Microbial Fuel cells, applications and biofilm characterization

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Biochemical Process Engineering
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Abstract

Since the 1900’s it has been known that microorganisms are capable of generating electrical power through extracellular electron transfer, by converting the energy found in organic compounds (Potter, 1911). Microbial fuel cells (MFCs) has garnered more attention recently, and have shown promise in several applications, including wastewater treatment (Yakar et al., 2018), bioremediation (Rosenbaum & Franks, 2014), biosensors (ElMekawy et al., 2018) desalination (Zhang et al., 2018) and as an alternative renewable energy source in remote areas (Castro et al., 2014). In MFCs catalytic reactions of microorganisms oxidize an electron donor, through extracellular electron transfer to the anode and with the cathode exposed to an electron acceptor, facilitating an electrical current (Zhuwei, Haoran & Tingyue, 2007; Lovley, 2006). For energy production in remote areas a low cost and easily accessible feed stock is required for the MFCs. Sweet sorghum is a drought tolerant feedstock with high biomass and sugar yields, good water-use efficiency, established production systems and the potential for genetic improvements. Because of these advantages, sweet sorghum stalks were proposed as an attractive feedstock (Rooney et al., 2010; Matsakas & Christakopoulos, 2013). Dried sweet sorghum stalks were, therefore, tested as a raw material for power generation in MFCs, with anaerobic sludge from a biogas plant as inoculum (Sjöblom et al., 2017).

Using sorghum stalks, the maximum voltage obtained was 546±10 mV, the maximum power and current density was 131±8 mW/m² and 543±29 mA/m² respectively and the coulombic efficiency was 2.2±0.5%. The Ohmic resistances were dominant, at an internal resistance of 182±17 Ω, calculated from polarization data. Furthermore, hydrolysis of the dried sorghum stalks did not improve the performance of the MFC, but slightly increased the total energy per gram of substrate. During the MFC operation, the sugars were quickly fermented to formate, acetate, butyrate, lactate and propionate with acetate and butyrate being the key acids during electricity generation.

Efficient electron transfer, between the microorganisms and the electrodes, is an essential aspect of bio-electrochemical systems such as microbial fuel cells. In order to design more efficient reactors and to modify microorganisms for enhanced electricity production, understanding the mechanisms and dynamics of the electron transport chain is important. It has been found that outer membrane C-type cytochromes (OMCs) (including omcS and omcZ discussed in this study) play a key role in the electron transport chain of Geobacter
*Geobacter sulfurreducens*, a well-known, biofilm forming, electro-active microorganism (Millo et al., 2011; Lovley, 2008). Raman microscopy is capable of providing biochemical information, i.e., the redox state of these c-type cytochromes (cyt-C) without damaging the microbial biofilm, allowing for in-situ observation.

Raman microscopy was used to observe the oxidation state of OMCs in suspended cultures, as well as in MFC biofilms. First, the oxidation state of the OMCs of suspended cultures from three *G. sulfurreducens* strains (PCA, KN400 and ΔpilA) was analyzed. It was found that the oxidation state can be used as an indicator of the metabolic state of the cells, and it was confirmed that PilA, a structural pilin protein essential for long range electron transfer, is not required for external electron transfer. Furthermore, we designed a continuous, anaerobic MFC, enabling in-situ Raman measurements of *G. sulfurreducens* biofilms during electricity generation, while poised using a potentiostat, in order to monitor and characterize the biofilm. Two strains were used, a wild strain, PCA, and a mutant, ΔOmcS. The cytochrome redox state, observed through the Raman spectra, could be altered by applying different poise voltages to the electrodes. This change was indirectly proportional to the modulation of current transferred from the cytochromes to the electrode. This change in Raman peak area was reproducible and reversible, indicating that the system could be used, in-situ, to analyze the oxidation state of proteins responsible for the electron transfer process and the kinetics thereof.

**Keywords:** MFC, Microbial fuel cell, Raman microscopy, BES, *Geobacter sulfurreducens*
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Acronyms

CE  Coulombic efficiencies

cytC  C-type cytochrome

ETC  Electron transport chain

HPLC  High-Performance Liquid Chromatography

MES  Microbial Electrosynthesis

MFC  Microbial Fuel Cell

OMC  Outer Membrane Cytochromes

PEM  Proton exchange membrane

RR  Resonance Raman

TCA  Tri-carboxylic acid

VFA  Volatile fatty acid
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List of attached papers

This licentiate thesis is based on the following research articles:


1 Introduction

1.1 Bio-electrochemical systems

It has been known, for more than a hundred years, that microorganisms can produce electrical energy via the oxidation of organic compounds (Potter, 1911). For a long time, this was mostly seen as a scientific curiosity, yet recent advances and a drive for finding more environmentally friendly methods have led to rapidly growing interest in the research field of microbial electrochemistry. This is a highly interdisciplinary field, combining microbiology, electrochemistry, materials science, engineering, and other related areas. This unique research field has led not only to a far better understanding of microbial electrochemistry, but also to a variety of practical applications, of which we are only seeing the beginning (Wang & Ren, 2013).

The research of microbial electrochemistry has led to a flexible application platform, i.e. microbial electrochemical systems which is a rapidly growing environmental technology that utilizes electron transfer between microorganisms and electron conductors. These electron conductors can either be naturally occurring conductive materials, or physical electrodes, typically made from graphite or metals (Schröder, Harnisch & Angenent, 2015; Wang & Ren, 2013).

Some of the applications that have been researched are: direct power generation (Sjöblom et al., 2017), chemical production (via microbial electrolysis cells or microbial electrosynthesis) (LaBarge et al., 2017; Jourdin et al., 2016; Chen et al., 2016), water desalination (Zhang et al., 2018), biosensors (ElMekawy et al., 2018) or wastewater treatment, including sulphate, nitrite or COD removal. (Feng et al., 2008; Puig, Serra, Vilar-Sanz, et al., 2011; Coma et al., 2013).

The microbial electrochemical systems offer some unique environmental technologies, such as an alternative electricity production from various organic substrates, discussed below, as well as carbon capture and storage in the form of microbial electrosynthesis (MES) cells. MES is not discussed in detail in this thesis, yet is believed to be a key part of the future of BES. The sequestration of CO₂ is seen as a possible method to mitigate climate change, caused by our over-use of fossil fuels (Boot-Handford et al., 2014). In MES cells CO₂ can therefore be used as a carbo feedstock to produce various key chemicals, commonly acetate,
using an external source of electricity, such as a renewable energy source (Wenzel et al., 2018).

The scope of the current work only includes the use of microbial fuel cells (MFCs) for direct power generation and the characterization of biofilms used in MFCs.

1.1.1 Microbial fuel cells

MFCs is one of the most researched systems in the microbial electrochemical systems platform (Wang & Ren, 2013). MFCs have shown promise in wastewater treatment, including some larger scale projects (Liang et al., 2018), bioremediation (Rosenbaum & Franks, 2014) or even as an alternative renewable energy source in remote areas (Castro et al., 2014). There is also an increase in research on MFC based biosensors (ElMekawy et al., 2018), as well as more intricate combined BESs such as combined osmotic MFC and up-flow microbial desalination cells (Sevda & Abu-Reesh, 2019).

In order to produce electricity, MFCs anaerobically oxidizes biodegradable substrates, such as waste materials or acetate, generating electrical current through extracellular electron transfer to the anode. A two-chambered MFC is one of the most common set-ups and is made up of a cathode chamber and an anode chamber, separated by a proton exchange membrane (PEM). A schematic of an H-cell MFC can be seen in Figure 1-1.

![Figure 1-1: Simplified Schematic of a typical three electrode, two-chamber MFC.](image-url)

Microorganisms are grown in the anode chamber, either as a biofilm or as a suspended culture, while the cathode is then exposed to an electron acceoptor to facilitate an electrical current. The two cells are typically separated by a PEM, allowing protons to migrate to the
cathode. The PEM is however one of the more expensive components of an MFC and several lower cost alternatives have been proposed, such as Fe₃O₄/PES nano composite membranes, or various ceramic membranes (Di Palma et al., 2018; Cheraghipoor et al., 2019; Ahilan, Wilhelm & Rezwan, 2018).

One of the most cost-effective electron acceptors typically used is oxygen, achieved by exposing the cathode to air. However, since the MFCs require anaerobic conditions, this can cause technical problems for lab scale applications, and therefore a strong electron acceptor such as ferro-cyanide is often used for many research purposes (Zhuwei, Haoran & Tingyue, 2007; Lovley, 2006; Logan & Regan, 2006; Thygesen et al., 2009).

For electron transfer via direct contact of the cell with the anode (either with pili or the cell’s surface), a well-established biofilm is one of the key factors of efficient electron transfer. There is a lot of work on designing electrodes to lower costs, enhance biofilm formation and improve electron transfer (Sonawane et al., 2017; Bhargavi, Venu & Renganathan, 2018). In order to design more efficient electrodes, it is important to understand the mechanisms and dynamics involved in the electron transfer (Lovley, 2008). This is evident when one looks at the improvements already achieved using improved electrode designs by, for example, the addition of Mn⁴⁺ or Fe³⁺ to graphite electrodes or the increase of the specific surface area by using porous or granular electrodes (Park & Zeikus, 2003; Jourdin et al., 2016; Liu et al., 2018). However, because they are cheap, inert and easy to shape, graphite electrodes are often been used for fundamental research purposes (Gregory, Bond & Lovley, 2004).

1.1.2 Microorganisms used for MFCs

Many microorganisms have been found that possess the capability of transferring electrons, derived from metabolic activity, to an anode. For example, members of Proteobacteria, Cytophagales, Firmicutes, Acidobacteria and yeasts are known to be capable of producing electricity (Franks & Nevin, 2010). A list of some of these organisms can be seen in table 1. Marine sediment, soil, fresh water sediment, wastewater, and activated sludge have all been found to be good sources for these electroactive microorganisms (Pisciotta et al., 2012; Caccavo et al., 1994; Zhuwei, Haoran & Tingyue, 2007).

Of these, some bacteria are particularly efficient in transferring electrons directly to the anode via specialized sets of cytochromes and other proteins including conductive pili (Lovley, 2012). Whereas other microorganisms require synthetic or self-produced mediators for electron transfer, without physically making contact with the electrode surface (Rabaey et al., 2005).
When using more complex feedstock, it might be advantageous to use a complex environmental inoculum (e.g. anaerobic sludge, manure or urban waste water) since the broad spectrum of bacteria could have a synergistic effect in the degradation of the complex organic matter and could lead to a more stable consortium (Ishii et al., 2015).

Table 1-1: A partial list of microorganisms that have been studied in MFCs

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonas hydrophila</td>
<td>Acetate</td>
<td>(Pham et al., 2003)</td>
</tr>
<tr>
<td>Clostridium beijerinckii</td>
<td>Starch, glucose, lactate, molasses</td>
<td>(Liu et al., 2015)</td>
</tr>
<tr>
<td>Candida melibiosica</td>
<td>Glucose, fructose, sucrose</td>
<td>(Sekrecka-Belniak &amp; Toczyłowska-Maminska, 2018)</td>
</tr>
<tr>
<td>Desulfovibrio desulfuricans</td>
<td>Sucrose</td>
<td>(Ieropoulos et al., 2005; Kang et al., 2014)</td>
</tr>
<tr>
<td>Erwinia dissolvens</td>
<td>Glucose</td>
<td>(Vega &amp; Fernández, 1987)</td>
</tr>
<tr>
<td>Methylomusa anaerophila</td>
<td>Methanol</td>
<td>(Amano et al., 2018)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Glucose, sucrose</td>
<td>(Feng et al., 2018)</td>
</tr>
<tr>
<td>Geobacter metallireducens</td>
<td>Acetate</td>
<td>(Min, Cheng &amp; Logan, 2005)</td>
</tr>
<tr>
<td>Geobacter sulfurreducens</td>
<td>Acetate</td>
<td>(Krige et al., 2019)</td>
</tr>
<tr>
<td>Gluconobacter oxydans</td>
<td>Glucose, ethanol</td>
<td>(Lee et al., 2002; Plekhanova et al., 2018)</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>Glucose</td>
<td>(Rhoads, Beyenal &amp; Lewandowski, 2005)</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>Glucose</td>
<td>(Vega &amp; Fernández, 1987)</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>Glucose</td>
<td>(Choi et al., 2003)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Glucose</td>
<td>(Rabaey et al., 2005)</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>Glucose</td>
<td>(Badea et al., 2019)</td>
</tr>
<tr>
<td>Shewanella oneidensis</td>
<td>Lactate</td>
<td>(Lamberg &amp; Bren, 2016)</td>
</tr>
<tr>
<td>Shewanella putrefaciens</td>
<td>Lactate</td>
<td>(Wu et al., 2018)</td>
</tr>
<tr>
<td>Streptococcus lactis</td>
<td>Glucose</td>
<td>(Vega &amp; Fernández, 1987)</td>
</tr>
</tbody>
</table>

1.1.3 MFC feedstocks, including sweet sorghum

MFCs have potential as green energy devices and it has several advantages over more traditional methods. MFCs can provide direct electrical energy from an extreme variety of organic matter, at ambient to low temperatures and at locations lacking electrical infrastructure (Rabaey & Verstraete, 2005). MFCs have, for example, been used to power lights at a latrine in Ghana, offering a decentralized sanitation solution (Castro et al., 2014). For a widespread use of MFCs it is important that a wide variety of low-cost raw-materials and inoculums can be utilized for electricity generation.

Many pure and complex feedstocks have been studied in MFCs. These include, but are not limited to, acetate, butyrate (Liu, Cheng & Logan, 2005a), glucose, xylose (Thygesen et al., 2009), lactate, starch, cellulose (Rismani-Yazdi et al., 2007; Toczyłowska-Mamińska et al., 2015), manure (Lin et al., 2016), cheese whey (Antonopoulou, Stamatelatou, et al., 2010), urine (Tremouli, Greenman & Ieropoulos, 2018), aquatic weeds (Kaur et al., 2018), brewery
waste (Feng et al., 2008), urban waste water (Rodrigo et al., 2007) and landfill leachate (Puig, Serra, Coma, et al., 2011).

Sorghum (Sorghum bicolor {L.} Moench) offers a high potential as a feedstock for fuels and chemicals, and has received a lot of attention as a bioenergy crop (Rooney et al., 2007). Sorghum has a high biomass yield per area and accumulate high levels of sugars in their stalks, which can further be hydrolyzed to utilize the cell wall biopolymers. Combined with its ability to withstand droughts, low water usage, established production systems and the potential for genetic improvements, sorghum is a highly attractive alternative feedstock for bioenergy production (Rooney et al., 2007; Li et al., 2018). Because sweet sorghums accumulate sugars in their stalks, these are of particular interest to the biorefinery industry. On a dry weight basis, sweet sorghum stalks contain about 50% soluble sugars (sucrose, glucose, fructose), 35% insoluble carbohydrates (hemicellulose and cellulose) and 3.2% lignin.

Sorghum is one of the most widely grown cereal crop in the world, mostly concentrated in Sub-Saharan Africa and India, where it is used as a food and feed grain, or even as a fuel source (Rooney et al., 2007). Relatively recently several countries or areas have also started commercialization of sorghum for biofuels, including, Brazil, The United States, Central America, China, Australia, India, Zimbabwe, Mozambique, Angola and the Philippines (Umakanth et al., 2019).

Sweet sorghum has been studied as a feedstock for the production of platform chemicals and biofuels, including ethanol (Matsakas & Christakopoulos, 2013), biogas (Ostovareh, Karimi & Zamani, 2015), butyric acid (Sjöblom et al., 2015), lipids (Matsakas et al., 2014), hydrogen (Antonopoulou, Gavala, et al., 2010) and 1-butanol (Sirisantimethakom et al., 2016). Sweet sorghum could, alternatively, be used as a raw material for electrical energy production in microbial fuel cells (MFCs).

Sweet sorghum stalks have not been studied in MFCs, as far as the author knows, and this study intends to examine the efficacy of dried sweet sorghum stalks when used as a substrate for direct electricity generation in an anaerobic sludge MFC.

By enzymatic treatment of the sweet sorghum, the amount of extractable sugars could potentially be increased significantly and the cellulosic part would be more susceptible to microbial degradation. In order to improve the electrical energy recovery, an enzymatic treatment of sorghum stalks with the commercially available Cellic® CTec 2 was also investigated.
1.2 Geobacter sulfurreducens

*G. sulfurreducens* is a gram negative, obligately anaerobic, metal and sulphur-reducing proteobacterium that is considered to be a well-known electroactive bacteria (Caccavo et al., 1994). It is often used as a model electroactive organism for MFCs, due to its tendency to form thick biofilms and ability to transfer electrons directly to electrodes without the need for external mediators. It is rod-shaped, non-fermentative that has flagellum and Type IV conductive pili. Type IV pili can generate motile forces and are also responsible for adhesion to surfaces or other cells.

During power generation, the oxidation of NADH, derived from organic matter oxidation, produces electrons, in association with proton pumping required for energy production. The subsequent electron transfer merely serves to translocate the electrons in order to avoid a build-up of electrons in the cell (Lovley, 2008; Mehta et al., 2005; Kim et al., 2008). Several physical or chemical electron acceptors can be used, and in this study three abiotic electron acceptors were used with *G. sulfurreducens*, i.e., fumarate and insoluble Fe(III)oxide for the suspended cultures along with a poised graphite electrode for the continuous on-line MFC system.

Fumarate is an electron acceptor that can be incorporated into cells and it has been shown that fumarate cannot be used as a carbon or energy source by *G. sulfurreducens*, since the succinate produced from formate reduction is excreted into the medium, and not oxidized in the tri-carboxylic acid (TCA) cycle (Galushko & Schink, 2000). Fumarate is reduced using a membrane bound fumarate reductase complex, FrdCAB, that also functions as a succinate dehydrogenase (Butler et al., 2006). Because fumarate is internalized, it has a comparatively simple electron transport chain (ETC), and its role in respiration has been extensively studied (Esteve-Núñez, Núñez & Lovley, 2004; Esteve-Nunez et al., 2005).

Insoluble Fe(III)oxide, on the other hand, is an extracellular electron acceptor and also the predominant form of Fe(III) in most soils and sediments. When Fe(III)oxide is used as an electron acceptor, electrons need to be transported outside the cell, leaving protons in the cytoplasm, in contrast to the use of fumarate which is taken up by the cell. It is, however, unlikely that this final electron transfer yields additional energy to the cell (Lovley, 2008). It is the translocation of these protons that dissipates the membrane potential and acidifies the cytoplasm, and this is theoretically the reason why growth rates are approximately 3 fold lower during Fe(III) reduction (Mahadevan et al., 2006).
1.2.1 *G. sulfurreducens* electron transport chain

Cyt-Cs play a key role in the ETC of *G. sulfurreducens*. It has an unprecedented number of putative cyt-Cs, with 111 coding sequences containing at least one match to the cyt-C motif that identifies heme groups and 73 cyt-Cs containing two or more heme groups. This extreme abundance in cytochromes is most likely due to the important role that they play in the ETC, and it also highlights the complexity of the direct electron transfer (Methé et al., 2003). Despite being one of the most studied systems, the mechanisms behind the different processes and how they are connected are still uncertain (Yates et al., 2018).

Several enzymes are believed to participate in the ETC, these include several outer membrane cyt-Cs (OMCs) (OmcS, OmcZ and OmcB) and the structural pilin protein PilA. A schematic seen in Figure 1-2 (Millo et al., 2011; Lovley, 2008). PilA, is a monomer that assembles into the electrically conductive pili found in *G. sulfurreducens*, which play a key role in long-range electron transfer through anode biofilms and are capable of forming a matrix with sufficient conductivity to account for electron flow through biofilm (Malvankar et al., 2011; Liu et al., 2019). The cytochrome OmcS is mainly localized along the pilA nanowires and is believed to be a key part of Fe(III) oxide reduction. It was believed that OmcS is important for the conductivity seen in the *G. sulfurreducens* pili, however recent studies have shown the presence of OmcS is not sufficient to confer the metallic like conductivity observed to pili (Liu et al., 2014; Malvankar et al., 2011). OmcZ has been found to participate in electron transfer through the biofilm bulk and is believed to be required for a low potential electron transport pathway (Richter et al., 2009; Peng & Zhang, 2017).

Specific information about the electron transfer to extracellular and intracellular electron acceptors was achieved by using four mutants of *G. sulfurreducens*. These include: the wild strain PCA (Caccavo et al., 1994); the strain KN400, which has enhanced capacity for current production, developed from PCA through selective pressure (the enhancement was associated with a greater abundance of electrically conductive pili) (Yi et al., 2009); a strain lacking PilA (ΔpilA); and finally a strain lacking the cytochrome OmcS, (ΔOmcS), which is believed to be important in Fe(III) oxide reduction. Counter-intuitively KN400 is known to have significantly lower levels of cyt-Cs when compared to the PCA strain (Yi et al., 2009).
1.3 Raman Spectroscopy

Raman spectroscopy relies on Raman scattering (inelastic scattering) of a monochromatic excitation light, usually from a visible, near infrared, or near ultraviolet laser (244-1064 nm). When the incident beam hits the sample there will be a large amount of elastic scattering (Rayleigh scattering), with no net energy transfer between the sample and the incident photon occurring. However, the light will also interact with the molecular vibrations, phonons or other excitations in the system resulting in a change of energy in the resulting photon. If the out-coming light has less energy (lower frequency) than the original photon, it is called stokes Raman scattering and if it has more energy (higher frequency) it is known as anti-Stokes Raman scattering (Amer, 2010b). Because the light interacts with the molecule, the shift in energy gives information about the vibrational modes in the system and can therefore be used for characterization.

However, in Spontaneous Raman scattering only a small fraction of the incident light produces an inelastic Raman signal which is useful for molecular characterization, while the majority of all incident photons undergo elastic Rayleigh scattering. Therefore, one of the most important parts of Raman spectroscopy is removing the intense Rayleigh scattered laser light from the signal in order to observe the weak inelastically scattered light from. The Rayleigh scattered light is filtered out using notch or edge filters, or a band pass filter. (Kudelski, 2009; Amer, 2010a).
Resonance Raman (RR) spectroscopy is particularly well-suited for examining the structure, environment and electronic properties of heme(s) in cyt-Cs. The RR spectra of cyt-Cs excited with visible excitations can provide vibrational information on the heme group without any interference from the protein or the solvent used. Furthermore, since the oxidation state of the heme group changes the vibrational modes of the molecule, resonance Raman spectroscopy can be used to monitor the oxidation state of heme groups (Desbois, 1994). An example of a raw and processed Raman spectra for *G. sulfurreducens* can be seen in Figure 1-3.

As discussed above *G. sulfurreducens* have a lot of cyt-Cs and the sheer abundance makes the cultures/biofilms visibly red. Four strong bands are known to be ascribed to the excitation of the heme groups found in the cyt-C, that are prevalent in the biofilm of *G. sulfurreducens* (at 747, 1133, 1310 and 1583 cm⁻¹) (Lebedev, Strycharz-Glaven & Tender, 2014; Virdis et al., 2014). This also compares well with the results obtained using cyt-C from horse heart (Pätzold et al., 2008). Because electron transfer is required for cell growth, and OMCs are directly involved in the electron transfer system, the Raman spectra can give information on the metabolic state of the biofilm, including the biofilm stability. In order elucidate specific ETC mechanisms, mutants with changes in key OMCs can be used. By measuring the Raman scattering of active and poised biofilms (specifically, the area of the spectra containing the cyt-C “fingerprint”), it would be possible to monitor the effect that poising the cell at different potentials has on the oxidation state of different biofilms.

![Figure 1-3](image)

**Figure 1-3**: An example of a raw and a processed Raman spectroscopy spectra from a suspended culture of *G. sulfurreducens* wild type strain PCA.
1.4 Thesis objectives

This thesis is focused on:

- The evaluation of sweet sorghum as a potential feedstock in MFCs for direct power generation, using anaerobic sludge as an inoculum.
- The evaluation of an in-situ Raman measurement technique for measuring an anaerobic, modular MFC designed for on-line monitoring and characterization *G. sulfurreducens* biofilms during electricity generation.
2 Experimental

Below is a short summary of experimental methods, please see Paper I & II for a more detailed description.

2.1 Microorganisms, inoculums and media

For all the sorghum experiments, a fortified anaerobic sludge (Biogas Boden, Sweden) was used as both the inoculum and the media. See Paper I for a more detailed description on the startup and operating procedures (Sjöblom et al., 2017).

*G. sulfurreducens* strain used in all Raman spectroscopy experiments, including PCA (ATCC 51573, DSMZ 12127), as well as modified versions of the strain (ΔOmcS, ΔOmcZ, ΔPilA and KN400), were obtained from Dr. Ashley Franks, La Trobe University, Bundoora, Australia. Inoculums and suspended cell cultures were grown in a slightly modified NBAF media and a freshwater media with acetate as the carbon source was used for all continuous stack MFCs. See Paper II for details a more detailed description of the media and start up procedures (Krige et al., 2019).

2.2 Bioreactor design and setup

2.2.1 Standard MFC used with Sorghum feedstock

A standard two-chamber, H-type, MFCs was used for all experiments with sorghum. This consisted of two 250 mL glass bottles connected with a glass tube and separated with a PEM (Nafion, N117). Both the anode and cathode were cylindrical graphite rods (75 mm long and 20 mm in diameter). The electrodes were connected to a 1000 Ω (Sjöblom et al., 2017).

2.2.2 3D-printed cuvette for suspended culture spectroscopy

Due to constraints caused by the focal distance of the objective of the Raman microscope and the need for anaerobic conditions, a standard flow through cuvette could not be used. Therefore, a 3D printed, flow-through cuvette in PETG, was designed and printed using a standard 3D printer, see Figure 2-1. Error! Reference source not found. (Prusa i3 MK3, Praha, Czech Republic). A thin cover-glass slide was then glued on top of the cuvette. A spherical inner volume was used in order to avoid any gas bubbles being trapped in the cuvette.
2.2.3 Continuous small-scale fuel cell for spectroscopy

Since no commercial product was available, a continuous MFC cell needed to be designed and built in order to allow for on-line Raman spectroscopy measurements. A two-chamber, modular reactor made using polycarbonate with stainless-steel tube-fittings and silicone gaskets, was designed, and built by Mercury engineering (Edenvale, South Africa). The chambers were separated with a PEM (Nafion N117). Each cell was fitted with solid graphite electrodes (70 × 22 × 10 mm), and a Ag/AgCl reference electrode was added to the anode chamber. A microscope slide was glued into window cut out of one cover plate. See Figure 2-1.

![Figure 2-1: A schematic and photo of a 3D-printed cuvette for suspended culture spectroscopy of G. sulfurreducens and photos of the stack MFC used for on-line Raman measurements.](image)

2.3 Analytical methods

For suspended cultures, using 3D printed cuvettes, the Raman spectra were collected using a Ramanscope III spectrometer in combination with a SENTERRA module allowing dispersive Raman microanalysis. Since the MFC stack did not fit onto the stage of the Ramanscope, all the Raman spectra for the stack MFC measurements were collected using an inverted microscope (Olympus IX71, Tokyo, Japan) coupled to a Raman spectrometer, Shamrock 303i (Andor Technology, Belfast, UK). Both Raman spectrophotometers were
equipped with a 532 nm excitation laser. For details on the collection methods and data analysis see Paper II.

Where applicable, biomass concentrations were determined using the optical density at 600 nm and the concentration of organic acids and sugars were determined using a Perkin-Elmer HPLC system with a Series 200 refractive index detector, as previously described (Sjöblom et al., 2015).
3 Summary of results

3.1 Paper I Sorghum as MFC feedstock

The aim of Paper I was to evaluate the effectiveness of dried sweet sorghum stalks as a raw material for direct electricity generation in anaerobic sludge inoculated MFCs. Dried sorghum stalks as well as a sorghum hydrolysate were evaluated and as a comparison the MFCs were also operated using glucose as a carbon source. A metabolic study was also done in order to better understand how the substrate is metabolized and how the sugars and the production and consumption of organic acids influence the electricity generation.

3.1.1 General performance of the MFCs

The MFCs were first started using glucose and reached 580-620 mV over a 1 kΩ resistance, with an initial lag phase of 40-96 hours before the voltage started to increase. When the cells were operational, glucose (3 g/L), dry sorghum stalks or the dry sorghum stalk hydrolysate was added. The voltage increased rapidly (10-30 min), quickly reaching a maximum, between 520-580 mV. The rapid increase implies that a stable electroactive consortium had been formed, the voltage response can be seen in Figure 3-1, and a summary of the results are given in Table 3-1. An additional test was conducted to determine whether a biofilm was present on the electrode. Under anaerobic conditions an active electrode was removed and rinsed with trace element solution, before being placed into a newly prepared anaerobic sludge cell, with 1.6g/L glucose. The voltage increased to 560mV within 30 minutes, instead of the normal lag phase of several days, thereby confirming some biofilm activity.

Figure 3-1: Representative voltage versus time graphs for the addition of glucose (3 g/L) followed by three consecutive additions of sorghum hydrolysate (0.73 g/L) (A) and dry sorghum stalks (0.73 g/L) (B). The small spikes are from measurements and the jagged initial part of the glucose graph is because of sparging.
There was no statistical difference in the maximum power output between the investigated substrates, even though different substrate concentrations were used (i.e. 3 g/L glucose and 0.73 g/L sorghum or hydrolysate). Liu, Cheng & Logan, (2005b) showed that the power of an MFC can be modeled using a Monod type equation, and that, given high enough substrate concentrations, it will be insensitive to the substrate concentrations. Since all the MFCs obtained virtually the same maximum voltage, it was assumed that the substrate concentrations were high enough to ensure that the MFCs were operating at their maximum power level. The lower concentrations did, however, lead to shorter current production times, allowing for more experimental repetitions.

Table 3-1: Summary of performance data for the MFCs, recorded with a 1000 Ω resistor. The mean ± standard deviation is presented from at least two individual experiments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Initial total soluble sugarsa (mg/L)</th>
<th>Mean voltage (mV)c</th>
<th>Max power density (mW/m²)</th>
<th>Max current density (mA/m²)</th>
<th>Coulombic efficiency (%)</th>
<th>Internal resistance (Ohm)d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry sorghum stalks (0.73 g/L)</td>
<td>289±13</td>
<td>546±10</td>
<td>131±8</td>
<td>543±29</td>
<td>2.2±0.5</td>
<td>182±17</td>
</tr>
<tr>
<td>Sorghum stalk-hydrolysate (0.73 g/L)b</td>
<td>294±12</td>
<td>541±7</td>
<td>128±5</td>
<td>561±42</td>
<td>2.5±0.4</td>
<td>184±13</td>
</tr>
<tr>
<td>Glucose</td>
<td>3000</td>
<td>566±0.7</td>
<td>136±16</td>
<td>602±29</td>
<td>5.3±0.7</td>
<td>199±8</td>
</tr>
</tbody>
</table>

a) For the sorghum material this refers to sucrose, fructose and glucose.
b) The sorghum hydrolysate was added in an amount equivalent to 0.73 g/L dry sorghum stalks.
c) The mean voltage for each run was calculated as the mean between the start and end voltage during the high part of the voltage graph.
d) The internal resistance was calculated from the slope of the voltage versus current graph with variable resistances ranging from 100 000-50 Ohms.

The coulombic efficiencies (CE) were quite low for all MFC runs, even though it was higher when glucose was used. However, a significant loss of the substrates to biogas production is believed to occur due to the competition between methanogens and electroactive microorganisms present in the sludge. There has been several studies on the suppression of methanogens in bioelectrochemical systems, via physical, chemical & biological pretreatment methods, in order to increase the CE (Jadhav et al., 2019). It is however expected that the CE decreases with more complex substrates, as shown by a decrease in CE when the degree of polymerization of cellulosic materials was increased (Ahmad et al., 2013).
The CE did not differ significantly between the sorghum stalks and the sorghum hydrolysate, however, the total electrical energy generated was slightly higher for the sorghum hydrolysate (42±8 J/g) compared to the dried stalks (36±3 J/g) and close to glucose (39±5 J/g). This would however be significantly higher if only the soluble sugars were considered (104 J/g and 91 J/g for the hydrolysate and dried stalks respectively), which would suggest that the microbial consortia could make use of other compounds in the sorghum material.

3.1.2 Metabolic study

To better understand the role of the various substrates and products on the electricity generation, the consumption of the sugars and the production and consumption of VFAs were monitored during electricity generation. A lower medium conductivity during the metabolic studies caused the MFCs to operate at a slightly lower performance than before, the voltages were therefore normalized relative to acetate.

The metabolic study was done using sorghum hydrolysate, Figure 3-2, and compared to pure glucose and sucrose, Figure 3-3, which were the main sugars present in the hydrolysate. In all the MFCs the sugars (sucrose, glucose or fructose) were consumed first, within 4-6 hours, coupled with a simultaneous production of formate, acetate, lactate and butyrate, as seen in Figure 3-2. The production of the VFAs is consistent with organic matter degradation in methanogenic environments (Stams, 1994). Because the VFAs contributed to the electricity generation, the main VFAs, which were acetate and butyrate, were also tested in an MFC, Figure 3-4.

The voltage quickly increased to its maximum value, as the sugars were consumed and the acids formed. A small peak was often observed as formate and lactate were consumed (within about 25 hours), after which it decreased slightly and stabilized. Thermodynamically, formate could generate higher voltages compared to acetate or lactate, and a more efficient ETC might be possible, but studies have shown that formate tends to generated lower power densities compared to acetate and lactate (Kiely et al., 2010).
Acetate and butyrate were then consumed more slowly, coinciding with electricity production, with a sharp voltage drop after their depletion. Unlike the MFCs with glucose and sucrose, the sorghum hydrolysate MFCs also produced propionate, which seems to have caused a long secondary plateau at 50-90 hours, Figure 3-2. Since propionate was the only detectable acid during this phase it seems feasible that it contributed to electricity generation at least in part. A similar plateau is observed in the glucose and sucrose MFCs, yet for a significantly shorter time Figure 3-3, yet there were no detectable VFAs during the secondary plateau.

Since this secondary plateau is only evident when sugars were used, and is not seen in Figure 3-4 where only acetate and butyrate were used, this could reflect microbial growth on the
sugars and a subsequent consumption of dead lysed cells following exhaustion of the major substrates.

With the use of sorghum, acetate was present at approximately twice the concentration of butyrate, whereas the reverse was true during electricity generation using glucose or sucrose. This might be due to a metabolic shift caused by the higher sugar concentrations, favoring butyric acid production. In a similar study, when the glucose concentration was increased above 3.5 g L\(^{-1}\) day\(^{-1}\), the electron transfer decreased and higher concentrations of VFAs were produced, with butyric acid being the main VFA (Rabaey et al., 2003).

![Figure 3-4: Normalized voltage and acid concentrations versus time for (A) acetate and (B) a mix of 0.8 g/L acetate and 0.4 g/L butyric acid.](image)

A large portion of the electricity was produced during the consumption of acetate and butyrate, and these slowly decreased during the operation of the MFCs, Figure 3-4. When acetate and a mix of acetate and butyrate were used as substrates, the maximum voltages were higher than the sugar substrates, yet there was no detectable difference in peak voltage between acetate or the mix of acetate and butyrate. The higher voltages might be due to the higher concentrations of the VFAs when compared to the sugars.

### 3.2 Raman spectroscopy

Raman measurements were done in three stages, first experiments were carried out using suspended cells grown in a serum bottle, to get preliminary Raman measurements and understand the effect of oxidation on the Raman spectra. Secondly, cells were grown in a chemostat, centrifuged and resuspended in a buffer solution, in order to obtain repeatable samples in the exponential phase, and eliminate the effect of substrate concentration. Lastly, on-line measurements were taken while poised at different voltages, using biofilms grown in a stack microbial fuel cell.
3.2.1 Serum bottle suspended cultures

Three strains were used for the initial suspended culture tests, namely KN400, PCA and ΔpilA. Of these strains, KN400 should have more abundant pili, and can be compared to ΔpilA, with PCA being the standard. The metabolites and Raman spectra were measured at different intervals for the duration of the cultivation. The Raman measurements were taken only after significant biomass was obtained.

Figure 3-5 shows the metabolite concentrations during a cultivation, as well as the absorbance (i.e., OD600) and the Raman peak areas for the strains ΔpilA and KN400. During growth of *G. sulfurreducens* on acetate and fumarate, the production and excretion of succinate is expected (Mahadevan et al., 2006; Pandit & Mahadevan, 2011).

There was a sharp drop in the peak area (4.1 ± 0.83-fold) after stationary phase was reached (i.e., when the limiting substrate has been completely consumed, fumarate in this case, and the primary metabolism ceases, ±70 h in this case). It therefore appears that the oxidation state of the cyt-Cs (measured via the Raman peak areas) is related to the metabolism of the culture, and is not merely related to the biomass concentration.

One way of understanding how this drop in Raman peak area is related to the primary metabolism is through the capacitor hypothesis (Esteve-Núñez et al., 2008). Theoretically, a capacitor-like electron “storage system” is produced when the cytochromes are charged during growth on the rich medium. There would be a continuous charge and discharge of the
cytochromes, since the system is dynamic, and when the fumarate is exhausted the primary metabolism stops resulting in the cessation of the charging of the cytochromes and a decrease in the peak area.

As seen in Figure 3-5, the maximum peak areas obtained for KN400 cultures were significantly lower than for that of PCA and ΔpilA. For example, the peak areas were on average 61% lower than the peaks for PCA, despite similar cell concentrations. This is most likely because cyt-Cs were much less abundant in KN400, compared to the PCA strain (Yi et al., 2009).

3.2.2 Chemostat Grown suspended cultures

Because of the significant differences in OMC expression in KN400 only PCA and ΔpilA were grown in chemostats. The aim of these experiments were to observe the electron transfer to an external electron acceptor (Fe(III)oxide), without conductive PilA and also to compare that to an electron acceptor that can be internalized, namely fumarate. The average peak areas of the sample suspended in wash buffer was used to normalize all results, as seen in Figure 3-6. The addition of Fe(III)oxide led to a 50%–80% decrease in the peak area, showing that the mechanisms involved in the electron transfer to an external source does not require pili to oxidize the OMCs. A possible explanation for this might be the direct transfer via OmcZ, since it has been found to participate in homogeneous electron transfer (through the biofilm bulk) (Richter et al., 2009).

It was also observed that the peak area decreases significantly more for PCA than for ΔpilA, suggesting that PCA has a higher capacity for electron transport when compared to ΔpilA. This is consistent with the presence of the conductive pili of the PCA strain, which are believed to be important for the reduction of external electron acceptors such as Fe(III) oxide.
3.2.3 On-line biofilm spectroscopy

Biofilms of two strains, the wild type PCA and a mutant, ΔOmcS, were grown and the Raman spectra of the biofilms were measured while poised at different voltages. It was demonstrated that the redox state of cyt-Cs, which is reflected in the Raman peak area, can be changed by controlling the poising potential. As a substitute for the ΔpilA strain used above, a ΔOmcS biofilm was used, since ΔpilA does not form electro active biofilms. Figure 3-7 shows how the Raman peaks increase with decreasing poised voltage. The area under the peak at 747 cm$^{-1}$ showed the largest response, and was therefore used as the main indicator of cytochrome oxidation state.

Figure 3-6: Normalized Raman peak areas of PCA and ΔpilA samples grown in a chemostat, suspended in a wash buffer (W.B.) and the same samples after the sequential addition of Fe(III)oxide and fumarate. (The error bars show the standard deviation of 6–9 Raman measurements).

Figure 3-7: Processed Raman spectra showing the changes in the strong peak at 745 cm$^{-1}$, which is related to the cytochrome oxidation state.
The increase of the Raman peak area, as the poised voltage decreases, can clearly be seen in both strains (i.e. Figure 3-8 and Figure 3-9), along with a subsequent decrease as the voltage was increased again. The decrease in poising potential serves to increase the resistance, this is believed to cause a build-up of a charge in the cytochromes i.e., the reduction of the cytochromes. When the resistance is lowered again the charge is released leading to the oxidation of the cytochromes. A similar response has been observed in the fluorescence response, where the fluorescence signal was lost when the cytochromes were oxidized (Esteve-Núñez et al., 2008).

The fact that the ΔOmcS MFC responds in a similar manner to the PCA MFC shows that ΔOmcS is not required for external electron transfer to an anode. This fits with the hypothesis that OmcZ, and not OmcS, facilitates electron transfer from the biofilm matrix to the anode (Lovley, 2011). Even though a similar peak area was obtained, ΔOmcS did not form as thick a biofilm as PCA.

Figure 3-8: The Raman peak area at 745 cm$^{-1}$ of PCA biofilm in a microbial fuel cell (MFC) stack, average of two measurements along with the current produced at the different poised level. The current is inversely related to the peak area.
The peak area of the ΔOmcS biofilm decreased drastically when the biofilm was poised at 300mV for the second time. The current produced also showed a relative decrease, yet not nearly as drastic as the Raman peak. This suggest that the biofilm was experiencing a decrease in activity at the end of the measurements. The change is however a lot smaller than that of the Raman peak area. It is therefore likely that this observation is the combination of a general decrease in MFC activity, as well as a localized inactivation of the biofilm, likely caused by the prolonged Raman spectroscopy. This requires further investigation, but a decrease in Raman laser power or exposure time might be required.
4 Conclusions and future work

Dried sorghum stalks were found to be an effective feedstock for direct use in MFCs and gave the same general performance as glucose or sucrose, when using anaerobic sludge as inoculum. A voltage of about 550 mV and a power- and current density of about 130 mW/m² and 550 mA/m² respectively, was achieved using Sorghum. Enzymatic treatment of the dried stalks was shown to be unnecessary since it did not improve the total energy obtained per gram of sorghum stalks. Sugars were quickly fermented in the MFCs, mainly to acetate and butyrate which were the key VFAs during electricity generation. Microbial growth and biogas formation might have led to a low CE for both the sorghum material and glucose, at around 2% and 5%, respectively.

Using suspended cultures, it was shown the Raman peak area can be used as an indicator of the cytochrome redox state of the cells. The measurements do, however, depend on the abundance of OMCs, as seen by the lower Raman peak areas observed when using KN400 when compared to PCA. Using chemostat grown cells, a higher capacity for electron transport was observed in the wild strain, when compared ΔpilA. Despite this, it was still shown that PilA is not required for external electron transfer to Fe(III)oxide.

Using a continuous, anaerobic MFC for on-line Raman measurements, it was shown that Raman can be used to monitor and characterize G. sulfurreducens biofilms during electricity generation. By monitoring the Raman peak area, it was shown that the redox state of the cytochromes could be modulated by applying voltage to the electrodes and was indirectly proportional to the current produced by the MFC.

It was also shown that OmcS is not required for direct electron transfer to a graphite electrode, by measuring the Raman spectra of a ΔOmcS biofilm in an MFC. Furthermore, Raman microscopy allows for the monitoring of biochemical information, i.e., the redox state of cyt-Cs, without destructive interference, such as staining the biofilm. This allowed for the biofilm and suspended cultures to be studied on-line in anaerobic conditions.

Finally, because the OMCs are an integral part of the ETC, the Raman measurements allows one to analyze the biofilm’s metabolic state as well as gain an understanding of the mechanisms involved the ETC. In order to utilize the Raman characterization method further, a recommendation for future work includes the kinetic study of Cytochrome redox reactions using the current resonance Raman setup with significantly shorter exposure times (ca. 1s). There might also be a possibility to utilize a three-dimensional holographic stimulated Raman
microscopy to observe the same kinetics. This should provide several benefits above the standard setup, including the ability of observing of a 3D section of biofilm, exposure times orders of magnitude smaller, and a high signal to noise ratio due to the stimulated nature of the signal. And finally, we are also working on 3D bio-printing Microbial electrosynthesis biofilms of *Sporomusa ovata*. This will include the use of carbon nanotubes to produce electrically conductive, bio-compatible, printable hydrogels.
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Research Paper

Direct electricity generation from sweet sorghum stalks and anaerobic sludge

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A B S T R A C T

Dried sweet sorghum stalks were valorized as a raw material for electricity generation in a two chamber microbial fuel cell using anaerobic sludge from a biogas plant as inoculum. The maximum voltage obtained on the sorghum stalks at an operating temperature of 35 °C was 546 mV with a maximum power- and current density of 131 mW/m² and 543 mA/m², respectively. The coulombic efficiency was 2.2%. Polarization data indicated that Ohmic resistances were dominant with an internal resistance of 182 Ω. The total electrical energy per gram of dried sorghum stalks was 165 J/g. Enzymatic treatment of the sorghum stalks did not improve the total electrical energy obtained. A metabolic study demonstrated that the sugars were quickly fermented to formate, acetate, propionate, lactate and butyrate with acetate and butyrate being the dominant acids during electricity generation.

1. Introduction

With origins in African agriculture over 6000 years ago the C4 grass Sorghum (Sorghum bicolor (L.) Moench) has received a lot of attention as an efficient bioenergy crop (Rooney et al., 2007). The combination of high biomass and sugar yield with good water use efficiency and tolerance to drought, established production systems and the potential for genetic improvements makes sorghums highly attractive as alternative feedstock for bioenergy production (Rooney et al., 2007). Sweet sorghum (Sorghum bicolor (L.) Moench) has received a lot of attention as a potential alternative to wheat (Rooney et al., 2007). As a disadvantage, sweet sorghum accumulate sugars in their stalks, which consequently are of particular interest to the biorefinery industry. On a dry weight basis, sweet sorghum stalks contain about 50% soluble sugars (sucrose, glucose, fructose), 35% insoluble carbohydrates (hemicellulose and cellulose) and 3.2% lignin (Matsakas and Christakopoulos, 2013). Due to the risk of microbial degradation it is advantageous to dry the stalks before storage (Matsakas and Christakopoulos, 2013). Sweet sorghum has mainly been studied as a feedstock for the production of platform chemicals and biofuels including ethanol (Matsakas and Christakopoulos, 2013), biogas (Ostovareh et al., 2015), butyric acid (Sjöblom et al., 2015), lipids (Matsakas et al., 2014), hydrogen (Antonopoulou et al., 2010a) and 1-butanol (Sirisantimethakom et al., 2016). An alternative use of sweet sorghum could be as a raw material for electrical energy production in microbial fuel cells (MFCs). A two chambered MFC is a common set-up and consists of a cathode chamber and an anode chamber separated by a proton exchange membrane (PEM) (Logan et al., 2006). Microorganisms are grown anaerobically in the anode chamber where they oxidize a substrate to release electrons and protons. The electrons pass to the cathode through an external circuit whereas the protons migrate to the cathode through the PEM. On the cathode surface the electrons and protons participate in the reduction of an electron acceptor and a current is established. The electron acceptor, or catholyte, can be a chemical such as oxygen but for many research purposes a strong electron acceptor such as ferrocyanide is used (Logan et al., 2006; Thygesen et al., 2009). Several pure compounds and complex feedstocks have been studied in MFCs including acetate, butyrate (Liu et al., 2005), glucose, xylose (Thygesen et al., 2009), lactate, starch, cellulose (Rismani-Yazdi et al., 2007; Toczyłowska-Mamińska et al., 2015), manure (Lin et al., 2016), cheese whey (Antonopoulou et al., 2010b), brewery waste (Feng et al., 2008), urban waste water (Rodrigo et al., 2007), etc. Sweet sorghum stalks have not been studied in MFCs as far as the authors know. Complex feedstocks contain a wide range of compounds which the microorganisms have to oxidize in order to generate electricity. For this reason it can be advantageous to utilize environmental inoculums such as anaerobic sludge, manure or urban waste water which contain a broad spectrum of microorganisms that could have synergistic effects in the degradation of the organic matter (Ishi et al., 2015). For example, the microbiota of thermophilic anaerobic sewage sludge have been found to consist of bacteria of the genus Clostridium, Caprothermobacter, Syntrophomonas, archaea of the genus Methanosarcina and...
**Methanobacterium** and fungi of the genus *Candida, Penicillium, Mucor, Saccharomyces* and *Trichoderma* (Ritari et al., 2012). Members of firmicutes, proteobacteria, acidobacteria and yeasts like *Saccharomyces cerevisiae* and *Hansenula anomala* are known to be capable of producing electricity (Franks and Nevin, 2010).

Certain bacterial species are particularly efficient in transferring electrons to the anode in a more direct sense involving specialized sets of cytochromes and other proteins including conductive pili (Lovley, 2012). Other microorganisms can utilize synthetic or self-produced mediators to assist in the electron transfer process without being in direct contact with the electrode surface themselves (Rabaey et al., 2005a). When electron transfer occurs by means of direct contact with the cell (with either pili or the cell’s surface) it is imperative that a biofilm forms on the electrode surface and hence there is a lot of work on trying to understand how the electrode should be designed in order to promote good biofilm formation and efficient electron transfer (Sonawane et al., 2017). For fundamental research purposes graphite electrodes have often been used because they are cheap, inert and easy to shape (Gregory et al., 2004). Microbial fuel cells have the potential to contribute to a more sustainable society providing direct electrical energy from a variety of organic matter at ambient to low temperatures at locations lacking electrical infrastructure (Rabaey and Verstraete, 2005). For a widespread use of MFCs in our society it is important that a broad range of raw-materials and inoculums for electricity generation can be exploited in these systems. In order to increase the number of potential feed-stocks for MFCs this study intends to investigate dried sweet sorghum stalks as a substrate for electricity generation using anaerobic sludge as a source of microorganisms. Enzymatic treatment of the sorghum stalks with the commercially available Cellic® CTec 2 was also investigated in order to improve the electrical energy recovery. By enzymatic treatment the amount of extractable sugars could potentially be increased and the cellulosic part could possibly be more susceptible for microbial degradation.

### 2. Materials and methods

#### 2.1. Microbial fuel cell set up

Two-chamber, H-type, MFCs were used for all experiments (Fig. 1). The chambers consisted of two 250 mL glass bottles connected with a glass tube. A proton exchange membrane (Nafion N117) was used to separate the chambers. The membrane was held in place and sealed by an O-ring and a chain clamp in the middle of the tube. Both the anode and cathode were constructed by embedding a 0.3 cm stainless steel rod, covered using heat-shrink tubing, into cylindrical graphite rods (75 mm long and 20 mm in diameter). The rods were glued in place using a silver conductive epoxy (Epotek 730-110, Epoxy technology inc., MA, USA). The electrode rod was fitted tightly in a rubber stopper along with a glass sample tube. The electrodes were connected using copper wires and a 1000 Ω resistor.

#### 2.2. Inoculation and operation

The sludge used for the experiments was collected from an anaerobic digester at a local biogas plant (Biogas Boden, Sweden), which was run at thermophilic conditions (55 °C). The experiments were started up using fresh anaerobic sludge samples. Sludge samples were analyzed using HPLC and no acetate, butyrate or glucose was detected. Prior to operation the sludge was filtered through nylon filter (pore size 200 μm) to remove larger solid particles and supplemented with 10 mL/L of a trace elements solution according to Antonopoulou et al. (2015). The fortified sludge (220 mL) was used as both the inoculum and the medium in the anode chamber and glucose, sucrose, acetate, butyrate or sweet sorghum was added as a carbon source. The dry sorghum stalks were added either as they were, at a concentration of 0.73 g/L, or at a concentration equivalent to 0.73 g/L dry matter (DM) after being hydrolyzed. The hydrolysis was performed at 20% w/v dry matter using the commercially available enzyme preparation Cellic Ctec 2 (Novozymes, Denmark) at an activity of 10 FPU/g DM, pH 5.0, a temperature of 50 °C and an incubation time of 18 h. Since the dry stalks and hydrolysate could not be pumped they were added to the dehydration process in order to increase the number of potential feed-stocks for MFCs this study intends to investigate dried sweet sorghum stalks as a substrate for electricity generation using anaerobic sludge as a source of microorganisms. Enzymatic treatment of the sorghum stalks with the commercially available Cellic® CTec 2 was also investigated in order to improve the electrical energy recovery. By enzymatic treatment the amount of extractable sugars could potentially be increased and the cellulosic part could possibly be more susceptible for microbial degradation.

![Fig. 1. Schematic figure of the two chamber, H-type MFC used in this study.](image)
anaerobic conditions. The cathode chamber was analyzed using a BioRad Aminex HPX87-P column, kept at 80 °C for the subsequent additions of the substrate. The voltage and energy per gram of substrate for the sorghum hydrolysate were based on three replicates within one experiment whereas the rest of the presented data are based on two replicates within one experiment. The data for the dry sorghum material is based on two replicates within one experiment. For the cases where three replicates were performed the mean ± standard deviation are presented. In the cases where duplicates were performed only the mean is presented.

### 3. Results and discussion

#### 3.1. General performance of the MFCs

The voltage for the startup experiments on glucose reached 580–620 mV over a 1 kΩ resistance with a background voltage of 40–70 mV. The performance of each individual MFC was reproducible, with an initial lag phase of 40–96 h before the voltage started to increase. For the subsequent additions of glucose (3 g/L), dry sorghum stalks and the dry sorghum stalk hydrolysate the voltage reached the maximum value, between 520 and 580 mV, within 10–30 min, suggesting that a stable electroactive consortium had been formed (Fig. 2a and b).

In order to confirm that there was a biofilm on the anode, the MFC was taken into an anaerobic box where the old electrodes were rinsed in sterile trace element solution, described in section 2.2, and transferred to a newly set up MFC using 1.6 g/L of glucose in fresh anaerobic sludge. When the system was reconnected to the voltmeter an increase from 520 to 560 mV could be observed within 30 min and a similar voltage graph as previously obtained on glucose was recorded (data not shown), clearly indicating that there was an electro active biofilm on the anode. However, this does not rule out that some planktonic microorganisms in the sludge could also contribute to electricity prepared. The sample concentrations of the acids and sugars were subsequently determined based on the reference graphs. The cell voltage across a 1 kΩ resistor was measured automatically every 30 s by an ADAM-4017 analog input module (Advantech, California, USA). The chemical oxygen demand (COD) of the samples were calculated by a standard test method for chemical oxygen demand (dichromate oxygen demand) of water (ASTM International, 2000). Briefly, the sample and standardized dichromate solution were refluxed for a digestion period of 2 h, after which the excess dichromate is titrated with a standard ferrous ammonium sulfate solution. Ortho-phenanthroline ferrous complex was used as an internal indicator.

#### 2.4. Calculations

Ohm’s law: \( V = I \times R \), where \( V \) is the voltage in volts, \( R \) is the resistance in ohms and \( I \) is the current in amperes, was used to calculate the current and the power, \( P \), in watts. The power was calculated according to \( P = V \times I \) and the total electrical energy \( E \) in joules was calculated as \( E = P \times t \) where \( t \) is the time in seconds. To get the stoichiometry of water (ASTM International, 2000). Brie

#### 2.3. Analytical methods

The concentration of organic acids and sugars were determined using a Perkin-Elmer HPLC system with a Series 200 refractive index detector, as previously described (Sjöblom et al., 2015). Brie

#### 2.2. Materials

The concentration of organic acids and sugars were determined using a Perkin-Elmer HPLC system with a Series 200 refractive index detector, as previously described (Sjöblom et al., 2015). Brie

**Fig. 2.** Representative voltage versus time graphs for the addition of glucose (3 g/L) followed by three consecutive additions of sorghum hydrolysate (0.73 g/L) (A) and dry sorghum stalks (0.73 g/L) (B). The small spikes are from measurements and the jagged initial part of the glucose graph is due to sparging.
generation through the production of mediators (Rabaey et al., 2005a, 2005b). The recorded mean voltages for the sorghum material did not vary notably, however, for glucose the voltage was somewhat higher (Table 1).

Comparing the maximum power and current from respective experiments revealed no apparent difference between the investigated substrates, even though glucose was used at a concentration of 3 g/L and the sorghum material was used at 0.73 g/L (Fig. 3a and b, Table 1).

The power of a MFC can be modeled according to a Monod type equation. If the substrate concentration is high enough the MFCs will operate at their maximum power and may be insensitive to changes in the substrate concentrations (Liu et al., 2005). The results therefore suggest that the substrate concentrations were high enough to ensure that the MFCs were operating at their highest power or the sorghum material resulted in a higher power generation compared to glucose. The internal resistance is known to be the limiting factor to the power output of MFCs, leading to similar maximum power outputs for the same type of MFCs, as discussed by Min et al. (2005). Major improvements of the performance could therefore only be made by changing the design of the MFC such as changing the distance between the electrodes, the medium conductivity etc. (Logan et al., 2006). The maximum power densities obtained for glucose and the sorghum materials was around 130 mW/m² in the current study (Table 1), which is higher to what has been found in the similar systems using waste water inclusions reporting 38 ± 1 mW/m² (Min et al., 2005) or 28–52 mW/m² for glucose (Thygesen et al., 2009). The higher power densities obtained for glucose in the current study compared to those found by Thygesen et al. (2009) can be attributed to a higher rate of substrate consumption as will be shown later in the metabolic study. The sugars were also fermented to acetate and other volatile fatty acids (VFAs) within 10–20 h which suggests that the measured power is more representative for a mix of volatile fatty acids where acetate and butyrate were dominating. In fact the data in Table 1 are very close to that reported for acetate in a similar system (Thygesen et al., 2009). However, regardless of the metabolic background the power quickly increased and stabilized at the reported values for respective substrates in Table 1.

### Table 1

Summary of performance data of the MFCs on glucose, dry sorghum stalks and sorghum stalk hydrolysate. The voltage was recorded with a 1000 Ω resistor. The mean ± standard deviation is presented when three replicates were made. When two replicates were made only the mean is presented.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Initial total soluble sugars (mg/L)</th>
<th>Mean voltage (mV)</th>
<th>Max power density (mW/m²)</th>
<th>Max current density (mA/m²)</th>
<th>Coulombic efficiency (%)</th>
<th>Internal resistance (Ohm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry sorghum stalks (0.73 g/L)</td>
<td>289</td>
<td>546</td>
<td>131</td>
<td>543</td>
<td>2.2</td>
<td>182</td>
</tr>
<tr>
<td>Sorghum stalk hydrolysate (0.73 g/L)</td>
<td>294 ± 12</td>
<td>541 ± 7</td>
<td>128</td>
<td>561</td>
<td>2.5</td>
<td>184</td>
</tr>
<tr>
<td>Glucose</td>
<td>3000</td>
<td>566 ± 0.7</td>
<td>136</td>
<td>602</td>
<td>5.3</td>
<td>199</td>
</tr>
</tbody>
</table>

* For the sorghum material this refers to sucrose, fructose and glucose.
* The sorghum hydrolysate was added in an amount equivalent to 0.73 g/L dry sorghum stalks.
* The mean voltage for each run was calculated as the mean between the start and end voltage during the high part of the voltage graph.
* The internal resistance was calculated from the slope of the voltage versus current graph with variable resistances ranging from 50 to 100,000 Ω.

#### 3.2. Coulombic efficiency

The coulombic efficiencies (CE) were quite low in the current study, with a value of 2.2–2.5% for the sorghum materials and about 5% for pure glucose (Table 1). However, considering that anaerobic sludge used for biogas production was used in the working chamber a substantial loss of the substrates to biogas production and growth of a variety of microorganisms present in the sludge could be anticipated. For example, in another study using a two-chamber H-type system, with cheese whey as substrate at 0.73 g COD/L, a CE of 1.9% was obtained and it was argued that the reason for this low value was due to growth of undesirable microorganisms present in the cheese whey and the production of gaseous compounds (Antonopoulou et al., 2010b). When glucose was used in the same study a CE of 28% was reported. In another study using a two chamber H-type system a CE of 7.4–9.9% was reported on glucose depending on the amount of humic acid used in the anode chamber (Thygesen et al., 2009). The fact that glucose showed a higher CE than the sorghum material in the current study, is also consistent with other studies on MFCs running on cellulotic materials. It has been shown that the coulombic efficiency decreased as the degree of polymerization of insoluble polysaccharide substrates increased (Ahmad et al., 2013). In the same study it was also concluded that the CE was higher for soluble polysaccharide substrates compared to insoluble polysaccharide substrates. The highest CEs are obtained when the substrate can be completely oxidized to CO₂. Pathways leading to incompletely oxidized substrate and microbial growth therefore led to lower CE. Obtaining high CEs utilizing environmental inclusions is therefore difficult and the highest reported values are between 65 and 89% following enrichment of electrogenic cells (Rabaey et al., 2003). Blocking biogas formation chemically by using 2-bromoethanesulfonate (Ganigué et al., 2015) could possibly be applied to increase the CE.

![Fig. 3. Voltage (open symbols) and total power (solid symbols) vs current using variable resistance from 50 to 100,000 Ω. (A) Two runs on glucose 3 g/L. (B) Two runs on dry sorghum stalks 0.73 g/L (diamond and square) and two runs on sorghum hydrolysate (triangle and circle).](image-url)
3.3. Electrical energy per gram substrate

The total electrical energy generated was 189 ± 37 J/g and 165 J/g for the sorghum hydrolysate and the dried stalks, respectively indicating that there was no major difference between the two fractions. Based on the total soluble sugars (sucrose, glucose and fructose) at the start of the experiments the total energy would be 469 ± 92 J/g and 416 J/g for the hydrolysate and dried stalks, respectively. Glucose showed a value of 241 ± 47 J/g suggesting that the microbial consortia also may have the ability to break down the cellulose part of the sorghum stalks and produce fermentation products such as acetate from which electricity could have been generated. For example, some Clostridial species are known to be cellulolytic (Lynd et al., 2002). The total energies per gram of substrate can be compared to a highly efficient MFC which gave 950 J per 0.08 g of glucose or 11875 J/g glucose (Rabaey et al., 2003).

3.4. Metabolic study

In order to better understand the effect of the substrates on the electricity generation in the MFCs a metabolic study was conducted to follow the consumption of the sugars and the production and consumption of organic acids during the operation of the MFCs. Because some of the VFAs, such as propionate, were formed in quite low amounts, the concentration of the sorghum material was increased to 1.2 g/L to better detect the VFAs during the metabolic study. During the metabolic studies the performance of the MFCs was slightly lower than that of the previous sections. This was caused by a lower conductivity of the medium and therefore only the voltages relative to acetate, which gave the highest voltage, will be given here.

3.4.1. Sorghum hydrolysate

For the sorghum hydrolysate, the sugars (sucrose, glucose or fructose) were consumed within 4–6 h with a drop in pH from 7.1 to 6.7 and concomitant production of formate, acetate, lactate and butyrate (Fig. 4).

This formate and lactate were completely consumed within about 15 h, with the production of propionate and more acetate. Butyric acid was also metabolized by the consortium during this time. As the sugars were consumed and the acids formed, the voltage quickly increased to its maximum value and often displayed a small, extra high peak which coincided with the consumption of formate and lactate after which it decreased slightly and stabilized. The small voltage peak at the start of the experiments can also be seen in the voltage graphs of Fig. 2 and may indicate the extra efficient electron transfer during the formate utilization. Thermodynamically formate could generate the highest voltage compared to acetate or lactate, but studies have shown that under identical conditions formate generated lower power densities compared to acetate and lactate suggesting alternative reasons for the initial high voltage observed in the current study (Kiely et al., 2010). Moreover, to date, there is no report on direct electrical generation from formic acid with exoelectrogens in the absence of exogenous mediators (Kiely et al., 2010). When the acetate and butyrate had been consumed at about 50 h the voltage dropped sharply to a plateau. Since propionate was the only detectable acid during this phase it seems feasible that it contributed to electricity generation at least in part. However, it should be noted that the propionate levels could have dropped to non-detectable levels any time between 50 and 90 h. Electricity production using propionate has also been shown by Desulfobulbus propionicus which is a bacterium of the group delta-proteobacteria that is enriched in marine sediments and known to generate maximum electricity on propionate and lactate (Schaetzel et al., 2008). The lower plateaus were most pronounced when sugars were used as substrates and could possibly also reflect a higher microbial growth on the sugars and a subsequent consumption of dead lysed cells following exhaustion of the major substrates. Because the inoculum was anaerobic sludge from a biogas plant, these results were expected and consistent with the degradation of organic matter in methanogenic environments. Under these conditions complex organic molecules are fermented to hydrogen, formate, acetate, lactate, propionate, butyrate and ethanol by fermentative microorganisms, whereas the acetogenic bacteria oxidize the more reduced compounds such as lactate, ethanol, propionate and butyrate to hydrogen, formate and acetate which can function as substrates for the methanogens (Stams, 1994). In addition, synthrophic acetate-oxidizing bacteria, which normally produce acetate through the Wood-Ljungdahl pathway, can shift pathway and instead oxidize acetate to H2 and CO2, particularly during degradation of protein rich materials (Müller et al., 2013). Electrons for electricity generation could be available for each oxidation step. From the voltage graph it is clear that the most efficient electron transfer occurs during the first 50 h with acetate and butyrate being the major contributors. Microorganisms which have been isolated from MFCs and are known to produce fermentation products belong to the genus Clostridia, Alcaligenes and Enterococcus (Rabaey and Verstraete, 2005). The production of butyric acid supports the inclusion of Clostridia in the sludge as several of these species have butyric acid and acetic acid as their major fermentation products (Vandak et al., 1995; Sjöblom et al., 2015). C. butyricum and C. beijerinckiis are known to be able to produce electricity on starch and Clostridia could hence be involved in the electricity generation (Niessen et al., 2004). The pH ranges used in the MFCs are also optimal for these species. Geobacter species may also be responsible for electricity generation as these can withdraw electrons from acetate and use electrodes as electron acceptors although it is questionable whether they can withstand the thermophilic environment where the sludge was taken from.

3.4.2. Glucose and sucrose

The main sugars in the sorghum stalks are sucrose, glucose and fructose and of these sucrose and glucose were tested individually in the MFCs in order to assess their contribution to electricity generation and how the consortium processed each substrate. The behavior of the MFCs on glucose and sucrose was very similar compared to each other and also compared to the sorghum hydrolysate, with a quick consumption of the sugars and concomitant production of formate, lactate, acetate, and butyrate (Fig. 5a and b).

Formate and lactate quickly dropped to undetectable levels whereas butyric acid and acetate continued to increase until about 25 h. However, in contrast to when the sorghum material was used as substrate, butyric acid was the dominant acid during electricity generation for both glucose and sucrose. The higher sugar concentrations...
compared to the experiments with the sorghum material may have promoted a metabolic shift that favors butyric acid production. For example, in a similar study it was found that when the glucose loading was increased above 3.5 g/L day the electron transfer decreased and the bacterial community accumulated higher amounts of VFAs where butyric acid was the dominant acid (Rabaey et al., 2003). The pH of the sludge was 7 and dropped to about 6.6 when the sugars were consumed and the acids formed, yet increased to about 7 again after the acids had been consumed. The system running on glucose showed a slightly higher normalized voltage (0.88 for glucose; 0.82 for sucrose) (Fig. 5a and b). Notably the normalized voltage for glucose and sucrose were somewhat lower compared to that of the sorghum hydrolysate (0.96) during the high part of the voltage graph. This supports the previous suggestion that a higher power generation could be obtained on the sorghum material, possibly because of its protein content. Similar to when the sorghum material was used, the voltage dropped to a short plateau at a lower voltage level when the butyrate and acetate had been consumed, before finally dropping to the background voltage. No detectable propionate was present at the low voltage phase suggesting that possible storage molecules and/or dead lysed cells were used for electricity generation during this time. In a comparable system running on glucose at 1.2 g/L and a domestic waste water inoculum production of formate, lactate and butyrate was not reported indicating that a different microbial consortium was active in those MFCs (Thygesen et al., 2009).

3.4.3. Acetate and a mix of acetate and butyrate

Because electricity was mainly produced during the consumption of acetate and butyrate these acids and a mixture of them were also investigated as substrates. When acetate and a mix of acetate and butyrate were used as substrates the maximum voltages were higher compared to when the sugars were used (Fig. 6a and b). There was no detectable difference in peak voltage between acetate and the mix of acetate and butyrate. Higher voltage from acetate compared to glucose has been observed in other studies as well (Thygesen et al., 2009; Rabaey et al., 2005b). However, as shown in Fig. 5a and b, the sugars are fermented mainly to acetate and butyrate and it might therefore be expected that the voltages obtained on the sugars would be the same as for the pure acids. The reason for the lower voltage obtained on the sugars could be that the concentration of the formed butyrate and acetate were lower compared to when the pure acids were used and/or that more complex metabolic pathways are involved when the sugars were used as substrates. Interestingly, when using pure butyrate as the substrate, it was noted that butyrate was converted to acetate, and when the butyrate levels reached non detectable levels the acetate was consumed during the course of the experiment (data not shown). Formation of acetate when using pure butyrate as the substrate is consistent with other studies and indicates the activity of acetogenic bacteria which can oxidize more reduced compounds to acetate (Liu et al., 2005). Electricity generation for butyrate could hence be produced during degradation of butyrate and/or following acetate oxidation. Interestingly the low plateau on the last part of the voltage graph was considerably smaller compared to when the sorghum hydrolysate or the sugars were used as substrates.

3.4.4. Relative power densities

Acetate and the mix of acetate and butyrate showed equal and highest power densities, followed by butyrate, sorghum hydrolysate, glucose and sucrose in decreasing order (Fig. 7).

The higher power density of acetate compared to glucose is consistent with other studies using a similar system (Thygesen et al., 2009). The lower power density obtained when using pure butyrate compared to acetate is consistent with the conversion of acetate to butyrate and the losses associated with this reaction. Other studies comparing butyrate and acetate as substrates for electricity generation has also found that acetate gives higher power densities compared to butyrate and suggested that this might be related to a lower uptake rate of butyrate compared to acetate (Liu et al., 2005). A similar argument could be
made for sucrose compared to glucose although according to Fig. 5a and b the consumption rates of glucose and formation rates of butyric acid and acetate are very similar. In contrast to glucose however, sucrose would have to be cleaved before it enters the metabolic pathways which costs energy.

4. Conclusion

Dried sorghum stalks could be used directly in the MFC and gave the same general performance as glucose or sucrose using anaerobic sludge as inoculum, reaching a voltage of about 550 mV and a power- and current density of about 130 mW/m² and 550 mA/m², respectively. Enzymatic treatment of the dried stalks didn’t improve the total energy obtained per gram of sorghum stalks. During operation of the MFCs the sugars were quickly fermented, mainly to acetate and butyrate which were the dominant acids during electricity generation. The CE for the sorghum material and glucose was low, at around 2% and 5%, respectively, presumably due to microbial growth and biogas formation.

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References


Paper II

On-Line Raman Spectroscopic Study of Cytochromes’ Redox State of Biofilms in Microbial Fuel Cells

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Abstract: Bio-electrochemical systems such as microbial fuel cells and microbial electrosynthesis cells depend on efficient electron transfer between the microorganisms and the electrodes. Understanding the mechanisms and dynamics of the electron transfer is important in order to design more efficient reactors, as well as modifying microorganisms for enhanced electricity production. Geobacter are well known for their ability to form thick biofilms and transfer electrons to the surfaces of electrodes. Currently, there are not many “on-line” systems for monitoring the activity of the biofilm and the electron transfer process without harming the biofilm. Raman microscopy was shown to be capable of providing biochemical information, i.e., the redox state of C-type cytochromes, which is integral to external electron transfer, without harming the biofilm. In the current study, a custom 3D printed flow-through cuvette was used in order to analyze the oxidation state of the C-type cytochromes of suspended cultures of three Geobacter sulfurreducens strains (PCA, KN400 and ΔpilA). It was found that the oxidation state is a good indicator of the metabolic state of the cells. Furthermore, an anaerobic fluidic system enabling in situ Raman measurements was designed and applied successfully to monitor and characterize G. sulfurreducens biofilms during electricity generation, for both a wild strain, PCA, and a mutant, ΔS. The cytochrome redox state, monitored by the Raman peak areas, could be modulated by applying different poise voltages to the electrodes. This also correlated with the modulation of current transferred from the cytochromes to the electrode. The Raman peak area changed in a predictable and reversible manner, indicating that the system could be used for analyzing the oxidation state of the proteins responsible for the electron transfer process and the kinetics thereof in-situ.

Keywords: microbial fuel cell; Raman spectroscopy; Geobacter sulfurreducens; cytochrome-C; Omc

1. Introduction

Electroactive biofilms have long been known to be capable of generating electrical power through extracellular electron transfer, converting the chemical energy found in chemical bonds of organic compounds. Microbial fuel cells (MFCs) have shown promise in wastewater treatment [1], bioremediation [2] or even as an alternative renewable energy source in remote areas [3]. In MFCs, under anaerobic conditions, catalytic reactions of microorganisms oxidize an electron donor through extracellular electron transfer to the anode. The cathode is then exposed to an electron acceptor to facilitate an electrical current [4,5]. The Gram-negative bacteria from the Geobacter genus are...
considered to be well-known electroactive bacteria, although several other micro-organisms show some electro-activity [6].

Efficient electron transfer between the microorganisms and electrodes is a key factor for the efficiency improvement of bio-electrochemical systems, such as MFCs and microbial electrolysis cells. Understanding the mechanisms and dynamics of the electron transfer is important in order to design electrodes in a more efficient way, as well as modifying microorganisms in an attempt to increase the current density [7]. This is evident when one looks at the improvements already achieved using improved electrode designs by, for example, the addition of Mn⁴⁺ or Fe³⁺ to graphite electrodes or the increase of the specific surface area [8,9].

*Geobacter sulfurreducens* is well known for its ability to form thick biofilms and transfer electrons to the surfaces of electrodes without the need for mediators [10,11]. Electrons are generated from the oxidation of NADH, which is derived from organic matter oxidation, in association with the proton pumping required for energy production. The subsequent electron transfer serves merely to dispose of the electrons.

In this study three abiotic electron acceptors were used, i.e., fumarate and Fe(III)oxide for suspended cultures and a poised graphite electrode for an on-line MFC system. Fumarate is an intra-cellular electron acceptor constituting a relatively simple electron transport chain and its role in respiration has been extensively studied [12,13]. Furthermore, it has been shown that *G. sulfurreducens* can neither oxidize fumarate nor use fumarate as a carbon or energy source since the succinate produced from formate reduction is not oxidized in the tri-carboxylic acid (TCA) cycle but excreted into the medium [14].

Insoluble Fe(III)oxide is a well-known electron acceptor for *G. sulfurreducens* and also the predominant form of Fe(III) in most soils and sediments. Under Fe(III)-reducing conditions, the TCA cycle is operated as a closed loop, producing eight electrons per molecule of acetate oxidized. In contrast to the use of fumarate as an electron acceptor, electrons are transported outside the cell, leaving protons in the cytoplasm. It is unlikely that this final electron transfer yields energy to the cell [7]. Translocation of these protons dissipates the membrane potential and acidifies the cytoplasm, which is theoretically the reason why growth rates are approximately 3 fold lower during Fe(III) reduction [15]. Several enzymes participate in the electron transport chain, including several outer membrane C-type cytochromes (OMC) (namely OmcS, OmcZ and OmcB, as seen in Figure 1) [7,16].

![Figure 1](Adapted from [7,17]).

In order to learn more about the electron transfer process, in-situ surveillance is a necessity. Several techniques have been used in an attempt to characterize the biofilm and shed light on the mechanisms and dynamics involved in electron transfer. These methods do however each have their own unique
advantages and disadvantages, and most require the removal of the biofilm from an anaerobic reactor to an aerobic environment, thus oxidizing the cytochromes. Some of which are discussed below:

Confocal Laser Scanning Microscopy can provide quite a lot of information, both on the biofilm structure and on the microbial activity or interaction (for example when stained with a live-dead stain [18]). However, most stains are toxic and kill the biofilm, therefore continuous measurements are not possible [19]. Using fluorescence in situ hybridization, which uses fluorescently labeled DNA-probes, specific DNA sequences can be targeted, which can be used to, for example, show interspecies electron transfer [20,21]. This method is, however, also not suited for continuous measurements. Electrochemical Impedance Spectroscopy (EIS) offers a non-destructive tool to analyze both the electrode interface and the electrochemical reactions involved, yet this gives limited information as to the internal cellular mechanisms [22]. DNA extraction and sequencing can be useful when analyzing biofilms grown from a consortium, yet it requires invasive methods such as the removal of parts of the biofilm or sections of the electrode [23,24]. Optical microscopy can also be used to obtain information on the localization of the biofilm, yet it is limited as to the amount of information that can be obtained [25]. MFCs are complex bio-electrochemical systems, and would therefore require more than a single technique to be completely understood.

C-type cytochromes are known to be good Raman scatterers, especially when a resonance excitation wavelength is chosen, and the sheer abundance of C-type cytochromes makes the cultures/biofilms visibly red. The genome of *G. sulfurreducens* contains 111 putative genes coding for C-type cytochromes, several of them being multi-heme cytochromes [26]. Four strong bands have previously been observed in the Raman spectra of *G. sulfurreducens* at 747, 1133, 1310 and 1583 cm$^{-1}$, which can be ascribed to the excitation of the heme groups of cytochrome-c that are prevalent in the biofilms [27,28]. This also compares well with cytochrome c from horse heart [29]. The cytochromes have also been shown to function as capacitors for the cells, storing electrons under some environmental conditions [30,31].

Four mutants of *G. sulfurreducens* were used, in order to get specific information about the electron transfer to extracellular and intracellular electron acceptors, i.e., the wild strain PCA [32], KN400, a strain with enhanced capacity for current production developed from PCA through selective pressure (this enhanced capacity was associated with a greater abundance of electrically conductive pili) [33], a strain lacking PilA (ΔpilA), the structural pilin protein which have been implicated in long-range electron transfer through anode biofilms, and a strain lacking the cytochrome OmcS that is localized on the pili (ΔOmcS), which is believed to be important in Fe(III) oxide reduction.

The aim of this paper was to evaluate a non-invasive method to analyze the biofilm of an MFC while the system is still active and electrically poised. Currently there are not many “on-line” systems for monitoring the activity of the biofilm and the electron transfer process without harming the biofilm [31,34]. By measuring the spectroscopic properties of active and poised biofilms (specifically, the Raman fingerprint OMCs), it would be possible to measure the effect that poising the cell at different potentials has on the oxidation state of different biofilms. Because the OMCs are directly involved in the electron transfer system, and the cells require electron transfer for growth, Raman spectra can give information on the metabolic state of the biofilm, as well as how stable the biofilm is, depending on the selected strain. This could also be combined with the use of different mutants in order to gain insight into specific electron transfer mechanisms.

In the current study, an anaerobic fluidic system enabling in situ Raman measurements was designed and applied to monitor and characterize *Geobacter* biofilms during electricity generation.

### 2. Results and Discussion

In order to obtain preliminary Raman measurements, experiments were carried out using cells suspended in a custom-made 3D printed flow-through cuvette. Secondly cells were grown in a chemostat, in order to obtain a repeatable sample in the exponential phase, and measurements were taken after resuspension in a buffer. Finally, biofilms were grown in a stack microbial fuel cell and
measurements were taken while the cell was poised at different voltages. Raman spectra were analyzed as described in Section 3.5.

2.1. Suspended Cells Raman Measurements

Three strains were used during the initial tests, namely KN400, PCA and Δ pilA. These strains were selected in order to observe the effect that the level of abundance of pili have on the oxidative state of OMCs. During these experiments, Raman measurements and analysis of metabolites were done at different time points during batch cultivation of *Geobacter* strains, where the Raman measurements were started once significant biomass was obtained. During the Raman measurements using different strains of *G. sulfurreducens*, very little difference was observed in how the strains reacted to the applied stimuli, showing proportionally similar decreases/increases in Raman peak area when, for example, fumarate was added.

Figure 2 shows an example of the metabolite concentrations, as well as the absorbance (i.e., OD600, a common method of measuring cell concentration [35]) and the Raman peak areas (measured when enough cell mass was obtained) for the strains Δ pilA and KN400 without the addition of supplementary fumarate. The production and excretion of succinate into the medium during growth on acetate and fumarate is consistent with previous metabolic studies on *G. sulfurreducens* [15,36]. For all the strains tested, the peak area dropped 4.1 ± 0.83 fold when the culture entered the stationary phase (i.e., when a substrate has been completely consumed, fumarate in this case, and the primary metabolism ceases, around 70 h in this case), suggesting that the peaks are related to primary metabolism and not only to the total biomass.

Despite the similar trends observed in the Raman peak areas from the different strains, the maximum peak area obtained was significantly lower for KN400 than for that of PCA and Δ pilA (as can be seen in Figure 3). For example, for the measured Raman peak area shown in Figure 3 the area at 747, 1133 and 1310 cm$^{-1}$ for KN400 were on average 39% that of the same peaks for PCA, despite the fact that the cell concentration was the same for each sample. The KN400 strain was specifically grown to be electroactive, yet in a previous study C-type cytochromes were found to be much less abundant in KN400, compared to the PCA strain [33], which would fit with the results obtained in the present study.

![Figure 2](image-url)  
**Figure 2.** Raman peak areas (Raman peak areas: 1314 cm$^{-1}$ ○, 1130 cm$^{-1}$ □, 749 cm$^{-1}$ ◻), metabolite concentrations (Fumarate ▲, Succinate ■ and Acetate ●) and OD600 (Absorbance ●) of Δ pilA and KN400 (KN400 is shown with a solid line and Δ pilA with dotted line) of an example run showing the significant difference between maximum Raman peak areas of the strains, as well as the significant decrease as stationary phase is reached.
The drop in Raman peak area, when the stationary phase was reached, could be explained by the capacitor hypothesis [31] where the cytochromes are charged during growth on the rich medium where high amounts of acetate (electron donor) and fumarate (electron acceptor) are present, resulting in a capacitor-like electron “storage system”. It is well known that fumarate reduction is coupled to NADPH and NADH oxidation and the reduced equivalents delivered into the electron transport chain. Since the system is dynamic, there is a continuous charge and discharge of the cytochromes. When the fumarate is exhausted the primary metabolism stops (even though acetate is still present (6–7 mM)) resulting in the cessation of the charging (reduction) of the cytochromes and a decrease in the peak area.

When the fumarate was close to being depleted (1–6% of initial concentration) and additional electron acceptor was added, an increase in the peak area was detected, indicating that there was an increase in metabolic activity that resulted in the eventual charging of the cytochromes. This increase is typically slightly delayed, occurring only after some minutes. Figure 4 shows a typical example of this, where fumarate was added to a sample in which the fumarate was close to being depleted (5.4% of the initial).

2.2. Chemostat Grown Biomass Raman Measurements

Two strains of *G. sulfurreducens* (PCA and ΔpilA) were grown in chemostats in order to observe the electron transfer without the conductive PilA to an external electron acceptor (Fe(III)oxide) and also to compare that to an electron acceptor that can be internalized, namely fumarate. The cells were centrifuged and resuspended in wash buffer, without an electron acceptor or donor. KN400 was not
selected because of the significant differences in OMC expression. The results were normalized with respect to the average peak areas of the sample suspended only in wash buffer (13,725; 6375 and 5930 for ΔpilA and 17,560; 8075 and 7655 for PCA, of the peaks at 749 cm\(^{-1}\); 1130 cm\(^{-1}\) and 1314 cm\(^{-1}\) respectively) as seen in Figure 5. There was a 50–80% decrease in the peak area when Fe(III) oxide was added. This shows that the mechanisms involved in the electron transfer to an external source does not require pili to effectively oxidize the OMCs.

This might be due to direct transfer using OmcZ which has been found to participate in homogeneous electron transfer (through the biofilm bulk) [37]. Furthermore, the decrease in the peak area is much more significant for PCA than for ΔpilA, suggesting a higher capacity for electron transport in the wild type when compared to ΔpilA. This is consistent with the presence of the conductive pili of the PCA strain which are used to reduce external electron acceptors such as Fe(III) oxide.

**Figure 5.** Normalized Raman peak areas of PCA and ΔpilA samples grown in a chemostat, suspended in a wash buffer (W.B.) and the same samples after the sequential addition of Fe(III) oxide and fumarate. (The error bars show the standard deviation of 6–9 Raman measurements).

### 2.3. Stack-MFC Raman Measurements

By using stack-MFC, it was demonstrated that we can modulate the redox state of the C-type cytochromes, which is reflected in the Raman peak area, by controlling the poising potential. The tests were done using two strains, the wild type PCA and a mutant ΔOmcS. ΔOmcS was used instead of the ΔpilA strain used above, since ΔpilA does not form electro active biofilms.

From Figure 6 the Raman peaks that showed the largest relative change in peak area are the peaks at 747 and 1133 cm\(^{-1}\), whereas the peaks at 1310 and 1583 cm\(^{-1}\) were smaller and showed less change at the different poised levels. The area under the peak at 747 cm\(^{-1}\) was calculated for each spectrum at the different poised values, from spectra like that seen in Figure 6. A clear increase in peak area is observed in Figure 7, as the poised potential decreases, followed by a decrease as the poised potential is then increased again. Similarly, a larger increase in peak area is observed when the cell is disconnected completely.

The clear increase in peak area when the resistance to current is increased shows the build-up of a charge in the cytochromes i.e., the reduction of the cytochromes. Subsequently, when the cell is poised at +300 mV the resistance is at its lowest, leading to the lowest peak area, and the oxidation of cytochromes. This is very similar to the fluorescence response observed by Núñez [31], where the fluorescence was lost when the cytochrome was oxidized.
not require OmcS for electron transfer to an external electrode. Since the current represents the activity of the entire biofilm it does suggest a slight decrease in the biofilm activity. This, however, requires further investigation, possibly a decrease in Raman laser power or exposure time. The general decrease can also be seen in the fact that current does not increase back to the original values as in Figure 8. The results do, however, show that *G. sulfurreducens* does not require OmcS for electron transfer to an external electrode.

The measurements were repeated using a ∆OmcS biofilm. The ∆OmcS mutant does not carry the OmcS protein located on the pilA nanowires. Although it was initially thought that OmcS is important for the conductivity of the pilA nanowires, studies have shown that the presence of OmcS on pili is not sufficient to confer conductivity to pili [38]. It was, however, not possible to achieve as thick a biofilm as when using PCA. However, as seen in Figure 8, a similar peak area was achieved during the measurements. The biofilm showed a similar response, with the peak area increasing as the current and poise voltage decreased.

However, the peak area decreased drastically when the biofilm was poised at 300 mV for the second time and the current was slightly lower than that of the first time the cell was poised at 300 mV. Since the current represents the activity of the entire biofilm it does suggest a slight decrease in the biofilm activity. The decrease in Raman peak area is, however, much more drastic than the decrease in current and we believe that this is a combination of the biofilm on the measurement spot being affected by the light source, as well as a general decrease in biofilm activity. This, however, requires further investigation, possibly a decrease in Raman laser power or exposure time. The general decrease can also be seen in the fact that current does not increase back to the original values as in Figure 8. The results do, however, show that *G. sulfurreducens* does not require OmcS for electron transfer to an external electrode.

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**Figure 6.** Processed Raman spectra showing the changes in the strong peak at 745 cm$^{-1}$, which is related to the cytochrome oxidation state.

**Figure 7.** The Raman peak area at 745 cm$^{-1}$ of PCA biofilm in a microbial fuel cell (MFC) stack, average of two measurements along with the current produced at the different poised level. The current is inversely related to the peak area.
Figure 8. The Raman peak area at 745 cm$^{-1}$ of ∆OmcS biofilm in an MFC stack, average of two measurements, as well as the current produced at the different poised levels. It can be seen that the current is inversely related to the peak area. A decrease in expected current as well as the expected peak areas can be seen at the last three poised levels.

3. Materials and Methods

3.1. Preparation of Measurement Cells

3D Printed cell: A flow-through cuvette was designed and printed in a black PETG plastic using a standard 3D printer (Prusa i3 MK3, Praha, Czech Republic). The cell had dimensions of 26 × 26 mm and 18 mm high with an 18 mm diameter cut-sphere as the containment area, with a total volume of 2.5 mL excluding the connecting tubes. A cover-slide was then glued to the cuvette using a silicone sealant. A schematic can be seen in Figure 9.

Stack microbial fuel cell: A two-chamber stack reactor was designed and built (Mercury engineering, Edenvale, South Africa) using polycarbonate for the main body and cover plates, with stainless-steel tube-fittings and silicone gaskets. The two chambers were separated with a proton exchange membrane (Nafion N117), each fitted with solid graphite electrodes, and the anode chamber was fitted with an Ag/AgCl reference electrode. A three-electrode configuration setup was used, consisting of two graphite cuboids (70 × 22 × 10 mm) serving as working and counter electrodes and an Ag-AgCl reference electrode. A window was cut out of one cover plate in order to fit a microscope slide directly over the anode.

Figure 9. Schematics of (A) the 3D printed cell and (B) the Microbial fuel cell used for Raman measurements.
3.2. Microbes, Media and Inoculation

*Geobacter sulfurreducens* strain PCA (ATCC 51573, DSMZ 12127), as well as modified versions of the strain (ΔOmcS, ΔOmcZ, ΔPilA and KN400), obtained from Dr. Ashley Franks, La Trobe University, Bundoora, Australia, was used in all studies. The strains ΔOmcS and ΔOmcZ have the genes for the outer membrane cytochromes S and Z removed, whereas ΔPilA has the the pilA gene removed. KN400 on the other hand is a variant of *G. sulfurreducens* with enhanced capacity for current production [33].

*G. sulfurreducens* inoculums were grown in a slightly modified NBAF media (pH of 6.8), with acetate (10 mM) and fumarate (40 mM) as the electron donor and acceptor, respectively [39]. The base composition of NBAF per liter of deionized water is 0.42 g of KH2PO4, 0.22 g of K2HPO4, 0.2 g of NH4Cl, 0.38 g of KCl, 0.36 g of NaCl, 0.03 g of CaCl2, 0.1 g of MgSO4·7H2O, 1.8 g of NaHCO3, 0.43 g of Na2CO3, 1.0 mL of 100 mM Na2SeO4, 10.0 mL of a vitamin solution, and 10.0 mL of NB trace mineral solution. The composition of the trace mineral solution per liter of deionized water is 2.14 g of nitriloacetic acid, 0.1 g of MnCl2·4H2O, 0.3 g of FeSO4·7H2O, 0.17 g of CoCl2·6H2O, 0.2 g of ZnSO4·7H2O, 0.03 g of CuCl2·2H2O, 0.005 g of AlK(SO4)2·12H2O, 0.005 g of H3BO3, 0.09 g of Na2MoO4·11H2O, and 0.02 g of N2WO4·2H2O [40].

The composition of the vitamin solution per liter of deionized water is 0.002 g of Biotin, 0.005 g of Pantothenic Acid, 0.0001 g of B-12, 0.005 g of p-aminobenzoic acid, 0.005 g of Thiocetic Acid, 0.005 g of Nicotinic Acid, 0.005 g of Thiamine, 0.005 g of Riboflavin, 0.01 g of Pyridoxine HCl and 0.002 g of Folic Acid [41].

In order to ensure that the electron acceptor is limiting, for the suspended cell Raman measurements, 15 mL of a 100 mM sodium acetate solution was added to 100 mL NBAF media. This resulted in a starting concentration of approximately 20 mM acetate and 32 mM fumarate after a 10% inoculation.

The microbial fuel cell stack was started with a 10% inoculation of a freshwater media, containing 20 mM acetate and 40 mM fumarate, as previously described [42]. After batch operation, the anode chamber was fed continuously using the freshwater media containing 10 mM acetate and no fumarate at a flow rate of 0.5 mL min⁻¹.

3.3. 3-D printed Cuvette Suspended Cell Raman Measurements

Two sets of Raman measurements were done using the printed cuvettes. Firstly, three *G. sulfurreducens* mutants (PCA, KN400 and ΔPilA) were grown in serum bottles using NBAF growth media containing a stoichiometric excess of acetate (approximately 20 mM acetate and 32 mM fumarate) to ensure that growth is limited by the electron acceptor [14]. Once sufficient biomass was obtained, samples were taken in an anaerobic box at regular intervals during the growth stage (exponential and stationary phase). After initial measurements, the kinetic effect of fumarate addition was studied by addition of additional fumarate (approx. 5 mM). This is illustrated in Figure 10.

![Figure 10. Schematic of the sampling process used for suspended cell Raman measurements.](image-url)
Secondly, in order to obtain controllable conditions, biomass was grown using a chemo-stat with an excess of electron donor (25 mM acetate and 40 mM fumarate), similar to the study of Nunez et al. [13,31]. Three 25 mL samples were then taken, centrifuged and the cells of each were re-suspended in 4 mL 50 mM phosphate buffer (pH 7). The samples were prepared in an anaerobic box to prevent the oxidation of the heme groups. No electron donor was added to the re-suspended cells.

After initial measurements, 5 mM Fe(III)oxide was added to the samples, in order to act as an extracellular electron acceptor, and measured again. Finally, additional fumarate (approx. 5 mM) was added, which acts as an electron acceptor that can be incorporated into the cell, and measured again (a schematic is shown in Figure 11).

![Figure 11. Schematic of the sampling process used for Chemostat grown biomass Raman measurements.](image)

### 3.4. Microbial Fuel Cell Set Up and Raman Measurements

The cell was sterilized by passing ethanol through the cell for approximately an hour followed by a rinse with 500 mL of sterile milli-Q water. After sterilization the cell was connected to two 250 mL bottles filled with freshwater medium with 20 mM acetate and 40 mM fumarate on the anode side. The media was recirculated through the reactor and the bottles were sparged continuously with 20:80 CO2/N2 to maintain anaerobic conditions. The cells were poised at 300 mV (using a MultiEmStat+ potentiostat, PalmSens, Netherlands) and the anode side was inoculated with 10% of the inoculum. At the point of maximum current production, the anode side bottle was switched to a continuous feed of freshwater media, containing only 10 mM acetate, at a flow rate of 0.5 mL/min. The feed was maintained for approximately 2–4 weeks before the biofilm was clearly visible as a pink layer.

During measurements, the MFC stack was initially poised at 300 mV, and then lowered to −150 mV and increased back to 300 mV in 150 mV intervals, taking two measurements at each poised voltage. The voltage was then changed directly to −300 mV, back to 300 mV and finally the potentiostat was disconnected.

### 3.5. Analytical Methods

The Raman spectra, taken using the printed cuvettes, were collected with a Ramanscope III spectrometer in combination with a SENTERRA module allowing dispersive Raman microanalysis. The system was equipped with a 532 nm excitation laser for use in the standard normal incidence sampling geometry. The data were accumulated in the range from 30 to 1550 cm⁻¹ using an incident laser power of about 20 mW. An integration time of 10 s with 10 co-addition scans was used to acquire the spectra.

Raman spectra for the stack measurements were collected using an inverted microscope (Olympus IX71, Tokyo, Japan) equipped with 532 nm excitation DPPS laser (Laserglow Technologies, Toronto, Canada) and coupled to a Raman spectrometer, Shamrock 303i (Andor Technology, Belfast, UK). The laser was operated at approximately 15 mW prior to the microscope objective, for 120 s.

The raw Raman spectra was processed in the following way: First the cosmic rays were removed using an automated tool. A chromatogram baseline estimation and denoising filter using sparsity was used to remove fluorescence and background noise. Finally, a Savitzky-Golay de-noising filter was used to smooth the signal. The smoothed curve was then integrated over a fixed window of 22 cm⁻¹ using Matlab R2018 to obtain the area below each peak. The peaks were compared to...
results from Virdis et al. and was found to have nearly identical peaks [34]. Four strong bands were observed at 744, 1131, 1317 and 1587 cm$^{-1}$ which can be ascribed to the excitation of the heme groups of cytochrome c that are prevalent in the biofilms [29,46].

Biomass concentrations were determined using the optical density at 600 nm. The concentration of organic acids and sugars were determined using a Perkin-Elmer HPLC system with a Series 200 refractive index detector, as previously described [47].

4. Conclusions

The 3D printed cuvette functioned well throughout all Raman measurements using isolated suspended cells from three strains. Raman spectra were obtained for the KN400, ΔpilA and PCA strains in suspended cultures, showing that the values of the Raman peak areas can be used as an indicator of the cytochrome redox state of the cells. This is further stressed by the observation of lower Raman peak areas when using KN400, due to the presence of less OMCs when compared to PCA. Furthermore, a higher capacity for electron transport in the wild type when compared to ΔpilA was also observed. It was also shown that PilA is not required for external electron transfer to occur.

An anaerobic fluidic system enabling in-situ Raman measurements was designed and applied successfully to monitor and characterize G. sulfurreducens biofilms during electricity generation. The redox state of the cytochromes, monitored by the Raman peak area, could be modulated by applying voltage to the electrodes and this is correlated with the modulation of current flowing between the cytochromes and the electrode. Through monitoring an MFC with a ΔOmcS biofilm it was also shown that OmcS is not required for effective electron transfer to an external electrode.

Raman microscopy was shown to be capable of providing biochemical information, i.e., the redox state of C-type cytochromes, without the need to interfere with the operation of the MFC, the removal of the electrode or the staining of the biofilm. Therefore, the biofilm and suspended cultures could be studied on-line in completely anaerobic conditions. Since these cytochromes are an integral part of the electron transport chain, Raman measurements can, for example, be used to give information on electron transfer to external electrodes. Hence, this study shows that Raman measurements can serve as a useful tool for elucidating the mechanisms involved in the electron transport chain, as well as to analyze the metabolic state of active biofilms.

Author Contributions: A.K., M.S. designed the research. A.K., M.S. and K.R. performed the experiments. A.K. analyzed the data. A.K., M.S., K.R., P.C. and U.R. wrote and revised the manuscript. M.S., K.R., P.C. and U.R. were responsible for supervision and funding acquisition. All authors have read and approved the final version.

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References


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