled by diffusion. The E *vs*. log v curves showed that for sweep rates up to 0.05 Vs^{-1} the peak potentials were independent of the sweep rate demonstrating a reversible electron transfer, while, between 0.05 and 1 Vs^{-1}, an irreversible electron transfer was noticed. The slope value of log i *vs*. log v curve for peak C (Figure 4.7C) in the entire sweep rate region was 0.53. Considering this value, it can be concluded that the limiting step

was diffusion. The relation between E and log v, for peak C, was identical to that observed for peak A and B, indicating an irreversible process. The calculated number of electrons, 1.1 and 0.99 for peaks B and C, which was approximately 1, showed that regardless of the eight electrons involved in the oxidation of acetate, redox mediators transfer one electron at a time [12, 28]. For peak A, it was not possible to calculate the number of electrons probably due to the existence of mixed processes control.

Table 4.1. Electrochemical data obtained during biofilm formation at 22 °C and 35 °C. (I – Irreversible process).

25 °C						
	Peak A (rev/slope)		Peak B (rev/slope)		Peak C (rev/slope)	
0 days	I	0.89	I	0.49	I	0.53
				Peak D		
18 days				I	0.50	
39 days				I	0.53	
35 °C						
	Peak A' (rev/slope)		Peak B' (rev/slope)		Peak C' (rev/slope)	
0 days	I	0.83	I	0.52	I	0.50
				Peak D'		
22 days				I	0.47	
39 days				I	0.52	
53 days				I	0.54	

At 35 °C (Figure 4.7D), the slope value of the log i *vs.* log v curve in the entire sweep rate region for peak A' was 0.83, demonstrating that this process was limited by both diffusion and adsorption step (mixed process). At the same temperature (Figure 4.7E and F), the slope value of the log i *vs.* log v curve in the entire sweep rate region for peak B' was 0.52 and for peak C' was 0.49. Considering these values, it can be concluded that the limiting step in the case of peaks B' and C' was also the diffusion step. The relation between Ep and log v showed that the peaks potential were dependent of the sweep rate demonstrating an irreversible electron transfer. The calculated numbers of electrons 1.2 and 0.8 for the peaks B' and C' were similar to those obtained at 22 °C.

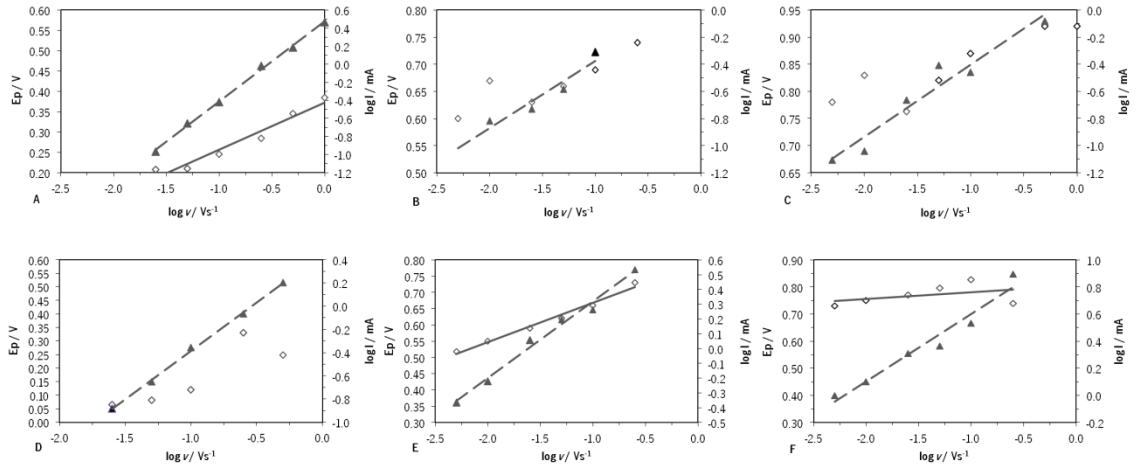

Figure 4.7. log I *vs.* log v (▲) and E versus log v (◊) curves for the oxidation peaks of a pure culture of *G. sulfurreducens* immediately after inoculation at 22 °C, peak A (A), peak B (B) and peak C (C) and 35 °C, peak A' (D), peak B' (E) and C' (F).

4.1.3.3.2. Biofilm of *G. sulfurreducens*

The voltammograms of carbon Toray after 39 days of biofilm growth at 22 °C for different scan rates are given in Figure 4.8. For lower sweep rates, a polarographic wave like oxidation was observed. At 5 mVs^{-1}, the oxidation started at 0.09 V *vs.* SCE and gradually increased to reach 5.5 mA at 0.470 V. This result shows that in biofilm different processes may occur in different active centres. In similar conditions, Wei *et al.* [22] presented an oxidation peak at the maximum potential of - 0.09 V *vs.* Saturated Hydrogen Electrode (SHE), which correspond to -0.33 V *vs.* SCE. The difference between both potentials with reference to SCE was higher, but the current density was lower (1.8 mA). This difference may due to the higher influence of diffusion limitation in the present study. Comparing the oxidation potential peaks obtained in the present study with published data for different types of cytochromes, OmcB can be established as the mediator responsible for heterogeneous electronic transference [12, 32].

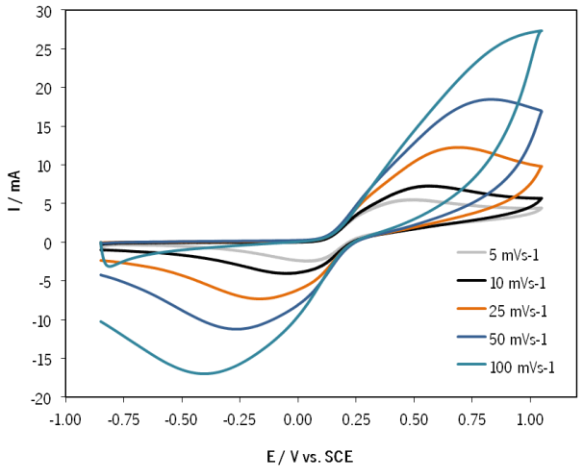

Figure 4.8. Voltammograms at different scan rates of carbon Toray with a *G. sulfurreducens* biofilm in the 39th day of growth at 22 °C.

In both biofilms, the shape of peak E suggests that this peak probably includes two oxidation processes attributed to peaks B and C observed in the presence of suspended bacteria. The data for these peaks were calculated by deconvolution using OriginPro 7.5 (OriginLab corporation). Figure 4.9 shows log i *vs.* log v curves for peaks B and C at 22 °C after deconvolution. For both peaks, the slope value was close to 0.5, for the entire sweep region, indicating that the kinetic was mainly diffusion controlled. This fact can be explained by limitation due to the diffusion of protons which are generated from acetate oxidation, acting as charge compensating ions in homogenous electron transfer within the biofilm [1]. From the E *vs.* log v curve it can be concluded that the electron transfer was irreversible. At 35 °C the results obtained were similar. The processes were controlled by diffusion and exhibited irreversible behaviour.

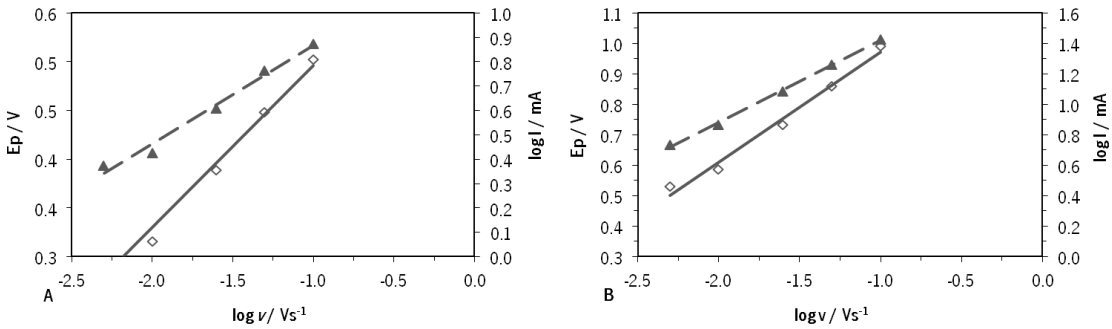

Figure 4.9. log i versus log v (▲) and E versus log v (◊) curves for the deconvoluted oxidation peaks, B (A) and C (B) of a *G. sulfurreducens* biofilm in the 39th day of growth at 22 °C.

4.1.4. Conclusions

These results contributed to a better understanding of the reaction mechanisms for acetate oxidation by *G. sulfurreducens*. In the presence of *G. sulfurreducens* an increase of current intensities was observed between - 0.5 V and 0.89 V *vs*. SCE with three well defined oxidation peaks, peak A, B and C at 0.16 V, 0.6 V and 0.8 V *vs.* SCE respectively. The processes occurring at lower anodic potentials, corresponding to a mediated heterogeneous electron transfer, can be attributed to the *c*-type cytochrome OmcB. The velocity of this process was enhanced by an increase of the temperature. From the voltammetric study it was concluded that the rate of the oxidation reactions of *G. sulfurreducens* biofilms in the logarithmic and stationary phases was limited by the diffusion. A mixed control with contributions of adsorption and diffusion was observed for *G. sulfurreducens* immediately after inoculation. The limitation of the reaction rate by diffusion and the calculated number of electrons which was around 1 may be explained by a transfer of one electron at a time through electron transfer mediators.

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4.2. Influence of the temperature in the outer membrane proteins expression and electrochemical behavior of *Geobacter sulfurreducens*

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Geobacter sulfurreducens has the ability to transfer electrons directly to an electrode. Different bacterial growth conditions, such as temperature, may influence the prevalence of certain proteins responsible for electron transport in the outer membrane. The aim of this work was to evaluate the influence of the growth temperature on the expression of outer membrane proteins and consequently on the kinetic parameters of the charge transfer reaction between the bacteria and the electrode surface.

G. sulfurreducens, cultured at 25 °C and 37 °C, was collected in the exponential phase for outer membrane protein extraction and cyclic voltammetry analysis. The proteins were analysed by two-dimensional gel electrophoresis. The results obtained at both temperatures showed that 13 proteins were differentially expressed. The identified proteins were related to membrane permeability, structural integrity, protein's synthesis and energy generation. From the identified proteins, only the LamB porin family was overexpressed at 37 °C. Cyclic voltammetry was used to compare the electrochemical behaviour of *G. sulfurreducens* grown at both temperatures. The results showed a dependency of the electrochemical behaviour of the bacteria, on its growth temperature. Three well-defined peaks were observed in voltammograms of bacteria grown at 25 °C while only two peaks were observed at 37 °C. The oxidation processes characteristic of *G. sulfurreducens* grown at 25 °C presented a mixed kinetic control and showed an irreversible behaviour in most sweep rates. At 37 °C, two distinct irreversible processes were observed, one controlled by diffusion and another by adsorption.

Keywords: Cyclic voltammetry, *Geobacter sulfurreducens*, Outer membrane proteins, Redox kinetics.

4.2.1. Introduction

Electroactive bacteria can use solid extracellular electron acceptors as final acceptors during respiration of organic compounds in anaerobic conditions. A good example of such kind of bacteria is *Geobacter sulfurreducens* that can transfer electrons directly to both reduce iron (III) oxides in the environment and transfer electrons directly to electrodes [1-3] . The Microbial Fuel Cell technology (MFC) is a novel approach for the production of electricity by electroactive bacteria [4]. High current densities were obtained in MFC inoculated with *G. sulfurreducens* when compared with other pure bacterial cultures [5, 6]. The understanding of the electron transfer mechanisms between the electroactive bacteria and the anode surface will certainly allow the optimization of this new and promising biotechnology for simultaneously treatment of contaminating waters and generation of electricity. The electron transfer from *G. sulfurreducens* to iron (III) oxides has been largely studied [7, 8] however, the charge transfer between the bacteria and the electrode is still misunderstood [9].

G. sulfurreducens does not require exogenous mediators to transfer electrons to extracellular electron acceptors, although two main mechanisms for electron transfer have been proposed: i) direct extracellular electron transfer (DEET) via redox-active proteins on the outer membrane surface [10-13], and ii) long-range electron transfer via pili [1, 9]. Several genetic studies suggested that outer membrane c-type cytochromes are involved in electron transfer to extracellular iron(III) oxides showing that iron (III) reduction is inhibited when the genes for these cytochromes are deleted. OmcB is an outer-membrane cytochrome present in *G. sulfurreducens* required for the reduction of soluble chelated iron (III) as well as insoluble iron (III) oxides [14]. OmcS and OmcE, two outer membrane c-type cytochromes, are required for growth of *G. sulfurreducens* on iron (III) and Manganese (IV) oxides [15]. The studies above mentioned suggested that electron transfer to iron (III) oxide requires additional and/or different proteins than those involved in the electron transfer to soluble chelated iron (III) [16]. In most recent studies, the deletion of the gene OmcZ greatly inhibited current production [17]; this fact corroborates the idea that the cytochromes *c*-type are required to facilitate electron transfer between bacteria and the anode surface [18] either directly or via pili in long distances. Co-immunoprecipitation studies showed that OmcS interacts with several proteins, namely OmcZ cytochromes [19]. This study suggested that the expression of other outer

membrane proteins (OMP) could cooperate and influence the electron transfer to electrodes. Gene expression patterns of current-consuming bacteria are very different from those corresponding to current-producing bacteria. The deletion of the genes involved in current production had no impact on consumption [20]. These results suggested that the routes for electron transfer from electrodes to bacteria may be different from those involved in current production [9].

According to literature, the electricity produced by *G. sulfurreducens* biofilms increased with biofilm thickness, which means that most of the cells contributed to the production of electricity without being in direct contact with the anode surface [11, 18]. It was suggested that long range electron transfer through anode biofilms takes place via conductive pili. The deletion of the gene PilA (responsible for the pili expression) caused only a decrease in power production without a complete inhibition due to the continuous transfer of the electrons from the cytochromes. Contrarily, the deletion of some outer membrane *c*-type cytochrome genes completely inhibits power production [11]. Thus, the expression of pili is favourable for the optimization of the electricity produced in a MFC. The formation of pili on the outer membrane of *G. sulfurreducens* cells could be induced during growth if the bacteria is cultured with acetate as electron donor and fumarate as electron acceptor at the suboptimal temperature of 25 °C [21].

The study of the relation between the expression of *G. sulfurreducens* OMP at different growth temperatures may provide some insights regarding the optimization of electron transfer processes and consequently electricity production in MFC. In fact, this approach was already used to describe the electron transfer mechanism underlying *G. sulfurreducens* grown in fumarate or soluble iron (III) reduction in batch and chemostat conditions [22], as well as to compare protein abundance in *G. sulfurreducens* grown on poorly crystalline iron (III) oxide or on soluble iron (III) citrate [16].

The OMP expressed at different growth temperatures may influence the electrochemical behaviour of the bacteria. The cyclic voltammetry (CV) is an electrochemical technique that can be used to assess the redox reactions between microorganisms and electrodes [23-29]. The application of a continuously time-varying potential to the working electrode results in the occurrence of redox reactions of electroactive species in the solution (faradaic reactions). The electroactive species might adsorb to the electrode, according to the applied potential,

originating a capacitive current due to double layer charging [30]. Previous electrochemical studies, confirm that *G. sulfurreducens* acts as catalysts for acetate oxidation, transferring the electrons directly to graphite anodes [31]. A recent study reported that the oxidation potential applied on electrodes influences the oxidation of those cytochrome proteins for which an energetically favourable interaction between electrode material and bacterial cells can be established [32].

The present work aims to study the influence of the growth temperature on the expression of the OMP of *G. sulfurreducens* and its effect in the electrochemical behaviour of these bacteria on carbon electrodes.

4.2.2. Materials and methods

4.2.2.1. Geobacter sulfurreducens growth

Geobacter sulfurreducens (DSM 12127) was obtained from DSMZ (Braunschweig, Germany) and cultured as suggested by Caccavo *et al*. [33]. *G. sulfurreducens* was grown in 100 mL bottles sealed with leak proof sealings, to ensure anaerobic sterilized microenvironment, at 25 °C and 37 °C with sodium acetate as electron donor and sodium fumarate as electron acceptor. The growth medium was prepared in ultra-pure water with the following composition per litre: 1.5 g of NH_4Cl, 0.6 g of Na_2HPO_4, 1.5 g of NaH_2PO_4, 0.1 g of KCl, 0.8 g of Na-acetate, 8.0 g of Na_2-fumarate, 2.5 g of $NaHCO_3$, 10 mL of vitamins solution (in a 1 L solution: 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyridoxine - HCl, 5.0 mg thiamine HCl . 2 H_2O, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg d-Ca-pantothenate, 0.1 mg vitamin B_{12}, 5.0 mg p-aminobenzoic acid, 5.0 mg lipoic acid), 10 mL of trace metals solution (in a 1 L solution: 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4 \cdot 7\ H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1.0 g NaCl , 0.1 g $FeSO_4 \cdot 7\ H_2O$, 0.18 g $CoSO_4 \cdot 7\ H_2O$, 0.1 g $CaCl_2 \cdot 2\ H_2O$, 0.18 g $ZnSO_4 \cdot 7\ H_2O$, 0.01 g $CuSO_4 \cdot 5\ H_2O$, 0.02 g $KAl(SO_4)_2 \cdot 12\ H_2O$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4 \cdot 2\ H_2O$, 0.025 g $NiCl_2 \cdot 6\ H_2O$, 0.3 mg $Na_2SeO_3 \cdot 5\ H_2O$) and 1 mL of a selenio-tungstate solution (in a 1 L solution: 0.5 g NaOH, 3 mg $Na_2SeO_3 \cdot 5\ H_2O$, 4.0 mg $Na_2WO_4 \cdot 2\ H_2O$). All components (except $NaHCO_3$ and Na_2-fumarate) were dissolved by heating the medium until boiling, cooling down to room temperature with ice, and simultaneously, bubbling a gas mixture N_2-CO_2 (80:20) from PraXair® in the solution to assure anaerobic conditions. Then, $NaHCO_3$ was added and the

pH was equilibrated at 6.8 with the gas mixture. The growth medium without fumarate was autoclaved for 20 min at 121 °C. Afterwards, the electron acceptor (50 mM of sodium fumarate) was added from a stock solution using a sterilized filter (0.22 μm) (medium 826 adapted from DSMZ method).

4.2.2.2. Proteomics

Preparation and analysis of *G. sulfurreducens* outer membrane protein extracts

Two cultures of *G. sulfurreducens* corresponding to growth temperatures of 25 °C and 37 °C respectively were centrifuged (4000 x *g* for 15 min at 4°C) during the exponential growth phase in order to collect the cells. After centrifugation, the pellet was diluted in Tris-HCl and disrupted by sonication (Ultrasonic Processor, Cole-Parmer, USA). The soluble and insoluble fractions were collected by centrifugation at 257000 x *g* for 60 min at 4°C [19]. The insoluble fraction, containing cytoplasmic membrane and outer membrane proteins (OMP), was separated by solubilisation of the first fraction in 1% (w/v) in sodium laurylsarcosine [34, 35]. The membrane proteins were resuspended in 50 mM Tris-HCl (pH 7.5) and loaded on a sucrose gradient (30 %, 50 %, and 70 %) in the same buffer and centrifuged at 82500 x *g* for 17 h at 4 °C in a Beckman SW28 rotor [16]. OMP layers were collected and diluted in 50 mM Tris-HCl (pH 7.5). The OMP were ultracentrifuged at 257000 x *g* for 60 min and the supernatant was collected and resuspended in the same buffer.

The protein concentration was measured according to the Lowry method [36], modified by Peterson [37], using the Sigma protein assay kit (Sigma Diagnostics, St Louis, MO, USA).

Two-dimensional gel electrophoresis

OMP patterns were analysed by two-dimensional gel electrophoresis (2-DE). Two hundred micrograms of proteins were added to isoelectric focusing buffer (IEF) composed of 8 M urea, 65 mM CHAPS, 10 mM DTT, 0.8 % w/v carrier ampholytes (pH 3.5–10; Sigma, St. Louis, MO, USA) with final volume, 300 μL [38]. The first-dimension gel separation was carried out with Immobiline Dry Strips L (pH 4–7, Amersham Pharmacia Biotech). The second dimension was obtained by SDS–PAGE using a 12.5 % (w/v) polyacrylamide resolving gel (width 16 cm,

length 20 cm, thickness 0.75 mm). After migration, proteins were visualized by silver nitrate staining [39].

2-DE Gel analysis

Spot quantification was achieved by computing scanning densitometry (ProXPRESS 2D, PerkinElmer Sciex). Gels were analysed using the Progenesis Samespot (Nonlinear Dynamics) software. For each experimental condition, at least three 2-DE gels of each condition were matched together to form a reference image. The two reference gels were then matched together so that the same spot in different gels had the same number. Protein spots from the two cultures of *G. sulfurreducens* were considered to display significant quantitative differences if they fulfilled the following criteria: p values ≤ 0.05 (t-test); detection threshold, average volume ≥ 20 (n = 3); differential tolerance, fold change ≥ 2 [40]. It was also considered the q value (q ≤ 0.05) to guarantee that no false positives were erroneously analysed.

Protein identification

Selected spots excised from the polyacrylamide gel complied the following criteria: volume varying with the incubation condition and displaying a high (average) value with a low coefficient of variation. Gel plugs were then dried using a SpeedVac centrifuge. Trypsin digestion was performed using an automatic digester (MultiPROBE II, PerkinElmer Sciex). After lyophilisation, the peptide extracts were resuspended in 10 μL of 0.2 % formic acid/5 % acetonitrile. Peptides were enriched and separated using a lab-on-a-chip technology (Agilent) and fragmented using an on-line XCT mass spectrometer (Agilent). The fragmentation data were interpreted using the Data Analysis program (version 3.4, Bruker Daltonic).

Bioinformatic tools for subcellular location

For protein identification, MS/MS peak lists were extracted and compared to the NCBInr protein database restricted to *G. sulfurreducens*, using the MASCOT Daemon (version 2.1.3) search engine. All searches were performed with no fixed modification and allowed for carbamidomethylation, oxidation and a maximum of one missed trypsin cleavage. MS/MS spectra were searched with a mass tolerance of 1.6 Da for precursor ions and 0.8 Da for fragment ions, respectively. If a protein was characterized by 2 peptides with a fragmentation

profile score higher than 25 the protein was validated. When one of the criteria was not met, peptides were systematically checked and/or interpreted manually to confirm or cancel the MASCOT suggestion.

4.2.2.3. Electrochemical Set up

A thermostated three-electrode glass cell with two compartments separated by an ion exchange membrane (Nafion 117, DuPont Co., USA) was used as electrochemical cell for the voltammetric study. A saturated calomel electrode (SCE) was used as reference electrode, a carbon Toray sheet (E-TEK division, USA) was used as working electrode and a platinum:iridium foil, 90:10, as counter electrode. The carbon Toray sheet (3 cm x 3 cm) was glued to a platinum wire (Alfa Aesor, Germany) using conductive carbon cement (Fluka, Sigma-Aldrich) and dried at room temperature during 24 h. Before each experiment, the anodic and cathodic chambers were filled with the supporting electrolyte (KCl, 0.1 M) and deaerated with pure argon (U Quality from L'Air Liquid). Prior to each experiment, the cleanness of the surfaces and solutions were tested by recording voltammograms in the supporting electrolyte and in medium alone. The electrochemical instrumentation consisted of a potentiostat/galvanostat from Amel Instruments coupled to a microcomputer through a AD/DA converter. The Labview software (National Instruments) and a PCI-MIO-16E-4 I/O module were used for generating and applying the potential program as well as acquiring data such as current intensities.

4.2.2.4. Electrochemical measurements

Cyclic voltammetric studies were carried out with carbon Toray anodes in the presence of enriched cultures of *G. sulfurreducens* (150 mgL^{-1} of protein) in the logarithmic growth phase, cultured at 25 °C and 37 °C. The Na-acetate concentration of the medium was adjusted to 20 mM with a stock solution using a sterilized filter (0.22 μm). In the cathode chamber, a supporting electrolyte (KCl 0.1 M) was used. The electrochemical assays were carried out at room temperature, in anaerobic conditions; the Argon stream was maintained over the solution during the measurements.

4.2.3. Results and Discussion

4.2.3.1. Differential protein expression in 2-DE gels

Different growth conditions may induce the expression of particular proteins that play important roles in the electron transfer process. In this work, the differential expression of *G. sulfurreducens* outer membrane proteins (OMP) was assessed at two distinct growth temperatures (25 °C and 37 °C).

The protein expression of *G. sulfurreducens* grown at 25 °C and 37 °C is shown in Figure 4.10 and protein identification is listed in Table 4.2. The experimental OMP characterization discriminated 13 proteins with $p<0.05$ and fold>2.

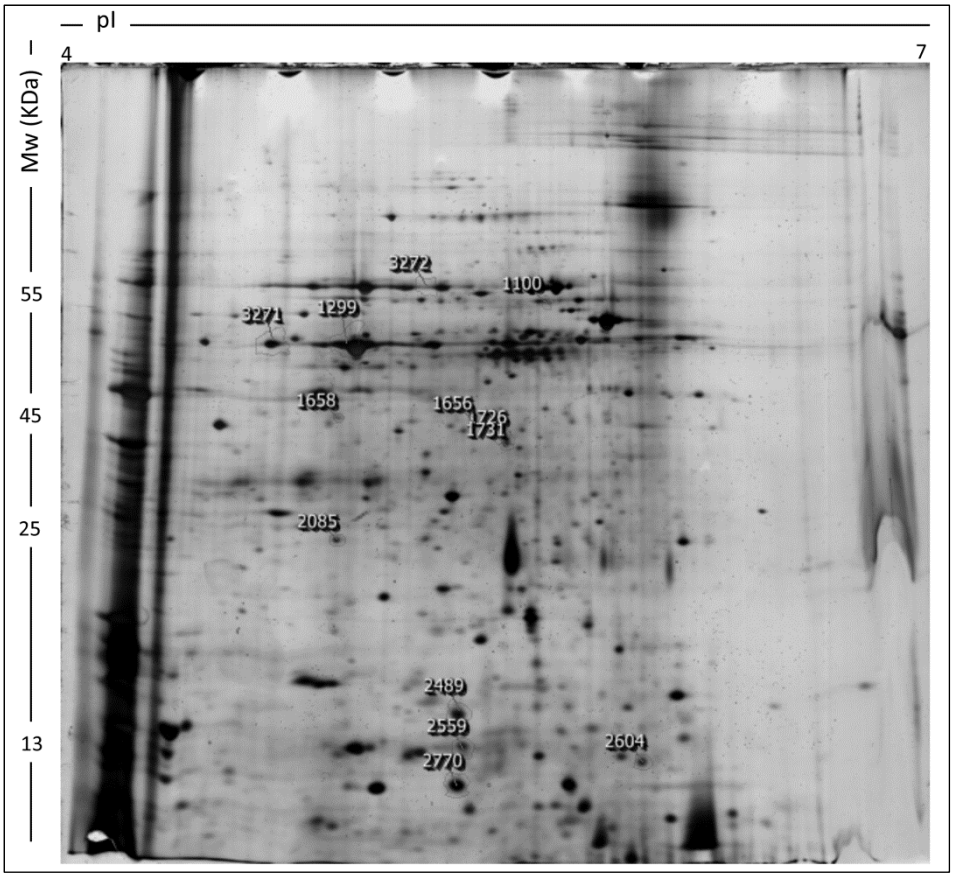

Figure 4.10. Comparison of the proteomes of outer membrane *G. sulfurreducens* cultured at 25 °C and 37 °C showing magnification views of the 13 identified protein spots whose intensity was altered in 25 °C cultured bacteria compared to 37 °C cultured ones. Gels were stained by silver nitrate.

The experimental OMP characterization discriminated 13 proteins differentially expressed ($p<0.05$ and fold>2) (Table 4.2) in *G. sulfurreducens* grown at different temperatures.

Table 4.2. Outer membrane proteins identified in *Geobacter sulfurreducens* cultured at 25 °C and 37 °C.

No. spot	Protein	Mean spot volume		pI	Mw (KDa)	Cov. (%)	Peptide match	GSU
		25 °C	37 °C					
1100	OmpA domain-containing protein	**5.68E5**	2.58E5	5.19	46.02	43	23	0360
1299	LamB porin family protein	9.97E6	**1.77E7**	6.25	48.85	55	236	3304
1656	Cytochrome c	**7.22E5**	1.34E5	7.25	45.36	3	1	2504
1658	LysM domain-containing protein	**3.51E5**	8.76E4	6.87	36.51	27	7	2551
1726	Hypothetical protein GSU0617	**1.06E6**	3.84E5	6.17	39.91	38	18	0617
1731	Hypothetical protein GSU0617	**5.47E5**	1.89E5	6.17	39.91	42	16	0617
2085	ABC transporter, ATP-binding protein	**9.72E5**	2.59E5	5.68	25.03	38	12	2686
2489	LamB porin family protein	**2.71E6**	1.17E6	6.25	48.85	49	36	3304
2559	OmpA domain-containing protein	**2.18E6**	1.03E6	5.54	20.79	25	11	0024
2604	50S ribosomal protein L7/L12	**1.89E6**	6.23E5	4.74	13.106	45	4	2864
2770	Hypothetical protein GSU3244	**6.08E6**	1.93E6	6.29	14.92	35	10	3244
3271	LamB porin family protein	5.41E6	**1.49E7**	6.25	48.85	68	99	3304
3272	F0F1 ATP synthase subunit alpha	**2.71E6**	1.41E6	5.72	54.72	44	30	0111

In the present study, the proteins OmpA domain-containing protein, LamB porin family protein and hypothetical protein GSU0617 were found as mass isoforms. The isoforms exhibited different apparent molecular masses and pI values, that reflect posttranslational modifications

[41]. The range of the membrane proteome alterations following growth at different temperatures is very low (about 3 %) i.e. 13 on 400 spots, which were discriminated on 2-DE gels. All the identified proteins are related with the outer membrane of *G. sulfurreducens* confirming a high efficiency in the OMP extraction protocol.

From the identified proteins, only the LamB porin family protein was underexpressed in bacteria that grew at 25 °C. Proteins of the LamB superfamily comprise channels involved in the diffusion of specific nutrients, like maltose and maltodextrins in *E. coli* and also facilitate the influx of various carbohydrates present in low concentrations in the extracellular environment [42]. LamB also functions as a glycoprotein in conditions of carbohydrate limitation [43]. Here, we show that the overexpression of this porin in *G. sulfurreducens* is not dependable on the carbohydrates in the surrounding environment and can be altered when the bacteria grows at 37 °C. This fact may represent that when *G. sulfurreducens* grows at higher temperatures its membrane permeability can be enhanced for the carbohydrates transport when nutrient concentration is insufficient.

Results from Table 4.2 also show that all OMP identified, with the exception of LamB, were overexpressed at 25 °C. The OmpA domain-containing protein is an OMP involved in the membrane biogenesis that belongs to the super-family of the Surface_Ag2 (Surface antigen). The function of this protein in *G. sulfurreducens* is not clear yet, however, the homologous protein in *E. coli* combined with the murein lipoprotein and peptidoglycan-associated protein contribute to the structural integrity of the outer membrane [44].

The LysM domain-containing protein, with transferase activity was also overexpressed in *G. sulfurreducens* grown at 25 °C. This protein is involved in bacterial cell wall degradation and may have a general peptidoglycan binding function [45, 46].

Two hypothetical proteins GSU0617 and GSU3244 were also overexpressed in bacteria that grew at 25 °C, and do not have any defined function in *G. sulfurreducens*.

The protein 50S ribosomal protein L7/L12 is a binding site for several of the factors involved in protein synthesis and appears to be essential for accurate translation. This ribosomal protein in association with some factors produces GTP hydrolysis-derived energy and is an example of a mechanochemical system, or molecular motor. Its quaternary structure has been conserved in eubacteria, eukaryotes, and archea and it is very well studied in some

organisms because the removal of this protein reduces the rate of protein synthesis [47]. The overexpression of this protein in *G. sulfurreducens* grown at 25 °C when compared to that at 37 °C might indicate a reduction in the synthesis of this protein at higher temperatures.

All the previously described proteins, with the exception of proteins with unknown function, are related with the membrane structure and functionality thus, the temperature of 25 °C seems to favor the *G. sulfurreducens* growth.

Some other proteins related with energy generation were also found overexpressed in *G. sulfurreducens* grown at 25 °C compared to 37 °C. The ABC transporter, ATP-binding protein, is a protein involved in the lipid A export and possibly in glycerophospholipid export and also in the biogenesis of the outer membrane. The transmembrane domains form a pore in the inner membrane and the ATP-binding domain is responsible for energy generation [48].

The F0F1 ATP synthase subunit alpha is a membrane-bound enzyme complex and ion transporter that combines ATP synthesis and/or hydrolysis with the transport of protons across the membrane. ATP-synthase is a multisubunit enzyme composed of two major parts: the F0 portion, an integral membrane complex involved in the translocation of protons, and the F1 portion, a large extramembrane complex [49]. This type of enzyme uses the proton gradient generated by the respiratory chain to synthesize ATP at the concentrations necessary to drive many of the cellular energy-requiring processes [49]. In a study of Ding *et al*. [16], F0F1 ATP synthase, that catalyzes the reactions of respiratory metabolism, were also found underexpressed during *G. sulfurreducens* growth on iron (III) oxides.

Cytochrome c is a well-described protein in *G. sulfurreducens* since it is a key protein for electron transport. Holmes *et al*. [11] suggested that this type of cytochrome is essential for electron transfer to electrodes. He also found that the expression of genes encoding this type of proteins is higher in cells growing on electrodes than that observed in cells growing with iron (III) citrate, a soluble electron acceptor. In this work, we showed that the cytochrome C expression is augmented when *G. sulfurreduscens* grows at 25 °C compared to 37 °C. This fact can change the electron transfer efficiency to electrodes. According with Ding *et al*. [16], when the genes for these cytochromes are deleted iron (III) reduction or electricity production is inhibited. Also, the presence of these types of proteins at the cell surface enhances the contact with extracellular electron acceptors. Furthermore, some cytochromes are also able to

accept electrons from inner membrane electron transfer components, acting as capacitors, and allowing electron transfer when *Geobacter* species are not grown in contact with iron (III) oxides [16].

The proteomic results showed that the growth temperature influences the expression of some OMP's related to electron transfer during respiration: namely, cytochrome c, F0F1 ATP synthase and ABC transporter ATP-binding protein were overexpressed in *G. sulfurreducens* grown at 25 °C. The other overexpressed proteins related to the membrane structure and functionality, at 25 °C, can be related to the growth of pili at suboptimal temperature as was suggested by Reguera *et al.* [21]

4.2.3.2. Electrochemical behaviour of *G. sulfurreducens* cultured at different temperatures

In order to study the difference in the electrochemical behaviour of *G. sulfurreducens* grown at two distinct temperatures (25 °C and 37 °C), cyclic voltammetry assays were carried out at 50 mVs^{-1}. The voltammograms of carbon Toray, in growth medium with and without *G. sulfurreducens*, at both 25 °C and 37 °C, are given in Figure 4.11 The voltammogram in growth medium alone at room temperature (Figure 4.11 – Blue line) exhibited an oxidation peak at 0.57 V *vs.* SCE with a moderate current intensity (0.273 mA) and a reduction peak at 0.22 V *vs*. SCE. The redox process observed with culture medium without *G. sulfurreducens* was probably due to the presence of trace minerals existing in the medium. This fact was confirmed by the voltammetric study of the individual components of the medium (data not shown).

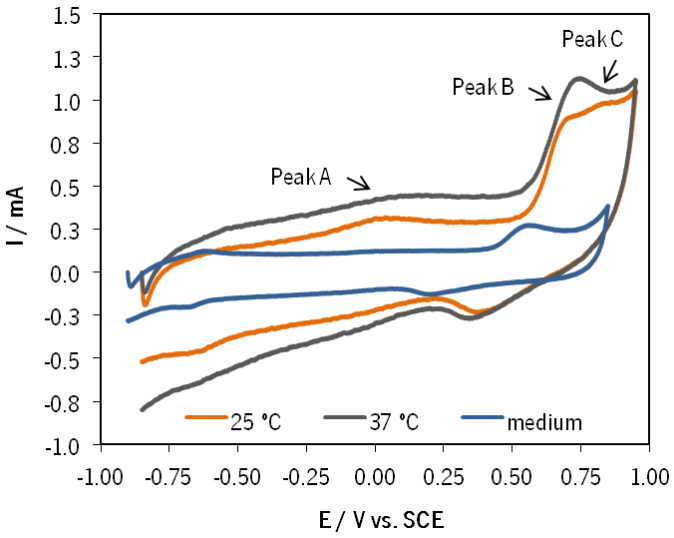

Figure 4.11. The voltammograms of carbon Toray in growth medium without (blue line) and with *G. sulfurreducens* cultured at 25 °C (orange line) and 37 °C (grey line) (0.05 Vs^{-1}; 150 mgL^{-1} of bacteria; pH 7; 20 mM acetate).

After the addition of *G. sulfurreducens*, an increase of the overall current intensities was noticed for the potential region between - 0.3 V and 0.9 V *vs.* SCE. For the bacteria cultured at 25°C, three peaks of oxidation during the positive sweep and one peak of reduction during the negative sweep were observed at 0 V (peak A), 0.69 V (peak B), 0.82 V (peak C) and 0.39 V (peak D) *vs.* SCE, respectively. The corresponding current intensities for the peaks A, B, C and D were respectively 0.31 mA, 0.87 mA, 0.98 mA and -0.226 mA (Figure 4.11 – orange line). For bacteria cultured at 37°C, the oxidation behaviour was different. Two oxidation peaks at 0.08 V (peak A') and 0.74 V (peak B') *vs.* SCE, respectively, were observed for this temperature (Figure 4.11 – grey line). The reduction peak D', with a current intensity of -0.27 mA, was noticed at 0.37 V *vs.* SCE. The three distinct oxidation peaks detected at 25 °C, can be attributed to independent or successive oxidation processes, which reveal the existence of three distinct redox couples, suggesting that different redox species present in the bacteria contribute to the current intensities [20, 25]. The oxidation currents observed in a large potential region are in agreement with the existing data on the availability of different groups of cytochromes in bacteria, which can be oxidized at different potentials [22]. The increase of the growth temperature from 25 °C to 37 °C increased the maximum current intensity of the peak B, making the peak C imperceptible in this case due to the overlap between these two

adjacent peaks. It can be concluded from these results that at 37 °C the process that occurs at lower potentials (peak B'), with a higher reaction facility, was privileged.

Comparing these results with the obtained in proteomic analyses, it was possible to verify that bacteria grown at 25 °C present more oxidations steps, 3 well defined peaks, when compared with bacteria grown at 37 °C, what is in agreement with the overexpression of proteins responsible for the respiratory process and energy production. Considering the suboptimal temperature of 25 °C required for pili production by *G. sulfurreducens*, it was expected higher current intensities of the peaks at this temperature. However, higher peak current intensities were obtained for *G. sulfurreducens* grown at 37 °C. This result may reflect that the voltammetric experiment was carried out in a bacterial suspension, in a short time interval, not allowing the biofilm formation on the anode. In this case, the presence of pili in suspended bacteria did not increase the current intensity.

4.2.3.3. Determination of the kinetic parameters

The kinetic parameters of the reactions were determined by cyclic voltammetry. A study was carried out in a sweep rate region between 5 mVs^{-1} and 1000 mVs^{-1} to investigate the rate-limiting step of the oxidation reactions in bacteria cultured at two temperatures (25 °C and 37 °C). The slope of the straight lines of the log i *vs.* log v, [(i), peak current intensity in mA and (v), potential sweep rate in mVs^{-1}] allowed to gain insights regarding the reaction limiting step. Slope values close to 0.5 indicate that the reaction is mainly diffusion controlled, while a slope value of 1.0 shows that adsorption is the limiting step [50]. To determine the reversibility of the charge transfer, the dependence of peak potential (Ep) on the log (v) was evaluated; the peak potential is dependent on scan rate for irreversible processes and independent in the case of reversible processes [50]. The curves representing the log i *vs.* log v and Ep *vs.* log v are depicted in Figures 4.12 and 4.13. A summary of the kinetic parameters of the redox reactions of *G. sulfurreducens* cultured at 25 °C and at 37 °C is presented in Table 4.3.

Table 4.3. Summary of electrochemical data of *Geobacter sulfurreducens* cultured at 25 °C and at 37 °C. (I – irreversible process; R – reversible process).

	Peak A			Peak B			Peak C		
	Ep 0.05 Vs^{-1}	Slp	R	Ep 0.05 Vs^{-1}	Slp	R	Ep 0.05 Vs^{-1}	Slp	R
25 °C	0 V	0.82	I	0.69 V	0.65	R/I	0.82 V	0.83	R/I
37 °C	0.08 V	0.99	I	0.74 V	0.50	I	-	-	-

4.2.3.3.1. *Geobacter sulfurreducens* cultured at 25 °C

For *G. sulfurreducens* grown at 25 °C, the slope value of the log i *vs.* log v curves in the entire sweep rate region for peaks A, B and C was respectively 0.82, 0.65 and 0.83, showing that these processes were limited by both diffusion and adsorption steps (mixed processes). For the peak A, the relation between E and log v (Figure 4.11A/Table 4.3) showed that the peak potential was dependent of the sweep rate revealing an irreversible electron transfer. At lower sweep rates, the peak potential of the peaks B and C were independent of the sweep rate depicted reversible processes, for higher sweep rates (≥ 0.05 Vs^{-1}), the peaks potentials exhibited irreversible behaviour.

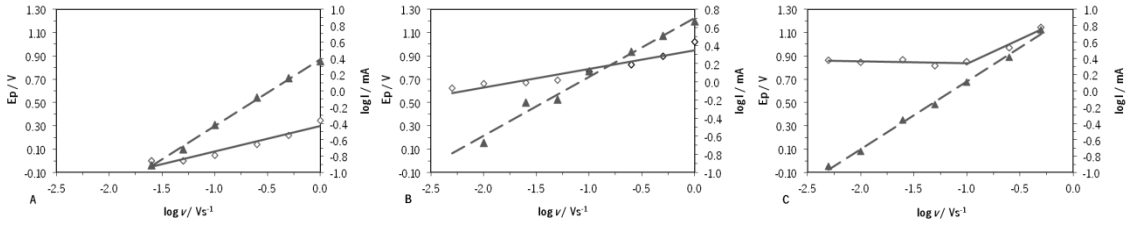

Figure 4.12. log I *vs.* log v (▲) and E versus log v (◊) curves for the oxidation peaks of a pure culture of *G. sulfurreducens* cultured at 25 °C, peak A (A), peak B (B) and peak C (C).

4.2.3.3.2. *Geobacter sulfurreducens* cultured at 37 °C

For *G. sulfurreducens* grown at 37 °C, the kinetic processes were different from those observed when the bacteria grew at 25 °C. In fact, the slope value of the log i *vs.* log v curve in the entire sweep rate region for peak A' was 0.99, suggesting that this process was limited by adsorption. For peak B', the calculated slope value was 0.50 suggesting that the reaction kinetic was controlled by diffusion. The E *vs.* log v curves in the entire sweep rate region for both cultures showed that the peak potentials were dependent of the sweep rate demonstrating irreversible electron transfer mechanisms.

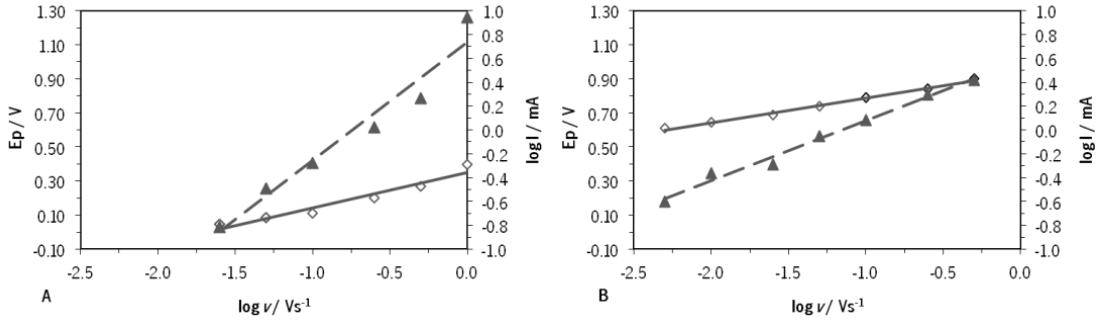

Figure 4.13. log I *vs.* log v (▲) and E versus log v (◊) curves for the oxidation peaks of a pure culture of *G. sulfurreducens* cultured at 25 °C, peak A (A), peak B (B) and peak C (C).

With the electrochemical study of the kinetic parameters it was possible to observe that in agreement with the previous results, the growth temperature also influences the limiting steps of the different oxidation processes observed in the voltammograms. The most evident difference observed in the two cultures of *G. sulfurreducens*, was the definition of the processes. Bacteria that grew at 25 °C present all the peaks limited by mixed processes whereas bacteria cultured at 37 °C present a peak limited by adsorption and another by diffusion. This limitation might be due to the overexpression of the LamB porin family protein in the outer membrane of the bacteria which are related to the diffusion of nutrients.

4.2.4. Conclusions

In this study it was possible to conclude that the growth temperature influences proteins expression in the outer membrane of *Geobacter sulfurreducens*. The differential expression of the outer membrane proteins is reflected in the electrochemical behaviour. The suboptimal temperature of 25 °C may induce the pili production, fact corroborated by proteomic and electrochemical analyses. Finally, *G. sulfurreducens* grown at 25 °C was a better inoculum for biofilm formation on anodes of microbial fuel cells.

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Chapter 5

Microbial Fuel Cells: wastewater treatment application

5.1. Optimization of a filter-press type Microbial Fuel Cell for electricity production and wastewater treatment

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Microbial fuel cell (MFC) technology is a novel approach for the production of bioelectricity using organic compounds (e.g. wastewater) in descentralized. In this context, a filter-press MFC was tested for electricity production and wastewater treatment. The cell, with 64 cm^2 of electrode area (carbon Toray) and 1 cm^3 of electrolyte volume, was operated with domestic wastewater in a batch mode (load 622 ± 10 mgL^{-1} COD) with recirculation in the anodic chamber. In the cathode compartment phosphate buffer with hexacyanoferrate was used. The results showed that the current density increased to 407 Wm^{-3} and this value was maintained during 4 days, resulting in a carbon removal of 83 %. Interruptions in the supply of fresh wastewater slightly decreased power density, while the increase/decrease of the recirculation did not influence power generation. The anode biofilm presented high conductivity, high activity and high diversity. The DGGE band-pattern of the DNA amplified for *Shewanellaceae* family showed the presence of several ribotypes which suggests the presence of different species or strains of *Shewanella*. This configuration is very compact and has a low internal resistance; thus, several units in series can increase the power density allowing the application of this technology for wastewater treatment and electricity generation.

Key Words: Carbon removal, Bioelectricity, Filter-press Microbial Fuel Cell.

5.1.1. Introduction

Municipal wastewater is characterized by a low concentration of organic carbon compared to industrial wastewaters and high volumes. Usually the activated sludge process is used in the treatment of municipal wastewater, consuming a lot of energy for aeration purposes [1, 2]. A novel approach for removing organic carbon from the wastewater under anaerobic conditions with the concomitant direct production of bioelectricity is based on the principal of the fuel cell, and is designated as Microbial Fuel Cell (MFC). The MFC might be an alternative treatment for low concentrated wastewater and presents considerable potential for small on-site applications in isolated and autonomous systems [3].

The MFC technology is based on the capacity of some carbon-oxidizing microorganisms to transfer electrons directly to an anode, thus generating electricity [4, 5]. This conversion is done through catalytic reactions operated by electroactive microorganisms under anaerobic conditions [6, 7]. Electroactive bacteria degrade/oxidize organic matter and have the particularity to transfer electrons, resulting from the respiratory process (where electrons are transferred gradually in a respiratory chain to store energy in the form of ATP), directly to external acceptors (instead of terminal electron acceptors, as for example oxygen, nitrate or sulfate) [8-11]. In the anodic chamber, electroactive bacteria, under anaerobic conditions, oxidize substrates and generate electrons as well as protons. The electrons are received by the anode and transported to the cathode through an external circuit; the protons are transferred to the cathode chamber by the proton exchange membrane (PEM) where they combine with oxygen to form water [5].

Among the electrochemically active microorganisms that can transfer electrons directly from the carbon source to an anode without the need of redox mediators, the most well known are *Shewanella putrefaciens*, a Gamma-proteobacterium [11, 12], *Geobacter sulfurreducens*, *Geobacter metallireducens* and *Desulfuromonas acetoxidans*, all Delta-proteobacteria [4, 11] and *Rhodoferax ferrireducens*, a Beta-proteobacterium [11, 13]. Other less studied electroactive bacteria are *Actinobacteria*, *Leptothrix* and *Desulfuromona* among others [14].

A conventional MFC consists of an electrochemical cell with anodic and cathodic compartments separated by a proton exchange membrane. Anode and cathode are

connected to each other using an external resistance. In a two-chambers MFC, the compartments can assume several designs, as for example the H-type MFC [15], the upflow MFC [16], the flat-plate MFC [17] among others. Other configurations are possible and were optimized to different applications, namely, the air-cathode MFC was used as a BOD sensor [18], single-chamber and tubular MFC were used in wastewater treatment [19, 20]. To date, the maximum potential reported in literature for a MFC was around 0.5 V to 0.8 V [21] and the power density varied in the range from 2.7 Wm^{-2} to 6.9 Wm^{-2}, which is similar to the power density generated in a hydrogen fuel cell [1, 22, 23]. A usual power optimization strategy consists in adapting the ratio electrode area vs. cell volume and the ratio anode area vs. cathode area. An increase of the surface area of the electrodes per reactor volume with a final cathode surface area of 280 m^2m^{-3} and a decrease of reactor volume with a final value of 2.5 mL permit to reach power densities of 1.55 kW m^{-3} with a MFC using oxygen as electron acceptor [24]. The use of a chemical electron acceptor, like ferricyanide or permanganate can also improve the cell output [25-27]. As an example, a volumetric power density of 2.15 kWm^{-3} was generated in a 0.335 mL reactor with a cathode area of 1920 m^2m^{-3} and ferric cyanide [28].

The main goal of the present work was to evaluate the feasibility of a filter press type MFC to simultaneously produce electricity and treat domestic wastewater. The specific objectives consisted in the assessment of the effect of recirculation flow rate and the competition caused by iron ions as soluble electron acceptor in the power density generated in the MFC.

5.1.2. Materials and methods

5.1.2.1. Wastewater collection and composition

Domestic wastewater was collected at a municipal wastewater treatment plant (Braga, Portugal), screened to remove coarse particles and, used as a source of organic matter and inoculum to start biofilm formation on the anode surface of the Microbial Fuel Cell (MFC). After collection, the wastewater was deaerated with nitrogen gas to establish anaerobic conditions, and kept at 4 °C for later use.

5.1.2.2. Microbial fuel cell description and operation

A Filter press type FM01-LC (laboratory-scale commercial), from Imperial Chemical Industries PLC (ICI) recently acquired by Akzo Nobel, was adapted as a two chambers microbial fuel cell (MFC). FM01-LC is a scaled-down version of the FM21-SP (21 dm^2) cell, used in the chloralkali production (chlorine and caustic soda). Carbon Toray supported on stainless steel sheets were used as anode and cathode without turbulence promoter meshes in order to provide more space for biofilm formation.

The volume and the electrode area for both anodic and cathodic compartments were respectively 1 cm^3 and 64 cm^2. The anolyte and the catholyte were separated by an ion exchange membrane (Nafion 117, DuPont Co., USA). Both solutions were pumped separately to the respective cell compartments by a two- heads Watson–Marlow peristaltic pump via Marprene tubs. The electrodes, both carbon Toray (E-TEK division, USA) sheet (4 cm x 16 cm), were pressed in stainless steel supports and connected to a resistance using copper coated wires (2 mm diameter).

To promote biofilm formation on the anode surface and consequently produce power density, domestic wastewater (with an average concentration of 622 ± 10 mgL^{-1} COD, 790 ± 20 μScm^{-1} of conductivity and pH 7 ± 0.2) was recirculated at 13 $mLmin^{-1}$ between the anode compartment and a 1 L stirred Erlenmeyer, at room temperature (≈ 22 °C). The cathodic chamber was filled with a phosphate buffer solution containing potassium hexacyanoferrate (50 mM) as electron acceptor which was also recirculated as described for the anodic chamber. The voltage difference between anode and cathode electrodes was measured across a fixed resistance of 1 kΩ every 30 min. Data were collected automatically and stored in a computer by a USB-9215A BNC connector datalogger (National Instruments) and a data acquisition software (Labview 6.0). The wastewater was replaced periodically to avoid organic carbon limitation. The biofilm formed on the anode is considered in steady-state when similar values of power density were obtained 3 times consecutively. Then, the power density production *vs.* carbon removal, polarization and power curves were obtained. The polarization curve, describing the voltage and the power density as a function of the current density [5, 29], was recorded using a series of resistances in the range of 31.1 kΩ to 10 Ω in the

beginning and in the end of the biofilm formation, without recirculation of the electrolytes. The internal resistance of the MFC (R_{int}) was calculated from the slope of the polarization curve in the region dominated by Ohmic losses [5, 29]. The open circuit voltage (OCV) was measured at infinite resistance.

The influence of the electrolyte recirculation break, flow rate and the presence of iron (III) oxides on power density was tested independently using wastewater with a composition identical to the one previously described. In order to evaluate the effect of the interruption of the recirculation, the pump was turned off in the stable phase of power generation for 2, 4 and 6 h. The power density was allowed to recover and reach the initial value between several tests. The effect of changing the recirculation flow (13 $mLmin^{-1}$) to higher (21.6 $mLmin^{-1}$) and lower (5.5. $mLmin^{-1}$) values on the production of power density was tested during 6 h. allowing after this period the recovery of the initial power density values.. To determine the influence of the presence of a second final electron acceptor in the production of power density, iron-citrate (20 mM) was added to the domestic wastewater. At the end of the experimental part concerning the study of the influence of the experimental parameters on the MFC, the cell was maintained in operation 3 more cycles to stabilize the activity of the biofilm and consequently the power density. Finally, the anode with biofilm was removed under anaerobic conditions and immediately used for the characterization of biofilm conductivity and microbial diversity.

5.1.2.3. Biofilm conductivity

Conductivity of the electrode with and without biofilm was determined using a constant volume support equipped with gold blocking electrodes and located within a Buchi TO 50 oven. The samples temperature were evaluated by means of a type K thermocouple placed close to the electrolyte film and impedance measurements were carried out at frequencies between 65 kHz and 500 mHz using an Autolab PGSTAT-12 (Eco Chemie), over a temperature range from 18 to 33 °C. Measurements of conductivity were effected during heating cycles. The reproducibility of recorded conductivities was confirmed by comparing the results obtained for a sample subjected to two heating-cooling-heating cycles. The excellent

reproducibility of the results obtained using this procedure demonstrated the correct operation of the support and the mechanical stability of the samples.

5.1.2.4. Analysis of biofilm bacteria viability

The anode biofilm was fluorescently stained with the LIVE/DEAD *Bac*Light™ Bacterial Viability Kit (L7012, Molecular Probes, Inc., The Netherlands) and examined by confocal laser scanning microscopy in a confocal system Olympus Fluoview 1000 with a microscope BX61 and the objective 100x. Lasers on 488 nm and 560 nm were used. Two- and three-dimensional images were prepared and biofilm thicknesses were calculated.

5.1.2.5. Analysis of biofilm bacteria diversity

Approximately 2 mL of a well homogenized biofilm sample was frozen at the time of sampling and stored at -20 °C. Total genomic DNA was extracted using a UltraClean™ Soil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to the protocol described by the manufacturer.

16S rRNA gene fragments were amplified by polymerase chain reaction (PCR) using 3 different sets of primers: i) U968-GC-F and L 1401-R, targeting conserved domains of total bacteria (BAC), ii) Geo564F and Geo840R, directed towards conserved regions of the gene within the *Geobacteraceae* family (GEO) [30] and, iii) She 120F and She 220R directed towards conserved regions of the gene within the *Shewanellaceae* family (SHE) [31]. The choice of the primer sets was based on the best-available primers in the literature, i.e. the primers that covered the widest range of microorganisms for each studied group. PCR amplification was performed in a 50 μL reaction mixture containing 5 μL of 10X PCR buffer (20 mM Tris–HCl (pH 8.4), 500 mM KCl), 3 mM $MgCl_2$, 200 μM of each of the four deoxynucleoside triphosphates (dNTP) (Invitrogen, Carlsbad, CA, USA), 1.25 U of Taq polymerase (Invitrogen, Carlsbad, CA, USA), 200 nM of each primer and 1 μL of appropriately diluted template DNA. All primers used were synthesized by STAB Vida (Oeiras, Portugal).

The thermocycling program used for the amplification of 16S rRNA gene fragments from total bacteria consisted in the following steps: an initial denaturation step of 5 min at 95 °C; 35

cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 40 s and, elongation at 72 °C for 90 s; and post-elongation at 72 °C for 5 min. The reactions were subsequently cooled to 4 °C. For the *Geobacteraceae* family, the 16S rRNA gene fragments were amplified using the following conditions: an initial denaturation step of 4 min at 94 °C; 35 cycles of denaturation at 94 °C for 30 s, a primer annealing step of 30 s by touchdown from 65 to 55 °C in 0.5 °C decrements over 20 steps, and an extension of 30 s at 72 °C. Reactions were finished with an extra 72 °C extension (3 min). For the amplification of gene fragments from the *Shewanellaceae* family, the thermocycling program was as follows: an initial denaturation step of 10 min at 94 °C; 40 cycles of denaturation at 94 °C for 30 s, annealing at 56.5 °C for 30 s and, elongation at 72 °C for 45 s; and post-elongation at 72 °C for 10 min. The reactions were subsequently cooled to 4 °C. A negative control (no DNA added) was included in all sets of amplification. The size of the PCR products was estimated using a 100 bp DNA ladder (Frilabo, Porto, Portugal) by electrophoresis in a 1 % (wt/vol) agarose gel stained with SBR green (Invitrogen, Carlsbad, CA, USA).

Denaturing gradient gel electrophoresis (DGGE) analysis of the amplicons was carried out using the Dcode system (Bio-Rad, Hercules, CA, USA) in gels containing 8 % (w/vol) polyacrylamide (37.5:1 acrylamide/bis-acrylamide). Denaturating gels were generated using a gradient mixer and a peristaltic pump by standard procedures. The gradient was made at an approximate rate of 4 mL min^{-1}. A linear denaturant gradient of 30–60 % was used for all analyses, where a denaturing strength solution of 100 % was defined as 7 M urea (Sigma–Aldrich, St. Louis, MO, USA) and 40 % formamide (Fluka Chemie, Buchs, Switzerland). Gels were run for 16 h at 85 V in a 0.5X TAE buffer (50X Tris acetate: 242 g Tris, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0) per liter) at 60 °C. Subsequently, DGGE gels were stained with silver as previously described by Sanguinetty *et al.* [32].

5.1.2.6. Analytical methods and calculations

Chemical analyses were performed according to standard methods [33]. The organic content of wastewater was assessed as chemical oxygen demand (COD), using chromate as the oxidant agent. The Fe^{2+} and Fe^{3+} were determined using a 10 % solution of 5-sulfosalicylic acid and a 25 % solution of ammonium hydroxide, 500 nm and 425nm, respectively, as described

by Karamanev [34]. pH was measured with a pH meter model 420A (Orion) and ionic conductivity (σ) with a conductimeter 522 (Crison).

The current intensity (i) was calculated according to the Ohm´s law, i=V/R, where V is the voltage and R the resistance. Current density (j) was calculated as j=i/Ao, where Ao is the projected surface area of the anode electrode. Power density (P) was calculated as the product of the current intensity and the voltage divided by the projected surface area (Ao) of the anode electrode (P=iV/Ao).

5.1.3. Results and discussion

5.1.3.1. Power density and carbon removal in the MFC

The power density was recorded during MFC operation and biofilm formation on the anode surface (Figure 5.1). The power density was around 0 Wm^{-3} during the first 10 d, corresponding to three consecutive batch cycles (data not shown). Then, it increased during 18 h to 407 Wm^{-3} and this value was maintained during 56 h (stable phase). Afterwards, the power density decreased abruptly due to carbon limitation. The COD removal in this first cycle was around 34 %. In consecutive operation cycles, the maximum power density obtained in the stable phase of the power generation curve was nearly constant. Nevertheless, the length of the stable phase and the COD removed increased gradually probably due to the development of the anode biofilm. After one month of operation of the cell, the power density in the stable phase decreased from 407 Wm^{-3} to 300 Wm^{-3}. Simultaneously, COD removal increased from 34 % to 87 % which is compatible with the discharge in the environment (75 % of COD removal) [35, 36].

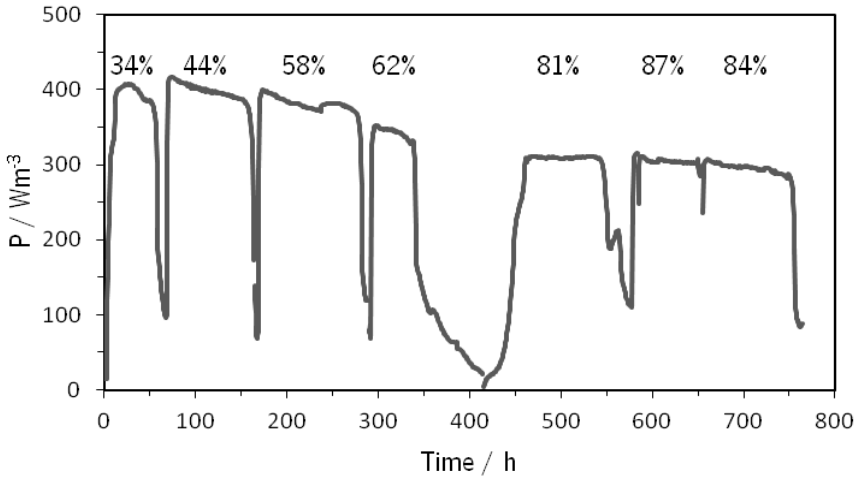

Figure 5.1. Power density along time and percentage of carbon removal.

The maximum power density obtained in the present study (407 Wm^{-3}) is in the range of values where a MFC is considered a competitive alternative to other processes used in wastewater treatment, as reported by Cheng and Logan [37]. The good performance of the MFC presented in this work might be due to the relatively high electrode area/electrolyte volume ratio. Comparing the filter press MFC with other types of MFC using ferrycianide as final electron acceptor presented in literature (Table 5.1), it was possible to conclude that the power densities were similar. As examples, Ringeisen *et al.* [38] obtained 500 Wm^{-3} in a MFC inoculated with *Shewanella oneidensi* and Rabaeuy *et al.* [25] obtained 216 Wm^{-3} with a mixed culture. An interesting fact is that the power densities generated by the present work remain higher than those obtained by a 6 two-compartment stacked MFC (275 Wm^{-3}) [39]. The material of the anode also influences the power density and the stability of the electroactive biofilm. Anodes with a high active surface area, for example the reticulated vitreous carbon [16, 38], the carbon brush [22], and carbon fibre [28], present a high power density per anode area. Another remark is that the power densities obtained in our study are higher than those mentioned in the literature for the MFC using carbon paper as anode material [17, 40]. It seems that the deposition of platinum on electrode surface increase the power densities [24]. The results obtained with a two-chamber stacked MFC using *Geobacter sulfurreducens* contradict the generally accepted opinion that mixed cultures produce higher power densities than pure culture [28], fact that could corroborate the idea that the power density is not only limited by the bacterial community but also by the cell design, in particular by the internal resistance. *G. sulfurreducens* is the only known electroactive bacteria that completely oxidize organic substrates to carbon dioxide in presence of an anode as electron acceptor [4, 41].

Table 5.1. Power densities of different MFC configurations.

Configuration	Biofilm	Electron donor	Electron acceptor	Electrode type	Maximum power density mWm^{-2}	Maximum power density Wm^{-3}	Reference
Two-chambers stacked MFC	Activated sludge	Glucose	FeCN	Plain Graphite	3600	216	[25] Rabaey *et al.*, (2003)
Two-chambers H MFC	*Geobacter sulfurreducens*	Acetate	FeCN	Plain Graphite	13.1	0.36	[41] Bond and Lovley (2003)
Single-chamber air-cathode MFC	Activated Sludge	Wastewater	Oxygen	Graphite felt	26	1.6	[19] Liu and Logan, (2004)
Two-chambers flat MFC	Mixed consortium	Wastewater	Oxygen	Carbon paper	309	77	[17] Min and Logan (2004)
Single-chamber air-cathode MFC	Mixed consortium	Acetate	Oxygen	Carbon paper	506	13	[40] Liu *et al.* (2005)
Upflow chamber MFC	Activated sludge	Sucrose	Oxygen	Reticulated vitreous carbon	170	8.7	[16] He *et al.* (2005)
Two-chambers stacked MFC	*Shewanella oneidensis*	Acetate	FeCN	Reticulated vitreous carbon	3000	500	[38] Ringeisen *et al.* (2006)
Two-chambers stacked MFC (6x)	Activated sludge	Acetate	FeCN	Plain Graphite	-	275	[39] Aelterman *et al.* (2006)
Single-chamber air-cathode MFC	Mixed consortium	Acetate	Oxygen	Carbon felt assemblies with Pt	2770	1550	[24] Fan *et al.* (2007)
Single-chamber air-cathode MFC	Mixed consortium	Acetate	Oxygen	Carbon felt	6860	-	[23] Fan *et al.* (2008)
Single-chamber air-cathode MFC	*Rhodopseudomonas palustris* DX-1	Acetate	Oxygen	Graphite brush	2720	1.8	[22] Xing *et al.* (2008)
Two-chambers stacked MFC	*Geobacter sulfurreducens*	Acetate	Oxygen	Carbon fibre	1880	2150	[28] Nevin *et al.* (2008)
Single-chamber air-cathode MFC	Mixed consortium	Lactate	Oxygen	Graphite felt	858	-	[42] Watson and Logan (2009)
Single-chamber air-cathode MFC	*Shewanella oneidensis*	Lactate	Oxygen	Graphite felt	148	-	[42] Watson and Logan (2009)
Filter press MFC	Mixed consortium	Wastewater	FeCN	Carbon paper	129	826	Present study

5.1.3.2. Performance of the MFC: polarization and power curves

The open circuit voltage (OCV) value corresponds to the maximum voltage that is possible to obtain from the cell under a set of experimental conditions. The measured OCV was 576 mV (Figure 5.2) at a power density around 400 Wm^{-3} and increased to 638 mV at the end of the experimental period characterized by a stable biofilm. The OCV values obtained in the present study are in the range of those reported in literature (0.5 V to 0.8 V) [21].

The polarization curves depicted in Figure 5.2 (grey line) show the relation between voltage (V) and current density (j) obtained by changing the resistance between the electrodes from 31.1 kΩ to 10 Ω. The shape of the curves confirmed the prevalence of ohmic losses generated by one or several of the following factors: membrane resistance, electrolyte resistance of the wastewater, and bacterial metabolism. In the present study, an initial step slope, occasionally observed in polarization curves due to activation losses, was not detected. The sharp final voltage drop might be due to mass transfer limitation losses due to the fast decrease of the carbon source concentration near the electrode surface. The maximum power density (P) obtained in the first power curve (Figure 5.2A, orange line) was 823 Wm^{-3}, which is equivalent to a current density of 1657 Am^{-3} (300 Ω). In the final power curve (Figure 5.2B, orange line), the maximum power density was 389 Wm^{-3} corresponding to ~~a~~ 697 Am^{-3} (800 Ω). This last value was very close to that determined during the operation of the MFC (308 Wm^{-3}). The internal resistance obtained from the slope of the first and last polarization curves in the linear region dominated by ohmic losses was 38 Ω and 7 Ω, respectively. These low values are due to the configuration of the cell that minimizes the distances between the electrodes and the membrane.

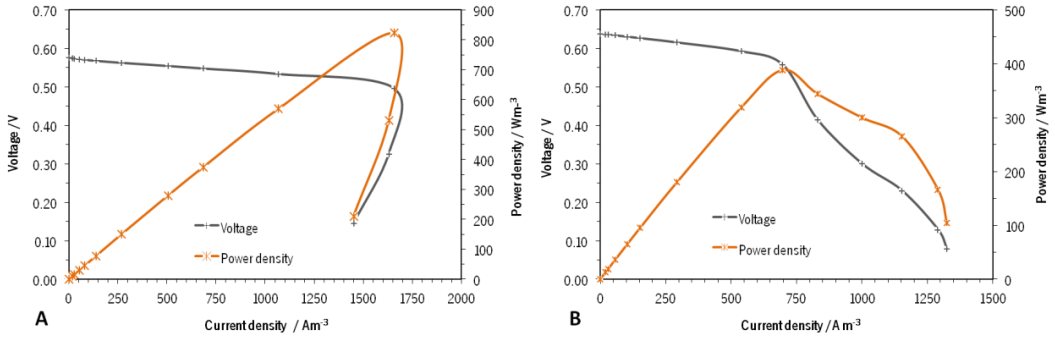

Figure 5.2. Polarization and power curves in the stable phase of biofilm formation (start (A) and end (B) of the optimization experiment).

5.1.3.3. Effect of operation conditions on power density

The influence of a recirculation break on power density is depicted in Figure 5.3. For a 2 h interruption interval, it was possible to observe a decrease of 6.2 ± 0.6 % (24 ± 3 Wm^{-3}) in the power density. A step increase in the period without recirculation to 4 h and 6 h caused further higher decreases of the power density of 9.3 ± 1.1 % (35.7 ± 5 Wm^{-3}) and 11.4 ± 1.2 % (43.8 ± 6.8 Wm^{-3}), respectively. These results showed that the decrease in power density due to a recirculation break was not proportional to the length of the interval and it was more pronounced in the first hours. Additionally, the cell was able to recover completely in less than 1 h after the restoration of the recirculation.

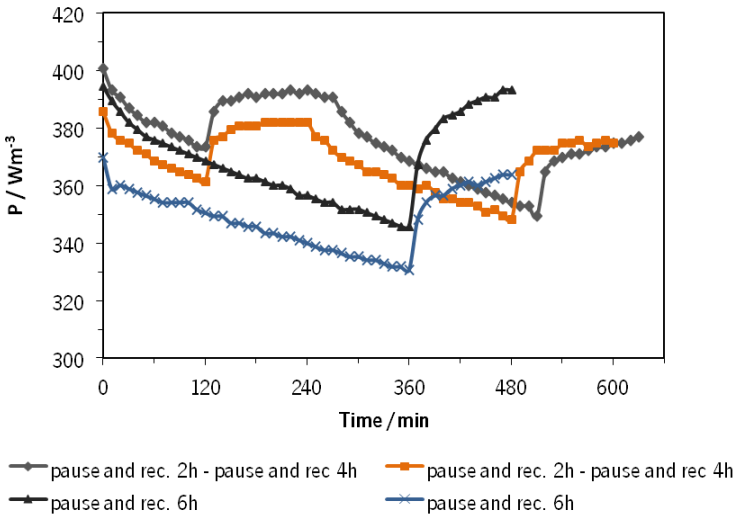

Figure 5.3. Influence of a recirculation break on power density (P).

Usually in the stable phase of power generation, the values of power density were around the same value, however, small variation can be observed due to the carbon consumption. The influence of the recirculation flow rate on power generation is depicted on Figure 5.4.

A two-fold increase of the recirculation flow rate from 13 $mLmin^{-1}$ to 21.6 $mLmin^{-1}$ caused only a slight increase of the power density of 0.58 % (2.6 Wm^{-3}) in the first 20 min. Then, a gradual slow decrease of the power density was observed for a long time period probably due to the consumption of carbon by the biofilm both recirculation flow rates. Decreasing the

recirculation flow rate from 13 $mLmin^{-1}$ to 5.5 $mLmin^{-1}$ had only a marginal impact on the power density which decreased by 0.67 % (2.4 Wm^{-3}) in the first 20 min; thereafter, the power density decreased gradually as explained before. These results suggested that the recirculation flow rates tested in the present study did not influence significantly the power generation because the flow regime did not change as confirmed by the Reynold numbers calculated for the flow rates tested (Laminar regime).

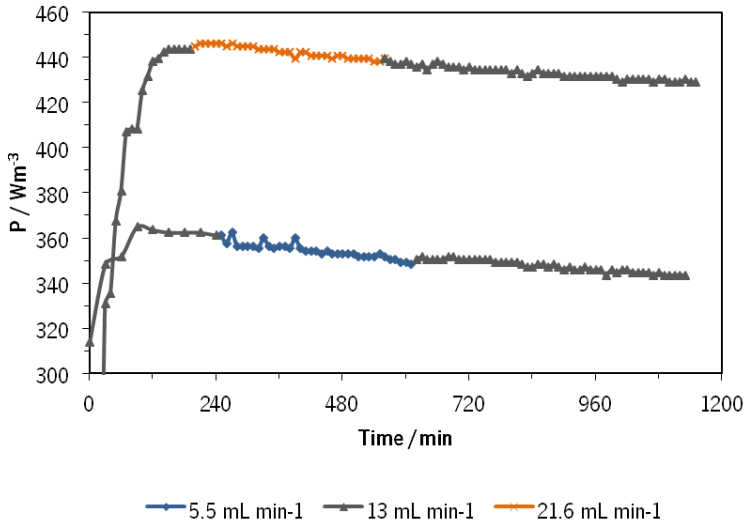

Figure 5.4. Influence of the recirculation flow rate on power density (P).

5.1.3.4. Influence of the presence of iron (III) ions on power density

Iron (III) salts is the main electron acceptor of electroactive bacteria in the environment [43]. In this regard, the competition between soluble iron (III) ions and the carbon electrode was assessed in the MFC (Figure 5.5). The results obtained showed a significant effect of the presence of iron (III) in the power generation of the MFC. In the first place, *G. sulfurreducens* transferred electrons to iron (III) reducing it to iron (II) and the power density was lower than the observed without iron (III) (Figure 5.5). Then, when only half of the initial iron (III) concentration remained in the medium, the power generation increased and reached the previous observed values (440 Wm^{-3}) while the iron (III) was completely reduced to iron (II).

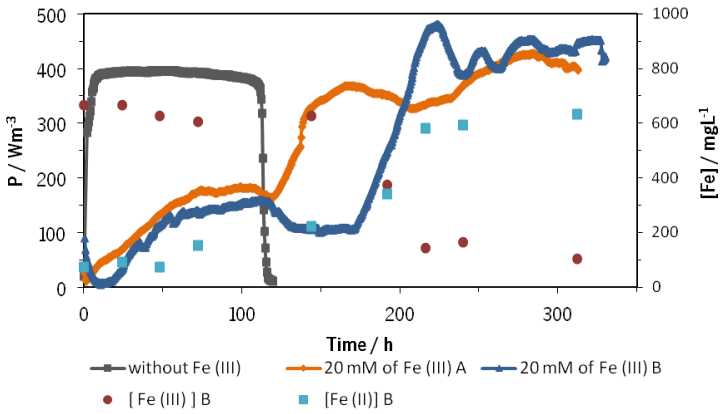

Figure 5.5. Influence of iron (III) (20 mM of Fe-citrate) in power generation.

5.1.3.7. Biofilm conductivity

The conductivity of the anode with and without biofilm over the temperature range from 18 to 33 °C is illustrated in Figure 5.6. These samples seem to show a linear variation of log (σ) with temperature. Electrochemical impedance spectroscopy measurements demonstrated that the anode biofilm conductivity is higher than that of the hydrated anode (without biofilm). The higher biofilm conductivity not only reduced the resistance but also lowered the activation energy barrier for electron flow through the biofilm.

The conductive behaviour of the biofim with temperature follows the Arrhenius equation in the measured temperature range:

$$\sigma = \sigma_0 \exp\left(\frac{-E_a}{RT}\right) \qquad (5.1)$$

where σ is the conductivity, σ_0, E_a , R and T are the pre-exponential factor, the apparent activation energy for transport, the gas constant (8.314 $J.mol^{-1}.K^{-1}$) and the temperature, respectively. The fitted value of the E_a for the anode biofilm is 14.3 $KJmol^{-1}$.

The morphology of the anode biofilm progressively developed from dispersed individual cells to tight aggregates and from rod-shaped cells to a filament-dominant structure. The relationship between anode microbial characteristics and electrochemical parameters was analyzed [44]. The results can suggest that the impedance measurement is affected by the diffusion of that species in terms of electrostatic and chemical interactions with the

surrounding biofilm. We assume an anomalous diffusion in the biofilm [45]. The anomalous diffusion theory used here to model the effect where some particles remain stuck for a long time due to interactions with the surrounding biofilm environment and diffusion is slower [45]. Impedance measurements indicated that the biofilm phase affects its intrinsic capacitance and resistance properties. The biofilm architecture and its corresponding porosity and overall thickness affects the measured impedance hence the resistance of the biofilm. Stoodley *et al.* [46] demonstrated the effect of electric fiels on the structure of the biofilm.

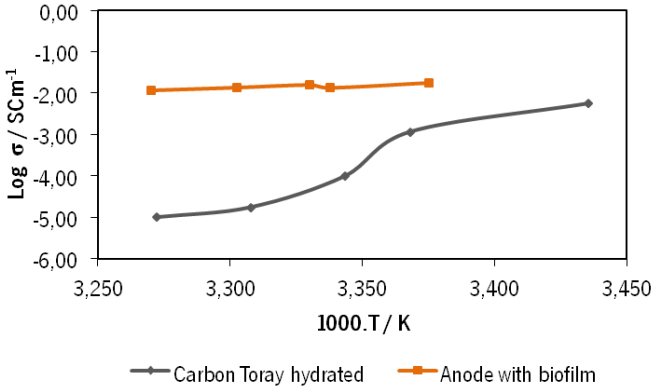

Figure 5.6. Variation of the conductivity of the anode without (grey line) and with a stable biofilm (orange line).

5.1.3.8. Biofilm bacteria viability

Confocal laser scanning microscopy images of the anode biofilm are depicted in Figure 5.7. No significant emission was obtained in the red channel, revealing that the whole biofilm was active. The live cells (green) formed a virtual monolayer on the anode surface, which suggested that bacteria colonized the anode and transferred electrons directly to the electrode surface [43]. This resulted in a thin biofilm with extensive pillar structures up to 13.5 μm in height. In the present study, the thickness of the biofilm formed in the carbon paper anode was lower than that of an *G. sulfurreducens* anode biofilm formed in solid graphite (50 μm) [28]. The structure of the anode surface may influence the thickness of the biofilm because the mesh of carbon fibres may enhance the transport of the electron donor [28]. The shear stress in the anode compartment (1 cm^3) can also influence the thickness of the biofilm.

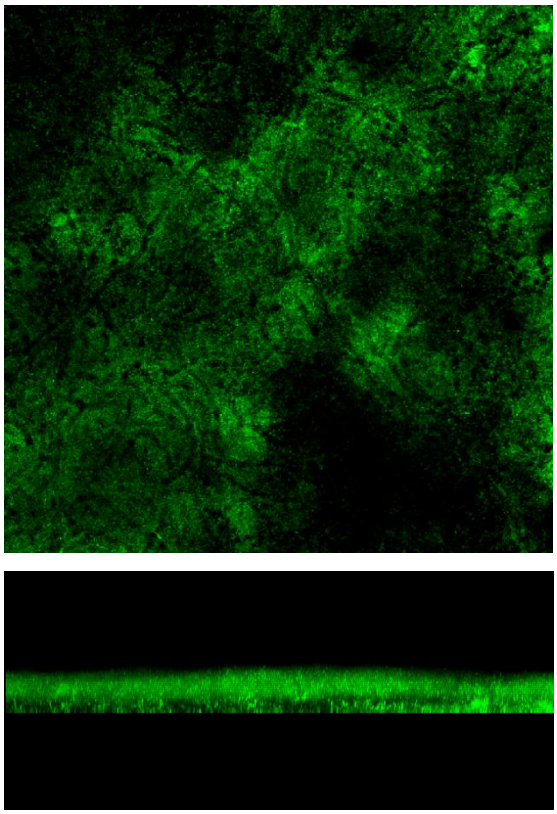

Figure 5.7. Anode biofilm stained with the LIVE/DEAD *Bac*Light™ Bacterial Viability Kit (L7012) and examined by confocal laser scanning microscopy. Live bacteria with intact cell membranes fluoresce green and dead bacteria with compromised membranes

5.1.3.9. Microbial diversity in the biofilm

The study of the diversity of bacteria in the biofilm was based on DGGE patterns of partial 16S rRNA gene amplicons. DNA extracted from the biofilm was used as template for amplification of the V6-V8 region of total bacteria (BAC), *Geobacteraceae* family (GEO) and, *Shewanellaceae* family (SHE). The amplicons were separated by DGGE and the obtained band-patterns are depicted in Figure 5.8.

The DGGE band-pattern (A) of the DNA amplified for total bacteria indicated the presence of various ribotypes which suggested a high microbial diversity. Additionally, the DGGE band-pattern (B) of the DNA amplified for the *Shewanellaceae* family showed the presence of several ribotypes which suggested the presence of different species or strains of *Shewanella.* Regarding the DGGE band-pattern (not shown) of the DNA amplified for the *Geobacteraceae* family it was not clear enough to conclude about the presence or absence of some

representatives of this family. PCR conditions need to be improved to amplify 16S rRNA gene fragments of this family.

The results are in agreement to literature studies that have found out a high microbial diversity in biofilms formed on anode surface of MFC using domestic wastewaters [47, 48]. Regarding the presence of *Shewanella* representatives it was expected since *Shewanella* species are widespread in nature, such as in marine, freshwater, sedimentary, and soil environments. Moreover, these species have the ability to reduce an extensive number of different electron acceptors using one or more proposed mechanisms of extracellular electron transport (EET) which favours its enrichment in MFC [49].

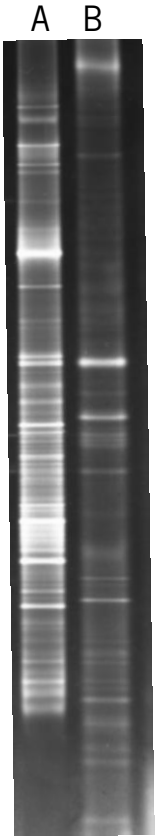

Figure 5.8. DGGE profiles of 16S rRNA fragments amplified from DNA extracted from anode biofilm using primers to total bacteria (A), and primers specific to *Shewanella* (B).

5.1.4. Conclusions

The main conclusions of the present work are the following:

- The power density of the MFC was marginally affected by a break in the recirculation system as well as the recirculation flow rate,
- The presence of a second soluble electron acceptor decreases significantly the power production,
- The anode biofilm presented high conductivity and diversity including members of *Shewanella* group that are well known for its electroactivity,
- The filter press Microbial Fuel Cell presented a good performance concerning both bioelectricity production (maximum power density of 823 Wm^{-3} and 87 % of carbon removal making it suitable process for use in isolated and autonomous systems. This configuration is very compact and several units in series can increase the power density.

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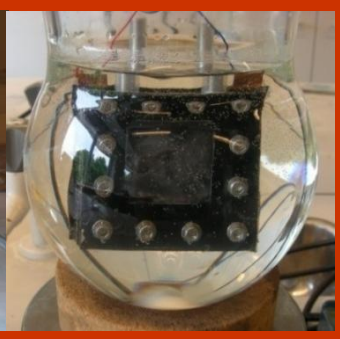

Chapter 6

Microbial Fuel Cells: sensor application

6.1. In situ microbial fuel cell-based biosensor for organic carbon

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The biological oxygen demand (BOD) may be the most used test to assess the amount of pollutant organic matter in water; however, it is time and labor consuming and is done ex situ. A BOD biosensor based on the microbial fuel cell principle was tested for online and in situ monitoring of biodegradable organic content of domestic wastewater. A stable current density of 282 ± 23 mAm^{-2} was obtained with domestic wastewater containing a BOD_5 of 317 ± 15 mgL^{-1} O_2 at 22 ± 2 °C, 1.53 ± 0.04 $mScm^{-1}$ and pH 6.9 ± 0.1. The current density showed a linear relationship with BOD_5 concentration ranging from 17 ± 0.5 mgL^{-1} O_2 to 78 ± 7.6 mgL^{-1} O_2. The current generation from the BOD biosensor was dependent on the measurement conditions such as temperature, conductivity, and pH. Thus, a correction factor should be applied to measurements done under different environmental conditions from the ones used in the calibration. These results provide useful information for the development of a biosensor for real-time in situ monitoring of wastewater quality.

Key words: submersible microbial fuel cell, biological oxygen demand biosensor, wastewater quality.

6.1.1. Introduction

Domestic wastewater commonly contains organic matter that includes nitrogenous compounds, proteins and amino acids, and non-nitrogenous compounds, carbohydrates and lipids. A detailed characterization of the organic matter in domestic wastewater is time and labor consuming; therefore, bulk parameters like biological oxygen demand (BOD) and chemical oxygen demand (COD) are routinely used as a measure of the degree of water pollution by organic carbon. The BOD is a measure of the quantity of oxygen used by microorganisms (e.g., aerobic bacteria) to oxidize the organic matter in a sample of water during a period of 5 days (BOD_5), while the COD is used as a measure of the oxygen requirement of a sample that is susceptible to oxidation by a strong chemical oxidant (e.g., potassium dichromate). COD values are always higher than BOD values. COD measurements can be made in a few hours, while BOD measurements take five days, but they do not differentiate between biologically available and inert organic matter [1-5].

An online biosensor to quantify BOD was developed using the microbial fuel cell (MFC) principle [6]. In a MFC, microorganisms growing on the surface of an anode electrode transfer electrons from the oxidation of organic matter present in the wastewater through an electrical circuit to a cathode electrode, thus generating an electrical current [7]. A biosensor based on the MFC principle does not need a transducer to read the signal and converts it to an electrical signal because the measured signal is already an electrical current [8]. This type of biosensor is an example of the successful application of an electrochemical method in the evaluation of wastewater quality. Several configurations based on MFC have been tested and optimized to be used as a BOD biosensor. The usual configuration was a mediator-less MFC, with two chambers separated by a cation exchange membrane and continuous wastewater flow in the anode chamber [6, 9-14]. The main drawback of this configuration is the complexity of the setup that is not suitable for in situ applications.

A very compact MFC configuration, known as submersible microbial fuel cell (SMFC), was developed by Min and Angelidaki [15]. This configuration uses an air-cathode which simplifies considerably the MFC configuration [16]. The aim of the present study was to adapt and test the SMFC configuration as an in situ BOD biosensor. The SMFC contained an anode electrode

connected to a rectangular cathode chamber and was submerged in anaerobic wastewater. The cathode chamber was continuously flushed with air. A proton exchange membrane was assembled between anode and cathode. The membrane-electrodes assembly was pulled together to reduce the internal resistance of the cell and placed on one side of the cathode chamber. Several experiments were carried out with domestic wastewater containing a BOD_5 concentration in the range of 17 ± 0.5 mgL^{-1} O_2 to 183 ± 4.6 mgL^{-1} O_2. The effect of temperature (11 ± 0.2 °C – 33 ± 0.3 °C), wastewater conductivity (1.1 ± 0.012 $mScm^{-1}$ - 13.4 ± 0.013 $mScm^{-1}$) and pH (6.0 ± 0.1 - 8.5 ± 0.1) were investigated.

6.1.2. Material and Methods

6.1.2.1. Wastewater collection and composition

Domestic wastewater was collected at the Lundtofte wastewater treatment plant (Lyngby, Denmark), screened to remove coarse particles and, used as a source of organic matter and inoculum to start biofilm formation on the anode surface of the BOD biosensor. The composition of the wastewater was: 470 ± 79 mgL^{-1} O_2 as COD, 317 ± 15 mgL^{-1} O_2 as BOD_5, 61 ± 13 mgL^{-1} total nitrogen, 12 ± 2.6 mgL^{-1} total phosphorous, 6.93 ± 0.16 pH units and 1.53 ± 0.04 $mScm^{-1}$ conductivity. After collection, the wastewater was sparged with argon gas to establish anaerobic conditions, and kept at 4 °C for later use.

6.1.2.2. BOD biosensor description

The BOD biosensor was constructed based on a previous Submersible Microbial Fuel Cell (SMFC) configuration described by Min and Angelidaki [15] (Figure 6.1A). The electrode system was very compact to minimize the internal resistance and comprised an anode electrode in direct contact with the proton exchange membrane that was hot-pressed to the cathode electrode. The anode was made of carbon paper (Toray carbon paper, E-TEK division, USA) with 9 cm^2. The cathode, with similar dimensions to the anode, was made of 5 % wet proofed carbon paper. The cathode's surface in direct contact with the proton exchange membrane (Nafion™ 117, DuPont Co., USA) was covered by a Pt catalyst (0.5 mg cm^{-2} with

20 % Pt, E-TEK). The electrodes were connected to a resistance with 1 kΩ using copper coated wires (2 mm diameter).

The electrode system (Figure 6.1B) was connected to one side of a rectangular cathode chamber made of non-conductive polycarbonate material. The other side of the cathode chamber was closed with a plate of the same material. The cathode electrode faced the inside of the chamber while the anode, in the opposite side, was in direct contact with the wastewater to be analysed. Air was continuously sparged inside the cathode chamber and functioned as the final electron acceptor. To assemble the BOD biosensor and to keep it tight, rubber gaskets and stainless steel screws were used, as depicted in Figure 6.1.

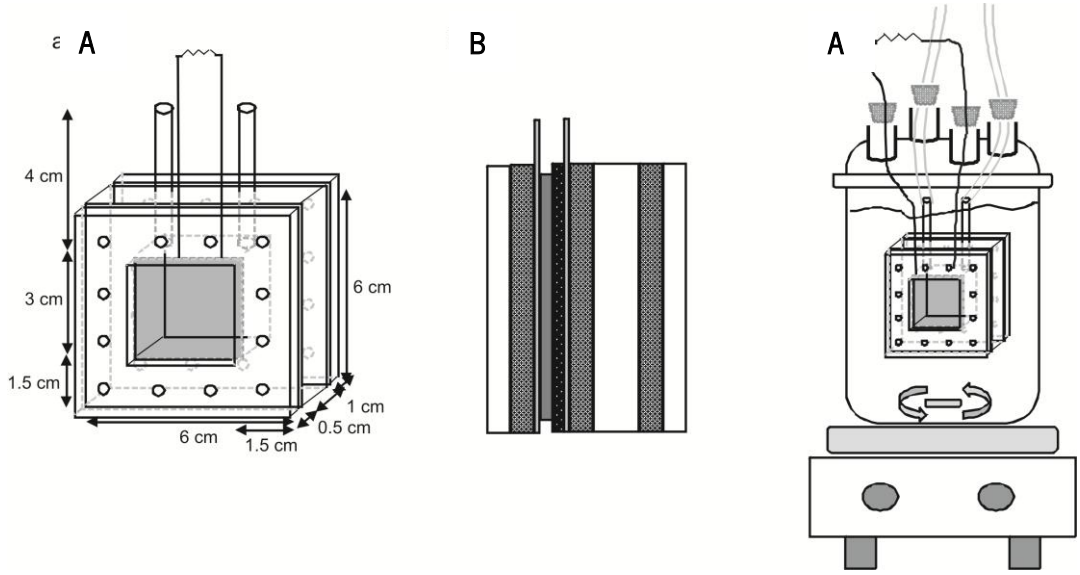

Figure 6.1. Schematic diagram of the submersible microbial fuel cell type BOD biosensor (A), the electrode system (B) and the experimental set-up (C). Polycarbonate support ▭, rubber gaskets ▩, wires ▭, anode ■ and cathode hot pressed with proton exchange membrane ▪.

6.1.2.3. BOD biosensor evaluation

Two biosensors were operated simultaneously at room temperature (22 ± 2 °C) during 3 weeks to promote biofilm formation on the surface of the anode. Subsequently, one of the two biosensors was discontinued and the biofilm was investigated by scanning electron

microscopy (SEM). The other was operated to evaluate current density as a function of BOD_5 concentration in wastewater. The influence of environmental parameters was also tested.

Biofilm formation

The BOD biosensor was immersed into 1 L domestic wastewater contained in a glass vessel tightly closed with a glass lid (1.5 L total volume), as described in Figure 6.1C The vessel was stirred at 200 rpm and the wastewater was initially sparged with argon gas for 10 min to obtain anaerobic conditions. Subsequently, the cathode chamber was continuously flushed with air (final electron acceptor) at a flow rate of 5 $mLmin^{-1}$. The voltage difference between anode and cathode electrodes was measured across a fixed resistance with 1 kΩ every 10 min using a multimeter (Fluke), and data were collected automatically by a data acquisition program and a computer. The wastewater was replaced periodically to avoid organic carbon limitation. The biofilm formed on the anode electrode reached steady-state when similar values of voltage were obtained 3 times consecutively. Then, the polarization curve (voltage (V) versus current density (j)) and the power curve (power density (P) versus current density (j)) were recorded using a series of resistances in the range of 22 kΩ to 100 Ω (domestic wastewater with a BOD_5 of 298.2 ± 4.2 mgL^{-1} O_2) [17]. The open circuit voltage (OCV), where i is the current intensity) was determined at infinite resistance. At last, one of the two biosensors was discontinued and the biofilm was investigated by SEM as described previously [18].

Effect of BOD_5 concentration and environmental parameters on current density

To evaluate maximum current density with different concentrations of BOD_5, five wastewater dilutions were prepared with tap water (17 ± 0.5 mgL^{-1} O_2 – 183 ± 4.6 mgL^{-1} O_2). The maximum voltage between anode and cathode electrodes was measured 2 times consecutively for each BOD_5 concentration across a fixed resistance with 1 kΩ, at which the maximum power was obtained from the previous polarization and power curves. Temperature (T), pH, and conductivity (σ) were tested independently using diluted domestic wastewater with a BOD_5 concentration of 143.5 ± 8.7 mgL^{-1} O_2, 1.1 ± 0.012 $mScm^{-1}$ and pH 6.5 ± 0.2. Temperature tested varied from 11 ± 0.2 °C to 33 ± 0.3 °C. The glass vessel was immersed in a water bath and temperature was adjusted with a thermostat. The wastewater pH was adjusted with 1 M HCl or NaOH solutions to obtain pH values in the range of 6.0 ± 0.1 to 8.5

± 0.1. The wastewater conductivity was adjusted with a KCl solution and varied between 1.1 ± 0.012 $mScm^{-1}$ and 13.4 ± 0.013 $mScm^{-1}$.

6.1.2.4. Analytical methods and calculations

The organic content of wastewater was assessed both as COD, using chromate as the oxidant agent, and BOD_5, using the Oxitop® method, as described in Standard Methods [19]. Initially, a correlation between BOD_5 and COD was experimentally determined for the Lundtofte domestic wastewater. The following least square regression equation obtained from the results was further used to convert COD into BOD_5 values, being both expressed in mgL^{-1} O_2: BOD_5 = 0.676 COD - 1.752 (R^2 = 0.986). pH was measured with a PHM 210 pH meter (Radiometer) and conductivity with a CDM 83 conductivity meter (Radiometer).

To determine the mass of volatile suspended solids per surface area of the electrode (mass of biofilm), two electrodes (similar surface area) with and without biofilm were dried at 105 °C and weighed. Next, the electrodes were burned at 550 °C and weighed again for comparison and quantification of biofilm biomass per cm^2.

The current intensity (i) was calculated according to the Ohm´s law, i=V/R, where V is the voltage and R the resistance. Current density (j) was calculated as j=i/Ao, where Ao is the projected surface area of the anode electrode. Power density (P) was calculated as the product of the current intensity and the voltage divided by the projected surface area of the anode electrode (P=iV/Ao).

6.1.3. Results and Discussion

6.1.3.1 Biofilm formation

When the BOD biosensor was immersed in domestic wastewater (317 ± 15 mgL^{-1} O_2 BOD_5), current kept increasing over time and, after a period of 3 weeks, it stabilized at a maximum value of 0.27 mA, corresponding to a current density of 282 ± 23 mAm^{-2}. Once the maximum current was reached, it could be maintained as long as domestic wastewater was periodically changed. The profile of current generation of a single-batch operation was characterized by a

steep increase of current after addition of domestic wastewater, followed by a plateau of maximum current with a length dependent on the concentration of BOD_5, and finally a decrease of current. SEM analysis performed after the enrichment period, when the current generation was stable, showed a confluent biofilm on the surface of the anode dominated by rod shaped microorganisms (Figure 6.2). The amount of biofilm formed on the anode was 5.2 ± 1.47 $mgcm^{-2}$, expressed as mass of volatile suspended solids per surface area of the electrode. Considering a wet biofilm density of 1.02 gcm^{-3}, the estimated biofilm thickness was 51 µm. The attached biomass determined in the present study was more than 10 times higher than previous reported values [20] but the thickness was of the same order of magnitude [20,21] suggesting the formation of a anode biofilm with high density.

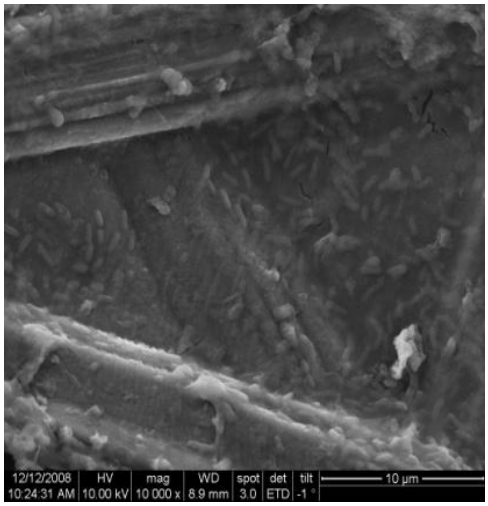

Figure 6.2. Scanning electron micrograph of anode surface inoculated with domestic wastewater in the stable phase of current density generation.

6.1.3.2 Performance of BOD biosensor

The open circuit voltage measured in the stable phase of current generation was 414 ± 6 mV using domestic wastewater with a BOD_5 concentration of 298.2 ± 4.2 mgL^{-1} O_2. The polarization curve depicted in Figure 6.3 (grey line) shows the relation between voltage (U) and current density (j) obtained by changing the resistance between electrodes from 22 kΩ to 100 Ω (current densities in the range of 20 mAm^{-2} to 522 mAm^{-2}). The shape of the curve confirmed the prevalence of ohmic losses generated by membrane resistance, electrolyte resistance of the wastewater, and bacterial metabolism. The initial steeper slope occasionally

observed in polarization curves due to activation losses was not observed in the present study. The maximum power density (P) obtained in the power curve (Figure 6.3 - black line) was 72 mWm^{-2}, which is equivalent to a current density of 283 mAm^{-2} (1 kΩ). These values are similar to the ones obtained in the stable phase of the SMFC power generation using and external resistance of 1 kΩ. In effect, the variation of resistance values did not cause any change in power density.

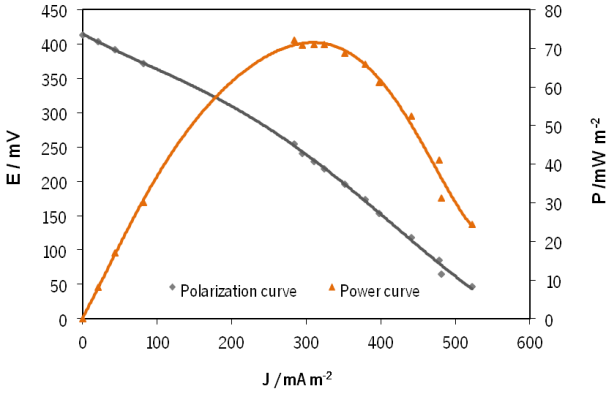

Figure 6.3. Polarization and power curves obtained by varying the external resistance between the electrodes, ranging from 22 kΩ to 100 Ω (domestic wastewater with a BOD_5 concentration of 298.2 ± 4.2 mgL^{-1} O_2 at 22 °C).

6.1.3.3. Current generation at various BOD_5 concentrations

The maximum current density generated from domestic wastewater with a BOD_5 concentration in the range of 17 ± 1 mgL^{-1} O_2 to 183 ± 5 mgL^{-1} O_2 was determined (Figure 6.4). The current density increased linearly with BOD_5 up to a concentration of 78 ± 8 mgL^{-1} O_2 ($j = 2.68\ i - 18.78$ with $r^2 = 0.996$). The range of BOD_5 concentrations that were evaluated with the BOD biosensor is in agreement by those published in literature [6, 9, 11, 12, 20] (Table 1). The maximum current density obtained in the present study was 222 mAm^{-2}, which is similar to those obtained by Gil *et al.* [9] and Kim *et al.* [6]. BOD_5 concentrations higher than 78 ± 8 mgL^{-1} O_2 resulted in further slow increase in the current density until reaching a plateau value. These experiments also revealed that the higher the BOD_5 concentration, the longer the BOD biosensor took to reach a new steady-state current (i.e., response time) after changing the strength of the wastewater. The response time for a BOD_5 concentration of 17 ± 1 mgL^{-1} O_2 was shorter than 30 min, however, about 10 h were needed for concentrations

higher than 78 ± 8 mgL^{-1} O_2. Several values are reported in literature for this parameter which encompasses the ones determined in the present study (Table 6.1). The main contribution of the proposed BOD biosensor compared to others is the fact that it is very compact and can be used in situ.

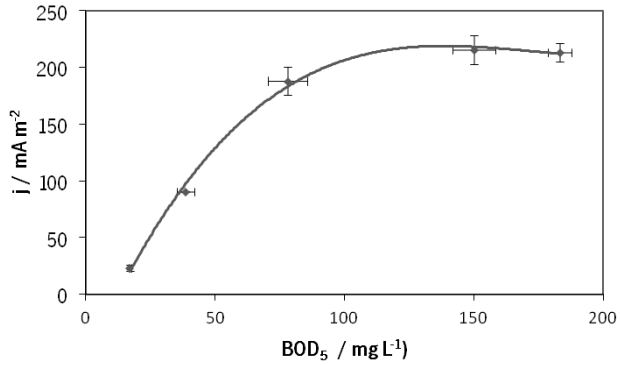

Figure 6.4. Current density (j) generation in the BOD biosensor as a function of initial BOD_5 concentration of domestic wastewater at 22 °C (R = 1 kΩ).

Table 6.1. MFC-type biosensors and their respective characteristics.

Configuration	Electron donor	Enrichment	Measuring range	Max concentration*	Maximum current intensity	Maximum current density	Response time	Reference
Mediator-less 1-chamber MFC (SMFC)	Wastewater	Wastewater (3 weeks)	17 – 183 mgL^{-1} O_2 BOD	78 mgL^{-1} O_2 BOD	0.2 mA (9 cm^2)	222 mAm^{-2}	30 min – 10 h	Present study
Mediator - less 2-Chamber MFC	Glucose and glutamate	Activated sludge (4 weeks)	20 - 200 mgL^{-1} O_2 BOD	100 mgL^{-1} O_2 BOD	6 mA (62 cm^2)	968 mAm^{-2}	60 min	Chang *et al.* 2004
Mediator - less 2-chamber MFC	Wastewater	Enriched culture of electrochemical active bacteria (5 weeks)	10 - 400 mgL^{-1} O_2 COD	50 mgL^{-1} O_2 COD	1.7 mA (56 cm^2)	304 mAm^{-2}	Not available	Gil *et al.* 2003
Mediator - less 2-chamber MFC	Wastewater	Enriched culture of electrochemical active bacteria	2.6 - 206 mgL^{-1} O_2 BOD	25 mgL^{-1} O_2 BOD	1.1 mA (56 cm^2)	196 mAm^{-2}	30 min - 10 h	Kim *et al.* 2003
2-chamber MFC C-type	Glucose and glutamate	Activated sludge (8 weeks)	20 - 200 mgL^{-1} O_2 BOD	200 mgL^{-1} O_2 BOD	2.9 mA (16 cm^2)	1812 mAm^{-2}	5 min - 10 h	Kim *et al.* 2006
2-chamber (MFC) C-type	Glucose and glutamate	Enriched culture of electrochemical active bacteria	50 - 100 mgL^{-1} O_2 BOD	Not available	1.9 mA	Not available	5 min	Moon *et al.* 2004
2-chamber MFC Oligotrophic	Glucose and glutamate	Oligotrophic microbes	2 - 10 mgL^{-1} O_2 BOD	10 mgL^{-1} O_2 BOD	8 µA (13 cm^2)	6.15 mAm^{-2}	60 min	Moon *et al.* 2005

*Maximum concentration of the interval where a linear relation between x and y was observed.

6.1.3.4 Effect of environmental conditions on current generation

The effect of changes in environmental parameters in the performance of the biosensor was assessed using domestic wastewater with a BOD_5 of 144 ± 9 mgL^{-1} O_2. The current density increased linearly with temperature (Figure 6.5A), about 6 $mA(m^2\ °C)^{-1}$, in the range of 11 ± 0.2 °C to 33 ± 0.3 °C. The maximum current density (288 mAm^{-2}) was obtained at pH 7.0. Minimum values were observed at pH 6 ± 0.1 and pH 8 ± 0.1, corresponding to current densities of 186 mAm^{-2} and 184 mAm^{-2}, respectively (Figure 6.5B). These results are consistent with literature studies regardless of the MFC configuration and organic carbon sources used [8]. The current density increased with the conductivity of domestic wastewater in the range of 1.1 ± 0.012 $mScm^{-1}$ to 7.51 ± 0.01 $mScm^{-1}$, from 199 mAm^{-2} to 316 mAm^{-2}, respectively, the effect being more important between 1.1 ± 0.012 and 2.1 ± 0.013 $mScm^{-1}$ (Figure 6.5C). These results suggested that a correction factor should be applied to measurements done under other environmental conditions. It is clear that further work is required to assess the long term operational stability of the BOD biosensor proposed in the present study. Also, the effect of competing electron acceptors present in the wastewater, such as oxygen, nitrate, and sulfate, on the generated current density should be determined.

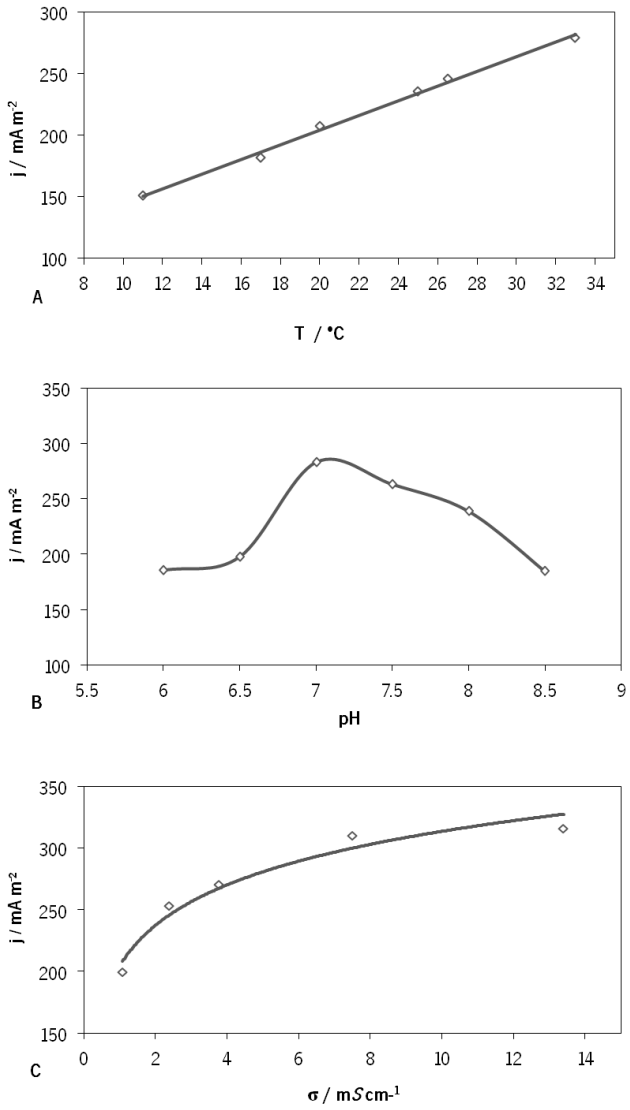

Figure 6.5. Effect of temperature (T) (A), pH (B) and, conductivity (σ) (C) on current density (i) generation in the BOD biosensor (144 ± 9 mgL^{-1} O_2).

5.1.4. Conclusions

A mediator-less submersible microbial fuel cell was tested as an in situ BOD biosensor. BOD_5 values of up to 78 ± 8 mgL^{-1} O_2 could be measured based on a linear relation between organic carbon content of the wastewater and current density, and the biosensor response time was lower than 10 h. The current density measured with the biosensor was affected by changes in pH and temperature and the measured values need to be corrected.

The biosensor developed and tested in the present work has an application in the field of wastewater with a medium to low concentration. Its main advantage is that no special anaerobic chamber (anode chamber) is needed because the sensor might be directly submerged in a wastewater channel or anaerobic reactor.

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Chapter 7

Conclusions and future perspectives

7.1. Conclusions

The integration of the knowledge about electron transfer in MFC will improve power generation. This thesis evaluates through cyclic voltammetry, biofilms characterization and operation conditions assessment, the possibility of MFC implementation in wastewater treatment with bioelectricity production in autonomous systems, as well as, the use of MFC as biological oxygen demand biosensor through the correlation between the power generation and carbon content of wastewater. In that regard, the present work was focused on the optimization of the bacterial electron transfer process to electrodes and on the advancement of wastewater valorization and monitoring technologies based on the MFC concept.

The kinetic studies of the redox processes during *G. sulfurreducens* biofilm formation on anode allow a better understanding of the reaction mechanisms for acetate oxidation by *G. sulfurreducens*. In the presence of *G. sulfurreducens* an increase of current intensities was observed between - 0.5 V and 0.89 V *vs*. SCE with three well defined oxidation peaks, peak A, B and C at 0.16 V, 0.6 V and 0.8 V *vs.* SCE respectively that can be attributed to independent or successive oxidation processes. The processes occurring at lower anodic potentials, corresponding to a mediated heterogeneous electron transfer, that can be attributed to the *c*-type cytochrome OmcB. The velocity of this process was enhanced by an increase of the temperature. From the kinetic study it was possible to conclude that the rate of the oxidation reactions of *G. sulfurreducens* biofilms in the logarithmic and stationary phases was limited by the diffusion. A mixed control with contributions of adsorption and diffusion steps was observed for *G. sulfurreducens* immediately after inoculation. The limitation of the reaction rate by diffusion and the calculated number of electrons which was around 1 may be explained by a transfer of one electron at a time through electron transfer mediators.

The proteomic study of outer membrane proteins of *G. sulfurreducens* cultured at two distint temperatures (25 °C and 37 °C) allows concluding that the growth temperature influences proteins expression in the outer membrane of *G. sulfurreducens*. Proteins related to electron transfer during respiration: namely, cytochrome c, F0F1 ATP synthase and ABC transporter

ATP-binding protein were overexpressed in *G. sulfurreducens* grown at 25 °C. The differential expression of the outer membrane proteins is reflected in the electrochemical behaviour. In the kinetic studies of these two cultures it was possible to verify that bacteria grown at 25 °C present more oxidations steps, 3 well defined peaks, when compared with bacteria grown at 37 °C, what is in agreement with the overexpression of proteins responsible for the respiratory process and energy production. The growth temperature also influences the limiting steps of the different oxidation processes observed in the voltametric studies. The most evident difference observed between the two cultures of *G. sulfurreducens*, was related to the kinetic of the processes. Bacteria that grew at 25 °C present all the peaks limited by mixed processes whereas bacteria cultured at 37 °C present a peak limited by adsorption and another by diffusion. This limitation might be due to the overexpression of the LamB porin family protein in the outer membrane of the bacteria which are related to the diffusion of nutrient. The suboptimal temperature of 25 °C may induce the pili production, fact corroborated by proteomic and electrochemical analyses. Finally, *G. sulfurreducens* grown at 25 °C was better inoculums for biofilm formation on anodes of microbial fuel cells.

An excellent performance for bioelectricity production and wastewater treatment was obtained in a filter press MFC with a mixed culture in the anode. The instantaneous power densities obtained 407 Wm^{-3} are in the range of that MFC became competitive with other wastewater treatments. The obtained low internal resistance values may be due to the configuration of the reactor that minimizes the distances between the electrodes and the membrane,. The carbon removal reached values of 83.7 % ± 4 %, which is compatible with the discharge in the environment. Their operation condition were optimized and was possible to conclude that the interruptions of electrolyte recirculation slightly decrease power density, however the increase/reduction of the influent flow did not influenced power generation. The presence of a soluble electron acceptor, namely iron (III) oxides significantly affected power generation. The anode biofilm presented high diversity being present species of the genus *Shewanella* and all of the biofilm cells were alive. This configuration is very compact and several units in series can increase the power density. This configuration can be used for bioelectricity production in isolated and autonomous systems.

The MFC principle can be applied in biosensors for biological oxygen demand monitorization. In a mediator-less submersible MFC the maximum power density obtained was around 72

mWm^{-2}, which is equivalent to a current density of 283 mAm^{-2}. BOD_5 values of up to 78 ± 8 mgL^{-1} O_2 could be measured based on a linear relation between organic carbon content of the wastewater and current density. The biosensor response time was lower than 10 h. The current density measured with the biosensor was affected by changes in pH, and temperature and the measured values need to be corrected. The biosensor developed and tested in the present work has an MFC application in the field of wastewater in a range from medium to low concentration. Its main advantage is that no special anaerobic chamber (anode chamber) is needed because the sensor might be directly submerged in a wastewater channel or anaerobic reactor.

This work demonstrates that MFC is an innovative technology for different applications, namely wastewater treatment and sensor for water quality monitorization.

7.2. Future Perspectives

A set of further research themes may be extracted from the results obtained with the MFC. The main ones are the following:

1. An increase in anode and cathode surface area will improve the power density of MFC technology. Therefore a deposition of carbon nanotubes in the surface of carbon electrodes may increase the active surface area and a higher volumetric power density would be expected. This strategy could be the goal of a research in a filter press-type of MFC with nanotube deposition on carbon electrodes. The cathode limitations influence the internal resistance, it is important to improve the catalytic activity of existing electrocatalysts and synthesize new possibly nonnobel metal catalysts for oxygen reduction reaction.
2. Oxygen is widely used as electron acceptor MFC systems due to its elevated oxidation potential and accessibility. To increase the rate of oxygen reduction, Pt catalysts are usually used for dissolved oxygen but, open-air cathodes, with air diffusion, can also lead to a higer power generation. Future research should be addressed in the implementation of air-cathodes in a filter press MFC.
3. CO_2 is considered to be the major greenhouse gas with a direct impact on climate change and it is of outermost importance that effective capture technologies are developed to reduce the amount of greenhouse emissions. MFC is a source of CO_2, however, new advances in the MFC technology can be implemented to promote environment CO_2 sequestration through biological conversion to organic matter by photosynthetic microorganisms, such as microalgae and cyanobacteria. These microorganisms can use CO_2 during the photosynthesis to produce carbon sources and using the cathode as electron donor. A detailed study of this strategy will allow the development of self-sustainable MFC that can simultaneously capture CO_2 from atmosphere.