THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Understanding extracellular electron transport of industrial microorganisms and optimization for production application

Frauke Kracke

Dipl.-Ing., Bio-chemical Engineering, Technical University Dortmund, Germany

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2016

School of Chemical Engineering

Advanced Water Management Centre

Centre for Microbial Electrochemical Systems

ABSTRACT

Microbial electrosynthesis (MES) and electro-fermentation are novel approaches to increase microbial production by stimulating the metabolism of the cells electrically. The technique is still in its infancy and while already hyped as promising method to convert CO_2 or cheap carbon sources and electrical energy into valuable chemicals and fuels, little is known about its true potential. This project uses a combination of *in silico* and *in vivo* approaches to gather novel information about the benefits and limitations of microbial electrosynthesis as well as its fundamental mechanisms.

Understanding microbial electron transport mechanisms is the key to optimization of any bioelectrochemical technology. Therefore, this work analyses the different electron transport chains that nature offers for organisms such as metal respiring bacteria and acetogens, but also standard biotechnological organisms currently used in bioproduction. Special focus lies on the essential connection of redox and energy metabolism, which is often ignored when studying bioelectrochemical systems. The possibility of extracellular electron exchange at different points in each organism is discussed regarding required redox potentials and effect on cellular redox and energy levels. Key compounds such as electron carriers (e.g. cytochromes, ferredoxins, quinones, flavins) are identified and analysed regarding their possible role in electrode-microbe-interactions.

Based on these findings a stoichiometric network analysis is performed analysing the effect of electrical stimulation on the microbial metabolism theoretically. *Escherichia coli* is used as model organism for production with the aim to identify target processes for MES. For the first time, 20 different valuable products were screened for their potential to show increased yields during anaerobic electrically enhanced fermentation. Surprisingly it was found that an increase in product formation by electrical enhancement is not necessarily dependent on the degree of reduction of the product but rather the metabolic pathway it is derived from. Contrary to the usual assumption a reduced product would always require electron supply by a cathode this study shows that a complex metabolic analysis is needed to identify the overall redox state of each process. A variety of beneficial processes is presented with product yield increases of maximal +36% in reductive and +84% in oxidative fermentations and final theoretical product yields up to 100%. This includes compounds that are already produced at industrial scale such as succinic acid,

lysine and diaminopentane as well as potential novel bio-commodities such as isoprene, para-hydroxybenzoic acid and para-aminobenzoic acid. Furthermore, it is shown that the way of electron transport has major impact on achievable biomass and product yields. The coupling of electron transport to energy conservation could be identified as crucial for most processes.

The *in vivo* approach of this project introduces the development of a standardised reactor platform suitable for reproducible, fully controlled electrically enhanced fermentations. Different organisms that are used in industrial bio-production were screened for their ability of electron uptake: *E. coli*, *Propionibacterium (P.) acidipropionici*, *P. freudenreichii*, *Citrobacter werkmanii* and *Clostridium (C.) autoethanogenum*. A growth-related current consumption was monitored for all tested strains, however in most cases the electron uptake was too small compared to substrate uptake to result in significant metabolic changes. An exception is presented by the GRAM-positive acetogen *C. autoethanogenum,* which shows a considerable metabolic shift from acetate towards lactate and 2,3-butanediol caused by extracellular electron supply. This is the first report of electro-activity of the close relative to *C. ljungdahlii.* In heterotrophic fermentations, extracellular electron supply cut acetate production by more than half while production of lactate and 2,3-butanediol increased thirty-five fold and three fold, respectively. Interestingly this metabolic response crucially depends on the redox potential at which the electrons are supplied, which is proven by the use of different mediator molecules. In agreement with the findings from the *in silico* study, this again is an indication for the importance of the metabolic pathway by which electrons enter the cellular metabolism for the success of microbial electrosynthesis.

This work applies a powerful metabolic modelling tool and studies electron transport mechanisms in the industrial alcohol producing *Clostridium autoethanogenum*. As such it adds an important piece of fundamental understanding of microbial electron transport possibilities to the research community and will help to optimize and advance bio-electrochemical techniques beyond the level of lab-scale studies.

Declaration by author

This thesis *is composed of my original work, and contains* no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted *to qualify for the award of any* other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the policy and procedures of The University of Queensland, the thesis be made available for research and study in accordance with the Copyright Act 1968 unless a period of embargo has been approved by the Dean of the Graduate School.

I acknowledge that copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate I have obtained copyright permission from the copyright holder to reproduce material in this thesis.

Publications during candidature

Peer reviewed papers:

- **Kracke, F.**, and Krömer, J. O. (2014). *Identifying target processes for microbial electrosynthesis by elementary mode analysis*. BMC Bioinformatics, 15(1), 410. doi:10.1186/s12859-014-0410-2

- Harnisch, F., Rosa, L.F.M., **Kracke, F.**, Virdis, B., and Krömer, J.O. (2015). *Electrifying White Biotechnology: Engineering and Economic Potential of Electricity-Driven Bio-Production.* ChemSusChem 8, 758-766. doi: 10.1002/cssc.201402736 Featuring a cover story and image: doi: 10.1002/cssc.201403199

- **Kracke, F.**, Vassilev, I., and Krömer, J.O. (2015). *Microbial electron transport and energy conservation – the foundation for optimizing bioelectrochemical systems*. Frontiers in Microbiology 6. doi: 10.3389/fmicb.2015.00575

Peer reviewed conferences:

- **Kracke, F.**, & Krömer, J. O.; "*Understanding benefits and limitations of Microbial Electrosynthesis: An elementary mode analysis of microbial production during electrically enhanced fermentation.*", 4TH INTERNATIONAL FUEL CELL CONFERENCE, September 2013 Cairns, Australia, oral presentation

- Averesch, K. F. G. , **Kracke, F.**, Lekieffre N., Krömer, J. O.; *"Parallel reactor system for the study of cathodic bioprocesses."*, 4TH INTERNATIONAL FUEL CELL CONFERENCE, September 2013 Cairns, Australia, poster presentation

- **Kracke, F.**, and Krömer, J.O.; "*Making the connection: Metabolic pathway study of microbial systems for electrosynthesis*", 5TH INTERNATIONAL MEETING ON MICROBIAL ELECTROCHEMISTRY AND TECHNOLOGIES, October 2015 Tempe, AZ, America, poster presentation

Publications included in this thesis

- **Kracke, F.**, & Krömer, J. O. (2014). *Identifying target processes for microbial electrosynthesis by elementary mode analysis*. BMC Bioinformatics, 15(1), 410. doi:10.1186/s12859-014-0410-2

This publication was slightly modified to fit the thesis and was incorporated as chapter 4.2.

Contributor	Statement of contribution
Frauke Kracke (Candidate)	Designed study (60%) Conducted the experiments (80%) Wrote the paper (100%)
Jens O. Krömer	Designed study (40%) Conducted the experiments (20%) Critically reviewed and edited paper (100%)

- **Kracke, F.**, Vassilev, I., and Krömer, J.O. (2015). *Microbial electron transport and energy conservation – the foundation for optimizing bioelectrochemical systems*. Frontiers in Microbiology 6. doi: 10.3389/fmicb.2015.00575

This publication was slightly modified to fit the thesis and was incorporated as chapter 4.1.

Contributor	Statement of contribution
Frauke Kracke (Candidate)	Designed the study (50%) Performed calculations (100%) Wrote the paper (70%)
Igor Vassilev	Designed the study (20%)

	Wrote the paper (30%)
Jens O. Krömer	Designed the study (30%) Critically reviewed and edited paper (100%)

- Harnisch, F., Rosa, L.F.M., **Kracke, F.**, Virdis, B., and Krömer, J.O. (2015). *Electrifying White Biotechnology: Engineering and Economic Potential of Electricity-Driven Bio-Production.* ChemSusChem 8, 758-766. doi: 10.1002/cssc.201402736

This publication was not completely incorporated in the thesis, however, a small section about the economic analysis of microbial electrosynthesis on the example of electric lysine production that was designed, calculated and written by Frauke Kracke and Jens O. Krömer was included in chapter 6.

Contributor	Statement of contribution
Frauke Kracke (Candidate)	Designed the study (10%) Performed calculations (20%) Wrote the paper (10%) Critically reviewed and edited paper (35%)
Falk Harnisch	Designed the study (40%) Performed calculations (20%) Wrote the paper (35%)
Luis F. M. Rosa	Designed the study (10%) Performed calculations (20%) Wrote the paper (15%)
Bernardino Virdis	Designed the study (10%) Performed calculations (20%)

	Wrote the paper (10%) Critically reviewed and edited paper (30%)
Jens O. Krömer	Designed the study (30%) Performed calculations (20%) Wrote the paper (30%) Critically reviewed and edited paper (35%)

- **Kracke, F.**, Virdis, B., Bernhardt, P. V., Rabaey, K., and Krömer , J. O. (2016). *Redox dependent metabolic shift in Clostridium autoethanogenum by extracellular electron supply.* Submitted to Biotechnology for Biofuels 03/2016

This submitted manuscript was modified to fit the thesis and was incorporated as chapter 4.4.

Contributor	Statement of contribution
Frauke Kracke (Candidate)	Designed the study (20%) Performed experiments (95%) Wrote the paper (100%)
Bernardino Virdis	Critically reviewed and edited paper (30%)
Paul V. Bernhard	Performed experiments (5%) Critically reviewed and edited paper (10%)
Korneel Rabaey	Designed the study (40%) Critically reviewed and edited paper (10%)
Jens O. Krömer	Designed the study (40%) Critically reviewed and edited paper (50%)

Contributions by others to the thesis

This thesis includes published work, which was drafted in collaborations as stated above. The design and mechanical setup of the bioelectrochemical system, which is presented in chapter 4.3.1, was performed in teamwork with Nicolas Lekieffre. Prof Paul Bernhardt synthesized and kindly provided the cobalt mediators used for electrically enhanced fermentations presented in chapter 4.3 and 4.4. Some fermentations that are reported under 4.3.2 were supported by the help of Kai F. G. Averesch and HPLC analysis was performed by Dr Manuel Plan, Metabolomics Australia. Helena Reiswich designed Figure 7, Figure 8 and Figure 10.

Statement of parts of the thesis submitted to qualify for the award of another degree

None.

ACKNOWLEDGEMENTS

Special thanks goes out to my supervisor Dr Jens O. Krömer: I really appreciate your always open door and ear. Your support, your academic know-how and maybe also the end-of-the-year-CEMES-BBQs certainly contributed to the success of my PhD. I also want to thank you, Dr Bernardino Virdis, for your positive attitude and valued feedback on my drafts and research plans.

I want to acknowledge the funding sources that I received throughout my PhD: The UQI fee waiver scholarship, the CEMES living allowance scholarship as well as generous support for scientific work and travel from CEMES. Especially, I would like to thank the UQ Graduate School for awarding me the travel grant GSITA in 2015, which enabled me to undertake an extensive trip that resulted in very important scientific connections for me.

Further, I want to thank all the researchers that showed interest in my work and assisted me during the past three years with valuable input in form of scientific discussions and technical support. Especially: Dr Kaspar Valgepea, Dr Esteban Marcellin and Dr Manuel Plan at AIBN, UQ, Brisbane; Dr James Behrendorff and Dr Michael Köpke at LanzaTech Inc., Skokie, USA; Prof Paul Bernhardt at the School of Chemistry and Molecular Bioscience, UQ, Brisbane; Dr Ashley Franks and his group at LaTrobe University, Melbourne; Nicolas Lekieffre, Prof Korneel Rabaey from University Ghent, Belgium; and many members of the ISMET community.

Surely my great appreciation goes to my family and friends, especially to you, Nils, and the rest of the awesome SMF crew from Brisbane:

Thanks for the last three years – it's been great!

Keywords

microbial electrosynthesis, electro fermentation, cathode, extracellular electron transport, bioelectrochemical system, redox metabolism, energy metabolism, metabolic modelling, bio-production, *Clostridium autoethanogenum*

Australian and New Zealand Standard Research Classifications (ANZSRC)

060104 Cell Metabolism, 40%

100302 Bioprocessing, Bioproduction and Bioproducts, 40%

100303 Fermentation, 20%

Fields of Research (FoR) Classification

1003 Industrial Biotechnology, 50%

0601 Biochemistry and Cell Biology, 25%

0605 Microbiology, 25%

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviation	Name
AA	Adipic acid
Ack	Acetatekinase
ADH	Alcohol dehydrogenase
ALD	CoA-dependent acetaldehyde dehydrogenase
AOR	Acetaldehyde:ferredoxin oxidoreductase
AQDS	Antraquinone-2,6-disulfonic acid
BA	Butyric acid
BDO	Butanediol
BES	Bioelectrochemical system
BM	Biomass
BMY	Biomass yield
BV	Benzyl viologen
CA	Chronoamperometry
CE	Counter electrode
CEMES	Centre for Microbial Electrochemical Systems (Brisbane, Australia)
COdh	Carbon monoxide dehydrogenase
CoS	Cobalt(III)sepulchrate
CV	Cyclic Voltammetry
DoR	Degree of reduction
EET	Extracellular electron transfer
efm	Elementary flux mode
EMA	Elementary mode analysis
EMP	Embden–Meyerhof–Parnas pathway
Fdh	Formate dehydrogenase
FolD	Methylene-H_4F dehydrogenase
GABA	γ-Aminobutyric acid
GC	Gas chromatography
HPA	Hydroxypropionic acid
HPLC	High-performance liquid chromatography
Hyt	Electron-bifurcating NADP- and ferredoxin-depended hydrogenase
MEC	Microbial electrolysis cell
Med	Mediator
MES	Microbial electrosynthesis
MES buffer	2-(N-morpholino)ethanesulfonic acid
MetFV	Methylene-H_4F reductase
MFC	Microbial Fuel Cell
MOPS	3-(N-morpholino)propanesulfonic acid
MS	Mass spectrometry
Nfn	Electron-bifurcating ferredoxin-depended transhydrogenase

NMR	Nuclear magnetic resonance spectroscopy
OD	Optical density
ORR	Oxygen reduction reaction
ox	Oxidised state
PA	Propionic acid
pABA	*para*-Aminobenzoic acid
PDO	Propanediol
PEEK	Polyether ether ketone
PFOR	Pyruvate:ferredoxin oxidoreductase
pHBA	*para*-Hydroxybenzoic acid
PQQ	Pyrroloquinoline quinone
Pt	Platinum
Pta	Phosphotransacetylase
PY	Product yield
RE	Reference electrode
red	Reduced state
ref	Reference
Rnf	Membrane-associated and energy conserving reduced ferredoxin:NAD^+ oxidoreductase
RO	Research objective
SHE	Standard hydrogen electrode
TCA	Tricarboxylic acid cycle
UQ	Ubiquinone
UQH_2	Ubiquinol
WE	Working electrode

Symbol	**Name**	**Unit**
μ	Growth rate	h^{-1}
E	Potential	V
$E^{0'}$	Redox potential; Formal potential	V
I	Current	A
M_w	Molecular weight	mol g^{-1}
ΔG	Gibbs free energy	kJ mol^{-1}

1 INTRODUCTION AND LITERATURE REVIEW

Currently, 95% of the feedstocks used globally in the chemical industry are based on non-renewable and unsustainable fossil fuels. Worlds increasing need to counteract climate change creates a fast growing demand for the development of commercial, sustainable bio-processes that provide replacement sources for conventional chemicals and fuels. Successful commercialisation requires bio-production with high yields and productivity to enable economic viability.[1, 2] Biotechnological production processes are often limited by the cell's need to generate reducing power. This requires the microorganisms to use some fraction of the provided feedstock as a source of electrons. Butanol, for example, is microbiologically produced from glucose at a consumption of ~3kg glucose per kg butanol.[3]

One novel and very promising way to increase yields of biological production is to stimulate the metabolism of the cells electrically and therefore direct electron flow to desired products. The technique, termed microbial electrosynthesis (MES), shows potential to increase the efficiency of microbial production by providing additional electron donors or acceptors to the cells.[4, 5] Arising from the research field of microbial fuel cells, which studies microbe-electrode interactions for many years, a broad basis of knowledge about the involved processes is readily available. However, the new focus of producing highly reduced chemicals in electrically enhanced fermentations raises several questions that haven't been addressed before. The intended conversion of a cheap and sustainable substrate into a value added product displays in most cases a reductive reaction, therefore, the microbial conversion will take place on the cathode while the power output in fuel cells investigated in depth the bacterial behaviour on an anode. First studies on the cathode confirmed the possibility of donating additional electrons to the microbial metabolism and its potential to increase production.[4, 6-9] But it was also found that the involved transfer mechanisms for electron uptake differ significantly from the known electron donating mechanisms.[10-12]

This project aims at exploring benefits and limitations of MES and gaining new insight into the mechanisms of electron transfer between microbes and cathodes. The following chapters review the history of electro-microbiology studies, discuss possible involved mechanisms of extracellular electron transport (EET), introduce computer-based tools to

simulate electrically enhanced fermentations and highlight questions that still remain unanswered, the driving force for this project.

1.1 BIOELECTROCHEMICAL SYSTEMS

Society's strong interest in the development of sustainable techniques for waste-treatment, energy and chemical production have opened a wide research field for bio-electro-chemical techniques. The culturing of electro-active bacteria in bioelectrochemical systems (BESs) has established a research platform for various exciting technologies. The application possibilities for electrode-bacteria-interferences include the production of electricity, waste and wastewater treatment, bioremediation and the production of valuable products from wastes.[13-15] In such BESs, the organisms interact with electrodes via the exchange of electrons, which are either supplied or removed through an external electrical circuit.

Figure 1 displays an overview of a BES setup and its operating options. The electrodes are immersed in a conductive aqueous solution containing substrates and/or products. At the anode an oxidation process occurs (e.g.: electrolysis of water $2\ H_2O \rightarrow O_2 + 4\ H^+ + 4\ e^-$) and the electrons travel via an external circuit to the cathode where a reductive reaction is carried out (e.g. hydrogen evolution: $2\ H^+ + 2\ e^- \rightarrow H_2$). Usually, an ion-permeable membrane is used to separate anolyte and catholyte to prevent unwanted interference of charged species. However, depending on the nature of the reactions occurring, a BES can also be operated without a membrane. The potential difference between the reference electrode and the working electrode determines whether the working electrode acts as anode or cathode. The electrical operation of the BES varies from power production, 'short-circuit-mode' where anode and cathode are connected without resistor, to the investment of electrical energy to favour reactions on the cathode. The actual power input or output and the theoretically calculated value of the correlated electrode reactions will always differ due to biological and electrochemical losses in the BES.[16] These energy losses display a combination of thermodynamic imperfections in the system such as heat losses, diffusion kinetics of ions in the liquid and activation overpotentials on the electrode surface due to non-ideal catalysis.[16-19] Microbial catalysis can be achieved on the anode or on the cathode as well as on both sides, which enables the operation of a fully biological cell.

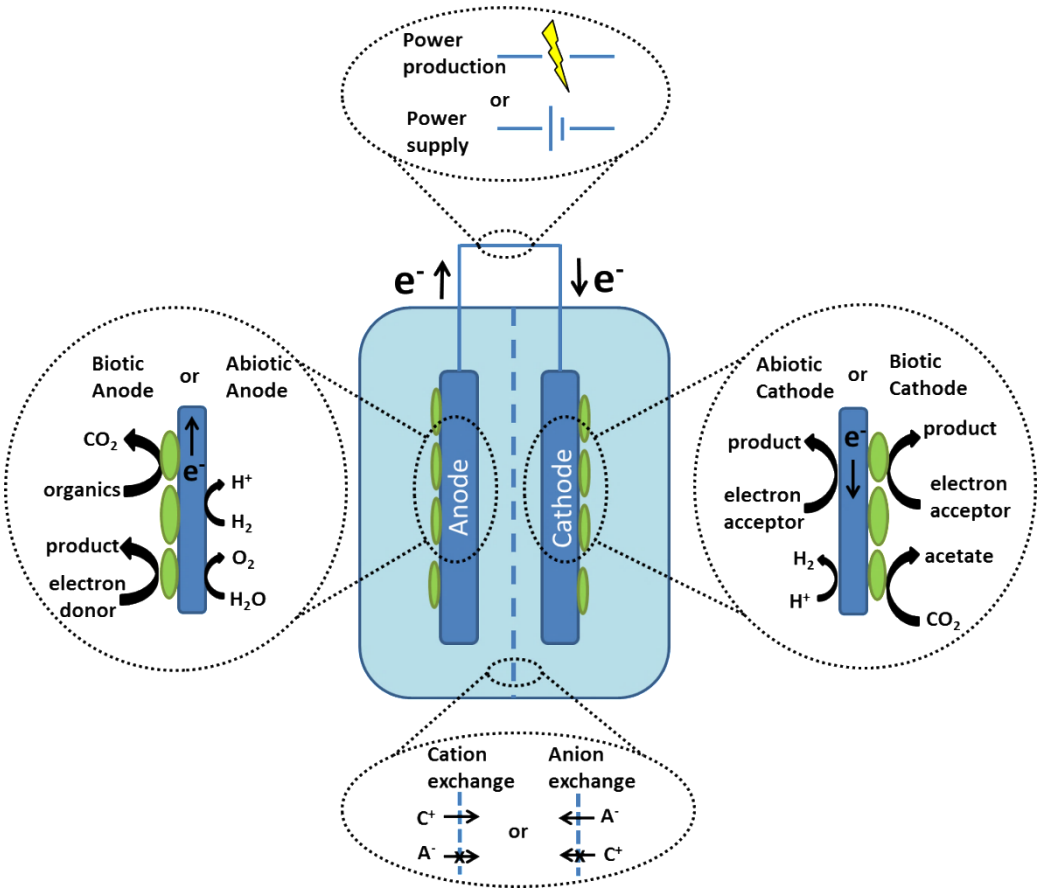


Figure 1: **Schematic principle of a bioelectrochemical system and its versatile operating options.** The given examples for anode and cathode reactions display only some possibilities of target reactions.

Initial research mainly focused on the application of BESs for power production. In so-called microbial fuel cells (MFCs) microbes donate electrons to electrodes and therefore generate an electrical current.[20, 21] Even though the results of hundreds of studies showing that microbial degradation of organic matter can be converted into electrical current are very interesting and promising,[21, 22] early optimism abated during recent years. Little increase in the power output of MFCs has been made and the processes remain not viable so far since they produce electricity at too high costs.[20, 23, 24] Another approach is presented by bioremediation of aquatic sediments and groundwater where metal-reducing microbes catalyse the transformation of organic contaminants to carbon dioxide.[25, 26] Within these systems the anodic oxidation by bacteria is coupled to production of chemicals on the cathode, usually hydrogen or methane, and they are referred to as Microbial electrolysis cells (MEC).[27, 28] In recent years another approach gets more and more attention which intends a shift in the current: Microbes are provided with additional electrons from electrodes to produce valuable chemicals at higher yields. Novel sophisticated techniques like wind turbines and photovoltaic cells enable

sustainable and cheap energy production and therefore allow bringing energy consuming technologies into focus. The electron driven applications this work focuses on are microbial electrosynthetic processes with the aim to produce value added multi-carbon molecules such as chemicals or fuels. Since these products are usually higher reduced molecules than the respective substrate the operation of the BES requires microbial growth on the cathode where electrons can be fed into the metabolism.

Microbial fuel cells

The ability of microbes to produce a measurable current flow was reported more than 100 years ago for *Saccharomyces cerevisiae*.[29] Initial research aimed for high power production in Microbial fuel cells (MFCs) that used the electron flow from a negative biotic anode to an electro-positive abiotic cathode.[30] An exciting technology in environmental engineering developed by aiming for sustainable waste(water) treatment where a mixed culture of bacteria oxidises a wide range of organic compounds and uses the anode as final electron acceptor.[22] Therefore an electrical current is developed. This process is coupled to oxygen reduction at the cathode which creates water as the final and clean product. Since the required cathode materials often included unsustainable catholytes such as cyanide, and/or expensive catalyst materials like platinum, new approaches followed that include a bio-cathode where bacteria catalyse the transfer of electrons to a terminal electron acceptor such as oxygen or nitrate. Therefore, a fully biological sustainable cell using cheap materials such as carbon or graphite can be generated that produces electricity from waste.[31] A big drawback of MFC technologies is the overpotential on the cathode that is observed for abiotic as well as biotic systems.[32, 33] That means to carry out the reduction reaction on the cathode a much higher potential is required than thermodynamically expected. To improve the performance of MFC the microbial communities were further investigated to optimize catalysing capacities. Community analysis of the formed anodic biofilms revealed a high species diversity of GRAM-negative and GRAM-positive species capable of extracellular electron transfer. However, the bacteria that show high current developments are usually GRAM-negative as GRAM-positive could not be found to show high capacities in extracellular electron transfer.[34-36] A breakthrough in MFC technologies was the isolation of the high current producing strain *Geobacter sulfurreducens* KN400, named by the TIME MAGAZINE as one of the 50 most important inventions in 2009.[26, 37]

The number of publications in the field of MFC is unceasingly rising and many groups from all over the world focus on different approaches to increase the power output of MFCs, including improvement of electrode materials, microbial cultures, feed composition, BES design, and cathode reactions.[23, 38-41] Despite various attempts to establish MFC technologies, currently only a few marine sediment MFCs have found practical use, providing electricity for low-power monitoring devices.[42] Full-scale applications of MFC are still limited due to their low power output. Further investigations leading to better understanding of the microbiological processes behind the current production is required to develop optimization strategies. Major problems that need to be addressed are proton accumulation within the biofilm and the overpotential at the cathode.[26, 43] Applications that are currently of interest are those that don't aim for current production but for wastewater treatment, degradation of specific pollutants and bioremediation (e.g. chlorinated compounds, denitrification, and uranium stabilization).[44-47] Even though MFCs are greatly limited for their application in industrial scale the initial research in this field has built a basis of knowledge about microbe-electrode-interactions that is the cornerstone for many recent advances in electro-microbiology. The demand of overcoming limitations of abiotic cathodes within MFCs created the first idea of microbes growing on cathodes.

Microbial electrolysis cells

The low current densities of MFCs and low prices for electricity encouraged a variation of the system that couples the anodic decomposition of organic wastes with the production of chemicals at the cathode. Initially, the cathodic reaction was the electrolysis of water ($2\ H_2O + 2\ e^- \rightarrow H_2 + 2\ OH^-$) what determined the name microbial electrolysis cells (MECs). But rapid developments of the system led to processes of hydrogen peroxide or methane production, which offer the advantage of generating valuable chemicals. The determining difference to an MFC is that an additional voltage needs to be applied, which supplements the low current generated on the anode sufficiently to overcome the cathodic overpotential and favour the desired reduction reaction. All electrons needed for the half-reaction at the cathode are generated by the anoxic oxidation of waste(water) at the anode. But to meet thermodynamic requirements for the reduction on the cathode an over-potential needs to be overcome which is set manually by a potentiostat or power supply.

FOLEY et al. performed a life cycle assessment of wastewater treatment by MEC and could find that contrary to the power production in an MFC the hydrogen peroxide production on the cathode of an electrolysis cell could turn the whole process into an overall net environmental benefit.[48] Laboratory studies report gas yields close to 100% for hydrogen and good efficiency in terms of electricity consumption.[49, 50] Various studies have been performed to demonstrate the benefits of MEC processes that combine wastewater treatment for example with phosphate recovery[51] or the removal of carbon and nitrogen with methane production.[52] First, pilot scale MFCs demonstrated the potential of MEC by removing sufficient compounds from wastewater to reduce the COD (chemical oxygen demand) below a critical level, reaching around 70% of energy recovery and constant production of hydrogen at ambient temperatures.[53]

Similar to fully biological MFCs, attempts are followed that involve microbial catalysis at both sides of the electrolysis cell and therefore include bio-cathodes.[54] Other approaches to study microbial activity on cathodes followed a concept of switching the operation conditions of functional bio-anodes by reversing the polarity of the working electrode. ROZENDAHL et al. converted like this an acetate and hydrogen consuming anodic culture into a hydrogen producing system on a cathode. [49, 55] Their findings strongly indicate the industrial potential for bio-cathodes even though the performance of the system needs to be improved by orders of magnitude.

1.2 MICROBIAL ELECTROSYNTHESIS AND ELECTRO FERMENTATION

Microbial electrosynthesis (MES) refers to the microbial production of multi-carbon compounds in a BES under current consumption. Initially, the term MES was used exclusively for the microbial reduction of carbon dioxide with the help of electricity [9, 56] But the research field was quickly widened by various processes that use biocatalysts for electricity-driven synthesis from other substrates than CO_2, often referred to as electro fermentation.[6, 57-60] Common in all studies is the aim of overcoming metabolic redox limitations by electron exchange between microbes and electrodes for increased production.

The electro-microbial production of chemicals and fuels is one of the most exciting potential applications for bio-electrochemical technologies offering a win-win-situation: On

the one hand, it provides an alternative production route to chemicals that would otherwise need to be synthesized from non-renewable resources. On the other hand, it enables the storage of electricity by converting electric energy into chemical energy permitting storage and delivery upon demand. MES can be powered by any source of electricity, but the initial idea was the use of solar power so an artificial photosynthesis is created (overall reaction: $CO_2 + H_2O \rightarrow$ organic compounds + O_2). The microbial process of converting solar energy into organic molecules is very efficient and offers many advantages over harvesting and processing plants.[42] The production of biofuels from plants requires big areas of arable land and additional energy input is needed while only a fraction of the processed biomass is recovered as actual fuel. The use of hybrid systems that combine the inorganic harvest of solar energy with the microbial production of chemicals could significantly improve efficiency and specificity.[57]

A big driving force of MES technologies is its potential as a carbon-capture process to significantly contribute to lower CO_2 emissions and therefore have a positive effect on climate change.[61] For a long time the use of CO_2 as raw material was deemed infeasible as the gas is difficult to capture from the atmosphere.[62] There is a dramatic shift of this opinion; however, as today's industry provides numerous gaseous waste streams rich in CO_2 making the compound easily available and allowing CO_2 utilizing technologies to get into focus. A young and award-winning company LANZATECH, for instance,specialises in gas fermentation processes in which the autotrophic acetogen *Clostridium autoethanogenum* converts the waste gas of steel mill plants into acetate and ethanol.[63] The electrons for CO_2 reduction in this process are provided by carbon monoxide and hydrogen that are also present in the waste gas. MES technologies could dramatically increase the usable spectrum of CO_2 rich gases by providing additional electrons from an electrode and therefore optimizing the electron to CO_2 ratio. The viability of such a process would be determined mostly by the price of electricity and the product value. This presents a key disadvantage of using carbon dioxide as sole substrate in a microbial electro-synthetic process as CO_2 is fully oxidised and therefore requires a large amount of electrons for its reduction to organic compounds. Hence many approaches regard organic molecules from other industrial waste streams, such as acetate, butyrate or lactate, as more beneficial substrates for MES.[64, 65] As the technology is still in its infancy we regard it as too early to rule out any possible production route for economic reasons so this thesis studies both cases: MES from CO_2 and electro-fermentation.

Since the products of interest are generally rather reduced carbon molecules, MES is a reductive process that occurs on the cathode. While electroactive cultures on the anode have been widely studied for decades, approaches of electrosynthesis show a rather short history with the first article appearing in 1974 that reports an increase of glutamic acid yield during glucose fermentation due to current supply.[8] In a following work, KIM et al. could observe a 26% increase in product yield and simultaneous lower co-product concentrations during electrically enhanced fermentation of *Clostridium acetobutylicum*.[57] Another study investigated a shift in the metabolism of *Propionibacterium freudenreichii* in the presence of electrically reduced electron carriers. Current supply via these mediators caused a product shift from acetate to propionate during fermentation.[6] More recently several groups are working on MES from carbon dioxide in mixed as well as in pure cultures where the main product is usually acetate[9, 66-68] and/or methane[69, 70] depending on BES operation conditions and microbial community. There are several approaches aiming at higher value products such as ethanol[60], butyrate[71] or for instance the increased microbial catalytic conversion from 6-bromo-2-tetralone to 6-bromo-2-tetralol in a mediated BES.[72]

To advance cathodic BES technologies a deeper understanding of the electron transport mechanisms into the cellular metabolism is needed. Therefore, it was of great importance when first in-depth studies of cathodic *Geobacter* biofilms reported that these bacteria are not only high current producers in anodic systems but can also directly accept electrons from solid state cathodes.[7] Another big step in microbial electrosynthesis research is represented by a study that presents an approach of fully biological BES for chemical production.[73] Here the electrons that are metabolised by *Sporomusa ovata* on the cathode by reduction of CO_2 to mainly acetate are simultaneously generated by an anodic biofilm of *Desulfolulbus propionicus* that oxidises sulfur to SO_4. These and other studies considered to represent important steps towards the production of value added products in BESs are summarized in the following **Table 1**.

Table 1: Overview of the most important studies towards microbial electrosynthesis, ordered by time.

organism	mediator	substrate	product	comments	reference
Brevibacterium flavum	NR[a]	glucose	L-glutamic acid	Reduced NR could increase the glutamic acid yield about 10%	Hongo 1979[8]
Clostridium (C.) acetobutylicum	MV[b]	glucose	butanol	The presence of electrically reduced MV increased butanol yields and inhibited acetone production	Kim 1988[57]
Propionibacterium (P.) freudenreichii	AQDS[c]; CoS[d]	glucose	propionic acid	Electrically reduced mediators enhance propionate production by shifting the metabolism	Emde 1990[6]
Actinobacillus succinogenes	NR	CO_2	methane	Studies of pure and mixed cultures that grow and produce H_2, methane and succinate respectively with NR as sole electron donor	Park 1999[74]
Trichosporon capitatum	NR	6-bromo-2-tetralone	6-bromo-2-tetralol	Increasing reaction rate in presence of applied potential and mediator is observed	Shin 2001[72]
Geobacter (G.) metallireducens	no	nitrate	nitrite	First evidence direct EET on cathode	Gregory 2004[7]
G. sulfurreducens	no	fumarate	succinate	First evidence direct EET on cathode	Gregory 2004[7]
Mixed culture	no	$NaHCO_3$	H_2, (methane)	First study that creates an electron consuming bio-cathode from an electron producing anodic culture	Rozendahl 2007[55]
Mixed culture	MV	acetate	ethanol	Mediated cathodic fermentation increased ethanol production and inhibited co-product formation	Steinbusch 2009[60]
Sporomusa ovata	no	CO_2	acetate, oxo-butyrate	*S. ovata* consumes electrons directly from a cathode to catalyse CO_2 fixation and acetate production	Nevin 2010[9]
Mixed culture	no	CO_2	methane, acetate, H_2	First report of simultaneous production of methane, acetate, and hydrogen	Marshall 2012[75]
Sporomusa ovata (cathode) *Desulfobulbus propionicus (anode)*	no	CO_2 (cathode) H_2S (anode)	acetate (cathode) SO_4 (anode)	First study combining anodic and cathodic biofilms for MES	Gong 2012[73]
Mixed culture	no	glycerol	1,3-propane-diol, ethanol	Investigation of community development on the cathode growing on glycerol	Dennis 2013[76]
Mixed culture (dominated by *Clostridium* species)	no	CO_2	butyrate, acetate, ethanol, butanol	Hydrogen-driven production of higher value products such as butyrate and alcohols	Ganigue 2015[71]
Mixed culture	no	CO_2	acetate	Highest reported production rates for acetic acid via MES with high electron recovery	Jourdin 2015[68]

[a]NR, neutral red; [b]MV, metylviologen; [c]AQDS, anthraquinone2,6-disulfonicacid; [d]CoS,cobalt(III)sepulchrate

The rapid development of new knowledge in this area during the last decade promotes the hypothesis that MES has the potential to become a key technology in future bioproduction processes. Obviously, the industrial interest in the field is constantly rising. The US DEPARTMENT OF ENERGY for example launched in 2009 a first major funding with the ELECTROFUELS PROGRAM OF THE ADVANCED RESEARCH PROJECTS AGENCY-ENERGY (ARPA-E). The organisation received 180 Mio USD in 2011 and 280 Mio USD in 2012 for the research area of advanced bio-production using electrochemical techniques and awarded 1.7 Mio USD to a project of THE UNIVERSITY OF MASSACHUSETTS at Amherst research group headed by DEREK LOVLEY aiming on development of "a microbial electrosynthesis process in which microorganisms use electric current to convert water and carbon dioxide into butanol at much higher efficiency than traditional photosynthesis and without need for arable land".[77]

Despite the growing interest, there are still several key challenges that need to be addressed before microbial electrosynthesis can take the step into full-scale processes. Similar to MFC processes the reactor design and engineering of the complete production process including feed stream pre-treatment and product separation will determine about scalability. Materials science recently shows promising progress in providing cheaper electrode materials that feature improved surface properties to increase electron transfer rates.[78-80] But before general process design steps can be approached a better understanding of the overall net benefits of possible processes of interest is needed. These need to feature the production of a high-value carbon-body from a ubiquitous available cheap source by the investment of a reasonable amount of electric energy. While the first published MESs display in general proof-of-concept-studies, interesting substrate and product combinations still have to be investigated. However, the major step to enable MES technologies beyond fundamental studies is the optimization of the microbial catalyst. Therefore, a better understanding of the electron transfer processes on the cathode is needed. Microbes that are especially good performing in presence of electrodes as electron donors need to be identified and the transfer mechanisms unveiled to be optimized.

1.2.1 AVAILABLE REACTOR SYSTEMS TO STUDY MES

To study electrically enhanced bio-production in depth a reactor platform fit for purpose is needed. Due to the fact that MES studies have only been intensified recently,

there are currently no commercially available systems designed specifically for the approach of electrically enhanced fermentations. The reactors used by different research groups vary significantly in their design (e.g. flow cell, tube-type, well-plate-type) and therefore in their performance which complicates comparison between different processes. **Table 2** gives a few examples of the variations in reactor and electrode design reported for important studies towards electrically enhanced production.

While different approaches may justify customised reactor designs to satisfy a specific purpose, a consistent but flexible setup usable for several different conditions is of great interest to enable comparison between different studies. A favourable system would be a two-chamber reactor since most production processes will be carried out on the cathode where oxygen is not desired. If an abiotic anode generates the electron supply that will most likely be performed by the oxidation of water and therefore oxygen production. A very recent report proposes a single chamber design for MES processes, which could greatly simplify engineering challenges regarding upscaling.[81] However, this system is not tested for many organisms and therefore a flexible lab-scale design would probably have the cathodic compartment separated by a cation-exchange membrane to guarantee anoxic conditions. The volume of a BES for MES studies is preferred to be rather small to minimize mass transfer limitations.[82] This would also benefit high throughput screening experiments to discover novel electroactive microbes on the cathode, which are desperately needed. Once promising processes come into focus a continuous flow reactor will also be of interest to study the long-term properties of microbial production on the cathode while optimizing the fermentation parameters for high performance.

The ideal BES setup is simple and flexible in its operation mode to allow the culturing of different strains, study of cathodic cultures, process development and optimization. It should feature the full process control of a conventional bioreactor (temperature, pH, stirring and gassing) as well as the additional implementation for electrodes controlled by a potentiostat. Here again, a certain level of flexibility is required to enable the use different electrochemical techniques (CV, CA etc.) as well the study of different electrode materials and use of mediators. Several BES designs have been developed already including strategies to minimize internal energy losses.[18] However, a standardised design is still missing from the research community.

Table 2: Varying reactor design of several example studies for microbial electrosynthesis and fermentation. Modified from Harnisch et al. 2015.[83]

substrate	product	microbial electro-catalyst	electrode and reactor design	reactor volume anode/cathode [L]	ref
H^+	H_2	naturally selected cultures from wastewater (bio-cathode)	graphite felt/two chamber reactor	0.25/0.25	55
H_2O	NaOH	selected cultures from brewery wastewater (bio-cathode)	graphite felt, stainless steel mesh/lamellar reactor naturally	1.02/0.61	84
Synthesis from CO_2					
CO_2	methane	microbial community from methane producing MEC	fibre brush anode, carbon cloth cathodes/single chamber MEC	0.25/0.25	85
CO_2	acetate, 2-oxobutyrate	*Sporomusa ovata*	unpolished graphite sticks/H-cell	0.2/0.2	9
CO_2	acetate, 2-oxobutyrate	*C. ljungdahlii, Sporomusa sphaeroides, Sporomusa silvacetica, C. aceticum, Moorella thermoacetica*	unpolished graphite sticks/H-cell	0.2/0.2	66
CO_2	methane, acetate, hydrogen	microbial community from brewery wastewater	graphite granules/glass chambers	0.15/0.15	75
CO_2	acetate, propionate butyrate	*C. acetobutylicum* with mediator NR	neutral red activated graphite felt/adapted bioreactor	0.05/0.5	86
CO_2	acetate	microbial consortium	NanoWeb RVC[a]/3-electrode dual chamber cell	0.01/0.25	68
Electro fermentations					
glycerol	ethanol	engineered *Shewanella oneidensis*	carbon fibre electrode/3-electrode cell with membrane	0.012	87
complex medium	l-glutamic acid	*Brevibacterium flavum* with mediator NR	Pt/shake flask	0.04	8
glycerol	1,3-propandiol, propionate, valerate	microbial consortium	graphite plates/cubic 2-chamber bioreactor	0.2/0.2	88
glucose, lactate	acetate, propionate	*P. freudenreichii* with mediator CoS	Pt electrodes/standard 3 electrode cell	0.005/0.1	6

[a] Flexible multiwalled carbon nanotubes on reticulated vitreous carbon

1.2.2 *IN SILICO* CHARACTERISATION OF MES

Mathematical modelling of metabolic networks became an indispensable tool in biotechnology predicting and analysing microbial pathways and therefore providing a systematic understanding of the involved processes.[89, 90]

The idea of overcoming metabolic redox limitations to optimize production is nothing new to biotechnology as it can be the crucial fact to decide about the viability of an industrial

biotechnology process.[91, 92] Therefore typically metabolic engineering practices are used to optimize the cellular metabolism for example by overexpression of key enzymes in the productive path or repression of undesired metabolic branches. It could be shown, that increasing the amount of redox cofactors such as NADH or NADPH available to the microorganisms is an effective way to increase the product yield of reduced products such as propane.[93] The approach of MES strategies to drive cellular redox reactions by direct interactions with a solid state electrode is, however, relatively new. While programs like OptKnock[94], OptForce[95] and EMILiO[96] help to develop knockout and inhibition strategies for microbial production processes, there is a lack of comparable tools to estimate the impact of electrical enhancement during fermentation.

In 2011 PANDIT et al. presented a first model that characterized the general role of bio-electrosynthesis in chemical production using a genome-scale metabolic model of *E. coli*.[97] Their model predicts the effects of additional electron supply on biomass growth, cellular ATP yield and production of succinate and alcohols in *E. coli*. It was found that electrochemical techniques could increase ATP yields as well as productivity within the model. As growth coupled production didn't channel all delivered electrons into the desired products they propose a dynamic fermentation strategy for MES that separates a primary growth phase without electrical enhancement from a latter productive phase under extracellular electron supply. Although this model covers the basic points of an electrochemically driven microorganism, it leaves several questions unanswered. Electrical enhancement is presented by an equation which implies the direct energy-free transition of electrons from the electrode to NAD^+ forming NADH which is to date especially for *E. coli* only speculative.[13, 98] Furthermore the given network, although claiming anaerobic conditions, contains reactions that use oxygen (O_2) which is not reasonable as it would get reduced on the cathode surface and therefore capture the electrons. Another disadvantage of the used model by PANDIT et al. is the linear programming, which reveals only one best solution for a given network and therefore does not enable detailed analysis of the predicted metabolic phenotype during MES. To this date, there are only two more modelling studies in the area. GALLARDO et al. followed the approach of PANDIT and developed a metabolic model for MES in which they assume that electrons directly reduce NAD^+ to NADH.[99] They use a genome-scale model of *Clostridium acetobutylicum* ATCC 824 and use constraint-based methods to investigate an increase of NADH levels on butanol production.[99] KAZEMI et al. recently presented a model for acetate production from CO_2 and electrons that estimates energy consumption and production based on an

idealised *Sporomusa ovata biofilm*.[100] Focus of this work is the modelling of process parameters such as the effect of substrate concentration, applied voltage, and achievable coulombic efficiencies while metabolic reactions are neglected. The electron feed is simply assumed to lead to acetate production in a stoichiometric ideal manner while cellular electron transfer processes are ignored. Hence, the work cannot provide any information about the actual metabolic effects caused by electrical stimulation. This knowledge gap was driving force to develop a theoretical approach to calculate the metabolic impact of different possible electron transport routes during electrically enhanced fermentation within this work.

The chosen tool for the presented *in silico* analysis of MES is elementary flux mode analysis (EMA) introduced by SCHUSTER et al.[101, 102] EMA calculates all possible solutions to a metabolic network model and determines a unique set of flux distributions, referred to as elementary flux modes (*efms*). To perform such elementary mode analysis, all essential pathways are defined by their individual metabolic reactions and enzymes, which are broken down to a stoichiometric matrix (S). Within this matrix, the rows represent each compound while a column corresponds to a reaction. The entries in the matrix are the stoichiometric factors of each metabolite for the corresponding reaction. EMA calculates flux distributions of a single cell in *steady state*. Internal reactions between metabolites are balanced as no accumulations or depletions are allowed while interactions with extracellular substrates or products are regarded by exchange reactions.[103] Therefore *steady state* conditions can be described with the flux balance equation: $S \cdot \vec{\vartheta} = \vec{0}$. Hereby $\vec{\vartheta}$ represents a vector with flux distributions of each reaction within *S* to fulfil the mass balance.[101, 104, 105]

Elementary mode analysis uses a convex analysis of the network by adding constraints that make sure irreversible reactions are followed in the appropriate direction. The finally obtained set of flux vectors are the *efms*.[106, 107] **Figure 2** illustrates the principle of EMA for a simple example network of three metabolites (A, B, C), three internal reactions (R1, R2, R3) and three exchange reactions (R4, R5, R6) of which one is reversible (R4). The corresponding elementary modes feature three essential properties: (i) they display a unique set of *efms* for the given network; (ii) each *efm* consists of a minimal set of reactions of which each is essential to fulfil the *steady state* condition within that mode; (iii) all *efms* together display the set of all routes through the metabolic network featuring property (ii).[105, 106]

To predict the phenotype of an organism during MES one can implement the electron transfer between microbe and electrode in a metabolic network by a corresponding reaction and calculate possible flux distributions by EMA. The definition of this EET reaction(s) has to be made based on current knowledge about the electron transport chains of the target organism. To draft a reconstruction of a microbial metabolism several databases are available providing information about genes, proteins, reactions, and pathways. EcoCyc, for example, was used in this study as it displays a bioinformatics database specific for *Escherichia coli* and includes detailed information about its metabolic pathways, enzymatic reactions, and regulatory network. Additionally, MetaCyc and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) represent comprehensive databases containing information about many different organisms and were consulted for the construction of metabolic networks within this work.

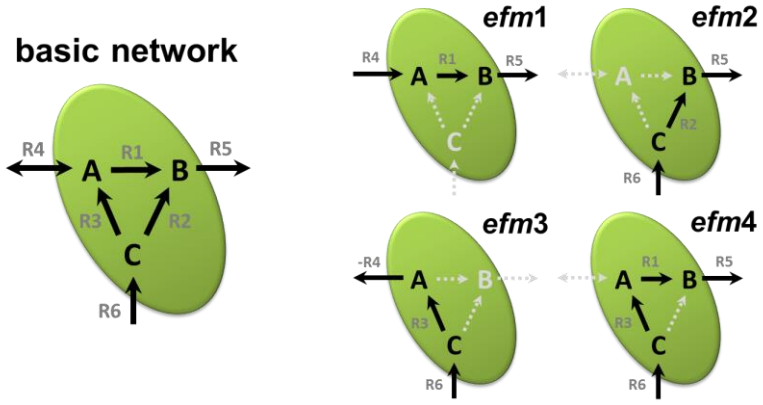


Figure 2: Simplified example of a basic metabolic network and its corresponding elementary flux modes (*efm*1-*efm*4). The basic network consists of the three metabolites A, B, C, their conversion reactions (R1-R3) and their exchange reactions (R4-R6) of which one is reversible (R4). The four elementary modes (black reactions on the right) display the operational options of the network in *steady state*.

As discussed earlier the choice of substrate and product is crucial for the viability of a BES as it determines the electrode potentials and therefore the required amount of electrons. On one hand product-substrate-combinations are required that maximise the benefit of electrical power, on the other hand, the amount of electrical energy that has to be invested still needs to provide an overall net benefit of the process.[64, 65] The EMA conducted in this project aims at identifying those beneficial processes by calculating various couples of cheap ubiquitous substrates (such as glycerol) and value added products such as fine chemicals, chemical building blocks or fuels. For the approach of identifying beneficial processes for MES, a simple tool was used that sorts these modes according to their yield for a desired product. By comparing the maximal product yields of

networks with and without extracellular electron transport one can identify the increase in product yield that is caused by providing additional electrons.

A useful tool to visualise the nature of the network is the plot of biomass against product yield. An example is given in **Figure 3** for isoprene production from glucose in *E. coli* on a cathode (calculated with *efm*tool[108] within this work). The *efm*tool draws a carbon balance around the import and export reactions of the network while internal accumulation and depletion of metabolites are not valid. Each solution that fulfils this requirement of balanced carbon is calculated as a single *efm* and is displayed as a dot within the diagram. Together all *efms* span the complete solution space for the given network. Within this solution space, one can determine maximum yields for certain products and reconstruct carbon fluxes within the network for example to study changes in by-product formation. The advantage of EMA over other modelling approaches is the calculation of ALL solutions rather than only one best solution (e.g. in flux balance analysis). Thereby not only theoretical maximum yields for production and biomass formation are assessable but the approach also enables comparison of all possible metabolic flux distributions, which presents a more holistic view of the impact of each EET model.[103]

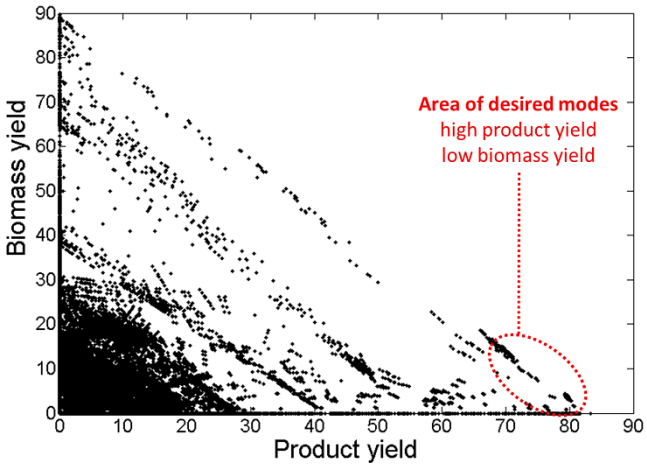


Figure 3: Example of biomass yield plotted against product yield for anaerobic isoprene production on a cathode in *E. coli* calculated by *efm*tool.

For the given example of isoprene production from glucose in *E. coli* the network contained 61 metabolites in 83 reactions (26 reversible) and 679 000 modes were calculated. Desired modes lie in the bottom right corner because they show high product yields with simultaneous low biomass production. To identify key reactions within MES processes a fraction of these desired modes featuring high product yield and low biomass production can be studied in detail. The tool enables to analyse the flux distributions for every mode, that means one can determine which pathways and reactions are highly active in a specific mode and which are not.

1.3 MECHANISMS OF MICROBIAL EXTRACELLULAR ELECTRON TRANSFER

The fact that some bacteria are able to electrically interact with solid state surfaces is known for over a century.[29] Specialist organisms like *Geobacter sulfurreducens* and *Shewanella oneidensis* were examined by various research groups and evidence for direct and indirect electron transfer could be found.[98, 109-111]

Even though a handful of organisms are known to be able to interact with electrodes the exact mechanisms involved in this extracellular electron transfer (EET) processes haven't been fully understood yet. It has been discovered that anodic EET can be performed either (i) direct by contact between electrodes and the cell surface or (ii) direct via special conductive cell structures such as pili or nanowires or (iii) indirect by mediating substances that act as electron carriers.[14, 35] There are several excellent reviews available that discuss the mechanisms of microbial EET in depth.[15, 43, 112-114] **Figure 4** shows simplified models of the different electron transfer routes between electrode and microbes.

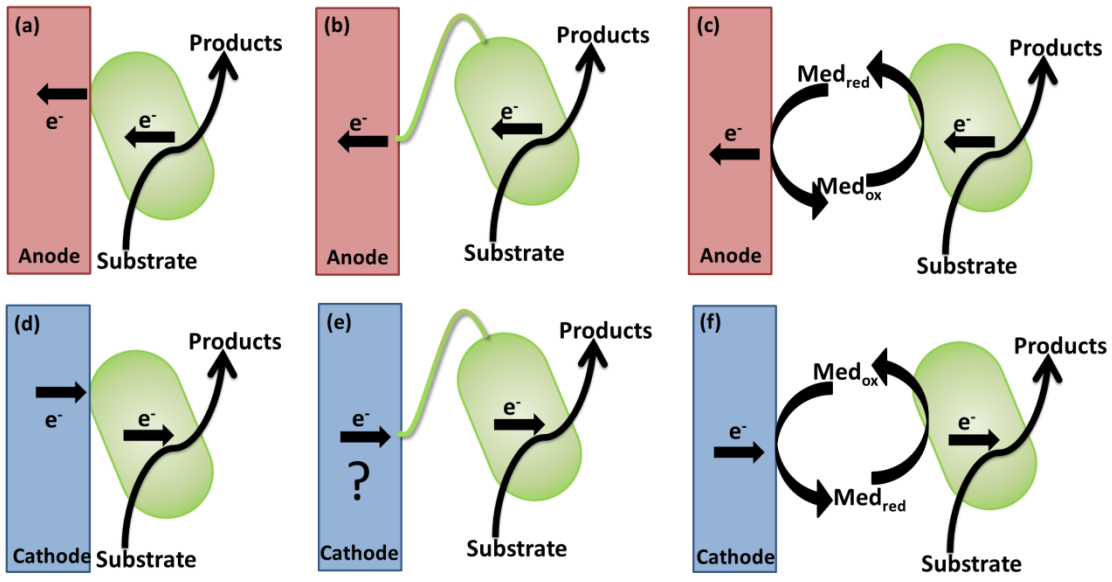


Figure 4: Extracellular electron transfer (EET) mechanisms in bioelectrochemical systems on anode (top) and cathode (bottom): direct EET by surface contact (a and d), direct EET via pili (b and e) and indirect via a soluble mediator (c and f).

The following chapters summarize briefly the current knowledge about these different EET mechanisms and highlight especially the use of mediators and processes on bio-cathodes as these lie in the focus of the presented work.

1.3.1 DIRECT ELECTRON TRANSFER

Direct electron transfer of bacteria in a bio-electrochemical system relies on a biofilm development on the surface of the solid state electrode. The model organisms that are studied in terms of direct anodic electron transfer are *Shewanella* and *Geobacter species*. Both organisms are found as dominant species in mixed cultures of MFC and various pure culture studies on solid state electrodes have been undertaken to gain insight in the involved transfer mechanisms.[35]

Geobacter as well as *Shewanella* species transfer an overflow of metabolic electrons through a chain of cell-embedded c-type cytochromes to final extracellular electron acceptors.[115, 116] This process of electron dumping to an extracellular acceptor is coupled with energy conservation and supports the growth of the organisms. The ability to use a solid surface as final electron acceptor of metabolic redox reactions gave these organisms the name *metal-respiring* or *electrode-respiring* bacteria. The terminal reductases, OmcA, and MtrC for *Shewanella* and OmcE and OmcS for *Geobacter sulfurreducens* are capable of transferring electrons either to solid acceptors such as metals or to soluble redox-compounds such as riboflavin. While *Shewanella* is known to excrete self-produced flavines as mediators, *Geobacter species* are not capable of synthesizing soluble electron shuttles, however, they are able to use them when provided externally.[117, 118]

Geobacter sulfurreducens' genome encodes for 111 different c-type cytochromes of which most are still uncharacterized but recent studies suggest that the diversity of cytochromes enables the organisms to adjust its redox activity according to the potential of the available terminal extracellular acceptor.[37, 119] This hypothesis was supported by a study on the cathode in which the research group around LOVELY for the first time confirmed direct electron transfer from a cathode to *Geobacter sulfurreducens* and *metallireducens* to achieve reductive reactions.[7] The observed potential on the cathode was much higher than the corresponding redox potentials of the two c-type cytochromes OmcS and OmZ that were identified to be essential for anodic EET of *Geobacter*. This indicates that different cytochromes or different transport mechanisms might be involved for reductive EET.[10]

Direct transfer of electrons between the electrode surface and microbes can also occur via pili, electronically conductive protein filaments that are often referred to as 'nanowires'. Both model organisms *Shewanella* as well as *Geobacter*, are found to form

pili while growing on an electrode surface. Recently it was found that the 'pili' of *Shewanella oneidensis*, in fact, are extensions of the outer membrane and therefore contain OmCs and facilitate EET.[120] The filament structures of *Geobacter* were investigated in depth as they are able to perform electric interspecies EET by transferring charge between different cells in a culture. Therefore, a conductive network is formed which enables electron transport from the electrode surface throughout the whole biofilm.[121-123] The bacterial filaments are conductive throughout their complete length and again cytochromes are supposed to be the electron transferring components as they are attached to the pili. There are two main theories about the electron transfer through pili. While the first proposes a metallic-like conductivity through the filament in which cytochromes represent only the entry/exit points the second theory claims an electron hopping between heme groups of cytochromes. Recent publications discuss new experimental data, evidence and doubts about both theories.[124-128]

To date direct electron transfer has mainly been reported and studied in GRAM-negative organisms. But recently direct EET was also observed for the GRAM-positive *Thermincola potens* and several multiheme c-type cytochromes could be located close to its cell wall or cell surface, which is very uncommon for GRAM-positive bacteria.[129, 130]

The direct EET in a BES is often regarded as the most desirable form of electron transport as it does not involve additional compounds and simplifies product removal by immobilisation of the microorganisms on the electrode surface. Nevertheless, this work focuses on mediated processes as they enable electron transfer to a wider range of organisms and avoid some experimental disadvantages like internal and external diffusion limitations, slow biofilm development, low conversion rates and low growth rates.[111, 114]

1.3.2 INDIRECT ELECTRON TRANSFER

Indirect or mediated electron transfer does not require the direct contact of microorganisms and the electrode surface but is performed by mediating molecules that transfer charge between electrode and organism. While there are several concerns for the use of mediators in large scale production as it complicates the product removal, has major diffusion limitations and can increase electrode overpotentials, it offers many advantages in lab environment by allowing the growth of electro-active cultures in suspension. Therefore, the BES can be operated like conventional fermentation processes

enabling high biomass production and a less challenging scenario in terms of reactor design. **Table 3** lists some advantages and disadvantages of the use of mediators in BESs.

Many bacteria, mostly GRAM-negative, are found to secrete endogenously redox molecules and it could also be observed that electrical stimulation can enhance this secretion.[21] Examples of bacteria-secreted electron shuttles are phenazines, flavines and quinones, but also primary metabolites like hydrogen- or sulfur-species can mediate charge transfer between solid state surfaces and the microbial metabolism.[15] The use of exogenously added mediators offers the possibility to control the amount of available redox equivalents and to enhance the EET of microbes that are not capable of self-secreting electron carriers. Requirements for properties of desirable electron shuttles are (i) low toxicity to the organisms and environmental friendliness, (ii) reversibility of the reduction reaction to allow reuse, (iii) stability and (iv) a proper redox potential that can be used by the bacteria. The latter point is still quite vague as the redox potential that is required for successful electron uptake still needs further investigation.

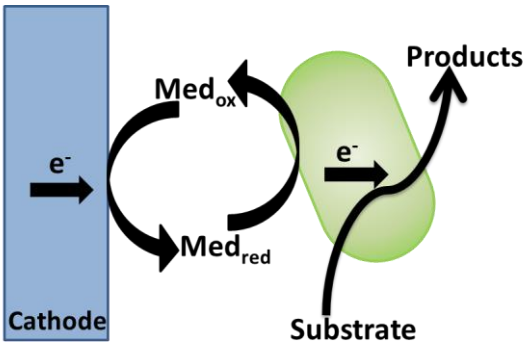


Figure 5: Mechanism of mediated EET in a BES.

Table 3: Overview advantages and disadvantages of using mediators in a bioelectrochemical system for microbial electrosynthesis.

Direct EET	H_2 mediated	Other mediators
+ Less negative potential required	+ Increased production rates	+ Enables fermentation in suspension
- Unknown electron transfer pathway	+ known metabolic pathway of electron (=H_2) uptake	- Unknown electron transfer pathway
- Very thin electric biofilms, growth limitation	- Low solubility of H_2	- Potential of being expensive and/or toxic
- Very low volumetric production rates	- Disturbance of biofilm development	- Potential complication of product separation
	- Local pH increases by proton consumption	

Currently, not many mediator molecules are known that are able to pass electrons between electrodes and cells without toxic effects and little is known about the exact mechanisms. Electron shuttles frequently used to mediate EET in BESs include neutral red (NR), methylviologen (MV), benzylviologen (BV), cobalt sepulchrate (CoS) and 2,4-anthraquinone disulfonate (AQDS).[6, 11] NR was observed to be able to act as a sole

electron donor for growth and succinate production of *Actinobacillus succiogenes* in a cathodic system.[74, 131] The following table 1.2 shows the standard electrode potentials (all $E^{'0}$ given vs. SHE) of known electron mediators as well as a selection of redox reactions that can occur in a BES and metabolic redox reactions in a microorganism. In an MFC the anodic oxidation of an electron donor is coupled to the cathodic reduction of an electron acceptor of a higher electrode potential and therefore current is produced. The oxidation on the anode could be for example represented by hydrogen ($E^{'0}$ = -0.421 V) and is coupled to the cathodic reduction which could be the conversion of fumarate to succinate ($E^{'0}$ = +0.031 V). As the resulting cell voltage is positive ($E_{cell} = E_{cathode} - E_{anode}$ = 0.452 V), and a power output is created.

Table 4: Standard redox potentials ($E^{'0}$) of a selection of redox reactions that could occur in a BES.

Half-reaction		Half-reaction	$E^{'0}$[V]*	Reference
1/2 O_2 + $2H^+$ +$2e^-$	↔	H_2O	+0.82	132
2 cytochrome a_{3ox} +$2e^-$	↔	2 cytochrome a_{3red}	+0.385	133
2 cytochrome c_{ox} +$2e^-$	↔	2 cytochrome c_{red}	+0.254	133
2 cytochrome b_{ox} +$2e^-$	↔	2 cytochrome b_{red}	+0.070	133
UQ + $2H^+$ +$2e^-$	↔	UQH_2	+0.045	133
Fumarate + $2H^+$ +$2e^-$	↔	succinate	+0.031	133
FAD + $2H^+$ +$2e^-$	↔	$FADH_2$	+0.031	133
glucose	↔	gluconate	-0.14	132
Oxaloacetate + $2H^+$ +$2e^-$	↔	malate	-0.166	133
AHDS + H^+ + e^-	↔	AQDS	-0.184	6, 111
Pyruvate + $2H^+$ +$2e^-$	↔	lactate	-0.185	133
Acetaldehyde + $2H^+$ +$2e^-$	↔	ethanol	-0.197	133
$[Co(sep)]^{3+}$ + H^+ +e^-	↔	$[Co(sep)]^{2+}$	-0.296	125
NAD^+ + $2H^+$ +$2e^-$	↔	NADH + H^+	-0.320	133
$NADP^+$ + $2H^+$ +$2e^-$	↔	NADPH + H^+	-0.324	133
NR_{ox} + $2H^+$ +$2e^-$	↔	NR_{red}	-0.33	131
cobalt sepulchrate$_{ox}$ + $2H^+$ +$2e^-$	↔	cobalt sepulchrate $_{red}$	-0.35	6
$[Co(AMMEsar)]^{3+}$ + H^+ +e^-	↔	$[Co(AMMEsar)]^{2+}$	-0.38	125
α-ketoglutarate + CO_2 + $2H^+$ +$2e^-$	↔	isocitrate	-0.380	133
BV_{ox} + $2H^+$ +$2e^-$	↔	BV_{red}	-0.398	134
$2H^+$ +$2e^-$	↔	H_2	-0.421	133
$[Co(cis\text{-diammac})]^{3+}$ + H^+ +e^-	↔	$[Co(cis\text{-diammac})]^{2+}$	-0.437	125
MV_{ox} + $2H^+$ +$2e^-$	↔	MV_{red}	-0.46	56
$[Co(trans\text{-diammac})]^{3+}$ + H^+ +e^-	↔	$[Co(trans\text{-diammac})]^{2+}$	-0.555	125
Acetate + $2H^+$ +$2e^-$	↔	acetaldehyde	-0.580	133
Succinate + CO_2 + $2H^+$ +$2e^-$	↔	α-ketoglutarate + H_2O	-0.670	133

* all standard potentials are given vs. SHE at pH7 and 25°C

In a BES for microbial production on the cathode, the corresponding anode reaction (together with energy losses in the system) determines the amount of additional energy that is needed to drive the reaction. The use of water as the electron donor ($E'^{0}_{(H2O/O2)}$ = +0.82 V) requires high energy inputs to drive cathodic catalysis. Nevertheless, the anodic reaction in this work will mainly be presented by the electrolysis of water on titanium electrodes as it is an easy and clean reaction. Additionally, the used reactor system and therefore the current consumptions will be very small. In real scale applications bio-anodes will be an advantage that can significantly reduce the required amount of electrical energy and do not require expensive electrode materials.[73]

1.3.3 Electron transfer at the Cathode

It was only within the last decade that processes with their key reaction on the cathode got into focus of the research community, hence not many studies have been published that investigate the mechanisms of cathodic EET. First investigations mainly focused on the oxygen reduction reaction (ORR) that happens on the cathode of a microbial fuel cell, to enable fully biotic and sustainable systems. Cournet et al. recently published a whole list of bacteria, including Gram-positive and facultative anaerobic organisms, that are able to catalyse that reaction on a solid state cathode.[135] The involved mechanisms, however, remain unclear. Huang et al. followed with a review of cathodic EET mechanisms that are observed in biological MFCs.[136] By studying ORR mechanisms of pure cultures on cathodes Freguia et al. could find evidence for direct as well as indirect transport mechanisms. The self-excreted redox- compound pyrroloquinoline quinone (PQQ) could be isolated from *Acinetobacter calcoaceticus* cultures and was identified to shuttle electrons from the cathode surface to the microbial metabolism, most likely via cytochromes. Whereas *Shewanella putrefaciens* cultures appeared to utilise outer membrane-bound redox compounds to perform direct electron transfer.[137] Dumas et al. could observe evidence for direct electron transport between graphite and stainless steel cathodes in fermentations of *Geobacter sulfurreducens* with fumarate as electron acceptor. Interestingly the observed potential on the active cathode differs considerably from the redox potentials of *Geobacters* c-type cytochromes that are known be the main catalysing enzymes in anodic EET, indicating a significant difference between anodic and cathodic electron transport mechanisms.[10] Stycharz et al. supported this hypothesis by

reporting current consuming *Geobacter* biofilms that did not show the high expression of outer membrane cytochromes or pili genes that is typical for anodic cultures.[12]

In natural environments the microbial uptake of electrons from solid state materials is observed especially in acidic environments such as mine drainage, where iron(II)- and sulphur-oxidising organisms catalyse the electron transfer between inorganic molecules such as Fe(II), S^0 or S^2 and oxygen. Similar to direct electron transfer on a bio-anode, outer-membrane c-type cytochromes could be identified as key compounds, which accept the initial electrons, pass them to the metabolic electron transport chain and finally transfer them to oxygen.[138] Therefore it might seem likely that c-type cytochromes play as well a crucial role for EET on bio-cathodes.

The first review on mechanisms of cathodic EET by ROSENBAUM et al. discusses known anodic mechanisms and their potential role on bio-cathodes.[98] They highlight a possible critical role of the combination of c-type cytochromes together with hydrogenases and natural mediators like PQQ in cathodic EET. The EET mechanisms of hydrogenase-containing bacteria have been widely discussed in the field of bio-corrosion processes as anaerobic organisms such as *Desulfovibrio sp.* have been found to corrode metal surfaces with reduced biogenic compounds like hydrogen sulphide and consume the hydrogen together with electrons from the metal via outer membrane hydrogenases. Again c-type cytochromes could be found to act as redox-partners.[139, 140] *Shewanella* has been reported to produce hydrogen in absence of external electron acceptors to equilibrate metabolic excess of reducing power.[141]

Two main differences to anodic EET could be identified so far (i) the uptake of additional electrons is contrary to the donation not necessarily energy conserving, (ii) the involved components seem to operate at significant different potentials.[11, 136] If outer membrane cytochromes accept electrons from a cathode they need to transfer them to redox partners in a more positive state. Therefore, the redox potential of the final electron acceptor can be regarded as crucial since it needs to be highly electron-positive (e.g. oxygen $E^{0'} = +0.820$ V) to leave enough potential difference between the electrode as electron donor and the final acceptor to allow energy-conserving reactions in the metabolism. This project aims to identify those possible beneficial end products for MES.

2 THESIS OVERVIEW

The literature review demonstrates the promising potential of the novel approach of microbial electrosynthesis but it also points out our currently limited knowledge of the underlying fundamentals. This was the rationale of this PhD, which aims to gather novel information about the microbial physiology during electrically enhanced fermentations for production and therefore advance the technology. To achieve that it was chosen to combine *in vivo* experiments to identify and characterise cathodic cultures for production with an *in silico* approach that enables the evaluation of different metabolic scenarios theoretically while current knowledge gaps remain.

The work can be characterised by the following three research objectives (ROs). First microbial electron transport chains are analysed with the aim to understand potential options for cathodic EET. The second objective is the development of a comprehensive modelling study to identify target processes for MES and simultaneously assess the effects of electrical interference on the cellular metabolism theoretically. Finally, RO3 describes the *in vivo* approach of in-depth study of cathodic cultures. **Figure 6** illustrates where the ROs of this work can provide novel information about a microbial electro-synthetic fermentation process.

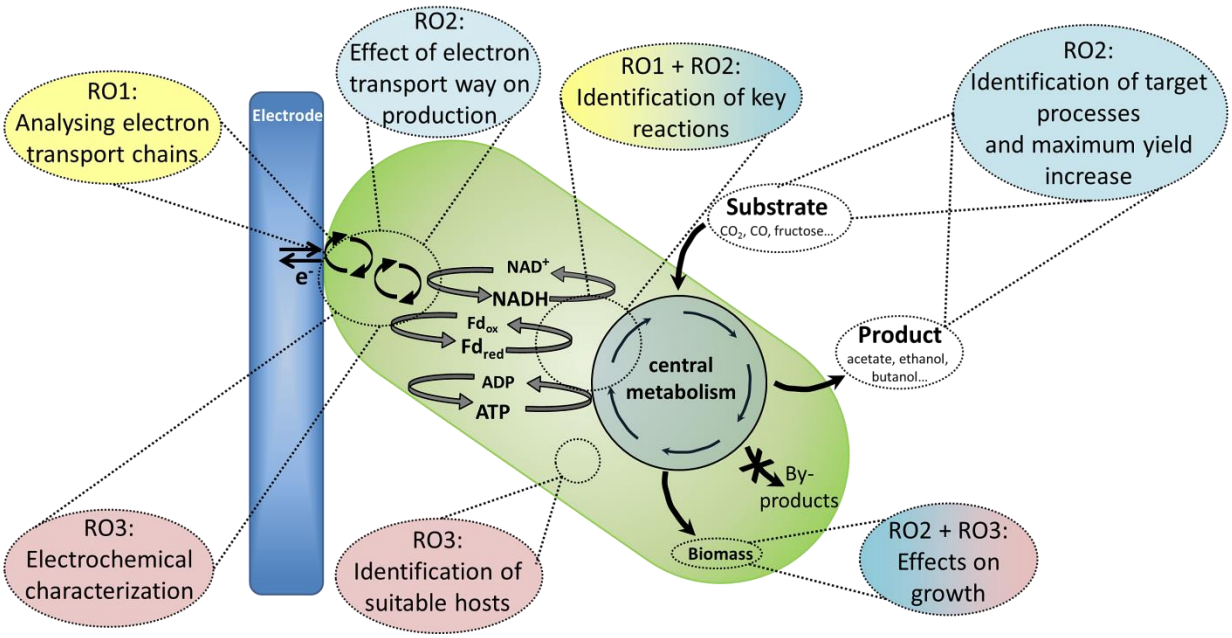


Figure 6: Schematic overview of the processes this work is investigating highlighted on the image of a cell in an electrically enhanced fermentation. Yellow: research objective (RO) 1: Studying cathodic electron transport mechanisms; Blue: RO2: Theoretical assessment of microbial electrosynthesis; Red: RO3: *In vivo* characterisation of the microbial metabolism during MES.

2.1 RESEARCH OBJECTIVE 1: STUDYING CATHODIC ELECTRON TRANSPORT MECHANISMS

The idea of microbial electrosynthesis resulted from the discovery that the microbial metabolism can be driven by an electrical current.[56] Central in this application is the ability of the microbial catalyst to interact with external electron donors and its metabolic properties of connecting electron transport and carbon metabolism. And here also lies the key challenge. A wide range of microbes has been discovered to be able to exchange electrons with solid surfaces or mediators but only a few have been studied in depth. In particular, electron transfer mechanisms from cathodes towards the microbial organism are poorly understood and the variety of mixed and pure cultures that are studied in electron consuming processes does not leave any conclusion on what metabolic features might be needed for optimal electron uptake. Therefore, the first objective of this work is to summarize and analyse microbial electron transport chains in regards to their EET properties.

The task is to analyse the different electron transport chains that nature offers for organisms such as metal respiring bacteria and acetogens, but also standard biotechnological organisms currently used in industrial bio-production. In each species, the connection between redox and energy metabolism should be studied to evaluate effects on energy yields when influencing the cellular redox state with bioelectrochemical techniques. The possibility of extracellular electron exchange at different points in each organism is to be analysed regarding required redox potentials and effect on cellular redox and energy levels. Key compounds such as enzymes and/or redox carriers that play important roles in metabolic redox reactions are to be identified. The aim is to analyse our current knowledge on electron transport processes and to use a theoretical approach to predict the impact of different modes of EET on the energy metabolism.

2.2 RESEARCH OBJECTIVE 2: THEORETICAL ASSESSMENT OF MES

A second major knowledge gap in the field of MES research is presented by the choice of target conversion. While the first published electrically enhanced fermentations display in general proof-of-concept studies, interesting substrate-product combinations still have to be investigated. Before general process design steps can be approached, a better

understanding of the overall net benefits of possible target processes is needed.[142, 143] These need to feature the production of a higher value carbon-body from a ubiquitous available cheap source by the investment of a reasonable amount of electric energy.

As pointed out in 0 mathematical modelling is an irreplaceable tool in biotechnology when estimating process benefits and/or metabolic processes. Therefore, the second objective of this project describes an *in silico* approach to reveal the theoretical potential of MES in bioprocess technologies. As the exact fundamental processes of microbe-electrode interaction remain unknown the objective is to design a metabolic network for a model organism and include model reactions for extracellular electron transfer. Elementary flux mode analysis (EMA) is chosen as a flexible stoichiometric pathway analysis tool that enables comparison of a large set of computed solutions. In **Figure 6** is highlighted where EMA can provide novel information about MES processes. The aim is to investigate a large set of interesting target products and determine the maximum theoretical possible process benefit if produced from different carbon sources under electrical enhancement. This allows quantification of the impact of bio-electrochemical systems on the bio-production of fuels and feedstocks and helps to prioritise research efforts.

2.3 RESEARCH OBJECTIVE 3: *IN VIVO* CHARACTERISATION OF THE MICROBIAL METABOLISM DURING MES

The third and final research objective is the *in vivo* study of the electrical impact on productive fermentations. As this requires a reactor platform fit for purpose the first task is the development of a bio-electrochemical system that provides control over all fermentation parameters as well as flexibility in its setup. The reactor system is to be used to characterize the behaviour of different organisms when exposed to a cathode or reduced mediator molecules. Monitored parameters include growth via spectrophotometric measurements, current consumption recorded by a potentiostat and chemical production analysed via HPLC or GC. The first aim of this objective is to identify strains that are able to take up electrons and to analyse the impact of electron supply on production. Therefore, a selection of industrial important bacteria should be chosen to be screened for electro-activity.

Depending on the performance of the different organisms during the first set of electrically enhanced fermentations, strategies for an in-depth characterisation will be followed. The aim is to get a deeper understanding of the electron uptake mechanisms for optimization purposes. Here the impact of redox potential at which electrons are supplied is to be studied by the use of different mediator molecules.

3 MATERIALS & METHODS

This chapter describes all materials and methods that were used to achieve the presented results in the following section 4 “research outcomes”.

3.1 MATHEMATICAL MODELLING

3.1.1 MODELLING TOOLS

The stoichiometric analysis of metabolic networks was performed based on the elementary mode analysis framework introduced by SCHUSTER et al.[101] The java implemented free software efmtool (version 4.7.1)[108] was used within MATLAB, MathWorks (version R2012a), to compute for each network the elementary flux modes which represent all possible and unique steady-state flux distributions the network could have based on reaction stoichiometry. It does not take regulatory or thermodynamic constraints into account (except reaction directions), which means that the theoretical maximum yields are the absolute maximum possible with given stoichiometric constraints and that *in vivo* yields will very likely be lower. Nevertheless, it represents the most reliable estimate of the capacity of a network. The operational mode that shows maximum possible carbon flux from substrate into product is referred to as *top mode*. Maximal theoretical yields for biomass or a certain product are obtained by calculating the carbon balance of all carbon containing substrates entering the network and the carbon-containing products leaving the network:

$$Yield_{product}\ [\%] = \frac{flux_{product} \cdot carbon_{product}}{flux_{substrate} \cdot carbon_{substrate}} \cdot 100\%$$

Where $flux_{product}$ is the reaction rate for products leaving; $flux_{substrate}$ the reaction rate for substrate uptake and $carbon_{product}$ and $carbon_{substrate}$ the number of carbon atoms in the product and substrate molecules, respectively.

Calculations were performed using a standard desktop computer or the high-performance computing cluster, Barrine, located at The University of Queensland's St Lucia campus.

3.1.2 *ESCHERICHIA COLI* MODEL

The basic *E. coli* core model used for the in silico analysis includes: Embden–Meyerhof–Parnas pathway/glycolysis, glycerol degradation, Entner–Doudoroff pathway, pentose phosphate way, tricarboxylic acid cycle, glyoxylic shunt, anaplerotic reactions, anaerobic fermentation, electron transport chain, import and export reactions and interaction with a soluble electron carrier. **Figure 11** and **Figure 12** in chapter 4.2 show a flux map of the corresponding network and the electrode reactions. For the production of industrial relevant products that are not metabolites of the main network, engineered pathway branches for production were implemented. Because oxygen will lead to abiotic current production in most cathodes, anaerobic conditions were assumed as a technical requirement.

The main network includes 57 metabolites, 75 reactions (24 reversible) and up to 215,000 *efms* per scenario were calculated. A link to the full network can be found in the appendix under 8.1.

3.2 BIO-ELECTROCHEMICAL SET UP AND TECHNIQUES

3.2.1 BIO-ELECTROCHEMICAL SET UP

The BES setup for the experimental characterisation of MES was developed by optimizing a commercially available analytical cell kit from BIOLOGIC (BIOLOGIC, SCIENCE INSTRUMENTS, France). The actual reactor vessel is a glass cell with working volume up to 150 mL while during fermentations a volume of 100 or 120 mL was used. The lid and all connections are removable to allow easy cleaning procedure. The lid and fittings to connect each probe and all electrodes were custom-made by a mechanic workshop at the University of Queensland from PEEK (Poly-ether ether ketone, purchased as raw material from E-PLAS engineered & industrial plastics, Australia). Inert Gas atmosphere was achieved by constant flushing of the reactor headspace with nitrogen (flow rate 0.1 L/min) and the respective off-gas was led through a condenser chilled to 4°C (custom made by LABGLASS, Brisbane) to minimize evaporation and stripping of volatile fermentation products.

The working electrode was carbon cloth (CCP20 FUEL CELL EARTH, Stoneham, USA) connected to a titanium wire (diameter 0.5 mm, purity 99.8%, ADVEND RESEARCH

MATERIALS, Oxford, England). The counter electrode was titanium mesh connected to titanium wire and was separated from the main reactor volume by a cation-selective membrane (surface area: 2.55 cm^2, CEM, Ultrex CMI7000, MEMBRANES INTERNATIONAL INC., USA). Titanium mesh and carbon cloth were cleaned by soaking three times in *iso*-propanol followed by rinsing with distilled sterile water before usage. The reference electrode was a commercial Ag/AgCl electrode in saturated KCl solution (BIOLOGIC SCIENCE INSTRUMENTS, France).

The pH controlling system was built by Nicolas Lekieffre, senior research assistant at CEMES, UQ. The analytical probe combines a pH probe and a PT100 Temperature probe (model F-235, BROADLEY-JAMES, USA) and was immersed in the reactor medium. The measured signal was amplified by optically isolated amplifiers (model PHT 4221, NEWPORT ELECTRONICS, USA) before feeding the controlling unit (compact RIO, NATIONAL INSTRUMENTS). This unit was regulated by LABVIEW software (NATIONAL INSTRUMENTS) on a personal desktop computer to control the acid and base pumps (model WPM, WELCO, Japan). The pH was set constant to the optimum pH for the respective organisms and was adjusted by adding 5 M sterile filtered NaOH and HCl. The parameters pH, temperature, time, volume of acid added and volume of base added were recorded electronically. Additionally, the EC software of the potentiostat recorded current and electrode potentials during the experiment.

Before every experiment the complete BES was autoclaved (121°C, 20 min, 2 atm) with either medium or buffer. Thermosensitive compounds were added later inside the biosafety cabinet (Class II Biological Safety Cabinet GELAIRE, Sydney, Australia). A detailed description of the development of the BES including pictures is given in chapter 4.3.1.

3.2.2 CYCLIC VOLTAMMETRY

Cyclic voltammetry (CV) experiments were performed in the BES described above as three-electrode system using either a modular five channel or sixteen channel potentiostat (Potentiostat/Galvanostat VSP or MPG2, BIOLOGIC SCIENCE INSTRUMENTS, France). The same electrodes as during electrically enhanced fermentations were used for CVs to determine the redox potentials of the mediators exactly at these conditions (WE: carbon cloth, CE: titanium mesh, RE: Ag/AgCl sat KCl).

The anodic solution was 10x concentrated PBS buffer while on the cathodic site the different mediators were added at 1 mM to the corresponding microbial media (omitting carbon source). CVs were run in a potential range from -1.0 V to 0.0 V vs. reference electrode at a scan rate of 1 mV s^{-1}. The recorded scans were taken after 5 initial cycles and display an average of three measurements with an overall maximum standard deviation of maximum ±2.5%. Midpoint potentials were determined by averaging the potential difference between oxidative and reductive peaks for each compound. Exact minima and maxima were determined with the EC-LAB software (Version 10.40, BIOLOGIC SCIENCE INSTRUMENTS, France).

3.2.3 CHRONOAMPEROMETRY

During electrically enhanced fermentations the BES were run in chronoamperometry (CA), whereby a constant potential of -0.603 V vs SHE was applied on the carbon cloth working electrode (-0.8 V vs RE). At this potential no abiotic hydrogen production was observed but full reduction of all tested mediators could be achieved (see **Figure 25** in chapter 4.4.3 for CVs of medium and different mediators). The potential was applied to prior to inoculation until a stable baseline current was achieved (< 100 μA). Cumulative electric charge, that is, the amount of electrons consumed (Coulombs), was calculated for each fermentation by integration of the current profile versus time logged by the potentiostat after subtraction of the corresponding baseline current.

3.3 CHEMICALS AND MEDIA

The mediators [Co(sep)]$(ClO_4)_3$, [Co(AMMEsar)]$(ClO_4)_3$ and [Co(*trans*-diammac)]$(ClO_4)_3$ were synthesised and kindly provided by Professor P. Bernhardt (School of Chemistry and Molecular Bioscience, University of Queensland, Brisbane, Australia). All other used chemicals were obtained from SIGMA-ALDRICH, USA or ALFA AESAR, USA in analytical grade.

To achieve anaerobic conditions the different media were boiled (omitting thermosensitive compounds such as vitamins) and cooled under constant inert gas bubbling (N_2).

E. coli media

Bacterial cultures of *Escherichia coli* were grown in lysogeny broth (LB) rich media or minimal media M9 with compositions given in **Table 5** and**Table 6**.

Table 5: Media compositions used in *E. coli* fermentations.

LB media			M9 Media		
tryptone	10	g	Na_2HPO_4	6.78	g
yeast extract	5	g	KH_2PO_4	3	g
NaCl	10	g	NaCl	0.5	g
Distilled water	1	L	NH_4Cl	1	g
			glucose	5	g
			$MgSO_4$ (1M)	1	mL
			Thiamine-HCl (10 g/L)	100	µl
			trace metal solution	1	mL
			Distilled water	1	L

To prepare the M9 media all salts till ammonium chloride were dissolved in 750 mL distilled water and the pH was adjusted to 7.4 with 10M NaOH, the volume was filled till 950 mL before autoclaving. After cooling down to room temperature the glucose was added as 25 mL of 20% sterile stock solution as well as magnesium, thiamine and the trace metal solution.

Table 6: Compositions of trace metal solution used for M9 media preparation.

Trace metal solution		
EDTA	15	g
$ZnSO_4$ $7H_2O$	4.5	g
$MnCl_2$ $2H_2O$	0.84	g
$CoCl_2$ $6H_2O$	0.3	g
$CuSO_4$ $5H_2O$	0.3	g
Na_2MoO_4 $2H_2O$	0.4	g
$CaCl_2$ $2H_2O$	4.5	g
$FeSO_4$ $7H_2O$	3	g
H_3BO_3	1	g
KI	0.1	g
Distilled water	1	L

To prepare the trace metal stock solution (composition **Table 6**), the pH was adjusted to 10 with NaOH to dissolve the EDTA and then the chemicals from top to bottom were added. Each chemical was fully dissolved before the next one was added. The final pH of this solution should be around 4. If required an acidification with sulphuric acid was implemented. The trace metal solution was filter sterilized.

P. acidipropionici media

Table 7: Complex medium (PAM) compositions used in *P. acidipropionici* fermentations.		
PAM		
Yeast extract	10	g
Peptone trypticase soy broth	5	g
K_2HPO_4	0.25	g
$MnSO_4 \cdot H_2O$	0.056	g
glucose	40	g
Distilled water	1	L

Propionibacterium acidipropionici and *P. freudenreichii* were grown in complex medium PAM (**Table 7**) or a more defined medium MSM which was modified from a receipt from Emde and Schink (**Table 8**).[6] PAM was prepared by dissolving all ingredients from **Table 7** in 920 mL distilled water. After autoclaving the glucose was added as 80 mL of sterile filtered stock solution (50 w%). MSM was enriched with 200 mM MOPS (3-(N-morpholino)propanesulfonic acid) to buffer the amounts of propionic acid that are produced during fermentation.

Table 8: Compositions for second medium (ESM) used in fermentations of *P. acidipropionici* and *P. freudenreichii*.

ESM			trace element solution SL-10 for ESM		
NH_4Cl	0.25	g	HCl (25%; 7.7M)	10	mL
KH_2PO_4	0.20	g	$FeCl_2 * 4\ H_2O$	1.5	g
NaCl	3.0	g	$ZnCl_2$	70	mg
$MgCl_2 * 6\ H_2O$	0.4	g	$MnCl_2 * 4\ H_2O$	100	mg
KCl	0.5	g	H_3BO_3	6	mg
$CaCl_2 * 2\ H_2O$	0.15	g	$CoCl_2 * 6\ H_2O$	190	mg
1 M MOPS (pH = 7)	200	mL	$CuCl_2 * 2\ H_2O$	2	mg
Glucose solution 50wt%	20	mL	$NiCl_2 * 6\ H_2O$	24	mg
Yeast extract (5 wt%)	20	mL	$Na_2MoO_4 * 2\ H_2O$	36	mg
Trace element solution SL10	1	mL	Distilled water	990	mL
Selenite-tungstate solution	1	mL			
7 vitamin solution	1	mL			
Distilled water	757	mL			

Vitamin solution for ESM			Selenite-Tungstate solution for ESM		
Vitamin B_{12}	10	mg	NaOH	500	mg
p-Aminobenzoic acid	80	mg	$Na_2SeO_3 * 5\ H_2O$	3	mg
D(+)-Biotin	20	mg	Na_2WO_4	4	mg
Nicotinic acid	200	mg	Distilled water	1000	mL
Calcium pantothenate	100	mg			
Pyridoxine hydrochloride	300	mg			
Thiamine-HCl * 2 H_2O	200	mg			
Distilled water	1000	mL			

To prepare the medium all components till calcium were dissolved in 757 mL distilled water and autoclaved (121°C, 20 min). After cooling down the MOPS buffer, glucose, yeast extract, trace elements, selenite-tungstate and vitamins were added from filter sterilised stock solutions under a biosafety cabinet (Class II Biological Safety Cabinet GELAIRE, Sydney, Australia). The filter sterilised solutions were stored at 4°C. The pH was adjusted to 7.2 with sterile NaOH. Final glucose concentration in the medium was 10 g/L. All media and stock solutions were purged with nitrogen prior usage to remove oxygen.

Citrobacter werkmanii media

Citrobacter werkmanii was grown in MSM medium, composition given in **Table 9**. The medium was prepared and autoclaved omitting glucose, glycerol and trace elements. These were added after autoclaving from sterile filtered stock solutions.

Table 9: Compositions of mineral salt medium (MSM) used in fermentations of *Citrobacter werkmanii*.

MSM			trace metal solution TM1 for MSM		
1 M MOPS (pH = 7.3)	200	mL	$Na_2EDTA * 2 H_2O$	6.37	g
K_2HPO_4	2.27	g	$ZnSO_4 * 7 H_2O$	1.0	g
KH_2PO_4	0.95	g	$CaCl_2 * 2 H_2O$	0.5	g
$(NH_4)_2SO_4$	0.67	g	$FeSO_4 * 7 H_2O$	2.5	g
Trace metal solution TM1	2	mL	$NaMoO_4 * 2 H_2O$	0.1	g
Glucose solution 3M	10	mL	$CuSO_4 * 5 H_2O$	0.1	g
Glycerol solution 9M	10	mL	$CoCl_2 * 6 H_2O$	0.2	g
Distilled water	778	mL	$MnSO_4 * H_2O$	0.52	g
			$MgSO_4 * 7 H_2O$	60.0	g
			Distilled water	1	L

Clostridium autoethanogenum media

The medium for *Clostridium autoethanogenum* fermentations was slightly modified from DSMZ medium 879. The vitamin and trace element solutions are identical to DSMZ medium 141 and all compositions are given in **Table 10**. The MES buffer was dissolved in 700 mL water and the pH adjusted to 5.9 with NaOH. All ingredients till the trace element solution were added, the volume adjusted to 960 mL and autoclaved. After autoclaving, vitamins, trace elements and fructose were added from sterile stock solutions and the medium was cooled under constant sterile N_2 bubbling to achieve anaerobic conditions. Cysteine was added as reducing agent directly prior to inoculation from a sterile anoxic stock solution. The pH of the trace and vitamin solution was adjusted to 6.5 – 7.

Table 10: Compositions of modified medium 879 used in fermentations of *C. autoethanogenum*.

Modified medium 879			Trace element solution 141		
MES	20	g	Nitrotriacetic acid	1.5	g
NH_4Cl	1	g	$MgSO_4$ * 7 H_2O	3	g
KCl	0.1	g	$MnSO_4$ * H_2O	0.5	g
$MgSO_4$ * 7 H_2O	0.2	g	NaCl	1	g
NaCl	0.8	g	$FeSO_4$ * 7 H_2O	0.1	g
KH_2PO_4	0.1	g	$CoSO_4$ * 7 H_2O	0.18	g
$CaCl_2$ * 2 H_2O	0.02	g	$CaCl_2$ * 2 H_2O	0.1	g
Yeast extract	2	g	$ZnSO_4$ * 7 H_2O	0.18	g
Trace element solution 141	10	mL	$CuSO_4$ * 5 H_2O	0.01	g
Vitamin solution 141	10	mL	$KAl(SO_4)_2$ * 12 H_2O	0.02	g
L-cysteine-HCl (100 mM)	10	mL	H_3BO_3	0.01	g
D-Fructose solution 50wt%	10	mL	Na_2MoO_4 * 2 H_2O	0.01	g
Distilled water	960	mL	$NiCl_2$ * 6 H_2O	0.03	g
			Na_2SeO_3 * 5 H_2O	0.3	g
Vitamin solution 141			Na_2WO_4 * 2 H_2O	0.4	g
Biotin	2	mg	Distilled water	1	L
Folic acid	2	mg			
Pyridoxine-HCl	10	mg			
Thiamine-HCl * 2 H_2O	5	mg			
Riboflavin	5	mg			
Nicotinic acid	5	mg			
D-Ca-pantothenate	5	mg			
Vitamin B_{12}	0.1	mg			
p-Aminobenzoic acid	5	mg			
Lipoic acid	5	mg			
Distilled water	1	L			

3.4 BACTERIAL CULTIVATION

3.4.1 ORIGIN OF BACTERIAL STRAINS

The *Escherichia coli* strain K12 MG 1655 was obtained from the culture collection of the Australian Institute for Bioengineering and Nanotechnology (AIBN, University of Queensland, Brisbane, Australia). *Propionibacterium acidipropionici* (DSM-4900) was also obtained from AIBN, kindly provided by Dr Esteban Marcellin as part of an industry linkage project with THE DOW CHEMICAL COMPANY (partially funded by ARC grant LP120100517). *Citrobacter werkmanii* (DSM-17579) was provided by the group of Prof Korneel Rabaey from the University of Ghent, Belgium, as part of a collaborative work. *Propionibacterium freudenreichii* (DSM-20271) and *Clostridium autoethanogenum* (DSM-10061) were imported from the LEIBNIZ INSTITUTE DSMZ-GERMAN COLLECTION OF MICROORGANISMS AND CELL CULTURES, Braunschweig, Germany.

3.4.2 CULTURING CONDITIONS

All strains were kept as glycerol stocks at -80°C. **Table 11** gives an overview of the different culturing conditions. *Escherichia coli, Propionibacterium acidipropionici*, *Propionibacterium freudenreichii* and *Citrobacter werkmanii* were anaerobically growth on solid medium by incubating agar plates in an anaerobic pouch system (Anaerogen™ compact, THERMO FISCHER SCIENTIFIC) or inside a vinyl anaerobic chamber (Type A, COY LABORATORY PRODUCTS, USA). The atmosphere inside the chamber was 20% CO_2, 4% H_2, N_2 to balance (COREGAS, Australia). From solid plates or from glycerol stocks (*Clostridium autoethanogenum* only) first pre-cultures of each strain were inoculated inside the anaerobic chamber in the corresponding medium. Culturing flasks were 125 mL serum bottles with rubber stopper screw cap (EDWARDS, Australia) filled with either 50 mL or 100 mL culture. Cultures of *Escherichia coli* and the *Propionibacteria* were incubated outside the chamber in a shaking incubator due to high cell densities (100 rpm, INFORS multitron standard shaker). The maintenance of anaerobic conditions in all flasks and media was confirmed by experiments of each medium containing resazurin (0.0001% w/v), however, all final experiments were performed omitting the redox indicator to prevent any interaction of resazurin with the electrodes. First pre-cultures were sedimented by centrifugation (4000 rpm, 5 minutes, 37°C or 30°C, respectively, Centrifuge SIGMA 3K30, DJB LABCARE Ltd, Buckinghamshire, England) and re-suspended in the corresponding fresh media (1:100). Cellular growth was monitored by measuring the optical density (OD) by UV-Vis Spectrophotometry (Genesys 10S, THERMO FISHER SCIENTIFIC) and exponentially growing cells were harvested again by centrifuging and washing in fresh media. This cell solution was used as inoculum for the main cultures in the BES. All centrifuging, washing and resuspending was performed under nitrogen atmosphere.

Table 11: Culturing conditions for the different strains used in this study.

	Escherichia coli	*Propionibacterium acidipropionici, P. freudenreichii*	*Citrobacter werkmanii*	*Clostridium autoethanogenum*
solid medium	LB, M9	PAM, ESM	LB	-
liquid medium	M9	PAM, ESM	MSM	Modified 879
temperature	37°C	30°C	37°C	37°C
incubation time	18 h	24 h	24 h	48 h
shaking	100 rpm	100 rpm	none	none
Wavelength for OD measurement	660 nm	600 nm	600 nm	600 nm

3.4.3 BES EXPERIMENTS

The electrically enhanced fermentations were conducted in 100 mL volume in the reactor system that is described in detail in the following section 4.3.1. The reactors were heated to the optimum temperature for each strain by a water bath and stirring was always 400 rpm (except at CV conditions). Additional mediating components were added to the cathodic side before inoculation under sterile conditions in concentrations of 1 mM. A constant potential of -0.8 V vs. the reference electrode (Ag/AgCl saturated KCl) was applied on the working electrode to reduce the mediator (corresponding to a potential of -0.603 V vs SHE). After 3-5 hrs the mediator was fully reduced and the chronoamperometry experiment started by adding the corresponding strain via a sterile three-way port of the reactor at the cathodic site. This port was also used for sampling (sample volume 3 mL) to measure growth, pH and metabolite production. First samples were taken prior inoculation, then directly after inoculation followed by regular sampling intervals depending on the growth rate of each culture.

Growth was monitored by photospectrometric measurement against cell-free media and 1 mL of each sample was centrifuged (15000 rpm, 5 min, 4°C) and the supernatant was frozen for HPLC analysis.

3.5 ANALYTICS

3.5.1 PHOTOMETRIC MEASUREMENTS

Optical density was measured in a UV-Vis spectrophotometer (Genesys 10S, THERMO FISHER SCIENTIFIC) at 660 nm (*E. coli*) or 600 nm (all other strains). Samples were processed immediately after extracting them from the BES and were diluted if necessary to stay within the linear range of the device, which is from OD = 0.005-0.3. Ideally, readings were taken at OD = 0.1.

3.5.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

To measure metabolite profiles fermentation samples were analysed via HPLC. Organic acids were detected from pure cell-free samples, sugar from 1/10 dilutions.

The used device (Agilent 1200 Series, Agilent Technologies, Santa Clara, USA) was a reverse phased chromatography and consisted of seven different modules (G1312A Binary pump, G1379B degasser, G1367B high-performance autosampler, G1330B FC/ALS (thermostat), G1365B column compartment (thermostat), G1365B multiple wavelength detector (MWD) and G1362A refractive index detector). The column Phenomenex Rezex RHM Monosaccharide H^+ had the dimension of 300 x 7.8 mm plus an up streamed guard column. The HPLC was driven with a mobile phase of 100% 0.008 N sulphuric acid with a flow rate of 0.6 mL/min. The maximum pressure was set to 80 bar and the column temperature to 70°C. 30 µl of every sample were injected and had a run time of 25 minutes. The wavelength of the MWD was 210 nm.

4 RESEARCH OUTCOMES

This section of the thesis is divided into 4 subchapters presenting the major findings that were made following the research objectives described in chapter 2. The first section 4.1 discusses characteristics of natural occurring electron pathways and analyses different options for EET. The following chapter 4.2 introduces elementary mode analysis as modelling approach to identify target processes for electrically enhanced production. Based on the electron transport chains analysed in 4.1 different models are developed to simulate electrode-microbe interactions. The dependency of theoretically achievable product yields on the electron transport way is discussed.

Chapter 4.3 introduces the *in vivo* approach of the project by reporting the development of a suitable reactor platform. This is followed by screening experiments of different organisms for electroactivity. The final chapter 4.4 connects all parts of the presented study and analyses electrified production in a cathodic BES on the example of *Clostridium autoethanogenum*. Metabolic modelling is used to develop theories of possible EET mechanisms that could explain the obtained *in vivo* results.

While each subsection includes a discussion of the presented results as well as a short conclusion, this chapter is followed by a general discussion of the research findings as chapter 5 and an outline of future perspectives and overall conclusions in chapter 6.

4.1 MICROBIAL ELECTRON TRANSPORT AND ENERGY CONSERVATION – THE FOUNDATION FOR OPTIMIZING BIOELECTROCHEMICAL SYSTEMS[1]

Whether one is considering current consuming or current producing bio-processes, crucial in each application is the performance of the microbial host. The ability and especially the efficiency of the organism to exchange electrons with an electrode and connect this extracellular electron transport to its cellular carbon metabolism significantly influences the overall process performance. Many applications in bio-electrochemical systems are so far restricted to lab-scale research projects as the electron transfer rates are simply too low to design a viable process scale-up.[50] In order to optimize and advance bioelectrochemical techniques a more thorough understanding of possible extracellular electron exchange mechanisms in both directions is needed.[144] Especially cathodic systems and the fundamentals of electron transport towards microbes are poorly understood.[98, 145] In this chapter the natural electron transport chains of different organisms are introduced and discussed according to their potential benefits and limitations if used in a BES. Special focus lies on the connection of redox and energy metabolism in each species.

4.1.1 VARIETIES OF MICROBIAL ELECTRON TRANSPORT CHAINS

In microbial electron transport chains, electrons are transferred from a low potential electron donor to an acceptor with more positive redox potential by redox reactions. These reactions are usually catalysed by membrane-bound compounds that use the energy difference between donor and acceptor to establish an ion gradient across the membrane, which in turn is used for ATP synthesis and thus converts the difference in electrical potential into chemical energy for the cell.[146]

In order to adapt to different environmental conditions microbes developed an enormous variety of electron transport chains.[147] Important systems catalysing these redox reactions include primary dehydrogenases that supply high energetic electrons from a donor such as NADH and usually couple electron transport to H^+ or Na^+ transport across the membrane.[146] Also involved in transmembrane ion transport are membrane-localised

[1] This chapter was modified to fit this thesis from the published work: **Kracke, F.**, Vassilev, I., and Krömer, J.O. (2015). *Microbial electron transport and energy conservation – the foundation for optimizing bioelectrochemical systems.* Frontiers in Microbiology 6. doi: 10.3389/fmicb.2015.00575

(multi-)protein complexes such as cytochromes and terminal oxidases (reductases) that transfer electrons to a final acceptor such as oxygen, nitrate or fumarate.[148-150] Most transmembrane reductases and oxidases function as ion pumps but some do not. Electron carrying co-factors such as quinones, flavines, heme, iron-sulfur-clusters or copper ions also play an important role in microbial electron transport. Some of these are soluble lipophilic molecules that shuttle electrons between the relatively large enzymatic complexes inside the membrane (e.g. quinones) while others are catalytic cofactors bound to proteins (e.g. heme groups of cytochromes).[151, 152] There are also membrane-bound complexes that use the exergonic electron bifurcation of soluble cellular redox compounds such as ferredoxin and NADH for transmembrane ion transport and therefore establishment of a motive force across the membrane that can drive ATP synthesis.[153-155]

The achievable energy gain (Gibbs free energy, ΔG) of each electron transport chain is depending on the redox potential difference (ΔE) of all reactions between electron donor and acceptor. Some bacteria incorporate several electron transport chains, which they can use sometimes even simultaneously in order to respond to different electron acceptors and donors available in the environment.[146, 156] Others are restricted to only one respiratory pathway.[157, 158] This diversity of microbial electron transport mechanisms illustrates the complexity of the approach of bioelectrochemical techniques. In order to interfere efficiently with the redox metabolism of an organism, one needs to understand the targeted site of extracellular electron transport and its metabolic impact.

A wide range of microbes has been discovered to be able to exchange electrons with solid surfaces (direct EET) and/or soluble mediators (indirect EET) but only a few have been studied in depth. In fact, the mechanisms of electron transport that are found in different species can differ significantly from one another. Dissimilatory metal-reducing bacteria are amongst the most studied being able to “respire” insoluble metals in anaerobic environments. The model organisms *Geobacter sulfurreducens* and *Shewanella oneidensis* were studied by various research groups for decades and evidence for direct and indirect electron transfer between the organism and electrodes could be found.[98, 109-111] For both bacteria outer-membrane cytochromes were identified as essential compounds to enable EET.[118, 159-161] However, there are several differences in their electron transport chains, for example *Shewanella* excretes soluble electron carriers while similar compounds are missing in *Geobacter spp*.[117, 162] Furthermore it could be shown that the redox chains catalysing an inward current rely on different mechanisms than current producing reactions.[10, 163] Another group of dissimilatory metal-reducing bacteria is

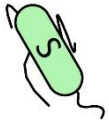
presented by the obligate anaerobe *Thermincola*, which were also found to be capable of directly transferring electrodes to anodes.[130, 164] Their EET mechanisms also seems to rely on cytochromes that in this case are cell-wall associated of the GRAM-positive bacteria.[129] Interestingly there are also organisms such as *Clostridium ljungdahlii*, which do not have any cytochromes but were tested positive on EET.[66, 165] The exact mechanisms by which electrons are transferred between the electrode surface and the microbial metabolism still remain unclear.[11, 145] Therefore we have a look at the native electron transport chains of several organisms that were studied in BESs. An overview is given in **Table 12** and the following sections discuss the mechanisms of the presented bacteria in detail.

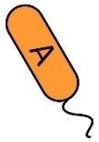
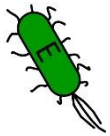
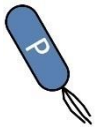
4.1.2 SPECIFIC EET IN METAL RESPIRING BACTERIA

Metal-reducing bacteria, as their name indicates, have the ability to interact with solid minerals, e.g. Fe(III) or Mn(IV), to obtain energy by using those minerals as electron acceptors and/or donors for their respiration process. Here a transport of electrons from a low redox potential donor to an acceptor with a higher redox potential can result in a proton gradient to drive ATP synthesis. That characteristic of breathing metals plays an important role in biogeochemical cycles and has the potential to be used in bioremediation and BES.[166, 167]

But how do the microorganisms respire insoluble metals and extract energy? This question has not been fully clarified in detail yet. In contrast to other respiration processes, where freely diffusible gas or readily soluble substances can easily enter the cell and be used as electron acceptors/donors, the major challenge for metal respiring bacteria is the interaction with the extracellular minerals, which cannot pass the cell membrane and do not have access to the periplasm nor cytoplasm. To overcome this barrier the bacteria need redox-active molecules in their outer membrane or have to excrete redox-active shuttle molecules to transfer electrons between the cellular interior and the extracellular metals.[147, 168] There exists a great diversity of mechanisms for such electron-shuttling pathways, which is explained in more detail in the section below by describing the EET of two model organisms.

Table 12: Electron transport characteristics and behaviour in bioelectrochemical systems of organisms discussed in this study. Coloured symbols are used for microbe identification in **Figure 9**. Blue: aerobes; green: facultative anaerobes; red-yellow: obligate anaerobes.

Organism	GRAM staining	Key components in electron transport chain and coupling to energy conservation	Experience in BES		Important references
			Anode	Cathode	
Shewanella oneidensis 	-	Mtr pathway: Proton gradient created by cytochromes (c-type), soluble electron carriers and membrane-bound NADH-hydrogenase; ATP via H^+-ATPase	Various studies, model organism; direct and self-mediated electron transfer	Direct use of electrons by thin biofilms for reduction of fumarate to succinate	[110, 169]
Geobacter sulfurreducens 	-	Branched OMCs system: Proton gradient created by cytochromes (c-, d-types), soluble electron carriers and membrane-bound NADH-hydrogenase; ATP via H^+-ATPase	Various studies, model organism; generation of comparatively high current densities by direct electron transfer through biofilms	Direct use of electrons by thin biofilms for reduction of fumarate to succinate	[7, 109]
Thermincola ferriacetica	+	Putative electron transport chain based on multiheme c-type cytochromes associated with periplasm and cell surface; ATP via H^+-ATPase	First proof of anodic current production by direct contact of a Gram-positive organism	No report	[164, 170]
Sporomusa ovata	-	H^+ gradient via membrane-bound cytochromes (b-, c-types) and quinones; ATP via H^+-ATPase	No report	Direct use of electrons from an electrode for CO_2 reduction to acetate and 2-oxobutyrate	[9, 171]
Moorella thermoacetica 	-	H^+ gradient via membrane-bound cytochromes (b, d-type), quinones and/or Ech-complex; ATP via H^+-ATPase	No report	Direct use of electrons from an electrode for CO_2 reduction to acetate at high columbic efficiencies (>80%)	[66]
Acetobacterium	+	Electron bifurcating ferredoxin reduction	No report	*A. woodii* was shown not to be	[66, 75]

woodii 		Na^+ gradient via membrane-bound Rnf complex (Ferredoxin:NAD^+-oxidoreductase), Membrane-bound corrinoids (No cytochromes, no quinones); ATP via Na^+-ATPase		able to directly accept electrons from a cathode; however was also determined as a dominant species in a cathodic mixed culture producing acetate from CO_2 and microbial and/or electrochemically produced hydrogen	
Clostridium ljungdahlii 	+	Electron bifurcating ferredoxin reduction H^+ gradient via membrane-bound Rnf complex (Ferredoxin:NAD^+-oxidoreductase) (No cytochromes, no quinones); ATP via H^+-ATPase	No report (however a close relative *C. acetobutylicum* was shown to be able to oxidate acetate under current production)	Direct use of electrons from an electrode for CO_2 reduction to acetate	34, 66
Escherichia coli 	-	H^+ gradient via membrane-bound cytochromes (a-, b-, d-, o-type) dehydrogenases, quinones, flavins (bound); ATP via H^+-ATPase	*E. coli* is able to produce current after long acclimation times without mediator, or on modified electrodes	No report	172-174
Pseudomonas aeruginosa 	-	H^+ gradient via membrane-bound cytochromes (a-, b-, c-, o-type), phenazines, flavines (soluble and bound), quinones and dehydrogenases; ATP via H^+-ATPase	Current production mediated by self-secreted phenazines	No report	175, 176
Corynebacterium glutamicum 	+	H^+ gradient via membrane-bound cytochromes (a-, b-, c-, d-type), quinones, flavins (bound) and dehydrogenases; ATP via H^+-ATPase	No report	Increased lactic acid production with a mediator in a cathodic system	177

G. sulfurreducens: branched OMCs system

The GRAM-negative obligate anaerobic δ-proteobacterium *G. sulfurreducens* is used as a model organism for investigating electroactive microorganisms.[178] Since its genome was sequenced it is easier to analyse the detailed molecular mechanism of EET and to construct molecular models. In its genome, more than 110 genes coding for putative c-type cytochromes have been identified, which likely play an important role in the electron transport pathway of this bacterium.[179] It is assumed that several multiheme c-type cytochromes enable the transport of redox equivalents between the cellular menaquinone pool and the extracellular insoluble metals to create a proton gradient for energy conservation.[178] The interaction between the cytochrome complexes in the electron transport chain is based on the redox potential of the different multiheme molecules of the cytochromes, whereby each heme has its own specific redox potential. In this way, wide windows of potential ranges are created that overlap with each other and allow a bio-energetic transfer of electrons.[180, 181]

Figure 7A illustrates a model of the EET mechanism with the participating proteins, which were assigned a central role. Here a diheme cytochrome c peroxidase, designated 'metal-reduction-associated cytochrome' (MacA), functions as a transmitter of electrons from the inner membrane to the triheme periplasmic c-type cytochrome (PpcA) in the periplasm. Following on PpcA passes the electrons to the outer membrane cytochromes, termed OMCs (e.g., OmcB, OmcC, OmcS, OmcZ), which transfer the electrons to the extracellular acceptor. The branched OMCs system is very complex and is still not fully understood. It seems that different OMCs are required to interact with different extracellular metals or electrodes.[178] For example, the octaheme cytochrome OmcZ is more abundant in biofilms grown on an electrode and a deletion of *omcZ* gene leads to a current decrease of more than 90% while there is no impact on the reduction of other electron acceptors as Fe(III) oxide.[182, 183] The dodecaheme cytochrome OmcB and the hexaheme cytochrome OmcS are essential for Fe(III) reduction while knock-out of the *omcB* or *omcS* genes results in no or hardly any effect on current generation through biofilms grown on an anode.[150, 184] The proposed model reflects a greatly simplified EET mechanism. In fact, the EET pathway is subjected to a complex regulatory mechanism and there exist many more homologous omc genes, which can replace lacking cytochromes genes in generated mutants.[185, 186] For instance OmcB is important for the reduction of soluble Fe(III), but a Δ*omcB* mutant can express homologs such as a dodecaheme OmcC, which allows the use of a parallel pathway to respire Fe(III).[187]

In comparison to the transport of electrons to the anode, much less is known about the mechanism by which the cell takes up electrons from the cathode. Due to the reverse electron chain pathway, the practicing shuttle molecules have to operate at different redox potentials. In contrast to current-generating biofilms, in current-consuming biofilms, a lower expression of OMCs such as OmcZ was detected. Deletion of those associated genes, which were essential for anodic biofilms, had no impact to cathodic biofilms. In further studies, it was observed that in current-consuming biofilms a gene (GSU3274) encoding a putative monoheme c-type cytochrome was strongly expressed. Mutants lacking this gene lost their ability to take up electrons from a cathode but did not show differences in EET to an anode. So it seems that GSU3274 plays a significant role in EET from a cathode.[12, 98]

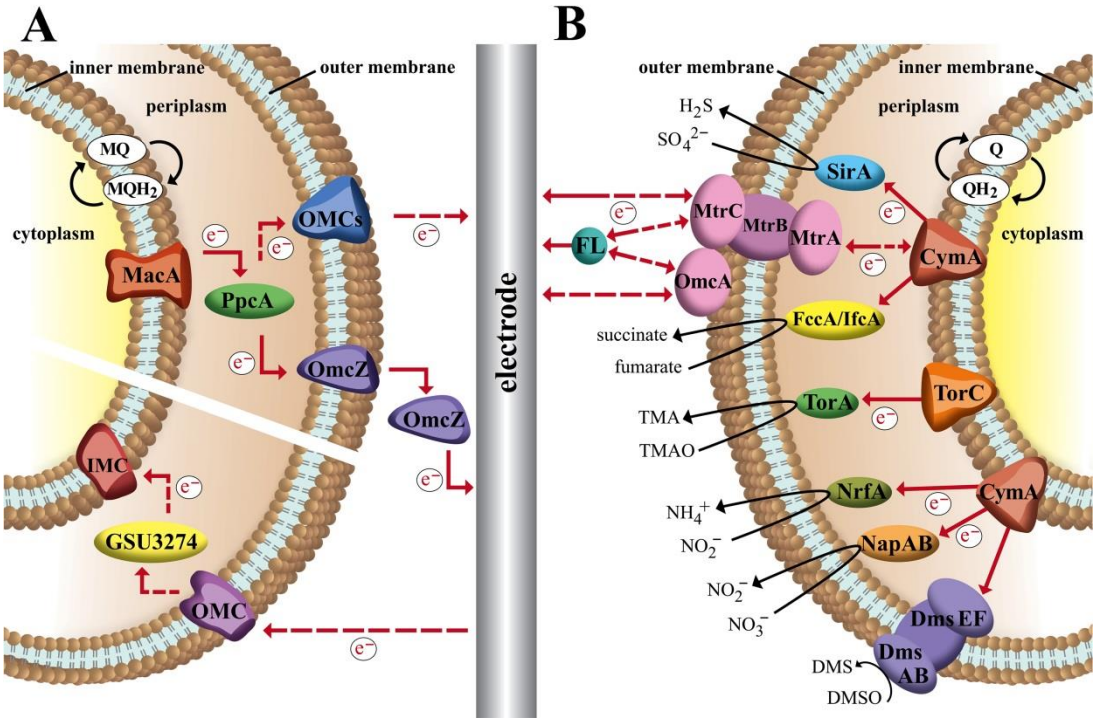


Figure 7: Schematic image of the proposed EET of two metal respiring bacteria and their interactions with an electrode in a bioelectrochemical system. Dashed arrows indicate hypothetical electron flow and solid arrows indicate experimentally proven electron flow. (A) Branched outer membrane cytochromes system of *Geobacter sulfurreducens*. Electrons can be transported between inner membrane, periplasm, outer membrane and an electrode via a chain of cytochromes and menaquinones (MQ). Terminal outer membrane cytochromes (OMCs) can vary depending on the environmental conditions. (B) Mtr-pathway and terminal reductases of *Shewanella oneidensis.* Quinones (Q) pass electrons to CymA or TorC, which transfer the electrons to terminal reductases or a MtrCAB complex. MtrCAB complex can interact with the electrode direct or via flavin molecules (FL). Reproduced from Kracke et al. 2015.[188]

G. sulfurreducens shows best EET performance as a biofilm grown on the electrode based on direct electron transfer. This bacterium possesses excellent biofilm formation properties and the thickness of the biofilm is linked with the amount of generated current in a linear correlation up to a certain thickness limit.[121] The transport of electrons in a

multilayer biofilm to an anode can be achieved by two combined mechanisms. One transfer way is based on secretion of non-diffusing 'mediators' (e.g. cytochromes such as OmcZ) into the biofilm matrix, which can act as electron shuttles.[183] The second way depends on 'nano-wires', which are electrically conductive appendages that enable physical connection between cells and/or cells and the surface of the electrode.[121] The mechanism of the EET in such nanowires has not been clarified yet. One model proposes that the nanowires are pili with a metallic-like conductivity, which is based on aromatic amino acid residues within the appendages, which enable electron delocalization due to π-stacking.[127, 189] Another theory describes a 'superexchange' model, in which the electrons are 'hopping' along a chain of redox active proteins to the final electron acceptor.[190]

In comparison to anodic-biofilms, it was reported, that cathodic-biofilms are less-developed and much thinner.[7, 163] The reason might be limiting redox surplus under non-autotrophic anaerobic growth conditions, which is enhanced by cathodic electron input as suggested by the *in silico* study presented in chapter 4.2 of this work.[191] However, this observation needs to be studied in depth and be validated experimentally for a better understanding.

S. oneidensis: characteristic Mtr-pathway

The second electroactive model organism of interest for BES technologies is the GRAM-negative facultative anaerobic bacterium *S. oneidensis.* The interesting characteristic of *S. oneidensis* is its ability to utilize a great variety of inorganic and organic compounds as a final electron acceptor in the absence of oxygen. As in the case for *G. sulfurreducens* that property is based on the interaction of a large number of multiheme cytochromes. The sequenced genome of *S. oneidensis* shows 42 putative cytochromes, of which 80% are located in the outer membrane.[192, 193] The first step of the electron transfer through the cell membrane is the oxidation of small electron carriers (quinols), which enable the transport of electrons between NADH-dehydrogenase and cytochromes in the inner membrane to create a proton gradient for energy conservation in form of ATP. That oxidation reaction can be catalysed by the tetraheme cytochromes TorC and CymA, whose sequence is very similar and both are attached to the inner membrane by a single α–helix. The next link in the electron transfer chain of TorC is a periplasmic reductase TorA, which can utilize the outer membrane permeable trimethylamine N-oxide compound as a terminal electron acceptor and reduces it to trimethoxyamphetamine.[194] In comparison to TorC, CymA can interact with different redox partners in the periplasm and

as a consequence outer membrane-crossing molecules like sulfite can be reduced by the octaheme redox partner SirA,[195] nitrite by pentaheme cytochrome NrfA,[196] nitrate by NapAB reductase[197] and fumarate by FccA and IfcA reductase.[198] Furthermore an octaheme cytochrome OTR was detected and showed in vitro capability of the reduction of a range of N- and S-oxides and oxyanions, but *in vivo* the function of OTR has not been confirmed yet.[199]

Additionally as a metal respiring bacterium like *G. sulfurreducens*, *S. oneidensis* is able to use extracellular terminal electron acceptors, e.g. electrodes, Fe(III) and Mn(IV) (see **Figure 7 B**). To overcome the outer membrane barrier the bacterium possesses a Mtr-pathway. Homologous genes for that pathway were also found in other dissimilatory metal reducing and oxidizing bacteria.[200] However, *S. oneidensis* Mtr-pathway is one of the best-investigated EET chains. It has been suggested that a decaheme cytochrome MtrA takes up electrons from CymA via an electron transport chain and passes them on to an extracellular decaheme cytochrome MtrC, which transfers the redox equivalents to final exogenous electron acceptors.[169, 201] Here MtrA, MtrC and a third element, MtrB form a complex, MtrCAB. MtrB is a porin molecule in the outer membrane and serves to organize and stabilize MtrA and MtrC to enable electron transfer. Besides MtrC a second decaheme cytochrome, OmcA was detected anchored as a lipoprotein in the outer membrane that was able to transfer electrons to exogenous electron acceptors as well.[161, 202] In addition to the *mtrCAB* genes the genome of *S. oneidensis* encloses homologs like *mtrFDE*. The homologous cytochromes can support the EET or can partly take over the activity of the MtrCAB complex depending on the electron acceptor and the growth conditions.[169] Likewise, the DmsABEF complex is based on homologs. Here the porin cytochrome complex DmsEF transfers the electrons to the DmsAB complex, which is then able to reduce DMSO to DMS.[203] The periplasmic tetraheme cytochrome STC has to be mentioned as well, which seems to support the transfer of electrons between the inner membrane and outer membrane, however, the exact mechanism remains unclear.[204] In contrast to EET to an anode, the information about taking up electrons from a cathode is limited. The interacting compounds in the cathodic process were not established yet. It was suggested based on *in vivo* studies, where electrons were provided from a graphite electrode to reduce fumarate, that an electron uptake is possible through a reverse Mtr-pathway.[110]

In contrast to *G. sulfurreducens*, *S. oneidensis* shows not only the ability of direct electron transfer, but can also perform indirect electron transfer due to excretion of redox

active mediators. Direct electron transfer is based on the physical connection with the electrode by forming a biofilm on the surface[117, 205] and through extensions in form of nanowires. While *Geobacter* nano-wires are assumed to be type IV pili, for *S. oneidensis* nano-wires it was shown, that their structure is similar to outer membrane vesicles and those nano-wires can be seen as extensions of the outer membrane and periplasm that include the multiheme cytochromes responsible for EET.[120] In the case of indirect electron transfer, *S. oneidensis* secretes flavin molecules that act as small diffusible shuttle molecules to transfer electrons between the electrode and outer membrane cytochromes[117] or as bounded cofactors for outer membrane cytochromes.[206] *G. sulfurreducens* is also able to produce flavin molecules, but here the flavins are preferentially bound to the outer membrane cytochromes and are not mobile like free shuttle units.[207]

4.1.3 CARBON RESPIRATION OF ACETOGENIC BACTERIA

Acetogenic bacteria, short acetogens, are anaerobic organisms that are able to assimilate CO_2 or CO via the Wood-Ljungdahl-pathway, also called carbonate-respiration or acetyl-CoA pathway. This autotrophic pathway offers the possibility to develop biotechnological processes that combine the usage of cheap ubiquitous substrates (i.e. syngas) with greenhouse gas reduction and therefore makes acetogens attractive hosts for biotechnology.[165, 208] This feature put the bacteria into focus of the research community trying to establish an artificial ‘photo’synthesis process by using CO_2 and electrons from an electrode.[9] Acetogens are also able to utilize a great variety of heterotrophic molecules such as sugars, glycerol and cellulose, which broadens the spectrum of possible substrates to waste streams from biodiesel industry (e.g. glycerol) or dairy industry (e.g. whey) and many more.[208] The main product is usually acetate (hence the name) but acetogens also bear the feature to naturally produce a broad spectrum of other chemicals, which are of industrial use either directly or as precursors such as ethanol, butanol, 2,3-butanediol or butyrate.[208] The mayor intermediate from carbon fixation via Wood-Ljungdahl-pathway is acetyl-CoA and is linked to other metabolic pathways such as Embden-Meyerhof-Parnas-pathway, therefore offering a great metabolic diversity for metabolic engineering of other production pathways.

In the Wood-Ljungdahl-pathway two CO_2 molecules are merged to form one molecule Acetyl-Coenzyme-A, which is either converted to acetate or assimilated in

biomass. The overall conversion of CO_2 to acetate uses one ATP (in the step of formyltetrahydrofolate synthetase) and creates one ATP by acetate kinase reaction. Therefore, no net energy gain can be achieved via substrate-level phosphorylation. Since acetogens are able to grow autotrophically, the pathway must be coupled to a chemiosmotic mechanism that provides additional energy. By calculating the Gibbs free energies of each reaction in the Wood-Ljungdahl pathway a net energy benefit of about -95 kJ/mol can be determined.[209] This energy could support the synthesis of 1-2 mol ATP via chemiosmosis as anaerobic bacteria require -60 to -80 kJ of free energy for the synthesis of 1 mol of ATP.[157] In recent years experimental evidence for membrane driven ATP synthesis in acetogens could be found, however, the sites and mechanisms of energy conservation differ between organisms. Over 100 different species of acetogens have been identified and despite their common feature of CO_2 assimilation via the Wood-Ljungdahl pathway, they are very diverse in terms of metabolism, phylogenetics or preferred habitat.[210] The best-studied organisms belong to the genera Acetobacterium and Clostridium and include a few species with fully available genome sequences (genome published: *Moorella thermoacetica*, *Clostridium ljungdahlii*, *Clostridium carboxidivorans*, *Eubacterium limosum*; under preparation: *Acetobacterium woodii*, *Clostridium aceticum*). Genetic and genomic tools are under intense development and promise fast advancing metabolic engineering platforms for acetogens.[211-214]

M. thermoacetica: cytochromes or Ech-complex?

From an energetic point of view and in regards to electron-transport properties acetogens are often divided into two groups: Na^+-gradient and H^+-gradient dependent species. *M. thermoacetica* (formerly *Clostridium thermoaceticum*) an example for the latter group was the model organism for HG WOOD and LG LJUNGDAHL for their studies of the acetyl-CoA pathway. They identified several membrane-integrated electron carriers menaquinone MK-7, a flavoprotein and two b-type cytochromes that are believed to play major parts in creating a proton gradient over the membrane.[157] Later such a proton motive force that could drive ATP synthesis in *M. thermoacetica* was measured, however, which components exactly transfer protons across the membrane remains unknown.[215] Genome studies of the organism revealed also membrane-bound components such as hydrogenases and NADH-dehydrogenase, which are known to transfer protons across the membrane in other organisms. Therefore, a membrane integrated electron transport chain via these complexes with H_2 as electron donor and methylene-THF as an electron acceptor is proposed.[216] However, so far no experimental data supports this hypothesis.

Recent studies also deliver evidence for electron-bifurcating enzymes that play important roles in electron transfer of autotrophic and heterotrophic pathways of *M. thermoacetica.*[217] The soluble complex HydABC oxidises hydrogen with simultaneous reduction of ferredoxin and NAD^+.[218] Additionally a second soluble transhydrogenase (NfnAB) catalyses the electron bifurcation from reduced ferredoxin and NADH to $NADP^+$. Furthermore the genome of *M. thermoacetica* codes for a membrane-bound energy-converting hydrogenase, called Ech-complex. For the methanogenic archaea, *Methanosarcina mazei,* the Ech-complex is responsible for the establishment of a proton gradient across the membrane, which leads to the theory that could also be the case in acetogens.[219, 220] This complex uses the excess energy that is freed from electron transfer from reduced ferredoxin to H^+ to transport ions across the membrane. A very recent report states that electron stoichiometry is only balanced with the involvement of the Ech-complex in energy conservation while the membrane-bound dehydrogenases and cytochromes play no major part.[219] As this hypothesis also lacks distinct experimental validation both theories were included in the summary of possible electron transport mechanisms of *M. thermoacetica* (**Figure 8 A**).

A. woodii and *C. ljungdahlii*: electron transport without cytochromes?

In 2010 NEVIN et al. reported that the electrons needed for CO_2 fixation via Wood-Ljungdahl pathway could be provided directly by an electrode, a breakthrough work in the field of bio-electrochemical techniques.[9] The acetogen *Sporomusa ovata*, a close relative to *M. thermoacetica* was able to directly accept electrons from a cathode and convert carbon dioxide to acetate and 2-oxobutyrate. Following studies showed similar abilities of other acetogens of the Sporomusa and Clostridium genera.[66] The acetogen *A. woodii*, however, was found unable to consume current and showed different behaviour compared to other acetogens in NEVINS experiments. *A. woodii* is an example strain for Na^+-dependent acetogens that typically lack cytochromes. It could be shown that the conversion of CO_2 to acetate via Wood-Ljungdahl-pathway is coupled to the generation of a sodium gradient across the cytoplasmic membrane.[158, 221] Since *A. woodii* does not contain cytochromes or quinones energy conservation must be secured by a different electron transport system. In 2008 evidence for a novel membrane-bound ferredoxin:NAD^+ oxidoreductase (Rnf complex) was reported that seems to be responsible for transmembrane Na^+ transport.[154] In this complex the electrons from reduced ferredoxin are transferred to NAD^+ to form NADH. Since the redox potential of ferredoxin ($E^{0'}_{Fd}$ = -500 to -420 mV) is more negative than that of the NAD^+/NADH couple ($E^{0'}_{NADH}$ = -320 mV), the

energy surplus (equivalent to -20 to -35 kJ/mol, three to four times more than released by the Ech-complex) is available for transmembrane ion transport.[222] This sodium gradient is harvested by the Na^+-dependent F_1F_0ATP synthase of *A. woodii*. It was suggested that some steps of the Wood-Ljungdahl pathway also add to the transmembrane ion gradient. The reduction of methylene-H_4F to methyl-H_4F was discussed as a likely site since the reaction is exergonic and coupled to a corrinoid iron sulphur protein.[215] But recent studies could identify the Rnf complex as the only membrane-bound electron transfer system and rather suggest a ferredoxin reduction by the methylene-THF reductase.[223]

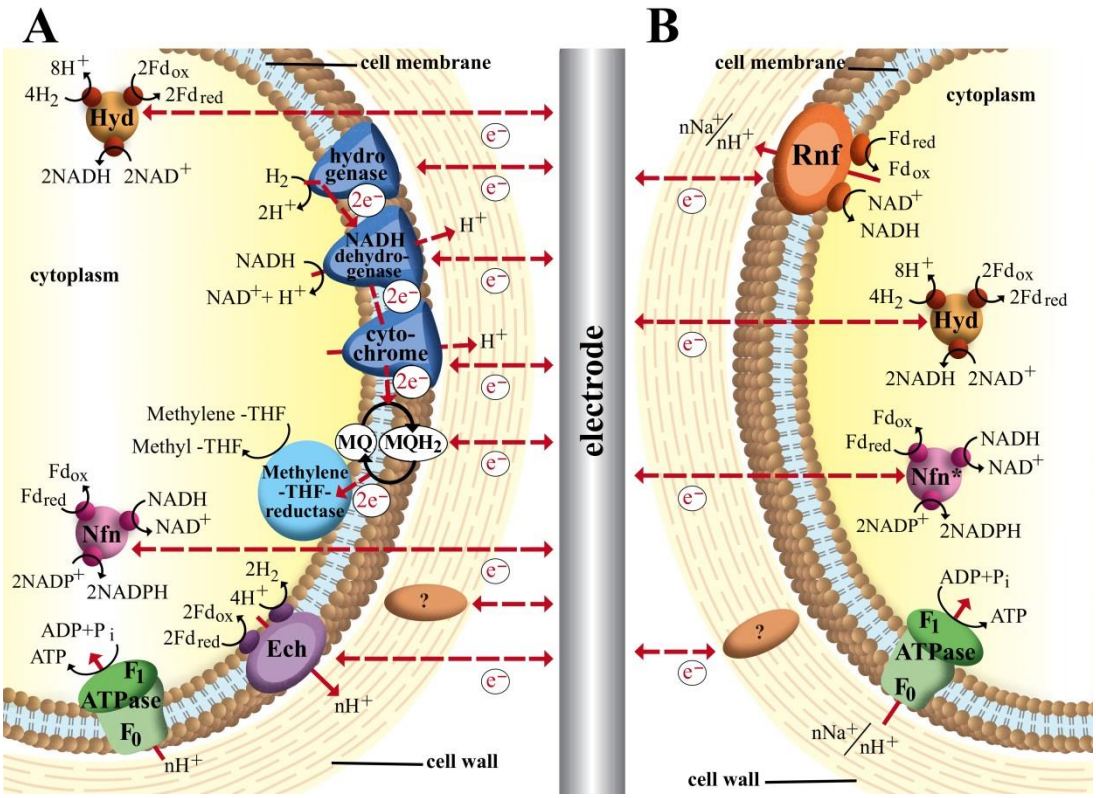


Figure 8: Schematic image of acetogenic electron transport chains and possible interactions with an electrode in a bioelectrochemical system. Dashed arrows indicate hypothetical electron and proton flow. (A) Electron transport mechanisms in *Moorella thermoacetica* via membrane-bound cytochromes and hydrogenases, menaquinones (MQ), soluble electron-bifurcating complexes (Hyd and Nfn) and proton pumping Ech-complex; (B) Electron transport of *Clostridium ljungdahlii* (H^+) and *Acetobacterium woodii* (Na^+) based on membrane-bound Rnf-complex and soluble electron-bifurcating complexes (* Nfn-complex is not found in *A. woodii*). "?" represents hypothetical cell-wall associated proteins that could facilitate electron transfer. Reproduced from Kracke et al. 2015.[188]

Interestingly a similar Rnf complex was detected in *C. ljungdahlii* even though its membrane gradient is proton based, which would put the organism into the H^+-acetogen-group together with *M. thermoacetica*. However, *C. ljungdahlii* does not contain any cytochromes and therefore it seems more reasonable to categorize anaerobic homoacetogenic organisms into Ech- and Rnf-containing groups with subgroups of Na^+- and H^+- dependent species.[165, 219] It could be shown that the Rnf complex is an electron

bifurcating ferredoxin:NAD^+ oxidoreductase and is essential for developing a proton gradient over the membrane under autotrophic as well as heterotrophic growth conditions.[224] With fructose as substrate and electron donor Rnf-deficient strains were growth-limited with significantly reduced ATP yields as a result of disruptions in the membrane gradient development. Autotrophic growth without Rnf complex was completely inhibited, indicating the Rnf-complex being a major if not the sole electron transport mechanism linked to energy conservation. The soluble electron bifurcating complexes HydABC and NfnAB are also found in *C. ljungdahlii* while *A. woodii* is believed to only use HydABC.[219] The proposed electron transport mechanisms for both organisms are shown in **Figure 8 B.**

4.1.4 OTHER RESPIRATORY PATHWAYS

The section above introduced typical electroactive microorganisms, which were used in BES. But what about other model and/or industrial microorganisms like *Escherichia coli* and *Corynebacterium glutamicum* or pathogenic microorganisms like *Pseudomonas aeruginosa*? Can we influence/manipulate their redox and energy metabolism? In order to answer this question, it is necessary to understand their respiratory pathways.

E. coli: model organism with respiratory flexibility

The respiratory system of *E. coli* is very well known and in many studies, the bacterium is used as a model for investigation of energetics and regulation of respiration. The respiratory chains show a great diversity and variability enclosing 15 primary dehydrogenases to oxidise electron donors and ten terminal reductases (or oxidases) to reduce electron acceptors (including isoenzymes).[225] Those primary dehydrogenases and terminal reductases are linked by three quinones: ubiquinone ($E^{0'}$ = +110 mV), demethylmenaquinone ($E^{0'}$ = +40 mV) and menaquinone ($E^{0'}$ = -80 mV). Depending on the enzymes various quantities of energy can be conserved due to the build-up of a proton gradient through proton pumps, or by arranging catalytic sites in a certain way to release the protons on opposite sides of the membrane to create a charge separation. The H^+/e^- ratios vary from 0 to 4 H^+/e^-.[132] Under aerobic conditions *E. coli* can conserve most energy by using quinol oxidases ($E^{0'}$ = +820 mV) to reduce O_2 to H_2O. Here O_2 represses the terminal reductases of anaerobic respiration. However, in the absence of O_2, energy can

be generated by nitrate reductases ($E^{0'}$ = +420 mV), nitrite reductase ($E^{0'}$ = +360 mV), DMSO reductase ($E^{0'}$ = +160 mV), TMAO reductase ($E^{0'}$ = +130 mV) or fumarate reductase ($E^{0'}$ = +30 mV) with nitrate delivering the most energy and fumarate the least energy.[132, 225, 226] So depending on which level or in which step the electron chain is targeted or manipulated, the metabolism will gain more or less energy.

Furthermore, it was demonstrated that *E. coli*, evolved under electrochemical tension in a microbial fuel cell, can generate current by using the electrode as an electron sink. It was proposed that the evolved cells possess the ability to excrete hydroquinone derivatives through a highly permeable outer membrane, which act as mediators to transport the electrons between cell and electrode.[181, 227] Another approach to obtain *E. coli* cells that show electroactivity is via metabolic engineering, which is discussed in 4.1.5.

P. aeruginosa: secretion of redox carriers

Another interesting microorganism is the GRAM-negative aerobic bacterium *Pseudomonas aeruginosa*. It is an opportunistic pathogen, which can live in various environments due its ability to catabolize a large number of substances.[228] Additionally the bacterium is a good biofilm-builder and has branched respiratory chains to use oxygen as an electron acceptor, involving five oxidases (bo_3 oxidase, aa_3 oxidase, cbb_3 oxidase 1, cbb_3 oxidase 2 and cyanide-insensitive oxidase), which are adapted to the varying availability of oxygen in the different biofilm stages due to biofilm thickness.[229] Preferentially *P. aeruginosa* obtains its energy from aerobic respiration, however, under anaerobic conditions the bacterium can also survive in presence of nitrate or nitrite by using reductases to reduce the N-molecules.[230]

When *P. aeruginosa* is cultivated in a microbial fuel cell, the bacterium shows an interesting behaviour. Instead of oxygen, nitrate or nitrite *P. aeruginosa* can use the anode as an electron sink to generate energy for an active growth.[176] The anode stimulates the production of phenazine derivatives, e.g. phenazine-1-carboxylic acid ($E^{0'}$ = -275 mV), phenazine-1-carboxamide ($E^{0'}$ = -150 mV) and procyanin ($E^{0'}$ = -32 mV). The secreted phenazine derivatives operate as soluble mediators, which significantly enhance the electron transfer between electrode and cells, resulting in an increased current generation. In addition, the diffusible phenazine derivatives enable the use of the electrode as an electron sink for cells in thick multilayer biofilms.[15, 176] Another attractive phenomenon is that in a mixed culture the secreted mediators can be used not only by *P. aeruginosa* itself

but also by other microorganisms, which generally are not able to produce redox active mediators.[231]

C. glutamicum: oxygen dependency

The third bacterium, *Corynebacterium glutamicum*, is an important GRAM-positive industrial microorganism, which has been widely used as a microbial cell factory for the production of various amino acids, nucleic acids and other chemicals in food, pharmaceutical, cosmetics and chemical industries.[232] *C. glutamicum* can utilize various carbon sources for energy conversion and oxygen as the preferable electron acceptor by using three oxidases. The bc_1 oxidase can take up electrons from menaquinol and pass the electrons to the aa_3 oxidase by forming a super-complex that has a low oxygen affinity. Whereas the third oxidase, a *bd* oxidase, has a high affinity to oxygen.[233] The bacterium can also survive under anaerobic conditions in the presence of nitrate. However, the growth is very limited, because the bacterium has a nitrate reductase, but lacks enzymes to degrade the toxic product of the nitrate reductase (nitrite).[234]

Manipulating the redox metabolism by cultivating *C. glutamicum* in a BES can result in higher yields of the target product.[177] Experiments demonstrated that the bacterium grown under a cathodic potential ($E^{0'}$ = -600 mV) using glucose as the carbon source showed a decreased growth rate and an increased lactate yield. Here the anthraquinone 2,6-disulfonate was added to the medium as an artificial mediator to shuttle electrons between cathode and cells. The mechanism how the redox metabolism is influenced by the artificial mediator is still unclear.[177]

4.1.5 MAKING THE CONNECTION: MICROBE-ELECTRODE INTERACTIONS

The previous chapter demonstrates the impressive diversity and complexity of microbial solutions for cellular electron transport. Similar to the ability to interact with many different electron donors and acceptors in the environment one can assume that microbes are also able to exchange electrons with electrodes via different cellular components and mechanisms. In order to achieve successful EET, the specific characteristics of the electron transport chains of the target organism should be considered. While it is questionable if there are groups or microbes that are better adapted for EET than others it is for certain that a bioelectrochemical approach is challenged by different conditions depending on the catalytic host. Organisms that feature outer membrane redox-

components might be able to perform direct electron transfer with electrodes while soluble intracellular complexes such as the electron bifurcating Nfn and Hyd complexes of acetogens are most likely only targetable via soluble mediators. For each organism, the specific redox-window of its electron transport chain(s) might dictate required potentials for use in BES. The following two sections discuss key components in electron transport chains of the presented organisms and the possible metabolic impact if EET at different sites can be achieved.

Key compounds in different electron transport chains

Figure 9 displays an overview of important redox reactions that are catalysed by the presented organisms. Actual environmental conditions in living cells differ from the standard biochemical conditions (25°C, 1 atm, pH=7), which might influence the actual redox potentials of each couple. The potential of the proton-hydrogen couple, for example, is -414 mV under standard conditions. In acetogenic environments, however, this potential lies closer to -350 mV as the partial hydrogen pressure is around ~200 Pa.[216] Many intracellular redox-carriers also show different redox potential than the corresponding pure compounds. The standard redox potential of ferredoxins with one or more iron-sulfur clusters, for example, lies around -400 mV.[235] Under physiological conditions in acetogens however, ferredoxins are usually >90% reduced and therefore reported to be able to catalyse reducing reactions at redox potentials as low as -500 mV.[235, 236] A similar effect shifts the true redox potential of the NAD^+/NADH couple to around -280 mV as the majority of molecules (>90%) are oxidised even though the $E^{0'}$ under standard conditions is -320 mV. Very close to that of $NADP^+$/NADPH (-324 mV), which in turn is shifted to -360 mV due to the intracellular ratio of $NADP^+$/NADPH being 1/40.[235] In these cases a redox-area, rather than one known midpoint potential, illustrates the potential range at which these reactions might occur inside the organisms. A fixed standard redox potential (dashed lines) or potential range for each reaction was allocated depending on availability in the literature. However, the reader might want to keep in mind that environmental conditions such as pH, redox potential of the solution and specific concentrations can result in a further redox potential shift. The ion couple Fe^{3+}/Fe^{2+} as an inorganic electron acceptor/donor is a good example for how the redox potential can be influenced by environmental conditions. The midpoint potential is about +770 mV at a low pH and in the absence of precipitation. However, depending on pH, concentration and in which form iron is available the midpoint potential can vary strongly.[237] For example, the midpoint potential of Fe_3O_4/Fe(II) is significantly lower (-314 mV), because magnetite is a less soluble

mineral. For the organic chelate complex, Fe(III)-citrate/Fe(II)-citrate, the solubility is higher and respectively the midpoint potential (+372 mV).[237, 238]

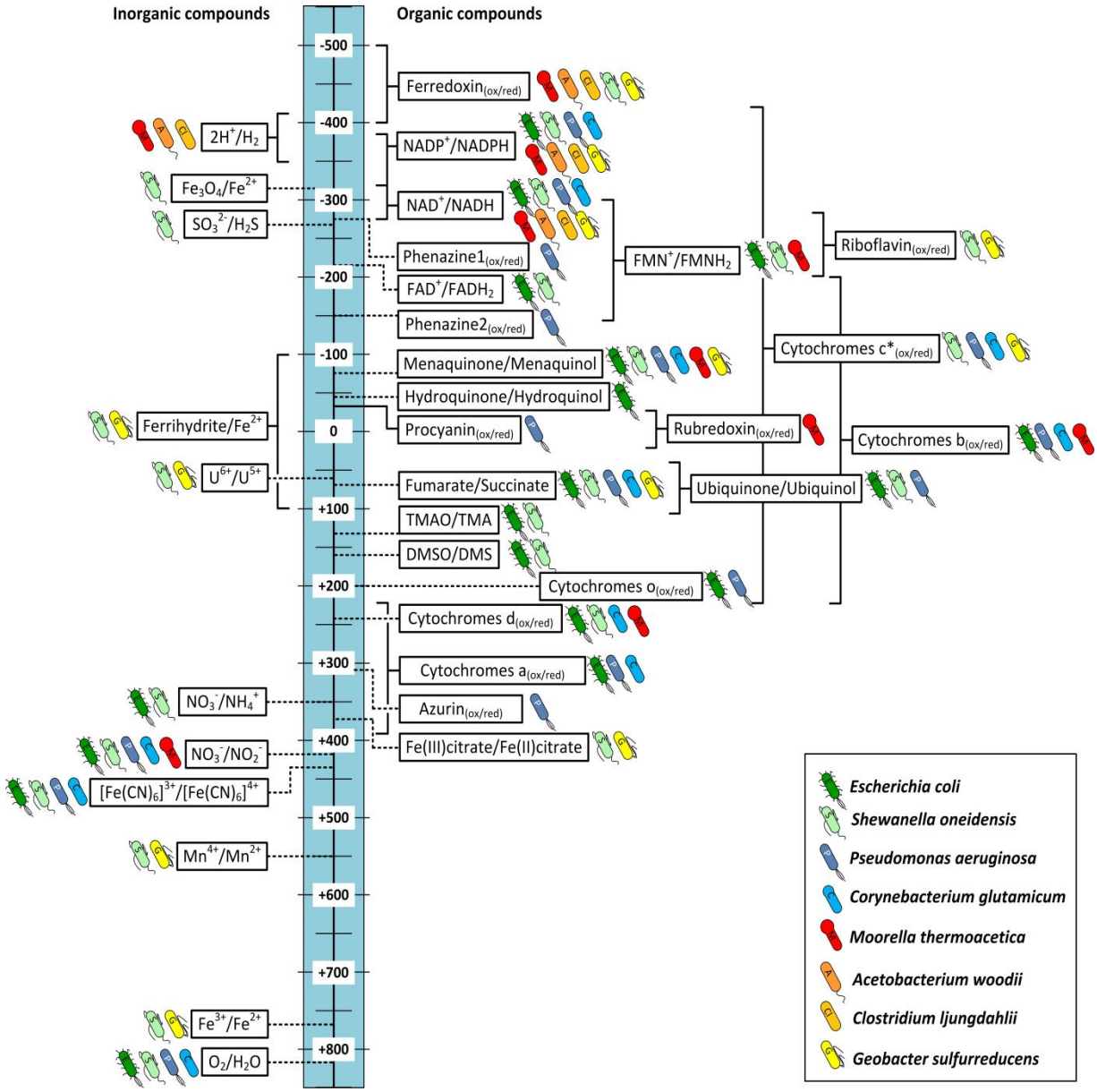


Figure 9: Redox potentials of important redox reactions in electron transport chains catalysed by the bacteria discussed in this study. Standard redox potential ($E^{0'}$ [mV, 25°C, pH = 7]) are indicated by dashed lines. If physiological or environmental conditions are known to shift the potential from the $E^{0'}$, redox windows are indicated (solid lines). The bacterial symbol behind each reaction shows the organisms that are known to catalyse the reaction naturally. Blue: aerobes; green: facultative anaerobes; red-yellow: obligate anaerobes; Phenazine1 = Phenazine-1-carboxylic acid; Phenazine2 = Phenazine-1-carboxamide. * c-type cytochromes can cover a broad range of redox potentials as indicated. Not all bacteria mentioned will cover the whole range. For detailed discussion refer to main text.

Some of the listed cellular redox compounds have been shown to play a major part in electron transfer between organisms and electrodes and therefore, others might as well. As discussed above direct EET in *Geobacter* and *Shewanella* can be achieved via a network of cytochromes with different midpoint potentials (e.g. PpcA: -170 mV,[239

OmcZ: -180 mV,[180] OmcS: -212 mV,[181] CymA: −200 mV, MtrA: −100 mV, MtrC: −138 mV[240]). However, for a better characterisation and a functional understanding of the cytochromes their wide potential windows should be considered, because the cytochromes are complex molecules not showing narrow midpoint potentials but more wide ranges of potentials suggested to be due to the multiheme molecules in the cytochromes. Each heme has its own specific redox potential and affects the potential of the neighbour hemes creating a specific wide window of potential ranges for the overall molecule.[161, 241] For example the potential windows of OmcZ, OmcS, CymA and MtrC are from -420 to -60 mV,[180] -360 to -40 mV,[181] -0.350 to -0.080 mV and -0.280 to ±0 mV,[240] respectively. It is assumed that the wide windows allow an overlapping of the redox potentials of the cytochromes in an electron transport chain and make a thermodynamic downhill process of electron transport along the chain possible. Furthermore, these wide potential ranges allow the interaction with a broad spectrum of external electron acceptors and donors.[161, 180, 240] However, the potential windows can vary depending on the environmental conditions. For the cytochromes of *S. oneidensis,* it was shown that the potential windows are shifted as a function of pH. It was observed that under higher pH conditions in most cases the redox potentials are shifted to more negative values.[240] **Figure 9** displays a broad potential range for c-type cytochromes based on the discussed literature and indicated all organisms that contain c-type cytochromes. However, this does not necessarily mean that all these bacteria are able to catalyse reactions in the full indicated redox potential window. *G. sulfurreducens*' genome encodes for around 110 different c-type cytochromes,[179] *S. oneidensis* for 42,[192] and *P. aeruginosa* for 27,[242] while *C. glutamicums* genome only encodes for a single c-type cytochrome.[242] Therefore the flexibility of the metal reducing organisms to adapt to different electron acceptors and donors is much higher.

In addition to the described cytochromes of *Geobacter* and *Shewanella* comparable complexes of other organisms might be able to perform EET via similar mechanisms. The membrane of the GRAM-positive acetogen *M. thermoacetica*, for example, contains b-type cytochromes that could be a possible site of interaction between electrode and organism.[243] In fact it could be shown that *M. thermoacetica* together with other cytochrome-containing acetogens is able to directly use electrons from a cathode for reduction of CO_2.[66] Interestingly the same study reports *C. ljungdahlii* is also capable of direct EET even though it is not known to be able to produce any cytochromes (see **Figure 8 B** and **Figure 9**).[165] Could this electron transport rely on other membrane-bound

enzymes? Or be mediated by an unknown soluble molecule or enzyme secreted by the organism? Since there is no evidence of such the actual mechanism of EET in *C. ljungdahlii* remains pure speculation to date. However for other microbes, self-excreted redox mediators are indeed an important way to exchange electrons with electrodes in both ways. *P. aeruginosa* for example produces several compounds of different redox potentials such as azurin ($E^{0'}$ = +310 mV), procyanin ($E^{0'}$ = -31 mV), phenazine-1-carbox amide ($E^{0'}$ = -150 mV) and phenazine-1-carboxylic acid ($E^{0'}$ = -275 mV). These enable the organism to adapt to different electron acceptors in the environment, including solid state electrodes.[176, 244] A very recent study of YANG et al. demonstrates that the concentration of such endogenous redox compounds can mediate electron flow in both directions and that the concentration directly correlates with achievable current densities.[245] By heterologous expression of a synthetic flavin biosynthesis pathway from *Bacillus subtilis* in *S. oneidensis* the secreted flavin concentration could be increased ~26 times, which increased power output as well as inward current of the organism in BES.[245] Another possibility could be the secretion of whole enzymes that facilitate electron flow from the electrode surface towards organisms. In a very recent study DEUTZMANN et al. were the first to report evidence of this mechanism of direct EET by cell-derived free enzymes in a cathodic BES using methanogens.[246] They could show that small surface associated enzymes such as hydrogenases and fumarate reductase were released from *Methanococcus maripaludis* cells and accumulated at the cathode surface where they catalysed the formation of small mediating molecules such as hydrogen or formate which in turn were immediately taken up by the cells.

The location of the target site of electron transfer decides if an organism might be able to perform direct EET or if an (endogenous or exogenous) mediator is required to transport the charge across the cellular membrane. Additionally, each organism can be allocated with a specific redox-potential-window in which its electron transport chains are able to operate, which is also visualised in **Figure 9**. It becomes obvious that compounds of facultative aerobe organisms such as *E. coli* and *S. oneidensis* are represented over a wide range of potentials. This visualises the high flexibility of both organisms to adapt their electron transport chains to multiple electron donors and acceptors. *G. sulfurreducens* even though a strict anaerobe still covers an impressive range of redox potentials with its many cytochromes and the ability to use soluble metals such as iron and manganese as final electron acceptors. The Rnf-complex containing acetogens *C. ljungdahlii* and *A. woodii*, however, are only able to transfer electrons in a limited potential window as they

do not contain any cytochromes and are restricted to only one way of respiration at relatively low potentials.

Transferring electrogenic capabilities between bacteria

To transfer the ability of one organism that is known to interact with an electrode in a specific way to another organism, synthetic biology tools can be used. It could be shown that expressing enzymes of the Mtr pathway of *S. oneidensis* inside *E. coli* increased current production of the optimized strain.[247, 248] In 2008 researchers could show that the expression of one single enzyme from *Shewanella's* Mtr-pathway is enough to transform *E. coli* into a metal respiring bacterium.[249] Expression of the cytoplasmic membrane tetraheme c-type cytochrome CymA enabled the mutant strain to reduce Fe(III) and sustain growth while the native strain lacked this ability. This was independent of the presence of periplasmic or outer membrane cytochromes for electron transfer. However, it could also be shown that activities of CymA for iron reduction were much lower in complete cells, indicating that diffusion limitations of solid state electron acceptors also play a significant role in this electron transport chain.[249] A very important follow-up study was able to find evidence for a connection of this transformed electron transport to intracellular carbon flow in *E. coli*.[250] Expression of CymA and Mtr cytochromes from *Shewanella oneidensis* in *E. coli* resulted in a strain that coupled current production to a shift in its metabolic fluxes towards more oxidised products.[250] The possibility to design a target electron flow via artificial complexes across the outer and inner membranes and their connection to cellular redox balance and carbon flow promises many possibilities to design optimized electroactive organisms. The above-cited example also shows that co-expression of multiple linked enzymes can greatly enhance the electron transfer rates.[250] Therefore the specific electron transport chains of the target organism and their possible connection to the enzymes to be introduced need to be considered when designing electroactive bacteria.

4.1.6 TARGETING ELECTRON TRANSPORT IMPACTS THE CELLULAR ENERGY METABOLISM

The achievable energy yield of an electron transport chain is dependent on the difference in electrical potential between electron donor and acceptor. Therefore, organisms that are able to respire in multiple ways will always choose available acceptors with the biggest potential difference to the donor (e.g. *E. coli:* $O_2 > NO_3^- >$ fumarate). To

compare energy efficiencies of different electron transport chains it is referred to the ratio of phosphate to oxygen (P/O quotient), which describes the ratio of mol ATP produced per mol oxygen reduced. The P/O ratio depends on the amount of ions that are transported across the membrane during the corresponding electron-transport chain and are available for ATP-synthesis via gradient-driven ATPase. It is also influenced by the efficiency of the specific ATPase as the amount of ions that are needed for synthesis of one mol ATP differ between organisms.[251] For example, the aerobic electron transport chain of *E. coli* transports up to 8 protons across the membrane with NADH as electron donor (2 e^-) and oxygen as final acceptor (see **Figure 10**).[132] With an ATPase that requires 3 protons for the conversion of one mol ADP to ATP a P/O ratio of ~2.7 is calculated.[252]

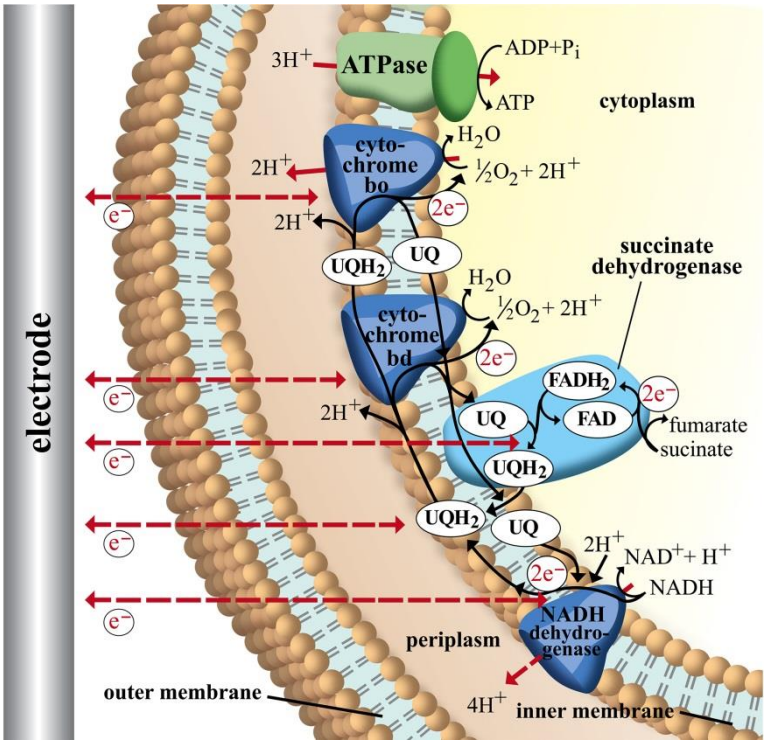


Figure 10: Schematic image of electron transport chains in *Escherichia coli.* NADH as electron donor via NADH dehydrogenase (NuoA-N), ubiquinone pool (UQ), succinate dehydrogenase and cytochromes *bd* (CydAB) and *bo* (CyoABCD). ATP is generated via F_1F_0-ATPase (3 H^+/ATP) from the membrane proton gradient. Possible sites of interaction with an electrode are indicated with dashed arrows. For detailed discussion and references refer to main text. Reproduced from Kracke et al. 2015.[188]

The ATP yield is an important factor for bacterial growth and can also significantly influence production if the metabolic pathway towards the target compound requires energy. Therefore here, similar to the P/O ratio, a hypothetical P/2e^- ratio is presented that reflects the ATP yield per pair of electrons transferred between the organism and an electrode in a BES. Some examples are presented in **Table 13**.

The electrode can either function as electron donor (cathode) or as final electron acceptor (anode). Since the exact sites of electrode interaction with the compounds of each electron transport chain remain unknown different scenarios are assumed. In column 2 of **Table 13**, the assumed site of electrode-bacteria interaction is given together with the corresponding hypothetical reaction. The P/2e^- ratio is calculated based on the resulting amount of protons carried across the membrane, which are assumed to be available to drive ATP synthesis via ATPase. An example is explained in detail in the following paragraph.

Table 13: Theoretical P/2e^- ratios for extracellular electron transfer between electrodes and different sites in the electron transport chains of *Escherichia coli*, *Moorella thermoacetica* and *Clostridium ljungdahlii*.

organism	Site of electron transfer	P/2e^-
Anodic electron transport		
E. coli[a]	Cytochrome *bo*	2.7
	Cytochrome *bd*	2
	Ubiquinone-pool ($UQH_2 \rightarrow 2e^- + UQ + 2H^+_{periplasm}$)	2
	FAD reduction ($FADH_2 \rightarrow 2e^- + FAD^+ + 2H^+_{cytoplasm}$)	0.6
	NADH oxidation ($NADH \rightarrow 2e^- + NAD^+ + 2H^+_{cytoplasm}$)	0
Cathodic electron transfer		
E. coli[a]	NADH reduction ($2e^- + NAD^+ + 2H^+_{cytoplasm} \rightarrow NADH$)	2.7
	FAD reduction ($2e^- + FAD^+ 2H^+_{cytoplasm} \rightarrow FADH_2$)	1.3
	Ubiquinone reduction ($2e^- + UQ + 2H^+_{cytoplasm} \rightarrow UQH_2$)	1.3
	Ubiquinone reduction ($2e^- + UQ + 2H^+_{periplasm} \rightarrow UQH_2$)	0.6
M. thermoacetica[b,c]	Ferredoxin reduction ($2e^- + Fd_{ox} \rightarrow Fd_{red}$)	0.75
	Hydrogen evolution ($2e^- + 2H^+_{cytoplasm} \rightarrow H_2$)	0.375
	NADH reduction ($2e^- + NAD^+ \rightarrow NADH$)	0
C. ljungdahlii[b]	Ferredoxin reduction ($2e^- + Fd_{ox} \rightarrow Fd_{red}$)	0.5
	Hydrogen evolution ($2e^- + 2H^+_{cytoplasm} \rightarrow H_2$)	0.25
	NADH reduction ($2e^- + NAD^+ + 2H^+_{cytoplasm} \rightarrow NADH$)	0

Column 2 gives the assumed site of electron transfer including hypothetical reaction. For following reactions and references refer to the main text and **Figure 8** *and* **Figure 10***. The corresponding amount of protons transferred across the membrane is assumed to be available for ATP synthesis. P/2e^- is mol ATP produced per pair of electrons transferred .*[a] 3 H^+ per ATP in ATPase; [b] 4 H^+ per ATP in ATPase; [c] Ech-complex based electron transport chain

Figure 10 shows a simplified image of electron transport chains in *E. coli* including possible sites of EET (dashed red arrows). As discussed above it could be shown that cytochromes of *G. sulfurreducens* are able to use electrodes as final electron acceptor. If

we assume the cytochromes of *E. coli* are also possible sites of EET we observe that the ATP yield of the electron transport chain depends on the specific cytochrome that is performing the electron transfer to the electrode. In the case of cytochrome *bo* transferring electrons to an anode as final electron acceptor 8 H^+ are transported across the membrane (4 H^+ from NADH dehydrogenase and 4 H^+ from UQH_2 and cytochrome *bo*). With an ATP synthase that requires ~3 H^+ per ATP[252], this leads to a P/2e^- ratio of 2.7. Running the electron transport chain with cytochrome *bd* as the final oxidase, however, leads to a P/2e^- ratio of 2 as fewer protons are transferred across the membrane (6 H^+ in total: 4 H^+ from NADH dehydrogenase and 2 H^+ from cytochrome *bd*). By including soluble redox carriers such as ubiquinones, FAD and NADH to the scenario one can address any site of *E. coli's* electron transport chain. The observation from this theoretical exercise is to internalize that the achievable ATP yield depends on the actual site of EET. If for example electrons are drawn directly from the cellular NADH pool of *E. coli* no energy gain via chemiosmotic coupling is possible as no proton gradient is established (see **Figure 10** and **Table 13**).

This becomes especially crucial in regards to cathodic electron transfer where processes often aim at microbial metabolism with electrons from electrodes as sole energy and redox source.[56, 66] As discussed in above the metabolism of acetogens for example relies on ATP synthesis via membrane-based electron transport complexes. Now we can pick again an example strain and theoretically discuss the metabolic impact of EET at different sites. *C. ljungdahlii* does not transfer electrons via cytochromes but was shown to interact with electrodes so we can assume EET via mediators or unknown membrane bound compounds towards its cellular redox compounds. In case electrons from a cathode would lead to hydrogen formation the organism could produce 2 mol of reduced ferredoxin per 4 mol of H_2 via electron bifurcation in the Hyd-complex (see **Figure 8**).[235] The exergonic reaction of reduced ferredoxin and NAD^+ is used for proton translocation in the Rnf complex (2 H^+/Fd_{red}) which in turn enables energy conservation via the membrane bound ATPase. With recent estimates of 4 protons per mol of ATP a P/2e^- ratio of 0.25 is calculated.[219] If it is possible to transfer electrons from a cathode directly to ferredoxin the ratio increases to 0.5 mol ATP per pair of electrons transferred. An electron input towards the NADH pool of the bacterium, however, would not deliver enough energy to establish a proton motive force and therefore ATP generation (see **Table 13**).

Remembering **Figure 9** one can draw the connection between required redox potential and target reaction in a specific electron transport chain of an organism to

optimize the conditions for a bioelectrochemical process. For a cathodic process of *C. ljungdahlii,* for example, the applied electrode potential needs to be at least low enough to allow hydrogen formation to enable ATP formation and therefore bacterial growth.

4.1.7 CONCLUSION ON THE ANALYSIS OF MICROBIAL ELECTRON TRANSFER CHAINS

The remaining question after the presented analysis of microbial electron transport chains is: Can we identify an ultimate organism for microbial electrochemical techniques? Requirements for an advantageous organism would be first of all high electroactivity. But depending on the target application different other features can be of crucial importance. Microbial electrosynthesis, for example, requires a strain that produces industrial relevant products preferably combined with the ability to utilise a variety of cheap substrates. Metabolic engineering tools should be available to optimize production and perform advanced strain designing. For application in bioremediation processes, the ability to break down a wide range of organic contaminants has priority together of course with the efficiency of extracellular electron transport. Other influencing factors are general characteristics that simplify process design such as high oxygen tolerance for anaerobes, no high-risk organism, no complicated fermentation conditions such as expensive media supplements, pressurised reactor etc.

The diversity of applications for BES makes it impossible to identify one organism that features all required properties. Still for each application, a specific species might outperform others. The highly flexible electron transport chains of metal respiring bacteria such as *Geobacter* and *Shewanella* make them excellent current producers over a wide range of potentials. Acetogens seem to be a very promising group of target organisms for microbial electrosynthesis with their ability to use CO_2 as sole carbon source. But this study also shows that the complexity of microbial electron transport possibilities bears many challenges for bio-electrochemical techniques. For the successful design of an electrically enhanced bioprocess the specific electron transport properties of the involved species needs to be considered. Environmental conditions such as applied potentials should be adjusted according to specific target sites for EET. Since these are unknown in most cases an analysis as presented in this manuscript will help to identify best- and worst-case scenarios for microbe-electrode interactions and identification of optimized windows for process parameters. Especially when looking at CO_2 as a feedstock, the available energy gain through EET will limit the feasibility of many organisms and constrain the list of feasible products. There is currently a lot of interest in microbial electrochemical

technologies; however, this study emphasises that without a deeper understanding of the underlying electron transfer mechanisms process development remains a trial and error exercise.

4.2 THEORETICAL ASSESSMENT OF MICROBIAL ELECTROSYNTHESIS[2]

The aim of the following chapter is to present a useful analysis tool, which is able to identify beneficial production processes for MES, and at the same time enables insight into the energy conservation possibilities during anaerobic electrically enhanced fermentation. Using an *in silico* approach to calculate the metabolic impact of different electron transport routes enables the evaluation of different mechanisms while current knowledge gaps remain.

Core networks of metabolic carbon pathways were created to determine the effect of electrical enhancement through different EET ways on production. The tool chosen for the metabolic analysis is elementary mode analysis (EMA), which determines all possible solutions of the metabolic matrix by calculating a unique set of so-called *elementary flux modes* (*efm*s) as introduced in 0.[102] Elementary mode analysis is based purely on stoichiometry of the reaction equations and *steady state* conditions of the organism. Therefore, the solution space can be regarded as outer boundaries of the metabolic possibilities. Here this is effectively used to determine the maximum theoretical possible advantage of EET on production and therefore one can assess the limitations of MES. Actual *in vivo* yields will lie inside the determined solutions space. However, they will usually be lower than the theoretical maximum yield and will depend on many factors such as thermodynamics, enzyme kinetics, gene regulation and product toxicity, which are not taken into account here.

4.2.1 MODELLING EET

Regardless of the major carbon metabolic pathway, the effect of electrical enhancement is typically assumed to result in an increase or decrease of intracellular redox factors such as NADH or NADPH.[98, 253] Electron transfer towards an anode is assumed to be coupled to energy conservation where the electrode functions as solid final acceptor during respiration.[114, 254] However the exact ratio and pathway of electrons and protons that are transported remains speculative. Furthermore, it is not known by which mechanisms non-metal-respiring organisms might transfer electrons to an anode and

[2] This chapter was modified to fit this thesis from the published work: **Kracke, F.**, & Krömer, J. O. (2014). *Identifying target processes for microbial electrosynthesis by elementary mode analysis.* BMC Bioinformatics, 15(1), 410. doi:10.1186/s12859-014-0410-2

whether that transfer promotes ATP generation or not.[34] Even though there is even less information available about cathodic electron transfer there is a general concept proposed that assumes the creation of a proton motive force by intracellular electron consumption, which is available for ATP synthesis.[4, 66, 97, 98] In mediated electrically enhanced fermentations of *Actinobacillus succinogenes* PARK and ZEIKUS observed an electron flow from the cathode into the product succinate.[74] Simultaneously, the electron transfer via the reduced mediator Neutral Red and the proton-pumping fumarate reductase complex of *A. succinogenes* induced proton translocation and therefore increased ATP synthesis.[131] While the activity of the proton pumping fumarate reductase of *A. succinogenes* is most likely solely responsible for the reported increase in proton flux through the ATPase complex, nowadays the theory about cathodic EET generally assumes that all electrons supplied by EET enter the cytoplasm as negative charge and catalyse intracellular, proton consuming reductions. Simultaneously, the proton consumption would lead to a proton gradient across the inner membrane that drives ATP synthesis.[4] But is this the only possibility? Observed is poor growth in very thin biofilms on cathodes,[7, 10] which seems to be surprising if cathodic EET could deliver redox power as well as energy (NADH and ATP). So the questions are: Are the protons involved in cathodic electron transfer generally available for ATP generation? And does electron transfer towards an anode always occur by the respiratory chain which thereby creates a proton gradient? What other ways of EET could occur and how would this impact production?

PANDIT et al. presented a first computational approach that characterized the general role of bio electrosynthesis in chemical production using a genome-scale metabolic model of *E. coli.*[97] Within their model, it is assumed that cathodic electrons enter the metabolism and directly reduce NAD^+ to NADH. Analogous to the theory discussed before the authors precariously assume the creation of a proton motive force that drives ATP synthesis even though the fumarate reductase of *E. coli* is, unlike the one of *A. succinogenes,* a non-proton-pumping enzyme.[255] Not surprisingly they report an increase of ATP yield caused by electron supply. This study regards extracellular electron transport coupled and uncoupled to ATP synthesis and discusses the properties of both options to boost the production of various valuable products. Four different electron transport scenarios for mediated cathodic and anodic EET are described in the following paragraph and are visualised in **Figure 11 A** and **B.**

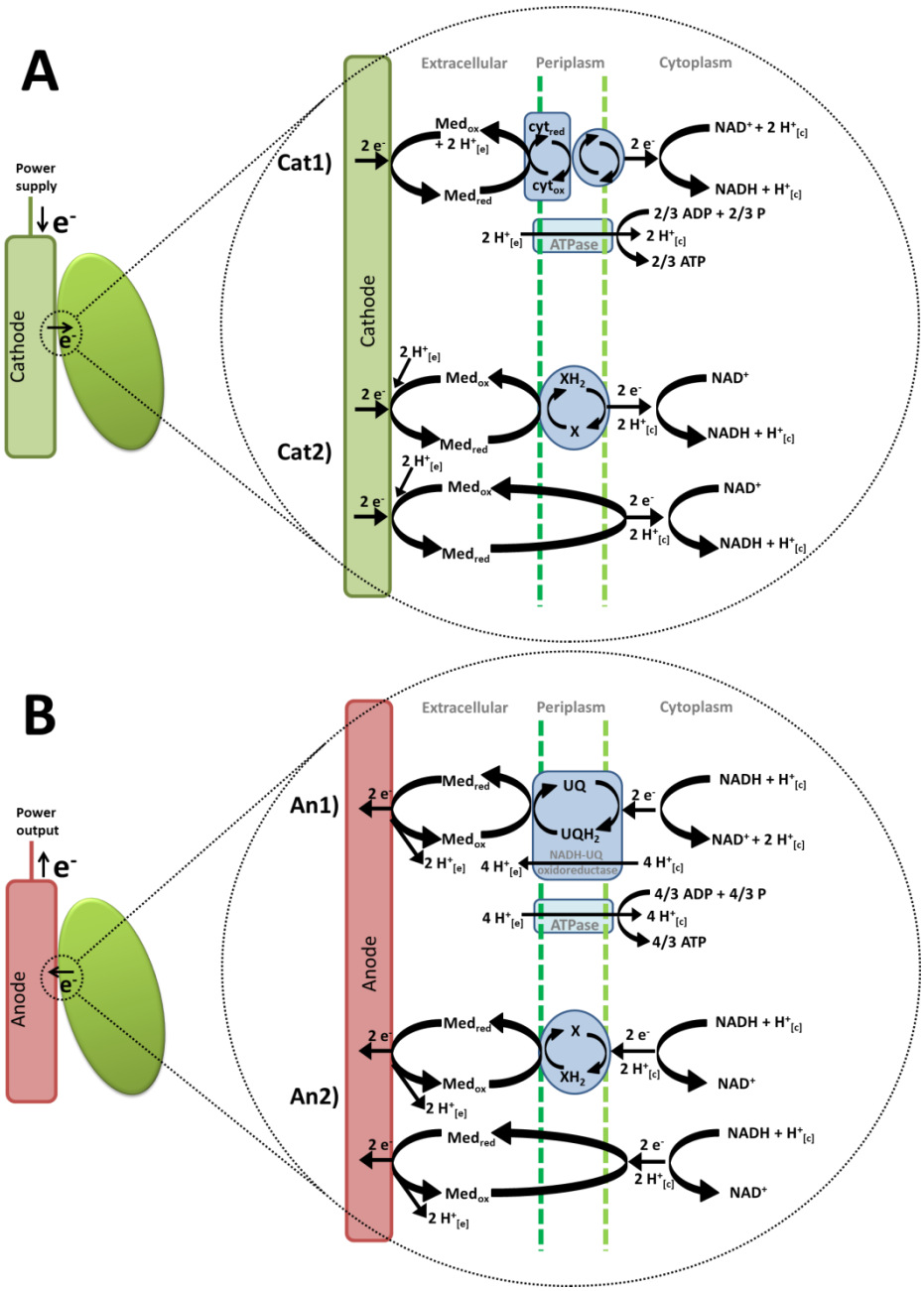


Figure 11: Models for extracellular electron transport mechanisms coupled and uncoupled to energy conservation. (A) Schematic image of two different electron transport mechanisms between cathodes and the microbial metabolism analysed within this study. *Cat1*) Electron transport via a mediator and a cascade of membrane-bound complexes (e.g. cytochromes) with simultaneous ATP generation; *Cat2*) Direct reduction of NAD^+ to NADH by electrons and protons by membrane-bound enzymes (e.g. hydrogenases) or diffusion of the mediator molecule. **(B)** Two different models for microbial interaction with an anode as electron sink. *An1*) Electrons from the quinone pool are transferred to the electron mediator by membrane-bound enzymes such as NADH-Ubiquinone oxidoreductase. As these complexes are proton pumping the created gradient can be used for ATP generation. *An2*) Electrons and protons are transferred simultaneously without creating a membrane potential.

Cathode 1 (*Cat1*): Mediator oxidation occurs on outer membrane cytochromes that transfer the electrons into the organism and finally onto NAD^+. Charge-imbalance creates driving force for 1 proton per electron to enter the cytosol and drive ATP-synthase (transport of 3 protons catalyses the generation of one molecule ATP).

Cathode 2 (*Cat2*): The mediator transfers its electrons and protons directly onto NAD^+ without creating a driving force for ATP synthesis. This could happen by diffusion into the cytoplasm or catalysed by enzymes such as hydrogenases.

Anode 1 (*An1*): Electrons from the Quinone-pool are transferred to the mediator molecule by membrane-bound cytochromes of the respiratory chain. Running the electron transport chain via Quinones creates a proton gradient that drives ATP synthesis.

Anode 2 (*An2*): The anode acts like an electron sink by directly accepting electrons from NADH. This could happen either catalysed by membrane-bound enzymes such as hydrogenases or by diffusion of the mediator into the cytoplasm.

Note that apart from the mediator diffusion model all models could theoretically also happen as direct electron transfer between the electrode surface and the cellular membrane.

4.2.2 IDENTIFYING TARGET PROCESSES FOR MES ON THE EXAMPLE OF *E. COLI*

The presented metabolic analysis was implemented exemplarily for the central carbon metabolism of *Escherichia coli* as model organism for industrial biotechnology. Tools for its genetic modification are well established which makes it an attractive host for the production of various compounds. Even though *E. coli* does not show a comparable electrical activity to *Geobacter* or *Shewanella* species, it was shown to be able to exchange electrons with electrodes via soluble mediator molecules.[256-258] Furthermore, recent studies report the successful transfer of functional molecules from the electron transport chain of *Shewanella oneidensis* into *E. coli* and therefore suggest that the microbe could be modified for optimized electron exchange mechanisms.[247, 259]

The following sections present calculated carbon yields for the production of biomass and various valuable compounds including carboxylic acids, alcohols and aromatics via electrically enhanced fermentation. This chapter focuses on electro fermentation from substrates other than CO_2, mainly glucose and glycerol, a process which is often referred to as “electro-fermentation”.[260, 261] Sugar fermentations are dominating in bio-industry and were therefore investigated to determine the potential of electrical enhancement to boost these processes.[262] As a second substrate of interest glycerol was chosen as it represents a cheap C-source often produced as a waste in

biodiesel production.[263] Its more reductive state compared to glucose suggests it could result in higher yields when converted into more reduced compounds and require less additional electrons.[264] A current review by JANG et al. summarises important C_2-C_6-products and their biological production.[262] All anaerobic production pathways were implemented in the presented metabolic network and the theoretical yields of each compound under electrical enhancement were analysed. **Figure 12** shows the metabolic fluxmap of the presented *E. coli* carbon network including all product pathways. A full list of all maximum product yields, with and without biomass formation as well as the number of computed *elementary flux modes* for each substrate and product combination can be found in the appendix under 8.1.

Impact of EET mechanism on Biomass yields

In absence of a final electron acceptor for the respiratory chain, the anaerobic formation of biomass is generally limited by the availability of energy and the overproduction of reduced redox equivalents.[265] This becomes clear by studying reaction R1 in Figure 12**,** which shows the coupling of biomass generation to ATP consumption and NADH formation. As a result, an anode as electron sink increases biomass yields, especially if the energy limitation is lifted by supplying extra ATP (*An1*). On the other hand, providing even more electrons through a cathode cannot significantly increase biomass yields. In fact, the addition of NADH to the anaerobic network leads to considerably less elementary flux modes as the network has fewer options to distribute carbon fluxes while retaining its redox balance (*efm* numbers in *Additional file 1* of the publication corresponding to this chapter, see appendix 8.1).[191]

The maximal carbon yields for biomass production that are achievable with the different electron transport pathways under anaerobic conditions in *E. coli* are summarized in **Figure 13**. For the use of glucose as substrate it can be seen that if redox power simultaneously provides additional ATP (*Cat1*) the biomass yield can be slightly increased, from maximal 26.5% to 32.5%, while the cathodic model that only supports NADH formation results in a minor yield decrease of about -0.6% (*Cat2*). The network with the anodic model *An2* acting as a pure redox sink is still ATP limited with a maximum achievable biomass yield of about 29.9%. However the anodic model that supports the creation of a proton motive force, *An1*, has the power to enhance biomass production to a maximal yield of 64.1% which equals an increase of about 37.5% and is close to the theoretical maximal biomass yield under aerobic conditions (71.5%).

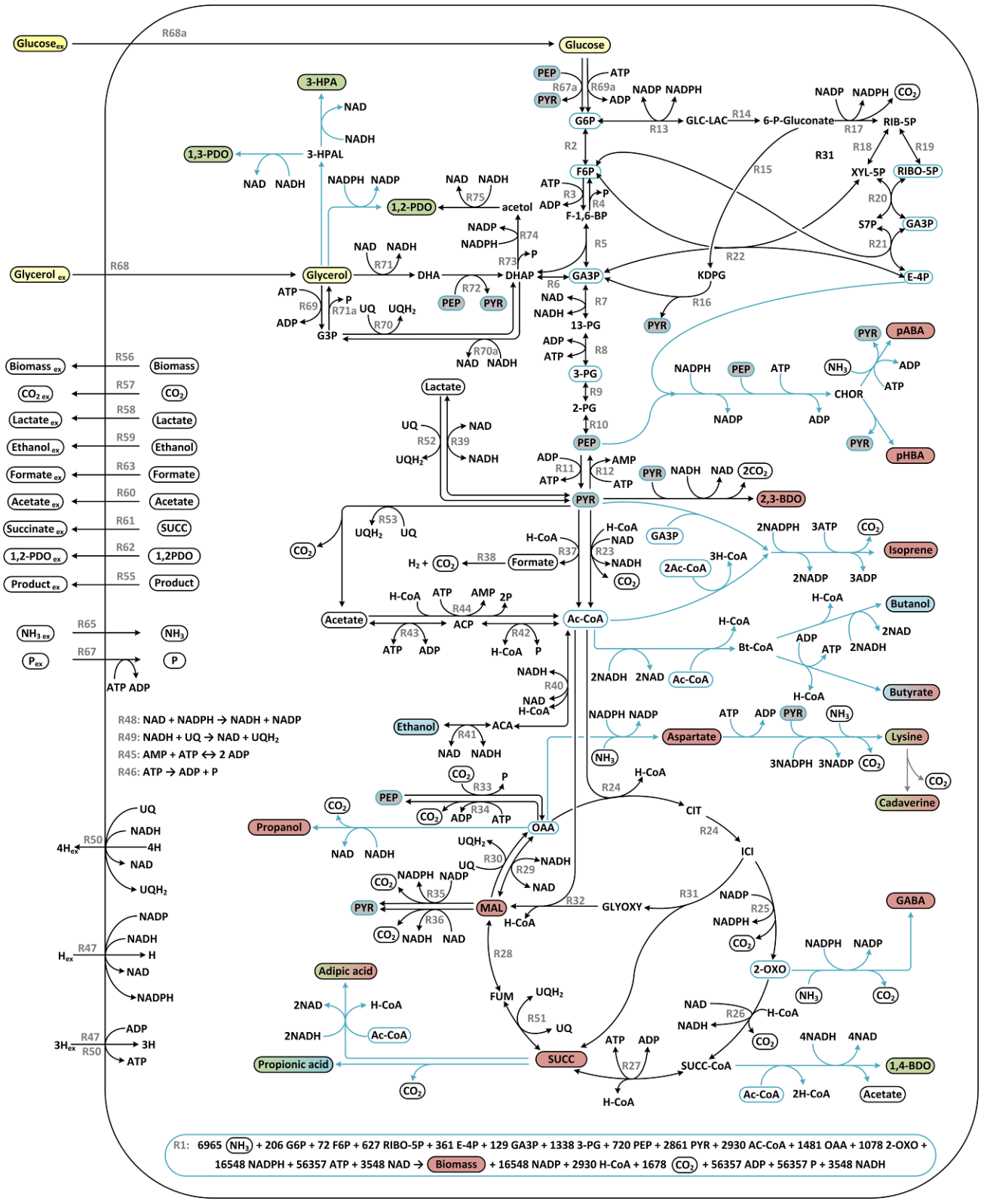


Figure 12: Metabolic fluxmap of the *E. coli* network including exchange reactions and production pathways. All reactions of the core network are shown with their respective number, RX, as given in additional file 1, see appendix 8.1. Production pathways are condensed to single reaction steps displayed in light blue. Required precursors for biomass formation (R1) are labelled with blue borderline. The substrates are highlighted in yellow while all target products are coloured according to their most beneficial electron-exchange-option. Increased production by anodes is displayed red, increased product yields by cathodes green while no benefit from either electron transport is shown blue. If a product shows increased yields on different electrodes depending on the substrate a mixed colour pattern was chosen. Key abbreviations are given in the supplementary information.

The use of fumarate as substrate was investigated as it is excessively used in literature that studies enhancement of succinate production by EET.[110, 266] This includes also the studies by PARK and ZEIKUS, which report for the first time the support of growth by electron supply through a cathode.[74, 131] As discussed earlier this might be a unique effect of the enzyme properties of *A. succinogenes*. Activity of its fumarate dehydrogenases complex creates a proton motive force and therefore ATP synthesis while this cannot be translated to other organisms such as *E. coli.*[255] The maximal biomass yield for *E. coli* grown anaerobically on fumarate was determined to be 13.6% for non-enhanced conditions while cathodic EET causes an increase to 19.3% and 14.2% for *Cat1* and *Cat2,* respectively. To create the amounts of ATP and NADPH needed for biomass formation from fumarate the metabolism is required to produce NADH. Without EET, the NADH is mainly produced by the malic enzyme and pyruvate decarboxylase so that in both cases one carbon is "lost" in the form of CO_2. NADPH is also created by a malic enzyme under CO_2 release (**Figure 12**). The ATP demand is fulfilled by a combination of running the electron transport chain with NADH as electron donor and fumarate as final electron acceptor and the acetate producing acetate kinase. This leads to a maximal possible biomass yield of 13.6% with the main by-products succinate (55%), CO_2 (19%) and acetate (13%). The assumption that cathodic EET results in an increase of available NADH (*Cat2*) reduces the by-product spectrum to carbon dioxide and succinate only. With fumarate as final electron acceptor, the electrons from NADH can enter the first step of the electron transport chain and create a proton motive force, which can drive the highly efficient ATPase. NADPH is created by the membrane-bound transhydrogenase driven by proton gradient. This reaction consumes 0.33 ATP equivalents per transhydrogenation (see reaction R47 in **Figure 12**). This results in a maximal biomass yield of 14.2% for *Cat2* with 6% CO_2 and 80% succinate as by-products. The extra ATP available in case of *Cat1* results in a biomass yield of maximal 19.3% with 9% CO_2 and 72% succinate. The high succinate formation in all cases points out that fumarate might be an interesting substrate to study electron transport but it is not considered a feasible feedstock for bioprocesses due to availability, price and the considerable amount of succinate as a by-product that is to be expected (see above).

Biomass yields calculated for growth on a further reduced substrate such as glycerol cannot be improved by providing additional electrons or protons as the breakdown of glycerol is highly limited by the availability of an electron acceptor. Usually, anaerobic growth with glycerol as the sole substrate is coupled to the production of hydrogen or

1,3-propanediol as this includes pathway branches that consume NADH created during biomass formation.[263, 264] If the cellular NADH level is further increased, redox balance can no longer be obtained and growth is inhibited. Still, growth on glycerol with additional electron uptake by a cathode is possible if it is coupled to a production pathway that balances NADH (e.g. propanediol and butanediol see following part of this work). *An2* increases the maximal achievable biomass yield on glycerol from 10.0% to 26.4%. Again *An1* results in a major increase of the max biomass yield up to 69.5% by providing additional ATP.

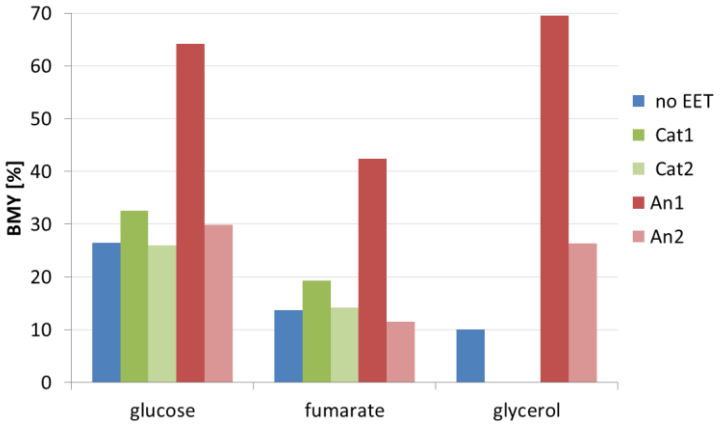


Figure 13: Theoretical maximum biomass yields on different substrates with and without electrical enhancement via different electron-exchange-models. *no EET*: no electrical enhancement, *Cat1*: cathodic electron supply coupled to energy conservation; *Cat2*: cathodic electron supply uncoupled to ATP formation, *An1*: anodic redox sink coupled to ATP generation; *An2*: anodic redox sink uncoupled to energy conservation

The here presented metabolic benefits of increased ATP availability and improved redox balance offered by an anode might be an explanation for the observed thick biofilms on anodic electrodes and poor growth of cathodic cultures.[7, 10]

4.2.3 Impact of EET mechanism on production

The decision if microbial electrosynthesis will become an important technique in bioindustry will strongly depend on the product yield increase that it can trigger. Therefore, it is important to understand the effects of different electron transport routes and energy conservation mechanisms that might happen during electrical enhancement. The degree of reduction (DoR) of a product is often used to describe the electron demand of its production. In fact, this is only useful for a direct conversion. The DoR is calculated by the formula given in **Table 14** and characterises a molecule by its oxidative or reductive state.

Table 14: Formula to calculate the degree of reduction (DoR) for substrates and products.[267]		
$C_aH_bO_cN_dS_eP_f$	C	+4
$DoR = \frac{4a + 1b - 2c - 3d + 6e + 5f}{a}$	H	+1
	O	-2
	N	-3
	S	+6
	P	+5

Figure 14 shows a selection of biotechnologically important substrates and products sorted by their DoR. Starting from sugars ($DoR_{glucose} = 4$) one would expect a benefit from additional electron supply for the production of all compounds with a DoR higher than 4, such as primary alcohols (e.g. $DoR_{ethanol} = 6$) or some carboxylic acids (e.g. $DoR_{butyric\ acid} = 5$). In fact, we observe an overall limited predictive power of the DoR as many products with a higher degree of reduction than the substrate show no increased yield with increasing availability of redox equivalents (e.g. ethanol). Contrary also substrate-product-combinations were found that benefit from extracellular electron supply even though their reductive state is equal (e.g. 3-hydroxy-propionic acid from glucose). Furthermore, it was observed that the production of two isomers of the same compound can benefit from opposing redox interference: While the production of 2,3-butanediol is increased in presence of an anode, 1,4-butanediol production benefits from additional electron supply by a cathode. Therefore, the presented stoichiometric approach is absolutely essential to determine the actual redox balance of a microbial conversion and identify substrate-product-combinations that could benefit from EET.

Figure 12 shows several products that benefit from the presence of a cathode *and* an anode depending on the used substrate: propionic acid, butyric acid, adipic acid, lysine and diaminopentane. The different response to electrical enhancement can be explained by the use of either glucose or glycerol as carbon source. All compounds mentioned above are derived from acetyl-CoA or intermediates of the tricarboxylic acid cycle (see **Figure 12**). To generate these metabolites glucose is broken down by glycolysis where glycerol only enters further downstream. At the end of glycolysis, the final metabolite pyruvate is generated with equimolar amounts of NADH and ATP if derived from sugar. With glycerol as the only carbon source, 2 mol NADH per mol pyruvate and ATP are created. Therefore, some production pathways that re-oxidise only one NADH per pyruvate consumed benefit from an anode on glycerol while a cathode might promote production from glucose. Fully redox-balanced production pathways such as for ethanol or butanol cannot be optimized by electrical enhancement (see **Figure 12**).

The results of all calculated productions and the effects of the cathodic and anodic electron transport models are summarised in **Figure 15** and *Additional file 1* of the publication corresponding to this chapter and are discussed in the following sections.

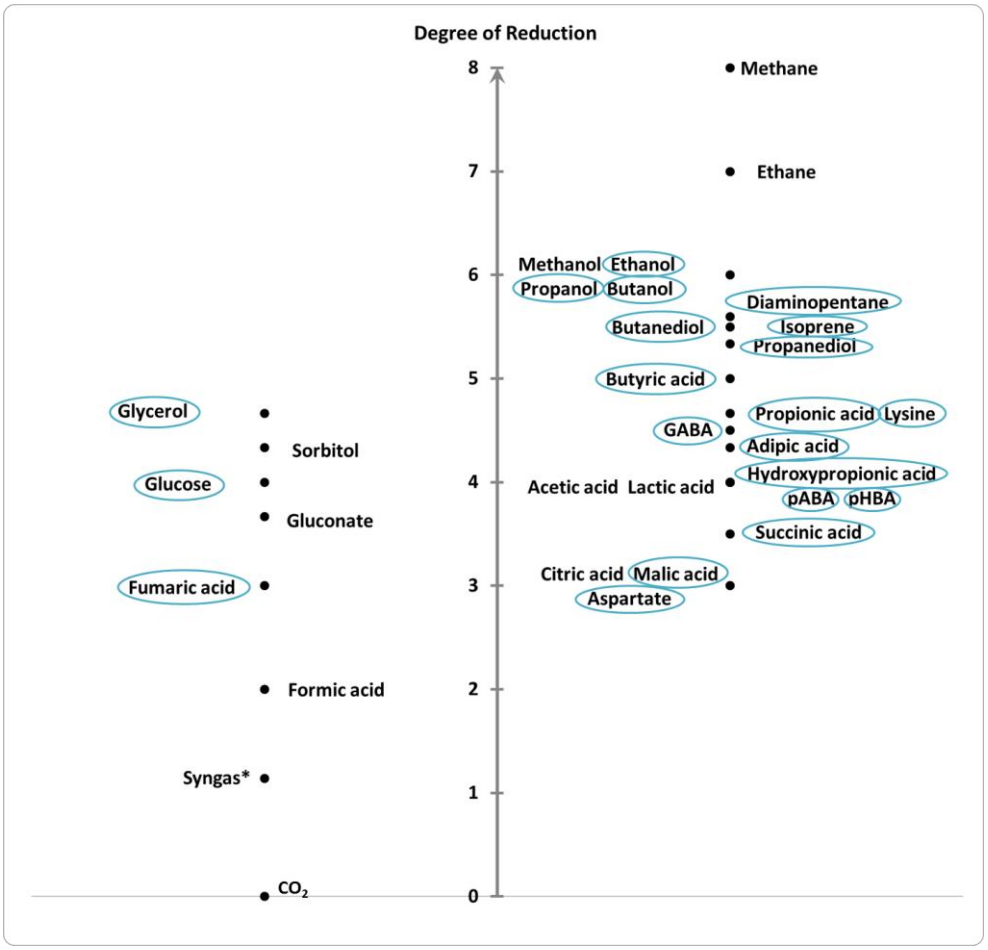


Figure 14: Degree of reduction of several industrial relevant substrates (left) and products (right). Highlighted are choices of substrates and products used in this study. * The given DoR of syngas refers to synthesis gas with an average composition of 40%CO, 30%CO_2 and 30%H_2.

Cathodic processes that promote microbial electro-reduction

The elementary mode analysis could identify several carboxylic acids and alcohols that show increased production from glucose and glycerol under extracellular electron supply by a cathode. Propionic acid and adipic acid are both derived from the tricarboxylic acid cycle intermediate succinate and show maximum achievable carbon yields of 100% on a cathode if produced from glucose (see **Figure 15 A**). Propionic acid is conventionally produced from petrochemicals and has many applications of industrial scale including food additives, perfumes, and pharmaceuticals. Sustainable microbiological production is most promising in natural producers such as *Propionibacterium* that are able to ferment a variety of carbon sources.[268, 269] It could be shown that the use of glycerol is advantageous

as its more reduced state compared to hexoses and pentoses benefits the overall metabolic redox state resulting in higher product yields and reduced by-product formation.[270] Accordingly the here performed metabolic analysis determined the maximum achievable yield of propionic acid from glycerol to 100% (see section 8.1 of the appendix). We propose that for glucose fermentation this maximum yield can also be achieved if additional electrons are provided by an electrode. The last step of propionic acid production in *E. coli* is the decarboxylation of succinate which results in a "loss" of one carbon atom in CO_2. Additional redox power allows the recirculation of CO_2 by the oxaloacetate forming phosphoenolpyruvate carboxylase as the increased availability of NADH enables the formation of malate and therefore succinate and PA (see **Figure 12**). The mechanism by which electrons are fed into the metabolism seems subordinate as for both cathodic models the max yield is 100%. However, comparison of the highest yields that enable simultaneous growth reveals a benefit from scenario *Cat1* where the additional ATP input enables a max product yield of 97.7% with a biomass yield of 2.1% and 0.2% CO_2 as the only by-products. Electron supply by *Cat2* by contrast results in a maximal growth-coupled product yield of 89.6% which is not much higher than the non-enhanced production (85.3%). A full list of all maximum yields for growth-coupled production of each substrate-product-combination is given in *Additional file 1* of the corresponding publication of this chapter (see section 8.1 in the appendix). Also the typical by-products of propionic acid fermentation succinate, CO_2 and acetate are detected in more than 85% of the 1,965 *efms* of *Cat2*. Whereas none of the 2,840 *efms* that use *Cat1* produces acetate as the influx of electrons and protons results in sufficient ATP production. EMDE and SCHINK reported similar results for *in vivo* fermentation of *Propionibacterium freudenreichii*, in which they observed an increased production of propionic acid in the presence of reduced mediators while acetate formation was inhibited.[6] According to the presented calculations, this would be an indication towards an EET mechanism as proposed for *Cat1* since the shift in product spectrum suggests that the cathode supports an alternative ATP source to acetate production. These results demonstrate that electrical enhancement could be a suitable technique to boost propionic acid production from glucose by reducing by-product formation. But they also highlight the importance of unveiling the actual connection between extracellular electron transport and energy metabolism for the viability of microbial electrosynthesis.

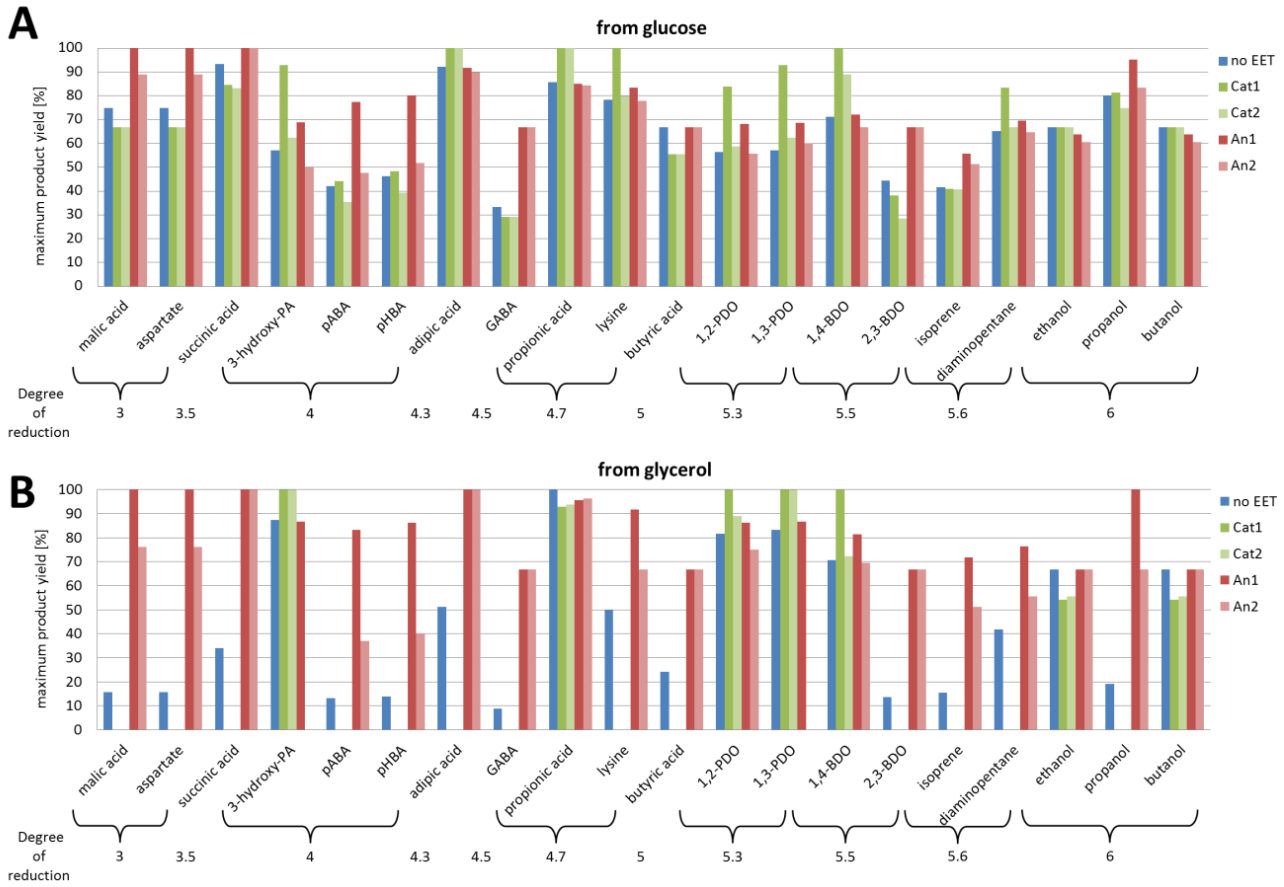


Figure 15: Theoretical maximum carbon yields for different products with and without electrical enhancement. (A) Summarizes all product yields for fermentation from glucose (degree of reduction$_{glucose}$ = 4) while **(B)** shows the maximum yields on glycerol (degree of reduction$_{glycerol}$ = 4.6). *no EET*: no electrical enhancement, *Cat1*: cathodic electron supply coupled to energy conservation; *Cat2*: cathodic electron supply uncoupled to ATP formation; *An1*: anodic redox sink coupled to ATP generation; *An2*: anodic redox sink uncoupled to energy conservation; BDO: butanediol, GABA: γ-aminobutyric acid, PA: propionic acid, PDO: propanediol.

The second group of products that benefit from additional electron supply by a cathode are compounds derived directly from glycerol or the upper branch of glycolysis such as 3-hydroxypropionic acid, 1,2-propanediol and 1,3-propanediol (see **Figure 12**). 1,2- and 1,3-propanediol (1,2-PDO; 1,3-PDO) are building blocks for polyesters and even though mostly produced chemically from propylene oxide and propenal, respectively, there are several emerging approaches for their microbiological production. DuPont Tate and Lyle BioProducts have already commercialized several corn-sugar-based 1,3-PDOs (http://www.duponttateandlyle.com). Reported yields of these glucose based fed-batch fermentations with engineered *E. coli* are around 60%,[271-273] which is close to the theoretical maximum product yield on glucose that was computed in this study for a non-enhanced network (57.1%). The here performed calculations suggest the maximum yield of this process (which is already on the commercial market!) could be increased up to 92.9% by electrical enhancement (see **Figure 15 A**). But the benefit achievable with

bioelectrochemical techniques for propanediol production is strongly dependent on the actual EET mechanisms. The product yield during anaerobic glucose fermentation is not only redox but also energy limited. The production of 1,3-PDO from both feedstocks can be summarised with the following equations 1 and 2.

(1) ½ glucose + $\sim P_i$ + 2 NADH + 2 H^+ → 1,3-PDO + H_2O + 2 NAD^+ + P_i

(2) glycerol + NADH + H^+ → 1,3-PDO + H_2O + NAD^+

The usage of glucose requires twice the amount of reducing equivalents and also a high energy phosphate bond ($\sim P_i$) such as ATP or PEP for the phosphorylation of sugar. Therefore, *Cat1* which provides simultaneously NADH and ATP causes a significant increase of the maximal 1,3-PDO yield to final 92.9% for glucose. If *Cat2* would represent the dominant mechanism the product yield could only slightly be increased to maximal 62.5% as the energy limitation would still remain. The production of 1,3-PDO from glycerol also benefits from electron supply by a cathode as seen in **Figure 15 B**. Here both cathodic models result in a maximum yield of 100%, yet the impact on the possible operational options for the network differs significantly for the different EET scenarios. **Figure 16** displays the plots of biomass against product yields for each *elementary flux mode* during production of 1,3-PDO. The transfer of electrons into the cellular metabolism via cytochromes (*Cat1*) displays for both substrates the most beneficial option (**Figure 16 C** and **D**). For glucose, the product yield is increased significantly and for glycerol not only the maximum achievable yield is improved but also the majority of the cathodic modes features high product and low biomass yields. The production pathway is the most efficient option for the network to maintain cellular redox balance as the internal NADH level is increased. This way cathodic electron supply is automatically coupled to product formation. For the production of PDO from glycerol, all modes that take up electrons via *Cat1* have product yields above 55%. Moreover 91% of the total 3,233 modes couple production to biomass formation, which would enable production during the electrochemically enhanced fermentation (**Figure 16 D**). For substrate-product combinations that show this behaviour, electrochemical techniques could offer the possibility to force the metabolism to operate in a desired mode(s) comparable to genetic engineering approaches. But also operational modes that show increased product yields without growth, offer an interesting perspective for microbial electrosynthesis. For the fermentation from glucose, it can be seen that all modes above 50% PDO-yield do not produce any biomass (*Cat1*) (**Figure 16 C**). This is also the case for both top modes (from glucose and from glycerol) with 100% product yield. The flux distributions of these

particular networks show a carbon flux that could be titled as “true catalysis”. The substrate is converted directly into the product by the addition of redox power from the electrode while no by-products are created. To realise “true catalysis” a two-part fermentation strategy could enable successful production by coupling a non-enhanced growth phase to a later electrically supported production phase.

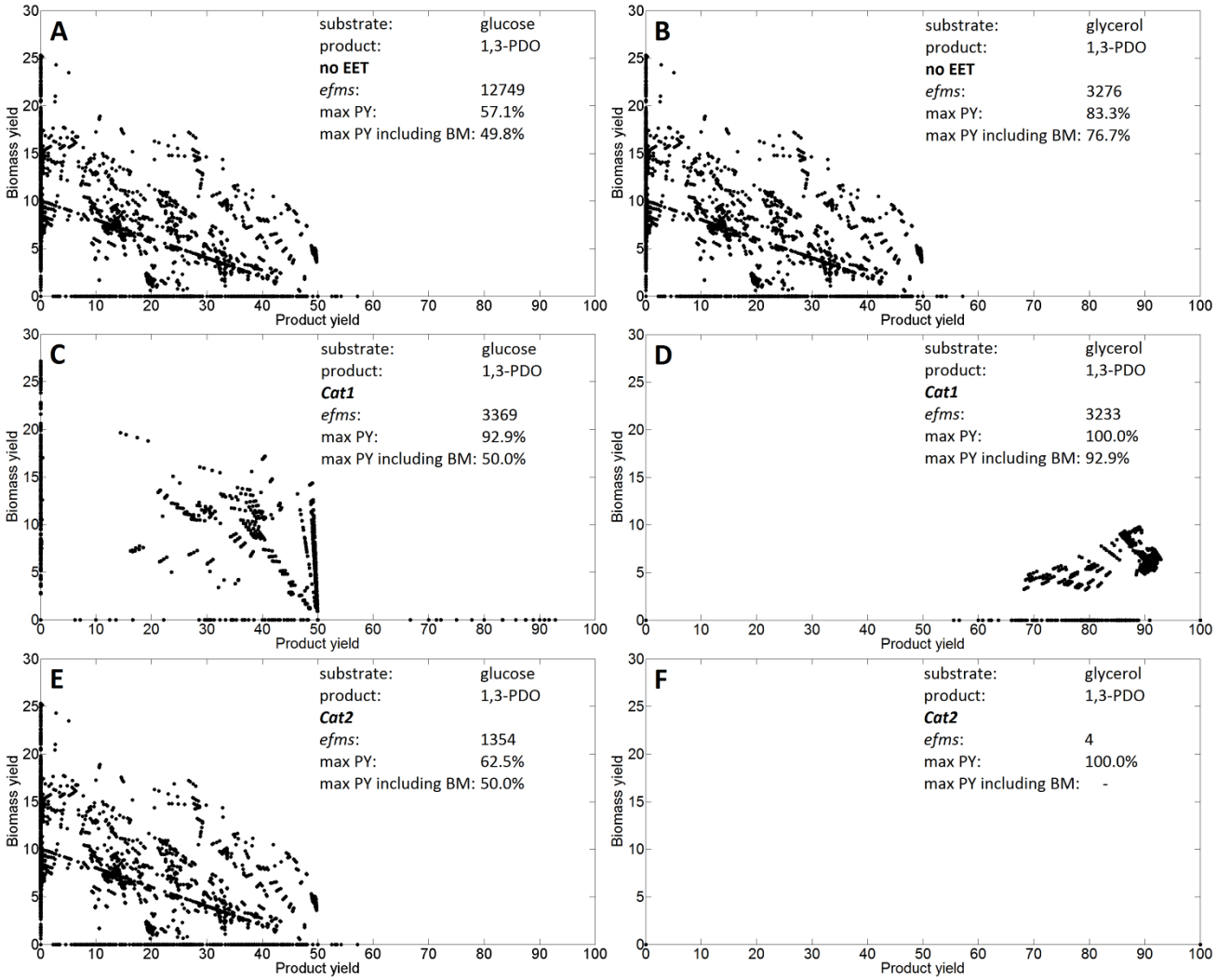


Figure 16: Plots of biomass and product yields of all elementary flux modes for anaerobic 1,3-propanediol production. Each data point in a plot represents the biomass and propanediol yield for a calculated elementary flux mode that uses the respective electron transport model on glucose or glycerol, respectively.

Anodic processes promote microbial electro-oxidation

The use of an anode in bio-electrochemical systems was widely studied in the research of microbial fuel cells, which create electricity as their main product. Substrates are usually mixed waste streams but also glucose and glycerol-containing media are used.[274-276] Since the power output of these systems is too small to become relevant on industrial scale anytime soon and because of the inherently low value of electricity, the

focus shifts towards higher value products.[19, 28] Several valuable compounds were identified that show increased production in presence of an anode (**Figure 15**). It can be seen that without electrical enhancement yields on glycerol are generally a lot lower than on glucose. This is due to the surplus of NADH created during glycerol degradation, which anaerobically becomes limiting if there are no NADH-consuming pathway branches such as PDO production.[277] In presence of an anode as electron sink the cellular redox state is optimized and product yields on glycerol can be increased to similar levels as on glucose. Products that benefit from anodic electron transport include glycolysis-derived products (isoprene, 2,3-butanediol), products from the tricarboxylic acid cycle (malate, succinate) and its derivatives (propanol, aspartate, γ-aminobutyric acid) and aromatics from shikimate pathway (pABA: *para*-aminobenzoic acid, pHBA: *para*-hydroxybenzoic acid) (see **Figure 12**). Again the actual EET mechanism is of great importance as the anode that promotes ATP synthesis (*An1*) triggers significant higher yield increases in many cases (see **Figure 15**).

2,3-butanediol (2,3-BDO) is an interesting example to study as the production is increased by an anode even though the substrates glucose and glycerol are further oxidised ($DoR_{2,3\text{-}BDO} = 5.5$, $DoR_{glucose} = 4$; $DoR_{glycerol} = 4.7$). It also shows reverse behaviour to its isomer 1,4-butanediol that benefits from a cathode, which is due to the different production pathways (see **Figure 12**). 2,3-BDO has applications in the food, pharmaceuticals as well as agrochemical markets and is still produced from fossil fuel feedstocks.[262] But there are several promising approaches for its microbiological production from sugars or glycerol.[278] It is metabolically derived from pyruvate via acetolactate and the recent progress in process optimization suggests bio-2,3-BDO will hit the industrial market soon.[279, 280] The degradation of glycerol and glucose creates in both cases equimolar ratios of reducing equivalents and pyruvate. Because the production pathway of 2,3-BDO requires only one NADH per two molecules pyruvate an overall surplus of NADH is accumulated. An anode as electron sink can help to optimize $NADH/NAD^+$ ratios and reduces the formation of by-products such as lactate or ethanol which are otherwise used as electron sink. In this case, the transport mechanism of electrons is subordinate as the limitation is purely stoichiometric. Increasing ATP levels cannot improve the maximum yield which is constrained by CO_2 formation due to decarboxylation steps during production. Therefore, the maximum theoretical yield for 2,3-BDO production on an anode (*An1* and *An2*) is 66.7% from glucose and glycerol, respectively.

However, many compounds are not solely redox limited in their production pathways and therefore the coupling of electron transport to energy conservation is of major importance as was shown before for 1,3-PDO production on a cathode. The here studied production of malic acid, propanol, isoprene, aspartate, pABA and pHBA on an anode benefits strongly from the proposed mechanism *An1* whereas a pure NADH-redox-sink (*An2*) results in a significantly smaller yield increase (section 8.1 in the appendix shows how to access the full table of theoretical yields for all products and EET models). The biggest difference between the two electron transport models is seen for production of the aromatics pHBA and pABA. These are used in sunscreens, dyes, liquid crystal polymers, polyurethanes and food additives and have also the potential to act as building blocks for aromatic polymers.[281] Even though purely synthesised from petrochemicals to date, there is potential for the bio-production of pHBA and pABA as microbes such as *E. coli* produce the aromatics via the shikimate pathway. This pathway requires phosphorylated Co-factors such as NADPH, ATP and PEP and therefore shows major possible flux increases by *An1*. pABA yields could theoretically be increased from 42.1% and 13.1% to 77.4% and 83.3% on glucose and glycerol, respectively. Solely the presence of an anodic electron sink by *An1* causes a shift of the theoretical maximum yield for pHBA production from 46.3% to 80.2% on glucose and 13.9% to 86.3% on glycerol (**Figure 15**). These promising results of redox optimization by electrical interference could offer a new basis for metabolic engineering towards these new products.

4.2.4 CONCLUSIONS ON THE ELEMENTARY MODE ANALYSIS OF MES

To understand benefits and limitations of microbial electrosynthesis a detailed understanding and analysis of the involved metabolic processes is needed. The presented analysis is the first published approach to methodically screen bio-production processes for their potential benefit from electrical enhancement and could successfully identify 18 target products with possible product yield increases between 7% and 84%. Even though *in vivo* yields will usually be lower than the here presented theoretical maximum yields these examples show a great potential of microbial electrochemical techniques to boost anaerobic glycerol and sugar fermentation. Contrary to the assumption reduced compounds such as bio-fuels and alcohols would always require electron input we revealed 12 production processes that show increased product yields on an anode while only 6 of the 20 studied products benefit from additional electron supply. Due to the anoxic

fermentation conditions required for an electrically enhanced process intracellular NADH levels are increased, so that in most cases an overall surplus of redox equivalents is accumulated. A benefit from cathodic EET is only seen for production pathways that purely rely on NADH input (e.g. 1,2-PDO, 1,3-PDO, 3-hydroxypropionic acid) while other products even though further reduced than the substrate are limited by redox surplus and/or energy requirements (e.g. propanol, 2,3-butanediol).

The accumulation of NADH during anaerobic growth could also explain the poor growth performance observed for cathodic cultures. The presented analysis shows that the presence of an anode promotes biomass formation while electron supply by a cathode limits the metabolic options of the organism during growth. This limitation could possibly be turned into a benefit by coupling growth to production as explained for the example of the anaerobic conversion of glycerol to 1,3-PDO on a cathode.

The elementary mode analysis does not only identify target processes but also highlights the major importance of electron transport mechanism and its coupling to energy conservation. For the majority of products, a crucial dependence of maximum achievable product yield and ATP availability was detected. Therefore, it is important to direct the focus of current research in the microbial electrosynthesis community towards fundamentals of electron transport as these are needed to be understood to design processes that approach the full potential of microbial electrochemical techniques.

4.3 *IN VIVO* CHARACTERISATION OF THE MICROBIAL METABOLISM DURING MES

The third objective of the presented project is the *in vivo* characterisation of industrially relevant organisms in a BES. The interest is to screen microbes that are used for the production of chemicals in biotechnology for their electroactivity. As outlined in 1.2.1 there is a lack of commercially available BES reactors and the design of reactors used in reported studies varies significantly, which complicates comparability. Here the development of a multi-reactor BES system and its use for the electrochemical characterisation of four different strains is presented. While the following chapter describes the BES set up and the characterisation of *Escherichia coli*, *Citrobacter werkmanii*, *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii,* the most interesting effect of extracellular current supply on production was found for *Clostridium autoethanogenum,* which is studied in more detail in chapter 4.4.

4.3.1 DEVELOPMENT OF A STANDARDISED REACTOR PLATFORM

The design of a bioelectrochemical system determines its performance as key parameters such as production rates directly depend on the ratio of cathode surface area to solution volume.[82] Within this study, the focus lies rather on process discovery than process optimization for the highest possible rates. Therefore, the BESs were designed to enable multiple simultaneous fully controlled fermentations with a high level of comparability. The cathode volume was chosen to be as small as possible to minimize mass transfer limitations within the system and enable the characterisation of slow growing organisms by not relying on a high biomass inoculum. On the other hand, the system volume needs to be sufficient to provide product concentrations that can be measured at an acceptable accuracy.[282] We chose commercially available small glass vials from BIOLOGIC (Science Instruments, France) with a working volume of 80-120 mL.

The set-up is a two chamber system divided by a cation exchange membrane (CMI-7000, MEMBRANES INTERNATIONAL INC., USA) with the anodic chamber inserted as a small glass tube with the membrane easily exchangeable at its bottom. Multiple parts on the reactor lid allow a flexible set up of probes and electrodes for each system. **Figure 17** shows an image of the first setup of BES designed by CEMES. The used electrode configuration in a three electrode setup with the working electrode being a carbon cloth

(6x3 cm) immersed in the main chamber. The counter electrode was a titanium mesh and an Ag/AgCl electrode in saturated KCl was used as reference electrode (RE). The RE was inserted in a separate glass tube with a vitreous glass frit at its end to prevent fouling and all electrodes were controlled by an either 5 or 16 channel potentiostat (BIOLOGIC, Science Instruments, France).

Figure 17: Pictures of the first (left and middle) and second generation (right) of bio-electrochemical system developed by CEMES. The system is suitable for anaerobic cathodic and anodic fermentations including electrochemical characterisation, controlling temperature, stirring, pH, reactor atmosphere and a three electrode setup connected to a potentiostat.

A three-way sampling valve provides sterile and anoxic sampling via a stainless steel needle and a pH probe can be inserted to measure the cathode chamber pH online. These probes can be connected to a pH control unit, which in turn controls acid and base pumps to maintain a certain pH level. Anaerobic conditions can be achieved by flushing the system with an anoxic gas. In this case, constant Nitrogen headspace flushing was adjusted at a flow rate of ~50 mL min^{-1} and stripping was minimised by cooling the reactor off-gas to 4°C. The suitability of the system for culturing strict anaerobes was tested by measuring the oxygen concentration of the described BES with an oxygen meter (PRESENS, PRECISION SENSING GMBH, Germany). At the described set up with constant nitrogen flushing an oxygen level below the detection limit of the system could be maintained (<15 ppb).

As a further optimization the Teflon reactor lids and probe-fittings lids provided by the manufacturer were replaced by custom made ones from PEEK (polyether ether ketone), which has better mechanical properties while being chemically inert over a large temperature range (see **Figure 17** right).

The small volume system was implemented for four reactors, which are temperature controlled by immersion into a water bath as can be seen on the left image in **Figure 18**. Based on the developed system the BES platform of CEMES was extended to two more reactor sets of four BESs each (see **Figure 18** right). Here a bigger reactor volume of 300 mL was chosen to favour in-depth studies such as -omics analysis, which require a high sample volume or long-term experiments for process optimization. Furthermore, a mass spectrometer was added to the analytical setup, which will enable the analysis of reactor off-gases in the future.

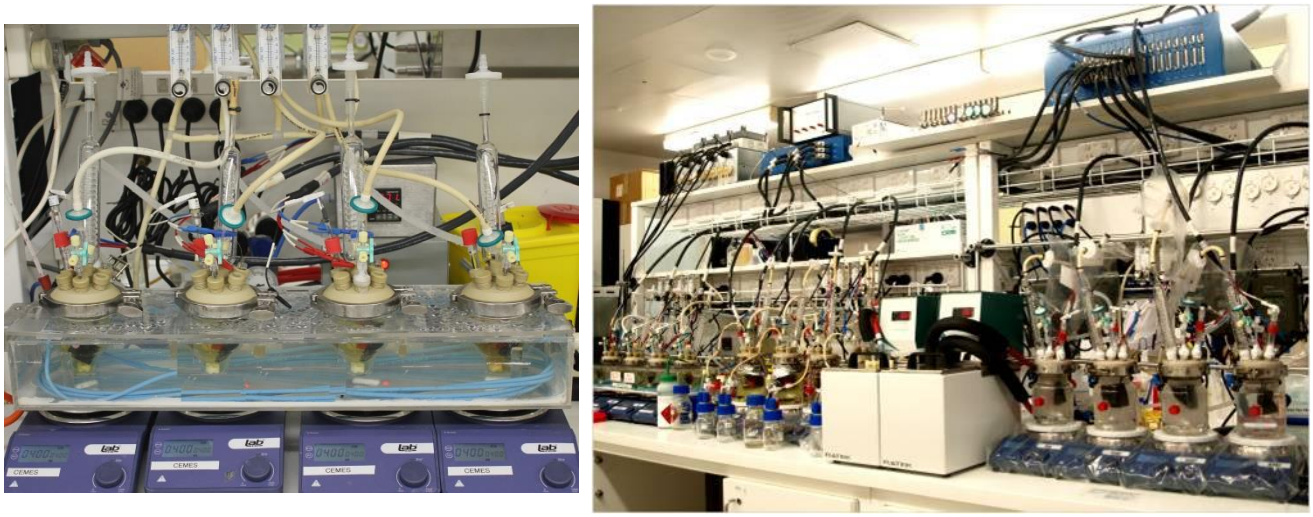


Figure 18: Pictures of the third generation of bio-electrochemical system developed by CEMES. Final design of small volume reactors used in this study (left) and total lab set up of twelve BESs (right).

4.3.2 SCREENING INDUSTRIAL ORGANISMS FOR ELECTRO-ACTIVITY

The small-volume BESs were used to screen different bacterial strains for electro-activity during anaerobic fermentation. The effect of mediated extracellular electron supply on different fermenting organisms was monitored: *Escherichia coli*, *Propionibacterium acidipropionici, Propionibacterium freudenreichii*, *Citrobacter werkmanii and Clostridium autoethanogenum*. These bacteria were chosen as they all are of industrial interest and at the same time present a variety of physiological properties (summarised in **Table 15**).

E. coli is probably the most studied prokaryote in biotechnology. Its fast growth and the large number of available tools for metabolic and genetic engineering make *E. coli* a frequent host for the production of heterologous proteins, fermentations products, bio-fuels and chemicals.[283-285] The organism was shown to be able to use an anode as final electron acceptor and therefore create a small electrical current. This current generation could be improved by electrode modifications or adding neutral red as mediator.[172-174] Another

approach to increase extracellular electron transfer of the organisms is by genetic modification as explained in 4.1.5 in more detail.[259]

Table 15: Industrial relevant organisms screened within this study for electroactivity.

Organisms	GRAM staining	Respiration	Industrial importance	Reported electro-activity
Escherichia coli	-	facultative anaerobic	Model organism in bio-technology, fast growing host organism for production of various compounds	Anode: current production after prolonged acclimation or in presence of a mediator or modified electrodes (neutral red)[172-174]
Propionibacterium acidipropionici	+	anaerobic	Widely used in probiotics and cheese industry as well as for production of propionic acid and vitamin B_{12}	none
Propionibacterium freudenreichii	+	anaerobic	Widely used in probiotics and cheese industry as well as for production of propionic acid and vitamin B_{12}	Cathode: Increased propionic acid yield from glucose in a BES with mediators[6]
Citrobacter werkmanii	-	facultative anaerobic	Promising host for sustainable 1,3-PDO production	none
Clostridium autoethanogenum	+	strict anaerobic	Sustainable ethanol pro-duction from waste gases	none

The second group of organisms that were studied in the BES are *Propionibacteria,* which are widely used in dairy industry and bio-industry for the production of propionic acid.[286] *Propionibacterium acidipropionici* is one of the most popular strains in this context as it produces the target product propionic acid at high yields and titers.[287] A second strain, *Propionibacterium freudenreichii*, was also tested as it was reported to increase its propionate production in a BES in an early study in the field of microbial electrosynthesis by EMDE and SCHINK.[6] This is also in agreement with the results of the elementary mode analysis presented in 4.2. In the same study, the cathodic production of 1,3-propanediol from glucose was identified as potentially beneficial (refer to **Figure 15**). In this context *Citrobacter sp.* are handled as promising candidates for sustainable industrial production as they naturally produce the compound at a high tolerance level.[272] *Citrobacter werkmanii* was identified to produce 1,3-PDO at especially high productivity and yield and therefore was chosen to be tested for electroactivity within this study.[288] So far no electrochemical activity has been reported, which is also the case for the last tested strain *Clostridium*

autoethanogenum. This anaerobic acetogen differs from the other bacteria as it is able to growth under autotrophic conditions forming ethanol and acetate from carbon monoxide in so-called syngas fermentation.[289] The company LANZATECH is commercializing the sustainable production of ethanol from waste gases with *C. autoethanogenum* and is permanently advancing process technologies and strain engineering tools for *Clostridia*.[290, 291] Currently there are no reports about an electro-activity of *C. autoethanogenum*, however it is a very close relative to *C. ljungdahlii,*[292] which amongst other acetogenic organisms was shown to directly accept electrons from a cathode.[9]

Escherichia coli

Neutral red (NR) has been reported to successfully mediate electron transfer between the cellular metabolism of *E. coli* and an anode as final electron acceptor.[172] Therefore here NR was used as mediator in first cathodic studies on *E. coli*. A typical current profile of such fermentation can be seen in **Figure 19**. Initially, the mediator was reduced inside the anaerobic reactor by applying a constant potential (phase a). The electrochemical potential required to achieve reduction of the mediator was determined by cyclic voltammetry prior to the experiment (data not shown). The successful reduction can also be visually monitored by the change in colour: Oxidised NR is red while it changes colour to yellow when reduced (see pictures of the BES inserted in **Figure 19**). After reaching a baseline current close to zero (phase b) the system was inoculated with anaerobically grown pre-culture. Immediately a reductive current could be monitored, which directly corresponds to bacterial growth (current was only monitored in the presence of cells and NR). After the exponential growth phase, the current profile reached a plateau before slowly decreasing back to the baseline.

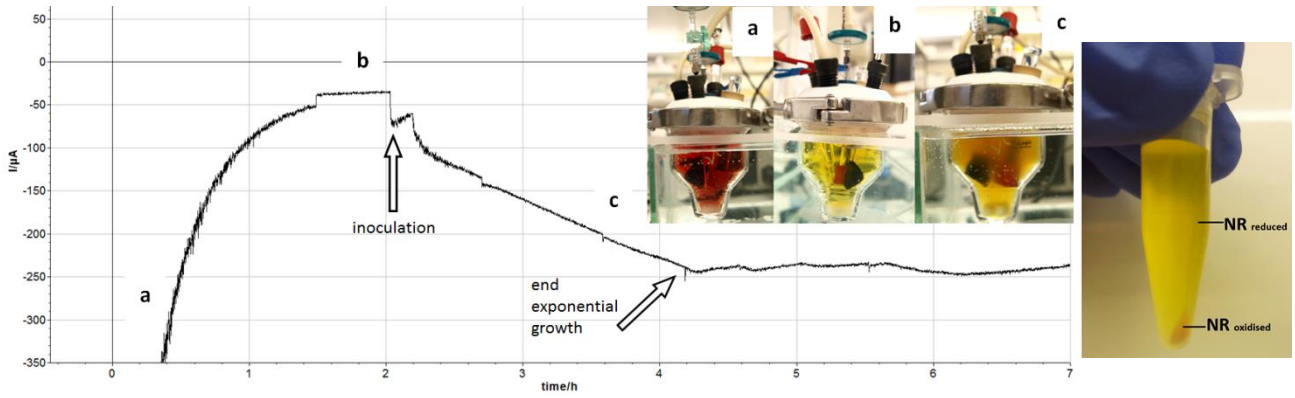


Figure 19: Left: Current profile of *E. coli* fermentation on glucose with 40 µM neutral red (NR) in a BES; chronoamperometry at -0.603V vs SHE. Inserted images show the BES in different phases of the experiment. **a)** mediator reduction; **b)** constant baseline current; **c)** reductive current during exponential growth. **Right: reactor sample during phase c after centrifugation.**

It seems the bacteria are able to accept electrons from NR, which in turn is re-reduced at the electrode thus creating the current. When the cells are separated from the medium by centrifugation they show a red colour while the supernatant is yellow (see **Figure 19** right). This could be a sign of oxidised neutral red inside the cells or at the cell surface. However, when comparing the metabolic profile of cathodic fermentation to control reactors (**Table 16**), one can see that the amount of transferred electrons is too small compared to substrate consumption to account for a significant change in metabolites. The BES consumed slightly higher amounts of glucose (17 mM compared to 15 mM) and correspondingly showed higher growth (maximum $OD_{BES} = 1.19 \pm 0.08$; maximum $OD_{control} = 1.07 \pm 0.04$) while growth rates were identical ($\mu_{max,BES} = 0.37 \pm 0.01\ h^{-1}$; $\mu_{max,control} = 0.37 \pm 0.01\ h^{-1}$).

Table 16: Metabolite profiles, glucose and electron consumption of anaerobic *E. coli* fermentation in presence of neutral red as mediator (t = 7.5 h).

	glucose consumption* [mM]	succinate* [mM]	lactate* [mM]	formate* [mM]	acetate* [mM]	ethanol* [mM]	electron uptake* [mM]
control	15.00 ± 1.05	0.31 ± 0.08	2.33 ± 0.25	15.10 ± 3.41	5.33 ± 0.41	8.26 ± 1.99	-
BES**	17.69 ± 1.48	0.55 ± 0.11	2.83 ± 0.29	18.02 ± 3.39	5.38 ± 0.44	10.21 ± 2.1	0.19 ± 0.04

*Given values are averages of triplicate reactors with corresponding standard deviation. **Concentration of neutral red was 40 μM; a constant potential of -0.603 V vs SHE was applied.

It seems the BES produced slightly higher amounts of reduced metabolites: 0.24 mM more succinate, 0.5 mM more lactate, 2.92 mM more formate, 0.05 mM more acetate and 1.95 mM more ethanol (see **Table 16**). However, the electron uptake was only around 200 μmol in average and therefore cannot account for a change in metabolites in the millimolar range. The recorded change is more likely due to the increased substrate uptake as there is no notable difference in the specific yields for each compound. Different strategies were followed to increase the electron uptake of *E. coli* (increasing mediator concentrations, different mediators and different potentials), but no significant change was achieved (data not shown). Clearly *E. coli* is able to interact with electrically reduced NR in a BES, however with the given standard deviations of metabolite concentrations it is not possible to reconstruct the electron flow.

A recently published study by HARRINGTON et al confirms the here made observations as it reports a very similar effect of NR on *E. coli* in cathodic sugar fermentations.[293] The scientists saw a slight increase in glucose consumption, growth and metabolite production in a BES with neutral red compared to the control reactor. However,

similar to the here presented results, it did not correspond with the small amount of electrons that were taken up. An explanation could be a change in the intracellular redox balance caused by electron supply via NR, which resulted in a general upregulation of overall metabolic activity. The effects are increased substrate consumption and metabolite production while overall electron fluxes remain low.

Propionibacterium acidipropionici and P. freudenreichii

To study the effect of extracellular electron supply on *Propionibacteria* in the BES, redox mediators were added to facilitate EET in suspension. The fermentation approach was identical to what was described for *E. coli* above. We tested neutral red as well as cobalt(III)sepulchrate ($[Co(sep)]^{3+}$), which had been reported as a successful mediator for electron uptake in a cathodic BES for *P. freudenreichii* previously.[6] EMDE and SCHINK reported an increased growth and production and propionic acid (PA) while acetate production was reduced. A growth-depended reductive current was recorded for both *Propionibacteria* strains in the BES. However, no growth-benefit or significant increase in production by the electrical treatment was observed.

For *P. freudenreichii* the maximum electron uptake with $[Co(sep)]^{3+}$ was 0.8 mM over 60 hours while 43 mM glucose were consumed. The BES consumed slightly more substrate, (43 mM compared to 42 mM in the control fermentation) while changes in the metabolite spectrum could not be detected as they lie within the standard deviation of triplicate fermentations. Similar to the case of *E. coli* described before the small uptake of electrons did not allow making any conclusions about the metabolic effect of the treatment.

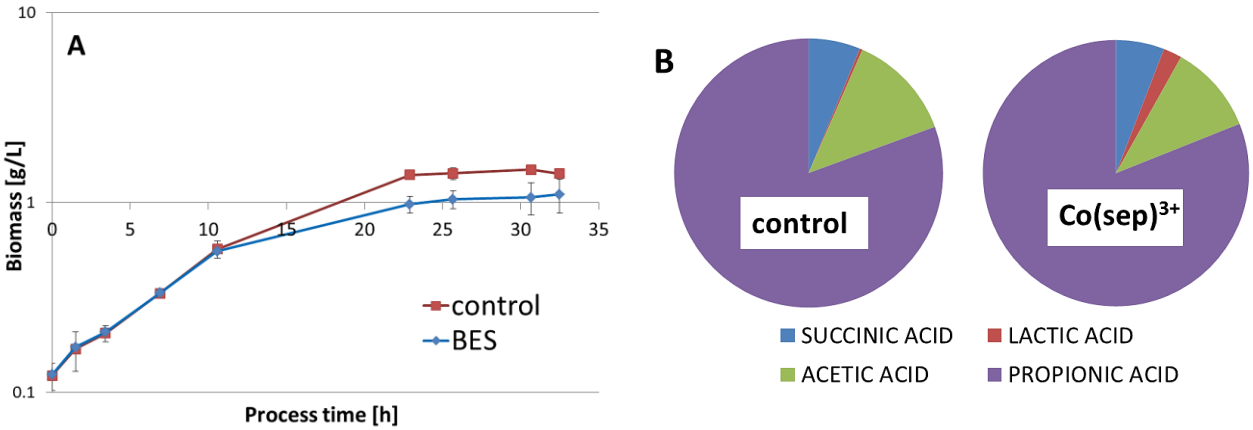


Figure 20: Comparison of *P. acidipropionici* fermentation data: control and with 40 µM $[Co(sep)]^{3+}$ as mediator in a BES. A) logarithmic growth profile; **B)** comparison of product composition of reference and electrically enhanced fermentation. Given values are averages of triplicate experiments and error bars correspond to standard deviation between repetitions.

In fermentations of *P. acidipropionici* in the BES a growth-related current was observed, however, contrary to what was reported by EMDE and SCHINK the growth was slightly inhibited by the mediator ($\mu_{control}$ = 0.141 h^{-1}; $\mu_{[Co(sep)]3+}$ = 0.137 h^{-1}). **Figure 20** shows characteristic fermentation data for *P. acidipropionici* and $[Co(sep)]^{3+}$. From the product spectrum it can be observed that slightly more PA is produced on the cathode ($Y_{PA,BES}$ = 1.37 ± 0.03 mM $mM_{glucose}^{-1}$; $Y_{PA,control}$ = 1.14 ± 0.02 mM $mM_{glucose}^{-1}$). Simultaneous lactic acid production seems to be enhanced by electron supply, which is also a metabolic redox sink (see **Figure 20 B**). The BES produced a titer of 1.3 ± 0.05 mM lactic acid while the control fermentations only contained 0.2 ± 0.03 mM. The current consumption was 1000 µmol electrons within 60 hours in this fermentation and therefore again cannot account for the total change in metabolites.

Citrobacter werkmanii

The elementary mode analysis presented in 4.2 identified the conversion of glucose or glycerol to 1,3-propanediol (PDO) as one of the most promising processes with theoretical possible yield increases up to 35.8% (see **Table 17**). It can also be seen, that this possible yield increase directly depends on the electron transport mechanism. On glucose, a theoretical maximum increase of +35.8% was calculated for *Cat1*, which describes electron transfer into the NADH redox pool coupled to ATP production via proton gradient (see chapter 4.2 for details). On the other hand, very little benefit (maximal +5.4%) in yield increase was computed if electron transfer is not coupled to energy conservation.

Table 17: Theoretical maximum yields for the anaerobic production of 1,3-propanediol from glycerol and glucose with and without electrical enhancement based on metabolic reaction stoichiometry. Calculations were done by elementary mode analysis based on a metabolic network of *Escherichia coli.*

		maximal theoretical product yield [%]			maximal yield increase [%]	
product	**substrate**	**no EET**	***Cat1**** **(NADH + ATP)**	***Cat2**** **(NADH)**	***Cat1**** **(NADH + ATP)**	***Cat2**** **(NADH)**
1,3-PDO	glucose	57.1	92.9	62.5	35.8	5.4
	glycerol	83.3	100.0	100.0	16.7	16.7

* *Cat1* describes electron feed coupled to ATP and NADH production; ***Cat2* describes electron feed into NADH pool only.

C. werkmanii was tested in a mediated BES as described above with glucose, glycerol and glucose-glycerol co-feed for electro-activity. Of all tested conditions a maximum electron consumption of ~200 µM electrons within 30 hrs could be found with

$[Co(sep)]^{3+}$ as mediator and glucose-glycerol co-feed (ratio 1:2). The average substrate uptake was with 80 ± 2.4 mM (about 30 mM glucose and 50 mM glycerol) much higher than the simultaneous electron uptake and therefore no metabolic shift was detectable.

4.3.3 CONCLUSION ON THE *IN VIVO* CHARACTERISATION OF DIFFERENT INDUSTRIAL STRAINS IN A CATHODIC BES

To summarize the work presented in this chapter we can state the development of a BES reactor platform was successful. The system showed satisfying performance in culturing different facultative and strict anaerobic (see following section 4.4) strains under extracellular electron supply with high reproducibility. Growth related current consumption was monitored for all tested strains, however, the electron uptake was small compared to substrate uptake. Most of the observed differences between electrically enhanced and control fermentations are very small and the results are not always conclusive. **Table 18** summarises the electron uptake of the different organisms.

Table 18: Summary of electron uptake and effect on production for the different organisms tested within this project.

Organisms	Maximum e^- uptake (fermentation time)* [µmol]	Maximum total current* [mA]	Effect on production	other comments
Escherichia coli	~200 (7.5h)	0.23 ± 0.03	none	Electron uptake too small to result in a significant change in the metabolite spectrum
Propionibacterium freudenreichii	~1000 (60h)	0.27 ± 0.09	none	Electron uptake too small to result in a significant change in the metabolite spectrum
Propionibacterium acidipropionici	~600 (32.5h)	0.25 ± 0.05	Slight increase in propionate but mainly lactate titer	Electron uptake too small to account for total change of metabolites
Citrobacter werkmanii	~200 (30h)	0.20 ± 0.03	none	Electron uptake too small to result in a significant change in the metabolite spectrum
Clostridium autoethanogenum	~2200 (100h)	1.15 ± 0.2	Significant increase in lactate and 2,3-butanediol production, decrease in acetate	Detailed study of electron uptake following

*given values are average of triplicate fermentations at the best-tested conditions; e^- uptake was determined by integration of the current profile after subtraction of baseline current.

For *E. coli* and *Citrobacter werkmanii* it could be seen that the consumed current is particularly small and therefore could not result in any significant change of metabolic

profile. Electron uptake was higher for the *Propionibacteria* and while for *P. freudenreichii* the standard deviation between repetitions was still higher than the electron uptake, an increase of propionate and lactate production by extracellular electron supply was monitored for *P. acidipropionici*. Unfortunately, the major shift was observed towards lactate while production of the target product propionate increased only by around 4%. Interestingly, the GRAM-positive *Clostridium autoethanogenum,* not discussed above, showed a significant shift in its metabolite profile when exposed to extracellular electron supply. The organism appeared as the most interesting candidate amongst the tested strains as a beneficial effect of decreasing acetate production could be observed while production of other metabolites increased. Therefore, *C. autoethanogenum* was studied in more detail and the results are reported in chapter 4.4 following.

4.4 IN-DEPTH CHARACTERISATION OF ELECTRICALLY ENHANCED PRODUCTION IN *C. AUTOETHANOGENUM*[3]

In the preceding chapter 4.3, the screening of different industrial relevant organisms for their electroactivity was reported. Here the first report of extracellular electron activity in *Clostridium autoethanogenum* is presented including a detailed study of the effect of this external electron supply on production.

4.4.1 ACETOGENIC ORGANISMS AS HOST FOR MES

As discussed in the introduction, acetogenic organisms are regarded attractive hosts for microbial electrosynthesis since they reduce carbon dioxide via the Wood Ljungdahl pathway (WLP), which is the most efficient microbial pathway for CO_2-fixation.[221, 294] The still low energy efficiency of WLP has beneficial effects, as it results in high electron recovery in products and low biomass production.[221] The main product is usually acetate but the central intermediate acetyl-CoA presents an excellent building block for various commodities.[165, 262] Tools for genetic manipulation of acetogenic organisms are rapidly evolving, offering a fast growing set of techniques for pathway engineering.[214] **Figure 21** displays a selection of substrates and products that are part of natural pathways in acetogenic Clostridia. Highlighted in blue are potential future processes that can be achieved via metabolic engineering or process design such as the use of electrons for CO_2 reduction in a BES. As discussed earlier many acetogens have already been reported to be electro-active and perform MES either via electrochemically produced hydrogen or by direct electron uptake from the electrode.[66, 98] The mechanisms of EET in acetogens remain to be uncovered, however, a detailed discussion of metabolic electron transfer and possible interactions with an electrode are discussed in chapter 4.1.

Amongst the first acetogens that were studied in BES processes, was *Clostridium ljungdahlii* and quickly it was titled “chassis organism” for electrified reduction of CO_2 as it is one of few acetogens with fully sequenced genome as well as first tools for metabolic engineering available.[165, 294] Here we report for the first time electron uptake in *Clostridium autoethanogenum*, a close relative to *C. ljungdahlii*.

[3] This chapter was modified from a manuscript draft submitted to Biotechnology for Biofuels (2016): **Kracke, F.,** Virdis, B., Bernhardt, P. V., Rabaey, K., Krömer, J. O. *Redox-dependent metabolic shift in* Clostridium autoethanogenum *by extracellular electron supply.*

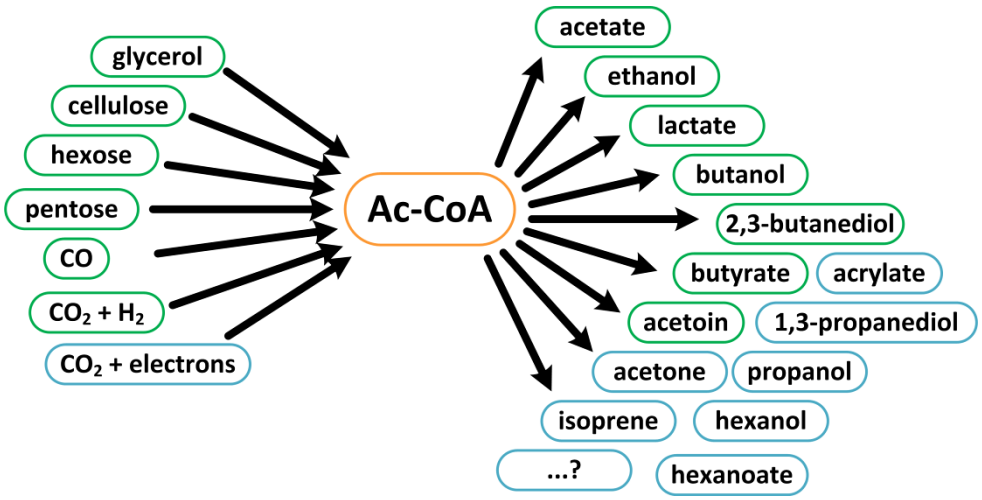


Figure 21: Acetogenic conversion of various substrates and products via the core intermediate acetyl coenzyme A (Ac-CoA). Green highlighted compounds are natural substrates and products of some acetogenic organisms while blue outline marks future products that could be achieved via metabolic or process engineering.

The attractive feature about *C. autoethanogenum* is its natural increased formation of ethanol from CO and CO_2/H_2, which presents a higher value product compared to acetate.[289] The biotech company LANZATECH is commercialising gas fermentations processes with *C. autoethanogenum* for the production of biofuels and constantly advances available tools for metabolic and genetic engineering as well as fundamental understanding of the acetogenic metabolism.[295, 296] Within this chapter we investigate the effect of extracellular electron supply at different potentials on the production of *C. autoethanogenum* in heterotrophic fermentations.

4.4.2 REDOX-DEPENDENT SHIFT OF METABOLIC FLUXES

The analysis of microbial electron transport chains (chapter 4.1) as well as the *in silico* analysis of different EET routes discussed in chapter 4.2 pointed out that the entry point of electrons in the microbial metabolisms can be of major importance for MES processes. Depending on the metabolic reaction that is directly connected to extracellular electron supply different effects on cellular redox and energy yields might be achieved. Therefore, we followed an approach of electron supply via mediators of different midpoint potentials to study the effect of production in *C. autoethanogenum*.

The chosen molecules are cobalt-based metal complexes that are successfully used in redox reactions with proteins.[297] These are very stable and biocompatible molecules that offer the advantage of having extremely similar chemical and physical

properties. Therefore, observed effects can be associated with high certainty to the only distinct difference between the molecules: the redox potential. The potential window was chosen around that of the H_2/H^+ couple, which acts as favourable electron donor in natural environments for *C. autoethanogenum*.[289] Under standard conditions the redox potential of the reaction between hydrogen and protons is -414 mV, however, the low hydrogen partial pressures in acetogenic environments shifts this potential more towards -350 mV (see discussion in section 4.1.5).[216] The three different molecules tested are $[Co(sep)]^{3+}$ ($Co^{3+/2+}$ -296 mV), $[Co(AMMEsar)]^{3+}$ ($Co^{3+/2+}$ -380 mV) and [Co(*trans*-diammac)]$^{3+}$ ($Co^{3+/2+}$ -555 mV) as shown in **Figure 22**.[297] Similar to the experimental approach of mediated cathodic fermentations described in the preceding chapter 4.3 the mediators were reduced inside the BES prior to inoculation. In all fermentations a constant potential of -603 mV vs SHE was applied, which was negative enough to reduce any of the mediators and did not support hydrogen evolution on the carbon cloth working electrode (as suggested by the absence of a catalytic wave for hydrogen evolution at that potential, determined by CV). Like this a baseline current (< 100 μA) was achieved with the mediator being present in its reduced form and any observed current after inoculation could be assigned to mediator oxidation by the organism.

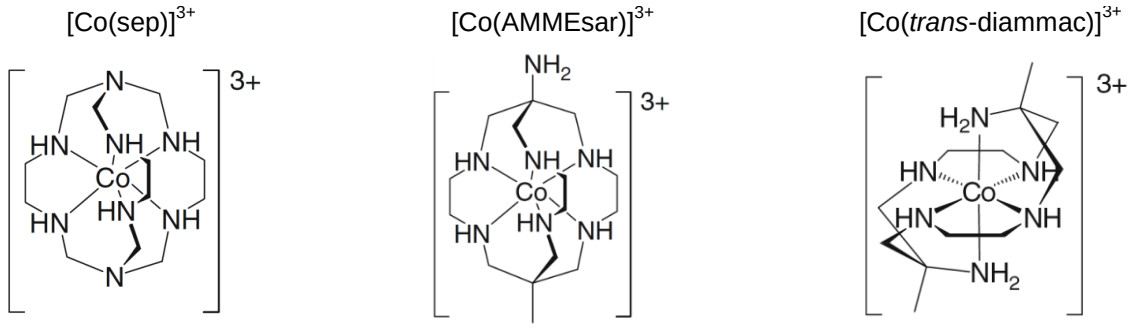


Figure 22: Chemical structures of the three cobalt-based mediators used in this study. *$E^{0'}$ vs SHE at 25°C and pH 7.[297]

In heterotrophic, anaerobic fermentations (C-source: fructose, 5 g L^{-1}) of *C. auto-ethanogenum* current consumption in presence of all three mediators was observed. No current development was detected in controls without mediator or without cells. **Figure 23** shows the metabolic profiles of mediated fermentations in the BES described in chapter 4.3.1. After inoculation varying lag phases were observed in the presence of the mediators (maximum 20 hours for [Co(*trans*-diammac)]$^{3+}$), therefore time zero was defined as the start point of fructose consumption. As can be seen from **Figure 23** very little change in the final end-products of fructose fermentation is made by $[Co(sep)]^{3+}$ and

[Co(AMMEsar)]$^{3+}$. The latter seems to slightly slow down substrate uptake and growth rate, final concentrations of metabolites, however, remain at similar levels.

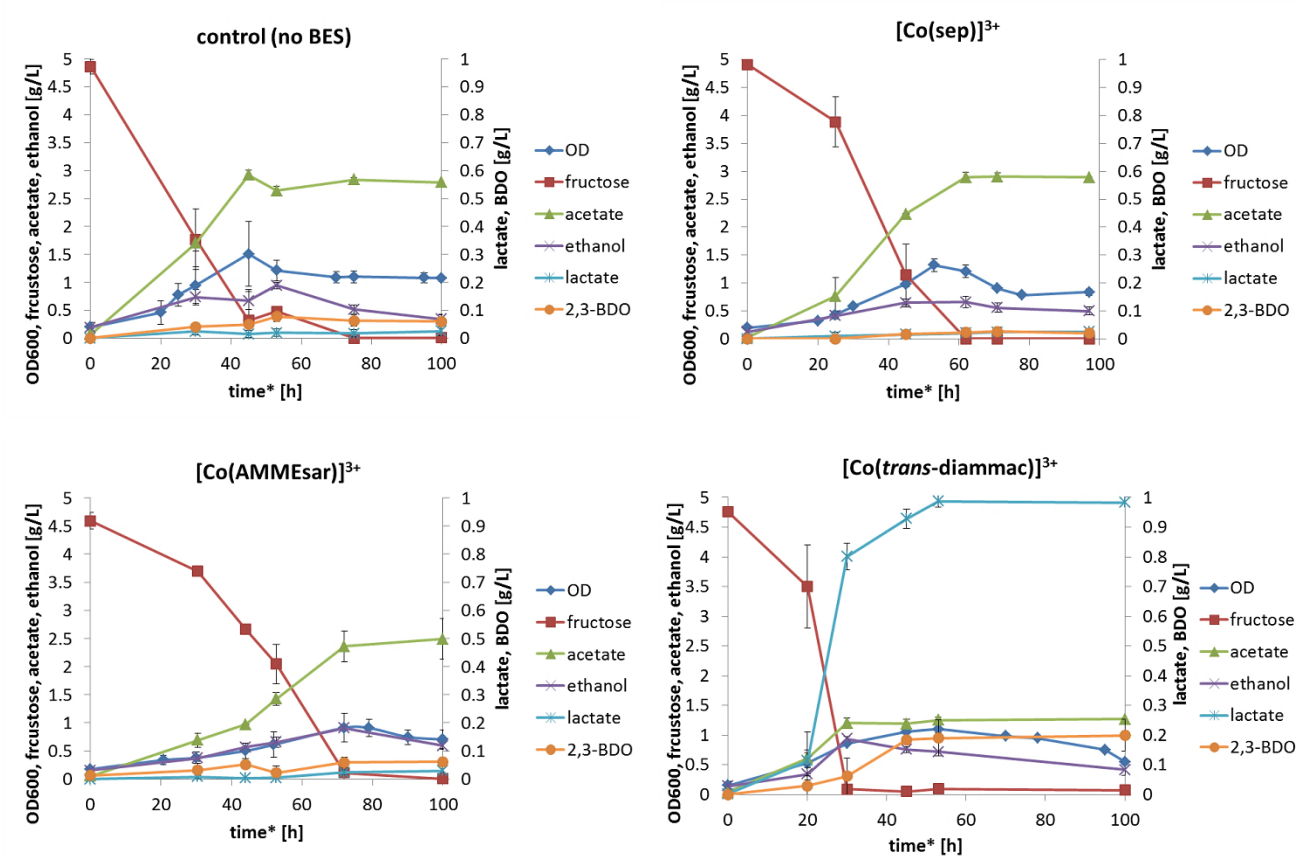


Figure 23: Metabolic profiles of heterotrophic anaerobic fermentations of *C. autoethanogenum* in a mediated bioelectrochemical system. Concentration of the mediators [Co(sep)]$^{3+}$, [Co(AMMEsar)]$^{3+}$ and [Co(*trans*-diammac)]$^{3+}$ was 1 mM and a constant potential of -0.603 V (vs SHE) was applied throughout the experiment. Control fermentations were performed in the same reactor set up with electrodes at open circuit. Shown data is average of triplicate fermentations with error bars displaying the corresponding standard deviation. *fermentation times are adjusted to time zero = start of fructose consumption as varying lag-phases in presences of the mediators were observed. 2,3-BDO: 2,3-butanediol; OD: optical density.

Only the low potential mediator significantly changed the metabolite spectrum. In presence of 1 mM [Co(*trans*-diammac)]$^{3+}$ acetate production drastically decreases (from 2.8 g L^{-1} to 1.3 g L^{-1}). Simultaneously, lactate and 2,3-butanediol production is enhanced from 0.03 g L^{-1} to 1 g L^{-1} and from 0.06 g L^{-1} to 0.3 g L^{-1}, respectively (see **Figure 23**). **Table 19** below summarises in detail the parameters of metabolite concentrations, yields, growth and electron consumption in fermentations with and without the three mediators. Similar to what was observed for the other tested organisms (see chapter 4.3.2) the amount of electrons consumed is relatively small compared to the substrate uptake and does not account for full changes in metabolite spectrum. A recent study on *Clostridium pasteurianum* DSM 525 reports a comparable effect of extracellular electron supply during heterotrophic fermentations.[298] Electron supply via a cathode significantly shifts the metabolite spectrum towards alcohols and lactate while acetate production decreases.

Similar to the observations for *C. autoethanogenum* made in this study, the current consumption is relatively small and the major share of electrons recovered in final end products originates from sugar. The authors report a direct correlation between electron uptake and changes in intracellular redox pools (NAD^+/NADH) and assume that a small current consumption stimulates redox-balancing pathways such as lactate production. This could explain the observations made for *C. autoethanogenum* in the presented study. In presence of [Co(*trans*-diammac)]$^{3+}$, an average electron uptake of 2.5 mM over 100 hours was observed. At the same time about 9.6 mM more lactate and 1.5 mM more 2,3-BDO were produced compared to control fermentations. The stoichiometric balance of electron uptake does not account for the shift in metabolite production, however, it seems that electron consuming pathways such as lactate and 2,3-butanediol production are induced by the electron supply. Interestingly this observation is only made in case of one mediator. While electron uptake was in the same range for all three molecules (2.8 mM, 1.3 mM and 2.5 mM for [Co(sep)]$^{3+}$, [Co(AMMEsar)]$^{3+}$ and [Co(*trans*-diammac)]$^{3+}$, respectively) only [Co(*trans*-diammac)]$^{3+}$ caused the discussed shift in carbon fluxes.

Table 19: Comparison of growth parameters, electron uptake, metabolite concentrations and yields of heterotrophic anaerobic fermentations of *C. autoethanogenum* in a bioelectrochemical system in presence and absence of three different Co-mediators.

condition	Control*	BES**	BES + [Co(sep)]$^{3+}$	BES + [Co(AMMEsar)]$^{3+}$	BES + [Co(*trans*-diammac)]$^{3+}$
growth rate [h^{-1}]	0.053 ± 0.006	0.053 ± 0.006	0.037 ± 0.006	0.033 ± 0.006	0.06 ± 0.017
OD_{600} max	1.51 ± 0.18	1.11 ± 0.03	1.30 ± 0.12	1.22 ± 0.22	1.09 ± 0.14
Current max [mA]	-	0.07 ± 0.03	1.34 ± 0.24	1.07 ± 0.19	1.18 ± 0.20
e^- uptake [mM]	-	0.09 ± 0.01	2.77 ± 0.74	1.28 ± 0.16	2.51 ± 0.12
Final concentration [mM]					
acetate	46.43 ± 5.24	42.36 ± 0.58	48.22 ± 0.21	40.9 ± 7.38	21.11 ± 0.96
lactate	0.28 ± 0.15	0.34 ± 0.04	0.34 ± 0.09	0.31 ± 0.11	9.87 ± 0.96
ethanol	17.63 ± 2.95	21.57 ± 0.54	13.83 ± 1.33	19.74 ± 0.61	19.04 ± 2.4
2,3-BDO	0.73 ± 0.14	0.78 ± 0.05	0.48 ± 0.17	0.68 ± 0.19	2.21 ± 1.21
Yield [$g/g_{fructose}$]					
BM	7.6 ± 1.2	6.86 ± 0.38	6.3 ± 0.36	7.74 ± 1.51	6.66 ± 1.64
acetate	54.57 ± 4.31	50.51 ± 1.08	58.9 ± 0.36	52.06 ± 8.18	24.82 ± 2.35
lactate	0.52 ± 0.27	0.60 ± 0.05	0.56 ± 0.21	0.56 ± 0.18	18.32 ± 2.27
ethanol	12.76 ± 2.09	19.19 ± 0.23	10.89 ± 2.02	17.71 ± 2.28	15.69 ± 0.58
2,3-BDO	1.31 ± 0.28	0.89 ± 0.04	0.69 ± 0.07	1.09 ± 0.52	4.02 ± 1.99

*control fermentations were performed in the same reactor set up with electrodes at open circuit. **in BES condition the working electrode was poised at -0.603 V vs SHE w/o mediator. Fermentation time = 100 h. All fermentations were performed in biological triplicates.

4.4.3 DISCUSSION OF POSSIBLE UNDERLYING EET MECHANISMS

The characteristics of the redox metabolism of *C. autoethanogenum* are comparable to its close relative *C. ljungdahlii*, which is discussed in detail in chapter 4.1.3. **Figure 24** shows a simplified flux map of the core metabolism of *C. autoethanogenum* and its redox and energy metabolism. The heterotroph has no cytochromes and cellular redox pools are connected by specialised electron bifurcating enzymes. An excellent recent study by MOCK et al. delivered many answers to open questions on bioenergetics of the organism by detailed determination of co-factors of metabolic redox reactions.[299] It was found that despite containing a variety of hydrogenases *C. autoethanogenum* seems to use only one active hydrogenase, which is electron bifurcating with ferredoxin and $NADP^+$ as electron acceptors (Hyt: $2\ H_2 + Fd_{ox} + NADP^+ \leftrightarrow Fd_{red} + NADPH + 3\ H^+$).[299] In cells grown on fructose only, activity of a second hydrogenase specific for ferredoxin was found, but the activity of the electron bifurcating enzyme was still about 7 times higher.[299] Accordingly most other redox reactions use NADPH or ferredoxin as co-factors (see **Figure 24**). The second important electron bifurcating enzyme is the Nfn complex that catalyses the reduction of $NADP^+$ with reduced ferredoxin and NADH. Energy conservation other than substrate-level phosphorylation via acetate-kinase reaction (Ack) is provided via the Rnf-complex, a membrane-associated reduced ferredoxin:NAD^+-oxidoreductase creating a proton gradient that drives ATP synthesis. The stoichiometric assumptions made for proton transport of Rnf complex and ATPase in **Figure 24** are based on the analysis of MOCK et al.[299] It could be shown that Rnf activity contributes significantly to cellular energy yields as deletion of the complex in *C. ljungdahlii* resulted in reduced growth on fructose and no growth on H_2/CO_2.[224]

In the here presented experiments a metabolic shift towards lactate and 2,3-BDO was observed in a bioelectrochemical system on fructose. Both products present NADH sinks (see **Figure 24**) and therefore one could speculate that cathodic electron feed results in an increase of cellular NADH levels as seen for *C. pasteurianum*.[298] However, that assumption alone cannot explain the observed different effect between the three tested mediators. The redox potentials of the three molecules in the BES set up and under the given conditions (carbon cloth working electrode, modified medium 879, pH = 5.9, 37°C) were determined by cyclic voltammetry as shown in **Figure 25**: $[Co(sep)]^{3+}$ -340 mV, $[Co(AMMEsar)]^{3+}$ -370.5 mV and $[Co(trans\text{-}diammac)]^{3+}$ -526.5 mV. The values are in good agreement with given mid-point potentials at pH = 6 in literature with deviations of -30 mV, +1.5 mV and -11.5 mV, respectively.[297]

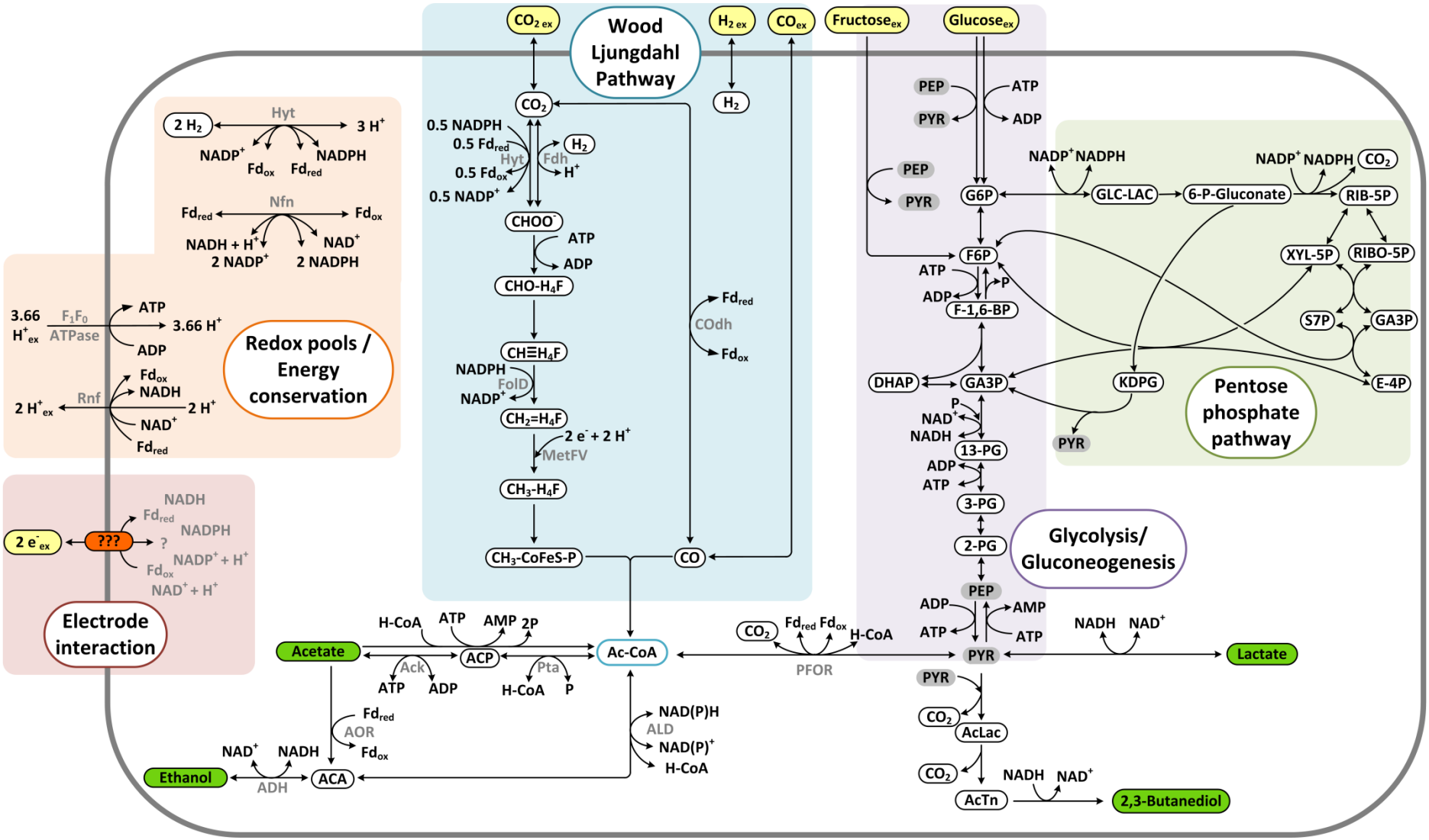


Figure 24: Metabolic fluxmap of the core carbon metabolism of *C. autoethanogenum* including redox and energy metabolism. Ack: Acetatekinase, ADH: alcohol dehydrogenase, ALD: CoA-dependent acetaldehyde dehydrogenase, AOR: acetaldehyde:ferredoxin oxidoreductase, COdh: carbon monoxide dehydrogenase, Fdh: formate dehydrogenase, FolD: Methylene-H_4F dehydrogenase, Hyt: electron-bifurcating NADP- and ferredoxin-depended hydrogenase, MetFV: Methylene-H_4F reductase (co-factors remain unknown), Nfn: electron-bifurcating ferredoxin-depended transhydrogenase, PFOR: Pyruvate:ferredoxin oxidoreductase, Pta: Phosphotransacetylase, Rnf: membrane associated and energy conserving reduced ferredoxin:NAD^+ oxidoreductase

Therefore, all three mediators should provide electrons at a potential low enough to feed into the cellular NADH pool (around -280 mV required, refer to the discussion in section 4.1.5). As discussed above the redox-pools of *C. autoethanogenum* depend on ferredoxin, H_2, NADH and NADPH. **Figure 25 B** shows the comparison of the measured redox potentials of mediators and cellular redox compounds and it can be seen that only a low potential electron supply such as provided in the case of [Co(*trans*-diammac)]$^{3+}$ can feed into the ferredoxin pool (-400 to – 500mV required). The Rnf complex is ferredoxin-dependent and the only known redox enzyme that is membrane associated in *C. autoethanogenum*.[299] In most cases of EET membrane-bound proteins facilitate the electron transfer and we have no reason to assume a free diffusion of the mediator molecules into the cytoplasm. Therefore, one possibility is an interaction of the redox mediators with the Rnf complex with only [Co(*trans*-diammac)]$^{3+}$ providing a low enough potential to reduce ferredoxin. If electrons would be directly transferred to the Rnf complex at a level comparable to ferredoxin, the enzyme could transfer the electrons further to NADH while transporting protons at the same time (see **Figure 24**). The resulting increase in NADH levels and proton gradient could provide an alternative ATP source to acetate production and therefore, be a driving force for the cell to shift carbon fluxes away from acetate in favour of NADH sinking products such as lactate and 2,3-BDO. However, further detailed studies are needed to elucidate the underlying mechanisms and provide evidence for EET routes in *C. autoethanogenum*.

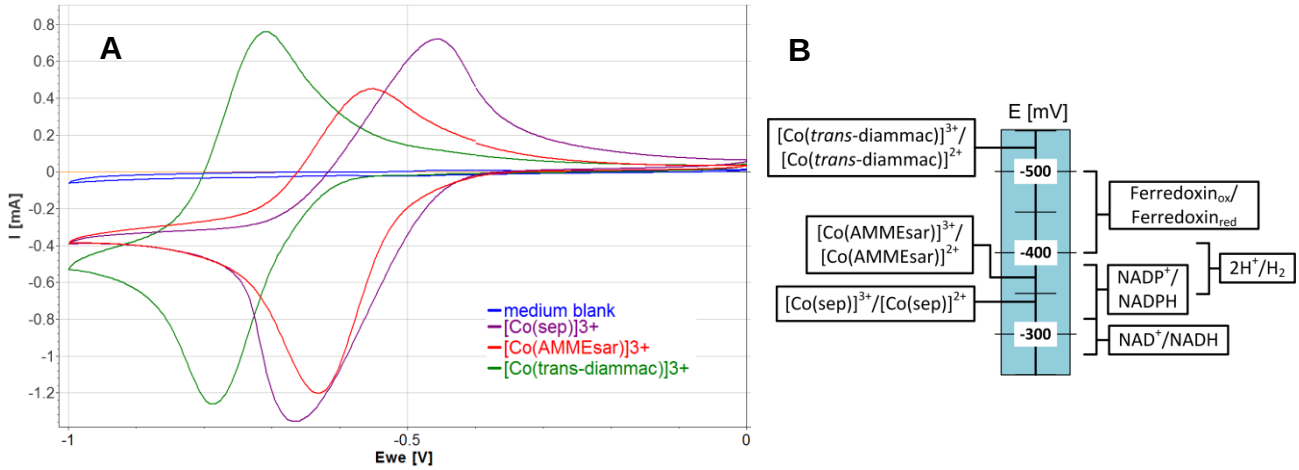


Figure 25: Redox potentials of the three mediators [Co(sep)]$^{3+}$, [Co(AMMEsar)]$^{3+}$ and [Co(*trans*-diammac)]$^{3+}$. (A) Cyclic voltammograms recorded in the bioelectrochemical system at 1 mV/s for 5 cycles, E_{WE} vs Ag/AgCl. **(B)** Schematic image of mediator redox potentials and redox windows for biological reduction of ferredoxin, NAD(P)$^+$ and H$^+$.

If indeed [Co(*trans*-diammac)]$^{3+}$ could successfully transfer electrons to drive the Rnf complex the mediator should be able to serve as sole electron source for autotrophic

growth on CO_2. First experiments following this approach showed small metabolic activity with the main product being acetate while little current was consumed (unpublished data). However, major growth limitations seem to occur. *C. autoethanogenum* is generally very difficult to grow on H_2 and CO_2 with a hydrogen threshold of >10%.[299] These difficulties have been linked to the energetics of the pathways with acetate and ethanol as final products; however, the exact bottlenecks remain unclear. When trying to replace hydrogen with a redox mediator as sole electron donor it is very likely that similar problems will complicate electrically mediated fermentations. The effects of cathodic electron supply mediated by the presented Co-molecules on autotrophic fermentations of *C. autoethanogenum* are the subject of ongoing studies.

4.4.4 Conclusion on electric fermentations of *C. autoethanogenum*

The presented study is the first to report on the electro-activity in the bio-chemical producing *C. autoethanogenum*. Production in acetogenic organisms is usually challenged by cellular energy limitations if the target product is not acetate. These results demonstrate a significant shift of carbon fluxes away from acetate towards electron dense products such as lactate and 2,3-butanediol by little electricity input.

The use of mediators with different redox potential revealed a direct dependency of the metabolic effect of the redox potential at which electrons are supplied. By analysing the electron bifurcating enzymes of *C. autoethanogenum* we hypothesize that electrons need to feed into the ferredoxin pool to cause the reported effect. Further studies are needed to unveil the exact processes of electron transfer. Nevertheless, the presented work demonstrates a promising concept of using redox-mediating molecules to provide electrons as a co-reducing power in heterotrophic fermentations to cause significant shifts towards reduced products at low electricity cost.

5 GENERAL DISCUSSION OF RESEARCH OUTCOMES

The main objective of the presented thesis was to analyze microbial electron transport chains with the aim to get a better understanding on how to channel cathodic electron feed towards target products.

In **Figure 26** the major research outcomes are summarized based on the schematic image from chapter 2 on research objectives (**Figure 6**). As highlighted in the introduction, MES has been demonstrated by proof of principle studies while major knowledge gaps about the underlying fundamental processes remain. The importance of understanding the mechanisms to advance bio-electrochemical technologies was emphasized in a recent review.[300] The study on microbial electron transport chains presented in chapter 4.1 addresses RO1 to investigate possibilities for EET routes for electron uptake. Microbial electron transport chains differ significantly between potential microbial hosts for MES such as metal reducing bacteria and acetogens. We show that the actual EET mechanism and therefore the process operation parameters directly depend on the special metabolic properties of the target species: Is it a GRAM-positive or –negative organism? Does it have outer membrane redox enzymes such as cytochromes? What are the theoretically required redox potentials for these enzymes? Questions like these often remain unasked even though they can help prioritize research efforts as discussed in this work. Being purely theoretical this part of the presented study cannot provide clear answers on which metabolic reactions are directly coupled to cathodic electron supply. However, the analysis shows the importance of cellular electron transport properties when designing BES processes. Furthermore, an estimation of effects on energy yields by electron supply is given as the redox metabolism that connects electron transport, carbon and energy metabolism also differs significantly between species. This is of major importance as it has a direct effect on recovery of electrons in the target product.

This rationale was pursued by developing a metabolic model for MES processes as proposed in RO2. The work presented in chapter 4.2 is one of the first modelling studies on MES, as the limited knowledge about the exact metabolic reactions makes it very difficult to design an accurate theoretical network.[97, 300, 301] Rather than just assuming one reaction of electrode-microbe interaction we propose different options for EET mechanisms and study their effect on production for the first time. The used approach of

elementary mode analysis offers the advantageous possibility of calculating every possible solution rather than one, which allows assessing the full metabolic capacities of the studied system. A drawback is usually the high computational capacity that is needed to process metabolic networks.[302] In the presented case the network was simplified to core carbon reactions thus minimizing the number of reactions, which resulted in EFM computations that could be performed on a normal desktop computer and in some cases with help of the high-performance computing cluster, Barrine, from The University of Queensland. A very recent study in the research area of metabolic network modeling reports the development of an advanced EMA tool, called FluxModeCalculator, which drastically reduces the required calculation cost.[303] This offers the possibility to complicate future models up to using full genome scale models while processing them on regular desktop computers.

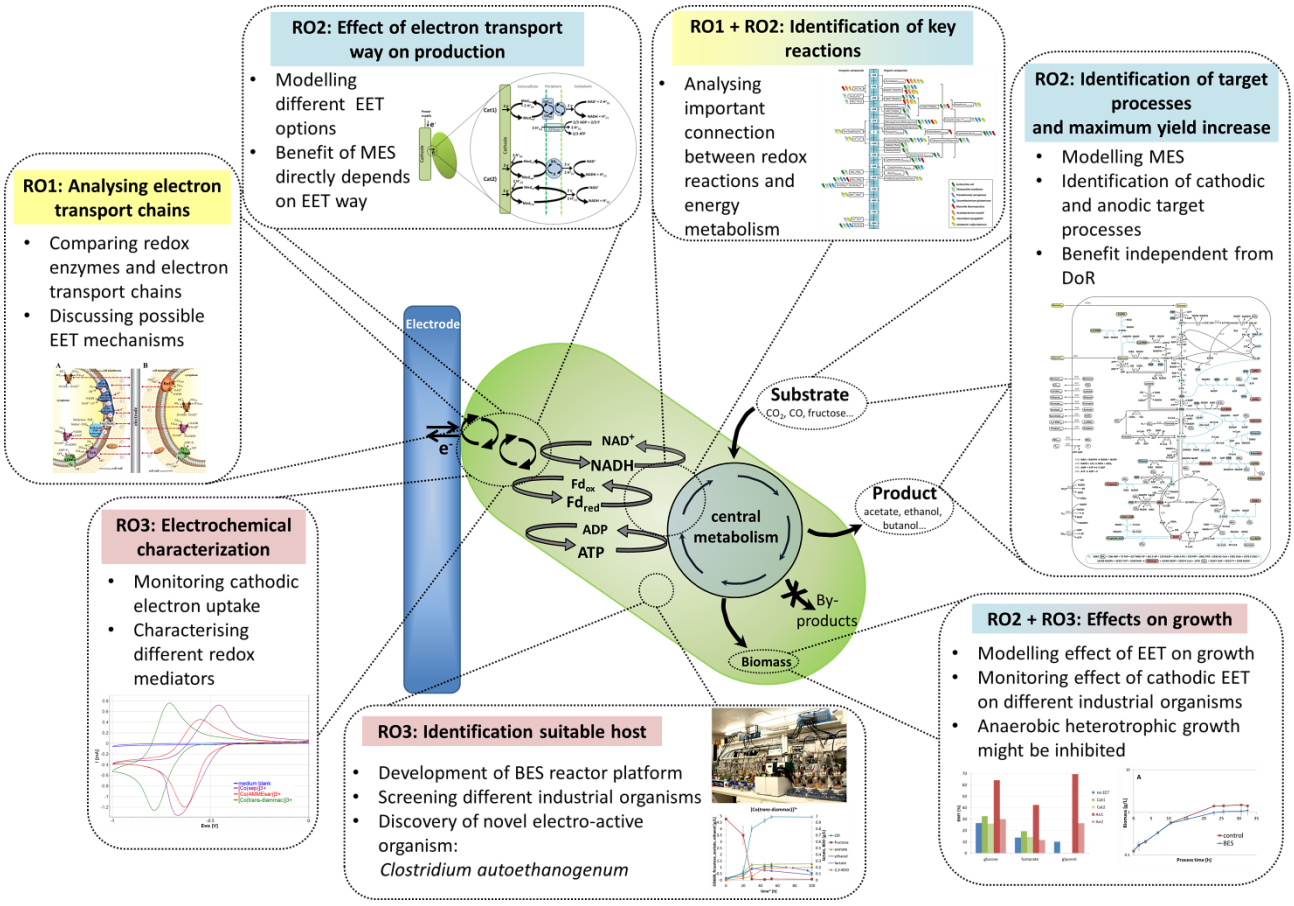


Figure 26: Overview of major research discoveries made according to each research objective (RO) on microbial electrosynthesis (MES). BES: bioelectrochemical system; EET: extracellular electron transfer; DoR: degree of reduction.

To address RO3, a BES reactor platform was developed and used for the *in vivo* screening experiments presented in chapter 4.3. We monitored current consumption for all tested organisms *Escherichia coli*, *Citrobacter werkmanii*, *Propionibacterium*

acidipropionici, *Propionibacterium freudenreichii,* and *Clostridium autoethanogenum*. However, the amount of electrons that were taken up was generally small compared to substrate consumption and let only in case of *C. autoethanogenum* to a significant metabolic shift. This might have various reasons. The chosen bacteria are no known electrogens such as *Geobacter* or *Shewanella* species. As discussed in the introduction electron transfer rates at the cathode are generally very low (see 1.3.3) and in our approach, a simultaneous electron source in the form of sugar is provided, which might be taken up at a higher rate. In *E. coli* fermentations we saw an example of the apparent differences between the mechanism of electron uptake and donation. While we observed a very low current consumption mediated by neutral red, the organisms is known to be able to use the mediator as final electron acceptor and create a much higher current under comparable conditions.[172, 173] *Propionibacterium acidipropionici* also consumed very little current, however in this case the electron uptake seemed to have an effect on cellular redox states strong enough to cause a shift in carbon flow. Unfortunately, the preferred electron sink seemed to be lactate rather than the target compound propionic acid. Nevertheless, the electro-activity of *P. acidipropionici* offers the possibility for further optimization of this process. For instance, metabolic engineering strategies could be followed to channel electron fluxes into propionic acid rather than lactate.

Following the predictions of the EMA study, the effect of extracellular electron supply on the production of 1,3-propanediol in *Citrobacter werkmanii* was tested. The modelling determined a possible yield increase of up to +36% on glucose and +17% for glycerol for an assumed EET model that connects redox and energy metabolism. Nevertheless, we could not achieve any increase in 1,3-PDO yield by electron supply via different mediators. This could be due to the very small electron uptake of maximal 7 $\mu M\ h^{-1}$. Also, other metabolic constraints that limit the 1,3-PDO production before stoichiometric limits are reached could play a role. Another explanation for not achieving any increase in product formation could be the underlying EET mechanism. If electron supply is uncoupled from energy conservation the *in silico* analysis predicts no improvement by electrical stimulation. However, further studies would be needed to determine the actual limitations for cathodic 1,3-PDO production *Citrobacter werkmanii*. In this project, in-depth characterisation concentrated on *C. autoethanogenum*, which showed a significant shift from acetate to lactate and 2,3-BDO induced by mediated electron supply.

The acetogen is a promising candidate for the industrial production of ethanol and acetate from waste gases.[289, 290] Even though tools for metabolic and genetic engineering of acetogens are under intense development, there are major challenges when trying to shift the production away from the major product acetate. Not only a large number of enzymes and regulatory mechanisms have to be engineered but also pathway energetics have to be met.[221] If carbon flux is channelled away from ATP generating enzymes such as acetate kinase a replacement energy source needs to be incorporated to provide a thermodynamically feasible scenario.[299] Here we observed a decrease of acetate production by 55% by the simple approach of supplying very little electrical energy in heterotrophic fermentations on fructose. The simultaneous use of a heterotrophic carbon source and gases, called mixotrophy, was recently proposed as an option to increase yields for electron dense products in acetogens.[304] In this concept the Wood Ljungdahl pathway is used to re-fix the two molecules of CO_2 from decarboxylation reactions during glycolysis. The electrons needed for this fixation are supposed to be provided from reducing equivalents created during glycolysis as well. However, as discussed before the reactions in WLP are NADPH and ferredoxin-dependent, while glycolysis produces NADH (refer to **Figure 24**). This could potentially be optimized by extracellular electron supply feeding into the ferredoxin pool. As discussed in section 4.4.3 we hypothesize that the electron supply by the low potential mediator $[Co(\textit{trans}\text{-diammac})]^{3+}$ could interact with the membrane bound Rnf complex of *C. autoethanogenum* and therefore contribute to intracellular NADH levels as well as the membrane gradient, which drives ATP synthesis. A direct connection to energy conservation together with the electron bifurcating enzymes could explain why little electron uptake (2 mM) results in a major metabolic shift (+10 mM lactate) away from the acetate kinase reaction. The presented results display a promising approach to improve production in *C. autoethanogenum* and further studies under autotrophic conditions are underway to fully elucidate the underlying fundamental processes.

6 CONCLUSION AND FUTURE PERSPECTIVES[4]

6.1 MAIN CONCLUSIONS

This work analyses the current knowledge on electron transport processes, uses a theoretical approach to evaluate benefits and limitations of MES technologies and studies the effects on electron uptake on production in the novel electrogen *C. autoethanogenum*.

We presented a detailed characterization of the metabolic features of potential hosts for microbial electrosynthesis and emphasize the importance that specific enzymes such as the diverse cytochromes of *Geobacter* species or the Rnf complex of acetogens have for the use in MES technologies. The *in silico* screening of selected metabolites for increased production by electron supply presents a pioneer study in the field and revealed two interesting phenomena: First of all, the degree of reduction of a product cannot predict whether its production might benefit from electron supply. Instead, the metabolic conversions that lead from one carbon source to a certain product decide if there is an electron surplus or electron need within the pathway. Secondly, we found that the theoretical yield increase that is possible for each product directly depends on the transport pathways by which electrons enter the metabolism.

The *in vivo* study on heterotrophic, cathodic cultures of different industrial organisms identified the ethanol producing acetogen *Clostridium autoethanogenum* as a novel electrogen. Extracellular electron supply by mediators cut acetate production by more than half while production of lactate and 2,3-BDO increased thirty-five fold and three fold, respectively. In agreement with the findings from the *in silico* study, we found that the redox potential at which the electrons are provided is of critical importance for this effect.

Understanding the underlying phenomena of cathodic EET is needed to increase electron uptake rates and optimize and advance the technology of MES towards application. The presented work adds an important piece of fundamental understanding of microbial electron transport possibilities to the research community and will help to prioritize research focus towards the metabolic electron flow upstream from the cathode.

[4] This chapter includes an economic feasibility study that was published as part of: Harnisch, F., Rosa, L.F.M., **Kracke, F.**, Virdis, B., and Krömer, J.O. (2015). *Electrifying White Biotechnology: Engineering and Economic Potential of Electricity-Driven Bio-Production.* ChemSusChem 8, 758-766. doi: 10.1002/cssc.201402736

6.2 FUTURE PERSPECTIVES

This work is concluded with a theoretical feasibility study on economics of MES to demonstrate there is a realistic market potential for sustainable electrified bioproduction processes. The section was drafted together with Dr Jens O. Krömer and was published as part of HARNISCH et al. 2015 and also featured on the cover of ChemSusChem 05/2015 (see appendix 8.3).[83] The production of lysine was chosen as model process and compare costs for bio-production based on sucrose and electric energy.

Lysine as a bulk chemical reached a global market volume of around 1.9 million tons per annum in 2013. The lysine price is between 1.6 and 2.4 $US per kg,[305] so very similar to any "value added" bulk chemical in the market. Hence, the production of lysine can be used as a model for other compounds (similar yields required, product value, scale, titer etc.). The typical bioprocesses are run at 250 kL scale in a representative fermentation facility. Normally, a parallel system is used (some processes running while others get harvested, cleaned, reconditioned, etc.) and, depending on batch time, two or three systems may run in parallel. Conservatively, we can assume that this facility would produce about 50 000 t per year. At a typical lysine titer of 120 g L^{-1},[306] one would need 1666 batches (à 30 h) per year with 30 tons of lysine equaling 205,212 mol lysine per batch ($M_{W,lysine}$ = 146.19 $gmol^{-1}$). This would mean that about six vessels would need to work non-stop; thus, the plant would probably need 18 fermenters to allow for downtime after and before the fermentation. Now, we can draw two scenarios for comparing sucrose-based and electricity-driven bio-production (both also being summarized in **Figure 27**, details can be found in the appendix under 8.2).

Scenario 1: Sucrose based bio-production

The theoretical yield of lysine per glucose is 620 mg lysine per gram of glucose[307] at a productivity of 4 g L^{-1} h^{-1}.[306] If we realistically assume that we could reach about 90% of the theoretical yield, we would get about 0.56 g lysine per gram glucose or 0.69 mol_{lysine} per mol_{hexose}. Assuming that the hexose would be provided in the form of sucrose, each batch would require 148,704 mol of sucrose ($M_{W,sucrose}$ = 342.3 g/mol), equaling to 50.9 t of sucrose.

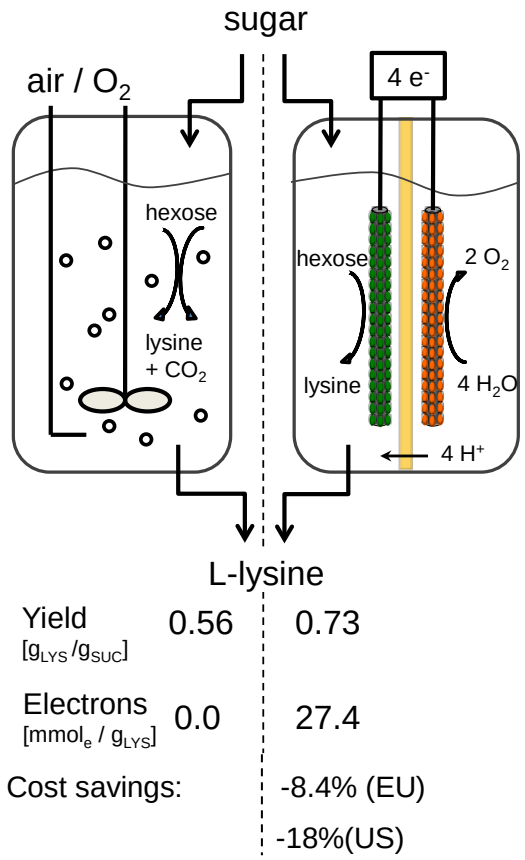


Figure 27: Example of lysine production from sucrose in a conventional fermentation process (left) versus bioelectrochemical system (right). Mass yields and electron demand were predicted using stoichiometric network analysis. The values assume that 90% of the theoretical maximum is achievable. Cost savings of 8.4% (in EU) and 18.0% (in US) could be anticipated if the necessary yields can be obtained. Modified from HARNISCH et al. 2015.[83]

Scenario 2: Cathode-driven bio-production

The theoretical yield of lysine on glucose and electrons (i.e., all redox-power is provided by the cathode) is 1 mol per mol hexose.[191] Again, we assume that it should be possible to reach 90% of the theoretical yield and that the system would reach a coulombic efficiency of 100%, the amount of sucrose needed to produce 30 t of lysine per batch is 39 t, that is, 114 006mol (refer to 8.2 in the appendix for calculations). Furthermore, when converting glucose to lysine the degree of reduction of the carbon does change and 4 mol of electrons are needed per mol of lysine, equaling 820 848 mol of electrons per batch that have to be delivered additionally as electricity. When assuming 365 days of operation to produce 50 000 t per year, the average production per day is 137 t per day, equaling to 937 kmol of lysine per day. Consequently, 3748 kmol d^{-1} of electrons are needed (amounting to a charge of 362×10^6 kC d^{-1}). When comparing this to the charge can be delivered in cathodic BES to date (current highest value: 102 kC d^{-1})[83, 308] one can clearly see that substantial effort in research and development is needed. When speculating on

this further, one can translate this into a continuous current flow of 4.2×10^6 A needed for production. When now looking at 3D-electrode materials, still possessing a good potential for further development, one can calculate the electrode area and electrode volume needed. For microbial anodes the highest current densities per projected surface area are roughly 100 A m^{-2};[79, 309] however, the highest reported current density for a biocathode is currently 37 Am^{-2} using RVC (reticulated vitreous carbon) electrodes with a surface density of 2,620 $m^2\,m^{-3}$.[308] When considering this number, an electrode surface area of 113×10^3 m^2 (equal to a RVC-electrode volume of 43 m^3) would be required. Although the reactor volume would also be determined by other parameters (vide supra), this already shows that BESs can come to a scale comparable to established fermenters.

Cost of both scenarios:

Overall, for the cathode-driven bio-production (*scenario 2*) 11.9 t less sucrose (50.9 t vs.39 t) would be needed to produce an identical amount (30 t) of lysine per batch, compared to *scenario 1*. However, 820,848 mol of electrons need to be acquired in *scenario 2*. First, let's consider the costs of sucrose (US: 38.75 ctUS per kg; EU: 45.85 ctUS/kg – see **Table 21** in the appendix). If the process runs in the USA the costs of sucrose per batch are 19.7 kUS$ for *scenario 1* and 15.1 kUS$ for *scenario 2*, resulting in a saving of 4.6 kUS$ per batch. For the EU the numbers differ slightly with 23.3 k$US for scenario 1 and 17.9 k$US for scenario 2 resulting in a saving of 5.4 k$US for electricity driven bio-production. However, this saving has to be used for electricity costs first. When considering a cell voltage of the BES of 1 V and the moles of electrons needed per batch, the costs of electric energy for electrosynthesis per batch are roughly 1.6 kUS$ in the USA and roughly 3.6 kUS$ in the EU (see **Table 22** in the appendix).

Summing up, this means for a comparison of both scenarios that, if lysine is to be produced in the EU using electricity rather than sucrose oxidation for generating redox-power, the substrate cost (sucrose and electrons) is 21.5 kUS$ per batch of 30 t lysine. In the USA, the total cost would be 16.7 k$US. This equates to a total saving on substrate cost of 8.4% (EU) and 18.0% (USA), respectively. At the current prices and assuming that 90% of the theoretical yields could be achieved, these savings equate over a 10 year period for our 50 000 t per annum plant to 30 million US$ (EU) and 50 million US$ (US), respectively. Depending on how far BES costs can be reduced this may or may not be a significant saving that warrants the investment.

6.3 RECOMMENDATIONS FOR FUTURE RESEARCH

The above presented case study of electrically enhanced lysine production demonstrates a realistic potential of MES technologies to optimize bioproduction processes. However, as discussed still many hurdles have to be overcome to achieve the required yields and electron transfer rates to enable economical feasible sustainable MES processes in reality. The investigations carried out in this thesis lead to the following research questions that should be focus of future studies in the field:

Advanced metabolic modelling of electrode-microbe interactions

The elementary mode analysis presented in chapter 4.2 was successfully used for the identification of target processes for MES at the example of *E. coli*. The approach can be transferred to other organisms to gather novel information about the theoretical impact of extracellular electron exchange on metabolic carbon fluxes. This can be used to develop production strategies for target compounds (e.g. identification of knockout targets). We already developed several metabolic models of different *Clostridia* strains to model carbon flow under autotrophic and heterotrophic conditions when additional electrons are provided (data not shown in this manuscript). The approach of modeling different EET scenarios can be further pursued by comparing experimentally determined parameters such as NAD^+/NADH ratios and ATP yields with the results of modeling studies for different EET routes. This could help prioritize further research efforts to unveil the underlying fundamental mechanisms of EET. The recent improvement of advanced tools in the field of flux mode analysis will also enable a much faster computation of the studied networks.[303] Thus, much larger metabolic networks can be processed, which enables the study of full genome-scale networks. This will present a more complex view of the metabolic processes during electrically enhanced fermentations.

Co-based compounds as redox mediators in electrified bioproduction processes

In the presented study macrocyclic cobalt-hexaamines were used to study the effect of redox potential in cathodic processes. While this study focused the underlying fundamental processes, the results indicate a great potential of the cobalt complexes to be used as electron shuttles in MES processes. The introduction discusses advantages and disadvantages of using mediators in electrically enhanced fermentations in more detail (see chapter 1.3.2). The here used compounds offer several distinct advantages: The compounds are highly biocompatible, show a low toxicity and therefore enable production

in bulk at high cell densities. In the presented fermentations with different organisms (see section 4.3.2) we did not observe a significant change in maximum growth yields at the concentrations of 1 mM while neutral red for instance inhibited anaerobic growth of *E. coli* already at concentrations of 100 μM (data not shown). Another advantage of using cobalt hexaamines as electron-transfer agents is their rapid electron transfer characteristics and high stability in the II and III oxidation states (at around neutral pH).[310] Therefore it would be of great interest to test their limitations in BES for production. First steps could be to determine toxicity thresholds for different organisms and exact electron transfer kinetics. The compounds have also been shown to facilitate hydrogen evolution directly at the cathode surface by decreasing the activation potentials.[310] Therefore these complexes could also improve MES processes aiming for CO_2 reduction mediated by electrically produced hydrogen.

The Co-hexaamines show a robust cycle between reductive and oxidative state with no decomposition of the molecule and are available at different midpoint potentials.[297, 310] They are thermo-stable (>200°C) and therefore potentially display no complication for downstream processing for instance if the target product is more volatile. Further studies are needed to identify possible beneficial MES processes and compatible organisms for these mediators and to investigate their potential use beyond lab-scale studies.

C. autoethanogenum as platform organism for MES

The results presented in section 4.4 of this thesis demonstrate for the first time the capability of electro-activity in *C. autoethanogenum*. As discussed the acetogen presents an attractive host for production of chemicals by its natural increased production of alcohols and is already used in industrial processes. It will be of great interest to further study the effect of extracellular electron supply on the production in *C. autoethanogenum* and to optimize the process.

This work emphasizes the great importance of understanding the electron transport mechanisms to advance MES technologies. Therefore, a first suggestion is to undertake detailed studies of the organism under BES and control conditions to shed light on the fundamental redox mechanisms. Measurements of intracellular $NAD(P)^+$/NAD(P)H and ATP levels could deliver information of shifts in specific redox pools depending on the potential of the used mediator. The results could be combined with stoichiometric modelling of different EET scenarios, comparable to what we presented for *E. coli,* to predict the way of electron uptake. We already developed a metabolic model for

C. autoethanogenum that is used for this purpose (data not shown). Furthermore analysis of the transcriptome and the proteome could be used to detect differences in protein expression and therefore indicate key enzymes for EET.

Another approach is presented by the use of knockout strains. A Rnf deficient mutant, for instance, could be tested for current consumption under mediated heterotrophic conditions to study the role of this important membrane complex in the process. In our discussion in chapter 4.4.3, we hypothesize that the low potential mediator [Co(*trans*-diammac)]$^{3+}$ mediates electron transport between the cathode and the intracellular ferredoxin pool potentially via Rnf complex. If this is the case electrons provided by [Co(*trans*-diammac)]$^{3+}$ should also enable autotrophic growth on pure CO_2 (see discussion in 4.4.3). The investigation of autotrophic *C. autoethanogenum* fermentations in a BES under different conditions are focus of ongoing studies in CEMES.

Based on the results presented in this work and ongoing projects research focus should shift towards process development. With novel knowledge about the underlying EET mechanisms, one can aim to increase electron transfer rates, for instance by overexpressing cellular key enzymes or optimizing BES conditions such as electrode materials, redox potential and mediator concentration. Metabolic engineered strains such as an acetate kinase deficient mutant, a Rnf overproducer or a strain modified for 2,3-butanediol production could open the field for sustainable production in *Clostridia* boosted by electric energy.

7 REFERENCES

1. Mohanty, A. K.; Misra, M.; Drzal, L. T., Sustainable Bio-Composites from Renewable Resources: Opportunities and Challenges in the Green Materials World. *J Polym Environ* **2002,** 10, (1), 19-26.
2. Demirbas, M. F.; Balat, M., Recent advances on the production and utilization trends of bio-fuels: A global perspective. *Energ Conserv Manage* **2006,** 47, (15–16), 2371-2381.
3. Jones, D. T.; Woods, D. R., Acetone-butanol fermentation revisited. *Microbiol. Rev.* **1986,** 50, 484-524.
4. Lovley, D. R., Powering microbes with electricity: direct electron transfer from electrodes to microbes. *Env Microbiol Rep* **2011,** 3, (1), 27-35.
5. Rabaey, K.; Rozendal, R. A., Microbial electrosynthesis — revisiting the electrical route for microbial production. *Nat Rev Micro* **2010,** 8, (10), 706-716.
6. Emde, R.; Schink, B., Enhanced Propionate Formation by *Propionibacterium freudenreichii* subsp. *freudenreichii* in a Three-Electrode Amperometric Culture System. *Appl. Environ. Microbiol.* **1990,** 56, (9), 2771-2776.
7. Gregory, K. B.; Bond, D. R.; Lovley, D. R., Graphite electrodes as electron donors for anaerobic respiration. *Environ. Microbiol.* **2004,** 6, (6), 596-604.
8. Hongo, M.; Iwahara, M., Electrochemiacal studies on fermentation. Application of electro-energizing method to L-glutamic acid fermentation. *Agric. Biol. Chem.* **1979**.
9. Nevin, K. P.; Woodard, T. L.; Franks, A. E.; Summers, Z. M.; Lovley, D. R., Microbial Electrosynthesis: Feeding Microbes Electricity To Convert Carbon Dioxide and Water to Multicarbon Extracellular Organic Compounds. *mBio* **2010,** 1, (2).
10. Dumas, C.; Basseguy, R.; Bergel, A., Microbial electrocatalysis with *Geobacter sulfurreducens* biofilm on stainless steel cathodes. *Electrochim. Acta* **2008,** 53, (5), 2494-2500.
11. Patil, S., A.; Haegerhaell, C.; Gortin, L., Electron transfer mechanisms between microorganisms and electrodes in bioelectrochemical systems. *Bioanal Rev* **2012,** 4, 159-192.
12. Strycharz, S. M.; Glaven, R. H.; Coppi, M. V.; Gannon, S. M.; Perpetua, L. A.; Liu, A.; Nevin, K. P.; Lovley, D. R., Gene expression and deletion analysis of mechanisms for electron transfer from electrodes to *Geobacter sulfurreducens*. *Bioelectrochemistry* **2011,** 80, (2), 142-150.
13. Franks, A., What's Current with Electric Microbes? *Journal of Bacteriology & Parasitology* **2012**.
14. Lovley, D. R., Electromicrobiology. *Annu. Rev. Microbiol.* **2012,** 66, (1), null.
15. Yang, Y.; Xu, M.; Guo, J.; Sun, G., Bacterial extracellular electron transfer in bioelectrochemical systems. *Process Biochem.* **2012,** 47, (12), 1707-1714.
16. Clauwaert, P.; Aelterman, P.; De Schamphelaire, L.; Carballa, M.; Rabaey, K.; Verstraete, W., Minimizing losses in bio-electrochemical systems: the road to applications. *Appl. Microbiol. Biotechnol.* **2008,** 79, (6), 901-913.
17. Bard, A. J.; Faulkner, L. R., *Electrochemical Methods: Fundamentals and Applications*. John Wiley & Sons, Inc: New York, 2001.
18. Hamelers, H. M.; Heijne, A.; Sleutels, T. J. A.; Jeremiasse, A.; Strik, D. B. T. B.; Buisman, C. N., New applications and performance of bioelectrochemical systems. *Appl. Microbiol. Biotechnol.* **2010,** 85, (6), 1673-1685.
19. Rabaey, K., *Bioelectrochemical systems: from extracellular electron transfer to biotechnological application*. International Water Assn: 2010.
20. Franks, A. E.; Nevin, K. P., Microbial Fuel Cells, A Current Review. *Energies* **2010,** 3, (5), 899-919.
21. Rabaey, K.; Boon, N.; Siciliano, S. D.; Verhaege, M.; Verstraete, W., Biofuel Cells Select for Microbial Consortia That Self-Mediate Electron Transfer. *Appl. Environ. Microbiol.* **2004,** 70, (9), 5373-5382.
22. Pant, D.; Van Bogaert, G.; Diels, L.; Vanbroekhoven, K., A review of the substrates used in microbial fuel cells (MFCs) for sustainable energy production. *Bioresour. Technol.* **2010,** 101, (6), 1533-1543.

23. Dewan, A.; Beyenal, H.; Lewandowski, Z., Scaling up Microbial Fuel Cells. *Environ. Sci. Technol.* **2008,** 42, (20), 7643-7648.
24. Wang, H.; Park, J.-D.; Ren, Z. J., Practical Energy Harvesting for Microbial Fuel Cells: A Review. *Environ. Sci. Technol.* **2015,** 49, (6), 3267-3277.
25. Williams, K. H.; Nevin, K. P.; Franks, A.; Englert, A.; Long, P. E.; Lovley, D. R., Electrode-Based Approach for Monitoring In Situ Microbial Activity During Subsurface Bioremediation. *Environ. Sci. Technol.* **2009,** 44, (1), 47-54.
26. Zhang, T.; Gannon, S. M.; Nevin, K. P.; Franks, A. E.; Lovley, D. R., Stimulating the anaerobic degradation of aromatic hydrocarbons in contaminated sediments by providing an electrode as the electron acceptor. *Environ. Microbiol.* **2010,** 12, (4), 1011-1020.
27. Call, D.; Logan, B. E., Hydrogen production in a single chamber microbial electrolysis cell lacking a membrane. *Environ. Sci. Technol.* **2008,** 42, (9), 3401-3406.
28. Wagner, R. C.; Regan, J. M.; Oh, S.-E.; Zuo, Y.; Logan, B. E., Hydrogen and methane production from swine wastewater using microbial electrolysis cells. *Water Res.* **2009,** 43, (5), 1480-1488.
29. Potter, M. C., Electrical Effects Accompanying the Decomposition of Organic Compounds. *Proc. R. Soc. Lond. B. Biol. Sci.* **1911,** 84, (571), 260-276.
30. Cheng, S.; Liu, H.; Logan, B. E., Increased power generation in a continuous flow MFC with advective flow through the porous anode and reduced electrode spacing. *Environ. Sci. Technol.* **2006,** 40, (7), 2426-2432.
31. Rabaey, K.; Read, S. T.; Clauwaert, P.; Freguia, S.; Bond, P. L.; Blackall, L. L.; Keller, J., Cathodic oxygen reduction catalyzed by bacteria in microbial fuel cells. *ISME J* **2008,** 2, (5), 519-27.
32. Freguia, S.; Rabaey, K.; Yuan, Z.; Keller, J., Sequential anode-cathode configuration improves cathodic oxygen reduction and effluent quality of microbial fuel cells. *Water Res.* **2008,** 42, (6-7), 1387-1396.
33. Zhao, F.; Harnisch, F.; Schröder, U.; Scholz, F.; Bogdanoff, P.; Herrmann, I., Challenges and constraints of using oxygen cathodes in microbial fuel cells. *Environ. Sci. Technol.* **2006,** 40, (17), 5193-5199.
34. Logan, B. E., Exoelectrogenic bacteria that power microbial fuel cells. *Nat Rev Micro* **2009,** 7, (5), 375-381.
35. Rabaey, K.; Rodriguez, J.; Blackall, L., L; Keller, J.; Gross, P.; Batstone, D.; Verstraete, W.; Nealson, K., H, microbial ecology meets electrochemistry: electricity-driven and driving communities. *ISME J* **2007,** 1, 9-18.
36. Dunaj, S. J.; Vallino, J. J.; Hines, M. E.; Gay, M.; Kobyljanec, C.; Rooney-Varga, J. N., Relationships between Soil Organic Matter, Nutrients, Bacterial Community Structure, And the Performance of Microbial Fuel Cells. *Environ. Sci. Technol.* **2012,** 46, (3), 1914-1922.
37. Yi, H.; Nevin, K. P.; Kim, B.-C.; Franks, A. E.; Klimes, A.; Tender, L. M.; Lovley, D. R., Selection of a variant of *Geobacter sulfurreducens* with enhanced capacity for current production in microbial fuel cells. *Biosensors Bioelectron.* **2009,** 24, (12), 3498-3503.
38. Jang, J. K.; Chang, I. S.; Kang, K. H.; Moon, H.; Cho, K. S.; Kim, B. H., Construction and operation of a novel mediator-and membrane-less microbial fuel cell. *Process Biochem.* **2004,** 39, (8), 1007-1012.
39. Liu, H.; Cheng, S.; Logan, B. E., Production of electricity from acetate or butyrate using a single-chamber microbial fuel cell. *Environ. Sci. Technol.* **2005,** 39, (2), 658-662.
40. Liu, H.; Ramnarayanan, R.; Logan, B. E., Production of electricity during wastewater treatment using a single chamber microbial fuel cell. *Environ. Sci. Technol.* **2004,** 38, (7), 2281-2285.
41. Logan, B. E.; Hamelers, B.; Rozendal, R.; Schröder, U.; Keller, J.; Freguia, S.; Aelterman, P.; Verstraete, W.; Rabaey, K., Microbial fuel cells: methodology and technology. *Environ. Sci. Technol.* **2006,** 40, (17), 5181-5192.
42. Lovley, D. R.; Nevin, K. P., A shift in the current: New applications and concepts for microbe-electrode electron exchange. *Curr. Opin. Biotechnol.* **2011,** 22, (3), 441-448.
43. Lovley, D. R., Bug juice: harvesting electricity with microorganisms. *Nat. Rev. Microbiol.* **2006,** 4, (7), 497-508.
44. Yang, Y.; Sun, G.; Xu, M., Progress of research on the microbial fuel cells in the application of environment pollution treatment--a review. *Acta Microbiol. Sin.* **2010,** 50, (7), 847.

45. Du, Z.; Li, H.; Gu, T., A state of the art review on microbial fuel cells: a promising technology for wastewater treatment and bioenergy. *Biotechnol. Adv.* **2007,** 25, (5), 464-482.
46. Rozendal, R. A.; Hamelers, H. V.; Rabaey, K.; Keller, J.; Buisman, C. J., Towards practical implementation of bioelectrochemical wastewater treatment. *Trends Biotechnol.* **2008,** 26, (8), 450-459.
47. Luo, H.; Xu, P.; Roane, T. M.; Jenkins, P. E.; Ren, Z., Microbial desalination cells for improved performance in wastewater treatment, electricity production, and desalination. *Bioresour. Technol.* **2012,** 105, 60-66.
48. Foley, J. M.; Rozendal, R. A.; Hertle, C. K.; Lant, P. A.; Rabaey, K., Life cycle assessment of high-rate anaerobic treatment, microbial fuel cells, and microbial electrolysis cells. *Environ. Sci. Technol.* **2010,** 44, (9), 3629-3637.
49. Logan, B. E.; Call, D.; Cheng, S.; Hamelers, H. V.; Sleutels, T. H.; Jeremiasse, A. W.; Rozendal, R. A., Microbial electrolysis cells for high yield hydrogen gas production from organic matter. *Environmental Science & Technology* **2008,** 42, (23), 8630-8640.
50. Logan, B. E.; Rabaey, K., Conversion of Wastes into Bioelectricity and Chemicals by Using Microbial Electrochemical Technologies. *Science* **2012,** 337, (6095), 686-690.
51. Cusick, R. D.; Logan, B. E., Phosphate recovery as struvite within a single chamber microbial electrolysis cell. *Bioresour. Technol.* **2012,** 107, 110-115.
52. Villano, M.; Scardala, S.; Aulenta, F.; Majone, M., Carbon and nitrogen removal and enhanced methane production in a microbial electrolysis cell. *Bioresour. Technol.* **2012**.
53. Heidrich, E.; Dolfing, J.; Scott, K.; Edwards, S.; Jones, C.; Curtis, T., Production of hydrogen from domestic wastewater in a pilot-scale microbial electrolysis cell. *Appl. Microbiol. Biotechnol.* **2012**, 1-11.
54. Jeremiasse, A. W.; Hamelers, H. V.; Buisman, C. J., Microbial electrolysis cell with a microbial biocathode. *Bioelectrochemistry* **2010,** 78, (1), 39-43.
55. Rozendal, R. A.; Jeremiasse, A. W.; Hamelers, H. V.; Buisman, C. J., Hydrogen production with a microbial biocathode. *Environ. Sci. Technol.* **2007,** 42, (2), 629-634.
56. Rabaey, K.; Rozendal, R. A., Microbial electrosynthesis—revisiting the electrical route for microbial production. *Nat. Rev. Microbiol.* **2010,** 8, (10), 706-716.
57. Kim, T. S.; Kim, B. H., Electron flow shift in *Clostridium acetobutylicum* by electrochemically introduced reducing equivalent. *Biotechnol. Lett.* **1988,** 10, 123-128.
58. Choi, O.; Um, Y.; Sang, B.-I., Butyrate production enhancement by Clostridium tyrobutyricum using electron mediators and a cathodic electron donor. *Biotechnol. Bioeng.* **2012,** 109, (10), 2494-2502.
59. Jain, H. S. J. Z. M., Electrically enhanced ethanol fermentation by *Clostridium thermocellum* and *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **2002,** 58, (4), 476-481.
60. Steinbusch, K. J. J.; Hamelers, H. V. M.; Schaap, J. D.; Kampman, C.; Buisman, C. J. N., Bioelectrochemical Ethanol Production through Mediated Acetate Reduction by Mixed Cultures. *Environ. Sci. Technol.* **2009,** 44, (1), 513-517.
61. Marshall, C. W.; LaBelle, E. V.; May, H. D., Production of fuels and chemicals from waste by microbiomes. *Curr. Opin. Biotechnol.* **2013,** 24, (3), 391-397.
62. Lim, X., How to make the most of carbon dioxide. *Nat. News* **2015,** 526, 628-630.
63. Peplow, M., Industrial biotechs turn greenhouse gas into feedstock opportunity. *Nat. Biotechnol.* **2015,** 33, (11), 1123-1125.
64. Desloover, J.; Arends, J.; Hennebel, T.; Rabaey, K., Operational and technical considerations for microbial electrosynthesis. *Biochem. Soc. Trans.* **2012,** 40, (6), 1233-1238.
65. Rabaey, K.; Girguis, P.; Nielsen, L. K., Metabolic and practical considerations on microbial electrosynthesis. *Curr. Opin. Biotechnol.* **2011,** 22, (3), 371-377.
66. Nevin, K. P.; Hensley, S. A.; Franks, A. E.; Summers, Z. M.; Ou, J.; Woodard, T. L.; Snoeyenbos-West, O. L.; Lovley, D. R., Electrosynthesis of Organic Compounds from Carbon Dioxide Is Catalyzed by a Diversity of Acetogenic Microorganisms. *Appl. Environ. Microbiol.* **2011,** 77, (9), 2882-2886.
67. Gildemyn, S.; Verbeeck, K.; Slabbinck, R.; Andersen, S. J.; Prévoteau, A.; Rabaey, K., Integrated Production, Extraction, and Concentration of Acetic Acid from CO_2 through Microbial Electrosynthesis. *ES&T Let.* **2015**.

68. Jourdin, L.; Grieger, T.; Monetti, J.; Flexer, V.; Freguia, S.; Lu, Y.; Chen, J.; Romano, M.; Wallace, G. G.; Keller, J., High acetic acid production rate obtained by microbial electrosynthesis from carbon dioxide. *Environ. Sci. Technol.* **2015**.
69. Villano, M.; Aulenta, F.; Ciucci, C.; Ferri, T.; Giuliano, A.; Majone, M., Bioelectrochemical reduction of CO_2 to CH_4 via direct and indirect extracellular electron transfer by a hydrogenophilic methanogenic culture. *Bioresour. Technol.* **2010,** 101, (9), 3085-3090.
70. Jiang, Y.; Su, M.; Zhang, Y.; Zhan, G.; Tao, Y.; Li, D., Bioelectrochemical systems for simultaneously production of methane and acetate from carbon dioxide at relatively high rate. *Int. J. Hydrogen Energ.* **2013,** 38, (8), 3497-3502.
71. Ganigué Pagès, R.; Puig Broch, S.; Batlle Vilanova, P.; Balaguer i Condom, M. D.; Colprim Galceran, J., Microbial electrosynthesis of butyrate from carbon dioxide. *© Chemical Communications, 2015, vol. 51, núm. 15, p. 3235-3238* **2015**.
72. Shin, H. S.; Jain, M. J.; Chartrain, M. C.; Zeikus, J. Z., Evaluation of an electrochemical bioreactor system in the biotransformation of 6-bromo-2-tetralone to 6-bromo-2-tetralol. *Appl. Microbiol. Biotechnol.* **2001,** 57, (4), 506-510.
73. Gong, Y.; Ebrahim, A.; Feist, A. M.; Embree, M.; Zhang, T.; Lovley, D.; Zengler, K., Sulfide-Driven Microbial Electrosynthesis. *Environ. Sci. Technol.* **2012,** 47, (1), 568-573.
74. Park, D. H.; Laivenieks, M.; Guettler, M. V.; Jain, M. K.; Zeikus, J. G., Microbial Utilization of Electrically Reduced Neutral Red as the Sole Electron Donor for Growth and Metabolite Production. *Appl. Environ. Microbiol.* **1999,** 65, (7), 2912-2917.
75. Marshall, C. W.; Ross, D. E.; Fichot, E. B.; Norman, R. S.; May, H. D., Electrosynthesis of Commodity Chemicals by an Autotrophic Microbial Community. *Appl. Environ. Microbiol.* **2012**.
76. Dennis, P. G.; Harnisch, F.; Yeoh, Y. K.; Tyson, G. W.; Rabaey, K., Dynamics of cathode-associated microbial communities and metabolite profiles in a glycerol-fed bioelectrochemical system. *Appl. Environ. Microbiol.* **2013**.
77. ARPA-E ARPA-E Budget http://arpa-e.energy.gov/?q=arpa-e-site-page/arpa-e-budget (01/06/2013),
78. Cheng, S.; Liu, H.; Logan, B. E., Increased performance of single-chamber microbial fuel cells using an improved cathode structure. *Electrochem. Commun.* **2006,** 8, (3), 489-494.
79. Flexer, V.; Chen, J.; Donose, B. C.; Sherrell, P.; Wallace, G.; Keller, J., The nanostructure of three-dimensional scaffolds enhances the current density of microbial bioelectrochemical systems. *Energy Environ. Sci.* **2013**.
80. Zhang, T.; Nie, H.; Bain, T. S.; Lu, H.; Cui, M.; Snoeyenbos-West, O. L.; Franks, A. E.; Nevin, K. P.; Russell, T. P.; Lovley, D. R., Improved cathode materials for microbial electrosynthesis. *Energy Environ. Sci.* **2012,** 6, (1), 217-224.
81. Giddings, C. G. S.; Nevin, K.; Woodward, T.; Lovley, D. R.; Butler, C. S., Simplifying Microbial Electrosynthesis Reactor Design. *Front. Microbiol.* **2015,** 6.
82. Rimboud, M.; Pocaznoi, D.; Erable, B.; Bergel, A., Electroanalysis of microbial anodes for bioelectrochemical systems: basics, progress and perspectives. *Phys. Chem. Chem. Phys.* **2014,** 16, (31), 16349-16366.
83. Harnisch, F.; Rosa, L. F. M.; Kracke, F.; Virdis, B.; Krömer, J. O., Electrifying White Biotechnology: Engineering and Economic Potential of Electricity-Driven Bio-Production. *ChemSusChem* **2015,** 8, (5), 758-766.
84. Rabaey, K.; Butzer, S.; Brown, S.; Keller, J. r.; Rozendal, R. A., High current generation coupled to caustic production using a lamellar bioelectrochemical system. *Environ. Sci. Technol.* **2010,** 44, (11), 4315-4321.
85. Cheng, S.; Xing, D.; Call, D. F.; Logan, B. E., Direct Biological Conversion of Electrical Current into Methane by Electromethanogenesis. *Environ. Sci. Technol.* **2009,** 43, (10), 3953-3958.
86. Jeon, B. Y.; Jung, I. L.; Park, D. H., Conversion of Carbon Dioxide to Metabolites by *Clostridium acetobutylicum* KCTC1037 Cultivated with Electrochemical Reducing Power. *Adv. Microbiol.* **2012,** 2, (03), 332.
87. Flynn, J. M.; Ross, D. E.; Hunt, K. A.; Bond, D. R.; Gralnick, J. A., Enabling Unbalanced Fermentations by Using Engineered Electrode-Interfaced Bacteria. *mBio* **2010,** 1, (5).

88. Zhou, M.; Chen, J.; Freguia, S.; Rabaey, K.; Keller, J. r., Carbon and electron fluxes during the electricity driven 1, 3-propanediol biosynthesis from glycerol. *Environ. Sci. Technol.* **2013,** 47, (19), 11199-11205.
89. Palsson, B., *Systems biology: properties of reconstructed networks*. Cambridge University Press New York: 2006; Vol. 1.
90. Westerhoff, H. V.; Palsson, B. O., The evolution of molecular biology into systems biology. *Nat. Biotechnol.* **2004,** 22, (10), 1249-1252.
91. Kjaergaard, L., The redox potential: its use and control in biotechnology. In *Advances in Biochemical Engineering, Volume 7*, Springer: 1977; pp 131-150.
92. Blank, L. M.; Ebert, B. E.; Bühler, B.; Schmid, A., Metabolic capacity estimation of *Escherichia coli* as a platform for redox biocatalysis: constraint-based modeling and experimental verification. *Biotechnol. Bioeng.* **2008,** 100, (6), 1050-1065.
93. Fasan, R.; Crook, N. C.; Peters, M. W.; Meinhold, P.; Buelter, T.; Landwehr, M.; Cirino, P. C.; Arnold, F. H., Improved product-per-glucose yields in P450-dependent propane biotransformations using engineered *Escherichia coli*. *Biotechnol. Bioeng.* **2011,** 108, (3), 500-510.
94. Burgard, A. P.; Pharkya, P.; Maranas, C. D., Optknock: a bilevel programming framework for identifying gene knockout strategies for microbial strain optimization. *Biotechnol. Bioeng.* **2003,** 84, (6), 647-657.
95. Ranganathan, S.; Suthers, P. F.; Maranas, C. D., OptForce: an optimization procedure for identifying all genetic manipulations leading to targeted overproductions. *PLoS Comp. Biol.* **2010,** 6, (4), e1000744.
96. Yang, L.; Cluett, W. R.; Mahadevan, R., EMILiO: a fast algorithm for genome-scale strain design. *Metab. Eng.* **2011,** 13, (3), 272-281.
97. Pandit, A. V.; Mahadevan, R., *In silico* characterization of microbial electrosynthesis for metabolic engineering of biochemicals. *Microb. Cell Fact.* **2011,** 10, (1), 76-76.
98. Rosenbaum, M.; Aulenta, F.; Villano, M.; Angenent, L. T., Cathodes as electron donors for microbial metabolism: Which extracellular electron transfer mechanisms are involved? *Bioresour. Technol.* **2011,** 102, (1), 324-333.
99. Gallardo, R.; Acevedo, A.; Quintero, J.; Paredes, I.; Conejeros, R.; Aroca, G., In silico analysis of Clostridium acetobutylicum ATCC 824 metabolic response to an external electron supply. *Bioprocess Biosystems Eng.* **2015**, 1-11.
100. Kazemi, M.; Biria, D.; Rismani-Yazdi, H., Modelling bio-electrosynthesis in a reverse microbial fuel cell to produce acetate from CO_2 and H_2O. *Phys. Chem. Chem. Phys.* **2015,** 17, (19), 12561-12574.
101. Schuster, S.; Hilgetag, C., On elementary flux modes in biochemical reaction systems at steady state. *J. Biol. Syst.* **1994,** 2, (02), 165-182.
102. Schuster, S.; Dandekar, T.; Fell, D. A., Detection of elementary flux modes in biochemical networks: a promising tool for pathway analysis and metabolic engineering. *Trends Biotechnol.* **1999,** 17, (2), 53-60.
103. Trinh, C. T.; Wlaschin, A.; Srienc, F., Elementary mode analysis: a useful metabolic pathway analysis tool for characterizing cellular metabolism. *Appl. Microbiol. Biotechnol.* **2009,** 81, (5), 813-826.
104. Klamt, S.; Stelling, J., Combinatorial complexity of pathway analysis in metabolic networks. *Mol. Biol. Rep.* **2002,** 29, (1-2), 233-236.
105. Papin, J. A.; Stelling, J.; Price, N. D.; Klamt, S.; Schuster, S.; Palsson, B. O., Comparison of network-based pathway analysis methods. *Trends Biotechnol.* **2004,** 22, (8), 400-405.
106. Schuster, S.; Hilgetag, C.; Woods, J.; Fell, D., Reaction routes in biochemical reaction systems: algebraic properties, validated calculation procedure and example from nucleotide metabolism. *J. Math. Biol.* **2002,** 45, (2), 153-181.
107. Stelling, J.; Klamt, S.; Bettenbrock, K.; Schuster, S.; Gilles, E. D., Metabolic network structure determines key aspects of functionality and regulation. *Nature* **2002,** 420, (6912), 190-193.
108. Terzer, M.; Stelling, J., Large-scale computation of elementary flux modes with bit pattern trees. *Bioinformatics* **2008,** 24, (19), 2229-2235.
109. Bond, D. R.; Lovley, D. R., Electricity Production by *Geobacter sulfurreducens* Attached to Electrodes. *Appl. Environ. Microbiol.* **2003,** 69, (3), 1548-1555.

110. Ross, D. E.; Flynn, J. M.; Baron, D. B.; Gralnick, J. A.; Bond, D. R., Towards Electrosynthesis in *Shewanella*: Energetics of Reversing the Mtr Pathway for Reductive Metabolism. *PLoS ONE* **2011,** 6, (2), e16649.
111. Thrash, J. C.; Coates, J. D., Review: Direct and Indirect Electrical Stimulation of Microbial Metabolism. *Environ. Sci. Technol.* **2008,** 42, (11), 3921-3931.
112. Lovley, D. R., The microbe electric: conversion of organic matter to electricity. *Curr. Opin. Biotechnol.* **2008,** 19, (6), 564-571.
113. Lovley, D. R., Live wires: direct extracellular electron exchange for bioenergy and the bioremediation of energy-related contamination. *E&E S* **2011,** 4, (12), 4896-4906.
114. Torres, C. I.; Marcus, A. K.; Lee, H.-S.; Parameswaran, P.; Krajmalnik-Brown, R.; Rittmann, B. E., A kinetic perspective on extracellular electron transfer by anode-respiring bacteria. *FEMS Microbiol. Rev.* **2010,** 34, (1), 3-17.
115. Fornero, J. J.; Rosenbaum, M.; Angenent, L. T., Electric power generation from municipal, food, and animal wastewaters using microbial fuel cells. *Electroanalysis* **2010,** 22, (7-8), 832-843.
116. Weber, K. A.; Achenbach, L. A.; Coates, J. D., Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. *Nat. Rev. Microbiol.* **2006,** 4, (10), 752-764.
117. Marsili, E.; Baron, D. B.; Shikhare, I. D.; Coursolle, D.; Gralnick, J. A.; Bond, D. R., *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proc. Natl. Acad. Sci. USA* **2008,** 105, (10), 3968-3973.
118. Shi, L.; Richardson, D. J.; Wang, Z.; Kerisit, S. N.; Rosso, K. M.; Zachara, J. M.; Fredrickson, J. K., The roles of outer membrane cytochromes of *Shewanella* and *Geobacter* in extracellular electron transfer. *Env Microbiol Rep* **2009,** 1, (4), 220-227.
119. Marsili, E.; Sun, J.; Bond, D. R., Voltammetry and growth physiology of *Geobacter sulfurreducens* biofilms as a function of growth stage and imposed electrode potential. *Electroanalysis* **2010,** 22, (7-8), 865-874.
120. Pirbadian, S.; Barchinger, S. E.; Leung, K. M.; Byun, H. S.; Jangir, Y.; Bouhenni, R. A.; Reed, S. B.; Romine, M. F.; Saffarini, D. A.; Shi, L., *Shewanella oneidensis* MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components. *Proc. Natl. Acad. Sci. USA* **2014,** 111, (35), 12883-12888.
121. Reguera, G.; Nevin, K. P.; Nicoll, J. S.; Covalla, S. F.; Woodard, T. L.; Lovley, D. R., Biofilm and nanowire production leads to increased current in Geobacter sulfurreducens fuel cells. *Appl. Environ. Microbiol.* **2006,** 72, (11), 7345-7348.
122. Snider, R. M.; Strycharz-Glaven, S. M.; Tsoi, S. D.; Erickson, J. S.; Tender, L. M., Long-range electron transport in *Geobacter sulfurreducens* biofilms is redox gradient-driven. *Proc. Natl. Acad. Sci. USA* **2012,** 109, (38), 15467-15472.
123. Virdis, B.; Harnisch, F.; Batstone, D. J.; Rabaey, K.; Donose, B. C., Non-invasive characterization of electrochemically active microbial biofilms using confocal Raman microscopy. *E&E S* **2012,** 5, (5), 7017-7024.
124. Boesen, T.; Nielsen, L. P., Molecular Dissection of Bacterial Nanowires. *mBio* **2013,** 4, (3).
125. Chen, K.-I.; McEwan, A.; Bernhardt, P., Cobalt hexaamine mediated electrocatalytic voltammetry of dimethyl sulfoxide reductase: driving force effects on catalysis. *J. Biol. Inorg. Chem.* **2011,** 16, (2), 227-234.
126. Malvankar, N. S.; Tuominen, M. T.; Lovley, D. R., Lack of cytochrome involvement in long-range electron transport through conductive biofilms and nanowires of *Geobacter sulfurreducens*. *E&E S* **2012,** 5, (9), 8651-8659.
127. Malvankar, N. S.; Vargas, M.; Nevin, K. P.; Franks, A. E.; Leang, C.; Kim, B.-C.; Inoue, K.; Mester, T.; Covalla, S. F.; Johnson, J. P., Tunable metallic-like conductivity in microbial nanowire networks. *Nat. Nanotechnol.* **2011,** 6, (9), 573-579.
128. Rollefson, J. B.; Stephen, C. S.; Tien, M.; Bond, D. R., Identification of an extracellular polysaccharide network essential for cytochrome anchoring and biofilm formation in *Geobacter sulfurreducens*. *J. Bacteriol.* **2011,** 193, (5), 1023-1033.
129. Carlson, H. K.; Iavarone, A. T.; Gorur, A.; Yeo, B. S.; Tran, R.; Melnyk, R. A.; Mathies, R. A.; Auer, M.; Coates, J. D., Surface multiheme c-type cytochromes from *Thermincola potens* and implications for

respiratory metal reduction by Gram-positive bacteria. *Proc. Natl. Acad. Sci. USA* **2012,** 109, (5), 1702-1707.
130. Wrighton, K. C.; Thrash, J. C.; Melnyk, R. A.; Bigi, J. P.; Byrne-Bailey, K. G.; Remis, J. P.; Schichnes, D.; Auer, M.; Chang, C. J.; Coates, J. D., Evidence for Direct Electron Transfer by a Gram-Positive Bacterium Isolated from a Microbial Fuel Cell. *Appl. Environ. Microbiol.* **2011,** 77, (21), 7633-7639.
131. Park, D. H.; Zeikus, J. G., Utilization of Electrically Reduced Neutral Red by *Actinobacillus succinogenes*: Physiological Function of Neutral Red in Membrane-Driven Fumarate Reduction and Energy Conservation. *J. Bacteriol.* **1999,** 181, (8), 2403-2410.
132. Unden, G.; Bongaerts, J., Alternative respiratory pathways of *Escherischia coli*: energetics and transcriptional regulation in response to electron acceptors. *BBA Bioenerg.* **1997,** 1320, (3), 217-234.
133. Karp, G., *Cell and Molecular Biology*. 6 ed.; Wiley: 2009; p 832.
134. Fultz, M. L.; Durst, R. A., Mediator compounds for the electrochemical study of biological redox systems: a compilation. *Anal. Chim. Acta* **1982,** 140, (1), 1-18.
135. Cournet, A.; Délia, M.-L.; Bergel, A.; Roques, C.; Bergé, M., Electrochemical reduction of oxygen catalyzed by a wide range of bacteria including Gram-positive. *Electrochem. Commun.* **2010,** 12, (4), 505-508.
136. Huang, L.; Regan, J. M.; Quan, X., Electron transfer mechanisms, new applications, and performance of biocathode microbial fuel cells. *Bioresour. Technol.* **2011,** 102, (1), 316-323.
137. Freguia, S.; Tsujimura, S.; Kano, K., Electron transfer pathways in microbial oxygen biocathodes. *Electrochim. Acta* **2010,** 55, (3), 813-818.
138. Castelle, C.; Guiral, M.; Malarte, G.; Ledgham, F.; Leroy, G.; Brugna, M.; Giudici-Orticoni, M.-T., A New Iron-oxidizing/O_2-reducing Supercomplex Spanning Both Inner and Outer Membranes, Isolated from the Extreme Acidophile *Acidithiobacillus ferrooxidans*. *J. Biol. Chem.* **2008,** 283, (38), 25803-25811.
139. Barton, L. L.; Goulhen, F.; Bruschi, M.; Woodards, N. A.; Plunkett, R. M.; Rietmeijer, F. J., The bacterial metallome: composition and stability with specific reference to the anaerobic bacterium *Desulfovibrio desulfuricans*. *BioMetals* **2007,** 20, (3-4), 291-302.
140. Hockin, S. L.; Gadd, G. M.; Barton, L.; Hamilton, W., Bioremediation of metals and metalloids by precipitation and cellular binding. *E&E S* **2007**.
141. Meshulam-Simon, G.; Behrens, S.; Choo, A. D.; Spormann, A. M., Hydrogen metabolism in *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.* **2007,** 73, (4), 1153-1165.
142. Rosenbaum, M. A.; Franks, A. E., Microbial catalysis in bioelectrochemical technologies: status quo, challenges and perspectives. *Appl. Microbiol. Biotechnol.* **2014,** 98, (2), 509-518.
143. Hallenbeck, P. C., The future of biofuels, biofuels of the future. In *Microbial Technologies in Advanced Biofuels Production*, Springer: 2012; pp 261-268.
144. Tremblay, P.-L.; Zhang, T., Electrifying microbes for the production of chemicals. *Front. Microbiol.* **2015,** 6.
145. Sydow, A.; Krieg, T.; Mayer, F.; Schrader, J.; Holtmann, D., Electroactive bacteria—molecular mechanisms and genetic tools. *Appl. Microbiol. Biotechnol.* **2014,** 98, (20), 8481-8495.
146. Anraku, Y., Bacterial electron transport chains. *Annu. Rev. Biochem.* **1988,** 57, (1), 101-132.
147. Hernandez, M.; Newman, D., Extracellular electron transfer. *CMLS* **2001,** 58, (11), 1562-1571.
148. Hannemann, F.; Bichet, A.; Ewen, K. M.; Bernhardt, R., Cytochrome P450 systems—biological variations of electron transport chains. *BBA-Gen Subjects* **2007,** 1770, (3), 330-344.
149. Vignais, P. M.; Billoud, B., Occurrence, classification, and biological function of hydrogenases: an overview. *Chem. Rev.* **2007,** 107, (10), 4206-4272.
150. Richter, K.; Schicklberger, M.; Gescher, J., Dissimilatory reduction of extracellular electron acceptors in anaerobic respiration. *Appl. Environ. Microbiol.* **2012,** 78, (4), 913-921.
151. Deller, S.; Macheroux, P.; Sollner, S., Flavin-dependent quinone reductases. *Cell. Mol. Life Sci.* **2008,** 65, (1), 141-160.
152. Dolin, M., Survey of microbial electron transport mechanisms. *The bacteria* **2012,** 2, 319.
153. Herrmann, G.; Jayamani, E.; Mai, G.; Buckel, W., Energy conservation via electron-transferring flavoprotein in anaerobic bacteria. *J. Bacteriol.* **2008,** 190, (3), 784-791.

154. Müller, V.; Imkamp, F.; Biegel, E.; Schmidt, S.; Dilling, S., Discovery of a Ferredoxin: NAD^+-Oxidoreductase (Rnf) in *Acetobacterium woodii*. *Ann. N. Y. Acad. Sci.* **2008,** 1125, (1), 137-146.
155. Schuchmann, K.; Müller, V., A bacterial electron-bifurcating hydrogenase. *J. Biol. Chem.* **2012,** 287, (37), 31165-31171.
156. Haddock, B. A.; Schairer, H. U., Electron-Transport Chains of *Escherichia coli*. *Eur. J. Biochem.* **1973,** 35, (1), 34-45.
157. Das, A.; Ljungdahl, L. G., Electron-transport system in acetogens. In *Biochemistry and physiology of anaerobic bacteria*, Springer: 2003; pp 191-204.
158. Müller, V., Energy Conservation in Acetogenic Bacteria. *Appl. Environ. Microbiol.* **2003,** 69, (11), 6345-6353.
159. Mehta, T.; Coppi, M. V.; Childers, S. E.; Lovley, D. R., Outer membrane c-type cytochromes required for Fe(III) and Mn(IV) oxide reduction in *Geobacter sulfurreducens*. *Appl. Environ. Microbiol.* **2005,** 71, (12), 8634-8641.
160. Shi, L.; Squier, T. C.; Zachara, J. M.; Fredrickson, J. K., Respiration of metal (hydr) oxides by *Shewanella* and *Geobacter*: a key role for multihaem c-type cytochromes. *Mol. Microbiol.* **2007,** 65, (1), 12-20.
161. Breuer, M.; Rosso, K. M.; Blumberger, J.; Butt, J. N., Multi-haem cytochromes in *Shewanella oneidensis* MR-1: structures, functions and opportunities. *J. R. Soc. Interface* **2015,** 12, (102), 20141117.
162. Holmes, D. E.; Chaudhuri, S. K.; Nevin, K. P.; Mehta, T.; Methé, B. A.; Liu, A.; Ward, J. E.; Woodard, T. L.; Webster, J.; Lovley, D. R., Microarray and genetic analysis of electron transfer to electrodes in *Geobacter sulfurreducens*. *Environ. Microbiol.* **2006,** 8, (10), 1805-1815.
163. Strycharz, S.; Woodard, T.; Johnson, J.; Nevin, K.; Sanford, R.; Löffler, F.; Lovley, D., Graphite electrode as a sole electron donor for reductive dechlorination of tetrachlorethene by *Geobacter lovleyi*. *Appl. Environ. Microbiol.* **2008,** 74, (19), 5943-7.
164. Marshall, C. W.; May, H. D., Electrochemical evidence of direct electrode reduction by a thermophilic Gram-positive bacterium, *Thermincola ferriacetica*. *E&E S* **2009,** 2, (6), 699-705.
165. Köpke, M.; Held, C.; Hujer, S.; Liesegang, H.; Wiezer, A.; Wollherr, A.; Ehrenreich, A.; Liebl, W.; Gottschalk, G.; Dürre, P., *Clostridium ljungdahlii* represents a microbial production platform based on syngas. *Proc. Natl. Acad. Sci. USA* **2010,** 107, (29), 13087-13092.
166. Lovley, D. R.; Holmes, D. E.; Nevin, K. P., Dissimilatory Fe(III) and Mn(IV) reduction. *Adv. Microb. Physiol.* **2004,** 49, 219-286.
167. Richardson, D. J.; Edwards, M. J.; White, G. F.; Baiden, N.; Hartshorne, R. S.; Fredrickson, J. K.; Shi, L.; Zachara, J. M.; Gates, A. J.; Clarke, T., Exploring the biochemistry at the extracellular redox frontier of bacterial mineral Fe(III) respiration. **2012**.
168. Hartshorne, R. S.; Reardon, C. L.; Ross, D.; Nuester, J.; Clarke, T. A.; Gates, A. J.; Mills, P. C.; Fredrickson, J. K.; Zachara, J. M.; Shi, L., Characterization of an electron conduit between bacteria and the extracellular environment. *Proc. Natl. Acad. Sci. USA* **2009,** 106, (52), 22169-22174.
169. Coursolle, D.; Gralnick, J. A., Modularity of the Mtr respiratory pathway of *Shewanella oneidensis* strain MR-1. *Mol. Microbiol.* **2010,** 77, (4), 995-1008.
170. Parameswaran, P.; Bry, T.; Popat, S. C.; Lusk, B. G.; Rittmann, B. E.; Torres, C. I., Kinetic, Electrochemical, and Microscopic Characterization of the Thermophilic, Anode-Respiring Bacterium *Thermincola ferriacetica*. *Environ. Sci. Technol.* **2013,** 47, (9), 4934-4940.
171. Tremblay, P.-L.; Höglund, D.; Koza, A.; Bonde, I.; Zhang, T., Adaptation of the autotrophic acetogen *Sporomusa ovata* to methanol accelerates the conversion of CO_2 to organic products. *Scientific reports* **2015,** 5, 16168.
172. Park, D. H.; Zeikus, J. G., Electricity generation in microbial fuel cells using neutral red as an electronophore. *Appl. Environ. Microbiol.* **2000,** 66, (4), 1292-1297.
173. Schröder, U.; Nießen, J.; Scholz, F., A generation of microbial fuel cells with current outputs boosted by more than one order of magnitude. *Angewandte Chemie International Edition* **2003,** 42, (25), 2880-2883.
174. Zhang, T.; Zeng, Y.; Chen, S.; Ai, X.; Yang, H., Improved performances of *E. coli*-catalyzed microbial fuel cells with composite graphite/PTFE anodes. *Electrochem. Commun.* **2007,** 9, (3), 349-353.

175. Hernandez, M. E.; Kappler, A.; Newman, D. K., Phenazines and other redox-active antibiotics promote microbial mineral reduction. *Appl. Environ. Microbiol.* **2004,** 70, (2), 921-928.
176. Rabaey, K.; Boon, N.; Höfte, M.; Verstraete, W., Microbial phenazine production enhances electron transfer in biofuel cells. *Environ. Sci. Technol.* **2005,** 39, (9), 3401-3408.
177. Sasaki, K.; Tsuge, Y.; Sasaki, D.; Kondo, A., Increase in lactate yield by growing *Corynebacterium glutamicum* in a bioelectrochemical reactor. *J. Biosci. Bioeng.* **2014,** 117, (5), 598-601.
178. Levar, C.; Rollefson, J.; Bond, D., Energetic and molecular constraints on the mechanism of environmental Fe (III) reduction by *Geobacter*. In *Microbial Metal Respiration*, Springer: 2012; pp 29-48.
179. Methe, B.; Nelson, K. E.; Eisen, J.; Paulsen, I.; Nelson, W.; Heidelberg, J.; Wu, D.; Wu, M.; Ward, N.; Beanan, M., Genome of *Geobacter sulfurreducens:* metal reduction in subsurface environments. *Science* **2003,** 302, (5652), 1967-1969.
180. Inoue, K.; Qian, X.; Morgado, L.; Kim, B.-C.; Mester, T.; Izallalen, M.; Salgueiro, C. A.; Lovley, D. R., Purification and characterization of OmcZ, an outer-surface, octaheme c-type cytochrome essential for optimal current production by *Geobacter sulfurreducens*. *Appl. Environ. Microbiol.* **2010,** 76, (12), 3999-4007.
181. Qian, X.; Mester, T.; Morgado, L.; Arakawa, T.; Sharma, M. L.; Inoue, K.; Joseph, C.; Salgueiro, C. A.; Maroney, M. J.; Lovley, D. R., Biochemical characterization of purified OmcS, a c-type cytochrome required for insoluble Fe(III) reduction in *Geobacter sulfurreducens*. *BBA-Bioenergetics* **2011,** 1807, (4), 404-412.
182. Nevin, K. P.; Kim, B.-C.; Glaven, R. H.; Johnson, J. P.; Woodard, T. L.; Methé, B. A.; DiDonato Jr, R. J.; Covalla, S. F.; Franks, A. E.; Liu, A., Anode biofilm transcriptomics reveals outer surface components essential for high density current production in *Geobacter sulfurreducens* fuel cells. *PLoS One* **2009,** 4, (5), e5628.
183. Richter, H.; Nevin, K. P.; Jia, H.; Lowy, D. A.; Lovley, D. R.; Tender, L. M., Cyclic voltammetry of biofilms of wild type and mutant *Geobacter sulfurreducens* on fuel cell anodes indicates possible roles of OmcB, OmcZ, type IV pili, and protons in extracellular electron transfer. *E&E S* **2009,** 2, (5), 506-516.
184. Kim, B.-C.; Postier, B. L.; DiDonato, R. J.; Chaudhuri, S. K.; Nevin, K. P.; Lovley, D. R., Insights into genes involved in electricity generation in *Geobacter sulfurreducens* via whole genome microarray analysis of the OmcF-deficient mutant. *Bioelectrochemistry* **2008,** 73, (1), 70-75.
185. Kim, B.-C.; Qian, X.; Leang, C.; Coppi, M. V.; Lovley, D. R., Two putative c-type multiheme cytochromes required for the expression of OmcB, an outer membrane protein essential for optimal Fe (III) reduction in *Geobacter sulfurreducens*. *J. Bacteriol.* **2006,** 188, (8), 3138-3142.
186. Schrott, G. D.; Bonanni, P. S.; Robuschi, L.; Esteve-Nuñez, A.; Busalmen, J. P., Electrochemical insight into the mechanism of electron transport in biofilms of *Geobacter sulfurreducens*. *Electrochim. Acta* **2011,** 56, (28), 10791-10795.
187. Leang, C.; Lovley, D. R., Regulation of two highly similar genes, omcB and omcC, in a 10 kb chromosomal duplication in *Geobacter sulfurreducens*. *Microbiology* **2005,** 151, (6), 1761-1767.
188. Kracke, F.; Vassilev, I.; Krömer, J. O., Microbial electron transport and energy conservation – the foundation for optimizing bioelectrochemical systems. *Front. Microbiol.* **2015,** 6.
189. Malvankar, N. S.; Vargas, M.; Nevin, K.; Tremblay, P.-L.; Evans-Lutterodt, K.; Nykypanchuk, D.; Martz, E.; Tuominen, M. T.; Lovley, D. R., Structural Basis for Metallic-Like Conductivity in Microbial Nanowires. *mBio* **2015,** 6, (2), e00084-15.
190. Strycharz-Glaven, S. M.; Tender, L. M., Reply to the 'Comment on "On electrical conductivity of microbial nanowires and biofilms"'by NS Malvankar, MT Tuominen and DR Lovley, Energy Environ. Sci., 2012, 5, DOI: 10.1039/c2ee02613a. *E&E S* **2012,** 5, (3), 6250-6255.
191. Kracke, F.; Krömer, J. O., Identifying target processes for microbial electrosynthesis by elementary mode analysis. *BMC Bioinformatics* **2014,** 15, (1), 410.
192. Heidelberg, J. F.; Paulsen, I. T.; Nelson, K. E.; Gaidos, E. J.; Nelson, W. C.; Read, T. D.; Eisen, J. A.; Seshadri, R.; Ward, N.; Methe, B., Genome sequence of the dissimilatory metal ion–reducing bacterium *Shewanella oneidensis*. *Nat. Biotechnol.* **2002,** 20, (11), 1118-1123.

193. Lower, B. H.; Hochella, M. F.; Lower, S. K., Putative mineral-specific proteins synthesized by a metal reducing bacterium. *Am. J. Sci.* **2005,** 305, (6-8), 687-710.
194. Dos Santos, J.-P.; Iobbi-Nivol, C.; Couillault, C.; Giordano, G.; Méjean, V., Molecular analysis of the trimethylamine N-oxide (TMAO) reductase respiratory system from a *Shewanella* species. *J. Mol. Biol.* **1998,** 284, (2), 421-433.
195. Shirodkar, S.; Reed, S.; Romine, M.; Saffarini, D., The octahaem SirA catalyses dissimilatory sulfite reduction in *Shewanella oneidensis* MR-1. *Environ. Microbiol.* **2011,** 13, (1), 108-115.
196. Gao, H.; Yang, Z. K.; Barua, S.; Reed, S. B.; Romine, M. F.; Nealson, K. H.; Fredrickson, J. K.; Tiedje, J. M.; Zhou, J., Reduction of nitrate in *Shewanella oneidensis* depends on atypical NAP and NRF systems with NapB as a preferred electron transport protein from CymA to NapA. *The ISME journal* **2009,** 3, (8), 966-976.
197. Simpson, P. J.; Richardson, D. J.; Codd, R., The periplasmic nitrate reductase in *Shewanella*: the resolution, distribution and functional implications of two NAP isoforms, NapEDABC and NapDAGHB. *Microbiology* **2010,** 156, (2), 302-312.
198. Maier, T. M.; Myers, J. M.; Myers, C. R., Identification of the gene encoding the sole physiological fumarate reductase in *Shewanella oneidensis* MR-1. *J. Basic Microbiol.* **2003,** 43, (4), 312-327.
199. Atkinson, S. J.; Mowat, C. G.; Reid, G. A.; Chapman, S. K., An octaheme c-type cytochrome from *Shewanella oneidensis* can reduce nitrite and hydroxylamine. *FEBS Lett.* **2007,** 581, (20), 3805-3808.
200. Shi, L.; Rosso, K.; Zachara, J.; Fredrickson, J., Mtr extracellular electron-transfer pathways in Fe(III)-reducing or Fe(II)-oxidizing bacteria: a genomic perspective. *Biochem. Soc. Trans.* **2012,** 40, (6), 1261.
201. Myers, J. M.; Myers, C. R., Role of the tetraheme cytochrome CymA in anaerobic electron transport in cells of *Shewanella putrefaciens* MR-1 with normal levels of menaquinone. *J. Bacteriol.* **2000,** 182, (1), 67-75.
202. White, G. F.; Shi, Z.; Shi, L.; Wang, Z.; Dohnalkova, A. C.; Marshall, M. J.; Fredrickson, J. K.; Zachara, J. M.; Butt, J. N.; Richardson, D. J., Rapid electron exchange between surface-exposed bacterial cytochromes and Fe(III) minerals. *Proc. Natl. Acad. Sci. USA* **2013,** 110, (16), 6346-6351.
203. Gralnick, J. A.; Vali, H.; Lies, D. P.; Newman, D. K., Extracellular respiration of dimethyl sulfoxide by *Shewanella oneidensis* strain MR-1. *Proc. Natl. Acad. Sci. USA* **2006,** 103, (12), 4669-4674.
204. Fonseca, B. M.; Paquete, C. M.; Neto, S. E.; Pacheco, I.; Soares, C. M.; Louro, R. O., Mind the gap: cytochrome interactions reveal electron pathways across the periplasm of *Shewanella oneidensis* MR-1. *Biochem. J.* **2013,** 449, (1), 101-108.
205. Marsili, E.; Rollefson, J. B.; Baron, D. B.; Hozalski, R. M.; Bond, D. R., Microbial biofilm voltammetry: direct electrochemical characterization of catalytic electrode-attached biofilms. *Appl. Environ. Microbiol.* **2008,** 74, (23), 7329-7337.
206. Okamoto, A.; Hashimoto, K.; Nealson, K. H.; Nakamura, R., Rate enhancement of bacterial extracellular electron transport involves bound flavin semiquinones. *Proc. Natl. Acad. Sci. USA* **2013,** 110, (19), 7856-7861.
207. Okamoto, A.; Saito, K.; Inoue, K.; Nealson, K. H.; Hashimoto, K.; Nakamura, R., Uptake of self-secreted flavins as bound cofactors for extracellular electron transfer in *Geobacter* species. *Energy Environ. Sci.* **2014,** 7, (4), 1357-1361.
208. Tracy, B. P.; Jones, S. W.; Fast, A. G.; Indurthi, D. C.; Papoutsakis, E. T., *Clostridia*: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. *Curr. Opin. Biotechnol.* **2012,** 23, (3), 364-381.
209. Diekert, G.; Wohlfarth, G., Metabolism of homoacetogens. *Antonie Van Leeuwenhoek* **1994,** 66, (1-3), 209-221.
210. Drake, H. L.; Gößner, A. S.; Daniel, S. L., Old acetogens, new light. *Ann. N. Y. Acad. Sci.* **2008,** 1125, (1), 100-128.
211. Banerjee, A.; Leang, C.; Ueki, T.; Nevin, K. P.; Lovley, D. R., Lactose-inducible system for metabolic engineering of *Clostridium ljungdahlii*. *Appl. Environ. Microbiol.* **2014,** 80, (8), 2410-2416.
212. Lee, J.; Jang, Y.-S.; Choi, S. J.; Im, J. A.; Song, H.; Cho, J. H.; Papoutsakis, E. T.; Bennett, G. N.; Lee, S. Y., Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for isopropanol-butanol-ethanol fermentation. *Appl. Environ. Microbiol.* **2012,** 78, (5), 1416-1423.

213. Lütke-Eversloh, T.; Bahl, H., Metabolic engineering of *Clostridium acetobutylicum*: recent advances to improve butanol production. *Curr. Opin. Biotechnol.* **2011,** 22, (5), 634-647.
214. Schiel-Bengelsdorf, B.; Dürre, P., Pathway engineering and synthetic biology using acetogens. *FEBS Lett.* **2012,** 586, (15), 2191-2198.
215. Ragsdale, S. W.; Pierce, E., Acetogenesis and the Wood–Ljungdahl pathway of CO_2 fixation. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **2008,** 1784, (12), 1873-1898.
216. Müller, V.; Frerichs, J., Acetogenic bacteria. *eLS* **2013**.
217. Huang, H.; Wang, S.; Moll, J.; Thauer, R. K., Electron bifurcation involved in the energy metabolism of the acetogenic bacterium *Moorella thermoacetica* growing on glucose or H_2 plus CO_2. *J. Bacteriol.* **2012,** 194, (14), 3689-3699.
218. Wang, S.; Huang, H.; Kahnt, J.; Thauer, R. K., A reversible electron-bifurcating ferredoxin-and NAD-dependent [FeFe]-hydrogenase (HydABC) in *Moorella thermoacetica*. *J. Bacteriol.* **2013,** 195, (6), 1267-1275.
219. Schuchmann, K.; Müller, V., Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria. *Nat. Rev. Microbiol.* **2014,** 12, 809-821.
220. Welte, C.; Krätzer, C.; Deppenmeier, U., Involvement of Ech hydrogenase in energy conservation of *Methanosarcina mazei*. *FEBS J.* **2010,** 277, (16), 3396-3403.
221. Fast, A. G.; Papoutsakis, E. T., Stoichiometric and energetic analyses of non-photosynthetic CO_2-fixation pathways to support synthetic biology strategies for production of fuels and chemicals. *Curr. Opin. Chem. Eng.* **2012,** 1, (4), 380-395.
222. Biegel, E.; Schmidt, S.; González, J.; Müller, V., Biochemistry, evolution and physiological function of the Rnf complex, a novel ion-motive electron transport complex in prokaryotes. *Cell. Mol. Life Sci.* **2011,** 68, (4), 613-634.
223. Poehlein, A.; Schmidt, S.; Kaster, A.-K.; Goenrich, M.; Vollmers, J.; Thürmer, A.; Bertsch, J.; Schuchmann, K.; Voigt, B.; Hecker, M.; Daniel, R.; Thauer, R. K.; Gottschalk, G.; Müller, V., An Ancient Pathway Combining Carbon Dioxide Fixation with the Generation and Utilization of a Sodium Ion Gradient for ATP Synthesis. *PLoS ONE* **2012,** 7, (3), e33439.
224. Tremblay, P.-L.; Zhang, T.; Dar, S. A.; Leang, C.; Lovley, D. R., The Rnf Complex of *Clostridium ljungdahlii* Is a Proton-Translocating Ferredoxin:NAD^+ Oxidoreductase Essential for Autotrophic Growth. *mBio* **2013,** 4, (1).
225. Berlyn, M. K., Linkage map of *Escherichia coli* K-10: the traditional map. *Microbiol. Mol. Biol. Rev.* **1998,** 62, (3), 814-984.
226. Ingledew, W. J., The Electron Transport Chain of *Escherichia coli* Grown Anaerobically with Fumarate as Terminal Electron Acceptor: an Electron Paramagnetic Resonance Study. *J. Gen. Microbiol.* **1983,** 129, (6), 1651-1659.
227. Qiao, Y.; Li, C. M.; Bao, S.-J.; Lu, Z.; Hong, Y., Direct electrochemistry and electrocatalytic mechanism of evolved *Escherichia coli* cells in microbial fuel cells. *Chem. Commun.* **2008**, (11), 1290-1292.
228. Baltch, A. L.; Smith, R. P., *Pseudomonas aeruginosa: infections and treatment*. Marcel Dekker, Inc.: New York, 1994.
229. Jo, J.; Price-Whelan, A.; Dietrich, L. E., An Aerobic Exercise: Defining the Roles of *Pseudomonas aeruginosa* Terminal Oxidases. *J. Bacteriol.* **2014,** 196, (24), 4203-4205.
230. Hassett, D. J.; Cuppoletti, J.; Trapnell, B.; Lymar, S. V.; Rowe, J. J.; Yoon, S. S.; Hilliard, G. M.; Parvatiyar, K.; Kamani, M. C.; Wozniak, D. J., Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. *Adv. Drug Del. Rev.* **2002,** 54, (11), 1425-1443.
231. Boon, N.; De Maeyer, K.; Höfte, M.; Rabaey, K.; Verstraete, W., Use of *Pseudomonas* species producing phenazine-based metabolites in the anodes of microbial fuel cells to improve electricity generation. *Appl. Microbiol. Biotechnol.* **2008,** 80, (6), 985-993.
232. Tatsumi, N.; Inui, M., *Corynebacterium glutamicum: biology and biotechnology*. Springer Science & Business Media: 2012; Vol. 23.
233. Bott, M.; Niebisch, A., The respiratory chain of *Corynebacterium glutamicum*. *J. Biotechnol.* **2003,** 104, (1), 129-153.

234. Nishimura, T.; Vertès, A. A.; Shinoda, Y.; Inui, M.; Yukawa, H., Anaerobic growth of *Corynebacterium glutamicum* using nitrate as a terminal electron acceptor. *Appl. Microbiol. Biotechnol.* **2007,** 75, (4), 889-897.
235. Buckel, W.; Thauer, R. K., Energy conservation via electron bifurcating ferredoxin reduction and proton/Na^+ translocating ferredoxin oxidation. *BBA Bioenerg.* **2013,** 1827, (2), 94-113.
236. Hess, V.; Schuchmann, K.; Müller, V., The ferredoxin: NAD^+ oxidoreductase (Rnf) from the acetogen *Acetobacterium woodii* requires Na^+ and is reversibly coupled to the membrane potential. *J. Biol. Chem.* **2013,** 288, (44), 31496-31502.
237. Straub, K. L.; Benz, M.; Schink, B., Iron metabolism in anoxic environments at near neutral pH. *FEMS Microbiol. Ecol.* **2001,** 34, (3), 181-186.
238. Thamdrup, B., Bacterial manganese and iron reduction in aquatic sediments. In *Adv. Microb. Ecol.*, Springer: 2000; pp 41-84.
239. Lloyd, J.; Leang, C.; HODGES, M. A.; Coppi, M.; Cuifo, S.; Methe, B.; Sandler, S.; Lovley, D., Biochemical and genetic characterization of PpcA, a periplasmic c-type cytochrome in *Geobacter sulfurreducens*. *Biochem. J* **2003,** 369, 153-161.
240. Firer-Sherwood, M.; Pulcu, G. S.; Elliott, S. J., Electrochemical interrogations of the Mtr cytochromes from *Shewanella*: opening a potential window. *JBIC* **2008,** 13, (6), 849-854.
241. Morgado, L.; Bruix, M.; Pessanha, M.; Londer, Y. Y.; Salgueiro, C. A., Thermodynamic characterization of a triheme cytochrome family from *Geobacter sulfurreducens* reveals mechanistic and functional diversity. *Biophys. J.* **2010,** 99, (1), 293-301.
242. Bertini, I.; Cavallaro, G.; Rosato, A., Cytochrome c: occurrence and functions. *Chem. Rev.* **2006,** 106, (1), 90-115.
243. Pierce, E.; Xie, G.; Barabote, R. D.; Saunders, E.; Han, C. S.; Detter, J. C.; Richardson, P.; Brettin, T. S.; Das, A.; Ljungdahl, L. G.; Ragsdale, S. W., The complete genome sequence of *Moorella thermoacetica* (f. *Clostridium thermoaceticum*). *Environ. Microbiol.* **2008,** 10, (10), 2550-2573.
244. Vijgenboom, E.; Busch, J. E.; Canters, G. W., In vivo studies disprove an obligatory role of azurin in denitrification in *Pseudomonas aeruginosa* and show that azu expression is under control of RpoS and ANR. *Microbiology* **1997,** 143, (9), 2853-2863.
245. Yang, Y.; Ding, Y.; Hu, Y.; Cao, B.; Rice, S. A.; Kjelleberg, S.; Song, H., Enhancing Bidirectional Electron Transfer of *Shewanella oneidensis* by a Synthetic Flavin Pathway. *ACS Synthetic Biology* **2015**.
246. Deutzmann, J. S.; Sahin, M.; Spormann, A. M., Extracellular Enzymes Facilitate Electron Uptake in Biocorrosion and Bioelectrosynthesis. *mBio* **2015,** 6, (2).
247. Jensen, H. M.; Albers, A. E.; Malley, K. R.; Londer, Y. Y.; Cohen, B. E.; Helms, B. A.; Weigele, P.; Groves, J. T.; Ajo-Franklin, C. M., Engineering of a synthetic electron conduit in living cells. *Proc. Natl. Acad. Sci. USA* **2010,** 107, (45), 19213-19218.
248. Pitts, K. E.; Dobbin, P. S.; Reyes-Ramirez, F.; Thomson, A. J.; Richardson, D. J.; Seward, H. E., Characterization of the *Shewanella oneidensis* MR-1 Decaheme Cytochrome MtrA: expression in escherichia coli confers the ability to reduce soluble Fe(III) chelates. *J. Biol. Chem.* **2003,** 278, (30), 27758-27765.
249. Gescher, J. S.; Cordova, C. D.; Spormann, A. M., Dissimilatory iron reduction in *Escherichia coli*: identification of CymA of *Shewanella* oneidensis and NapC of *E. coli* as ferric reductases. *Mol. Microbiol.* **2008,** 68, (3), 706-719.
250. TerAvest, M. A.; Zajdel, T. J.; Ajo-Franklin, C. M., The Mtr Pathway of *Shewanella oneidensis* MR-1 Couples Substrate Utilization to Current Production in *Escherichia coli*. *ChemElectroChem* **2014**.
251. Møller, J. V.; Juul, B.; le Maire, M., Structural organization, ion transport, and energy transduction of P-type ATPases. *BBA-Rev. Biomembranes* **1996,** 1286, (1), 1-51.
252. Noguchi, Y.; Nakai, Y.; Shimba, N.; Toyosaki, H.; Kawahara, Y.; Sugimoto, S.; Suzuki, E.-i., The energetic conversion competence of *Escherichia coli* during aerobic respiration studied by 31P NMR using a circulating fermentation system. *Journal of Biochemistry* **2004,** 136, (4), 509-515.
253. Venkata Mohan, S.; Velvizhi, G.; Vamshi Krishna, K.; Lenin Babu, M., Microbial catalyzed electrochemical systems: A bio-factory with multi-facet applications. *Bioresour. Technol.* **2014**.

254. Rittmann, B. E.; Torres, C. I.; Marcus, A. K., Understanding the distinguishing features of a microbial fuel cell as a biomass-based renewable energy technology. In *Emerging environmental technologies*, Springer: 2008; pp 1-28.
255. Iverson, T. M.; Luna-Chavez, C.; Cecchini, G.; Rees, D. C., Structure of the *Escherichia coli* Fumarate Reductase Respiratory Complex. *Science* **1999,** 284, (5422), 1961-1966.
256. Erickson, B.; Nelson; Winters, P., Perspective on opportunities in industrial biotechnology in renewable chemicals. *Biotechnology Journal* **2012,** 7, (2), 176-185.
257. Thompson, B. G.; Gerson, D. F., Electrochemical control of redox potential in batch cultures of *Escherichia coli*. *Biotechnol. Bioeng.* **1985,** 27, (10), 1512-1515.
258. McKinlay, J. B.; Zeikus, J. G., Extracellular iron reduction is mediated in part by neutral red and hydrogenase in *Escherichia coli*. *Appl. Environ. Microbiol.* **2004,** 70, (6), 3467-3474.
259. TerAvest, M. A.; Ajo-Franklin, C. M., Transforming exoelectrogens for biotechnology using synthetic biology. *Biotechnol. Bioeng.* **2015**.
260. Maciel Filho, R., Development of routes to acrylic and propionic acid production by fermentative process from sugar. **2010**.
261. Angenent, L. T.; Rosenbaum, M. A., Microbial electrocatalysis to guide biofuel and biochemical bioprocessing. *Biofuels* **2013,** 4, (2), 131-134.
262. Jang, Y.-S.; Kim, B.; Shin, J. H.; Choi, Y. J.; Choi, S.; Song, C. W.; Lee, J.; Park, H. G.; Lee, S. Y., Bio-based production of C2–C6 platform chemicals. *Biotechnol. Bioeng.* **2012,** 109, (10), 2437-2459.
263. Clomburg, J. M.; Gonzalez, R., Anaerobic fermentation of glycerol: A platform for renewable fuels and chemicals. *Trends Biotechnol.* **2012**.
264. Gonzalez, R.; Murarka, A.; Dharmadi, Y.; Yazdani, S. S., A new model for the anaerobic fermentation of glycerol in enteric bacteria: Trunk and auxiliary pathways in *Escherichia coli*. *Metab. Eng.* **2008,** 10, (5), 234-245.
265. Clark, D. P., The fermentation pathways of *Escherichia coli*. *FEMS Microbiol. Lett.* **1989,** 63, (3), 223-234.
266. Mehta-Kolte, M. G.; Bond, D. R., *Geothrix fermentans* Secretes Two Different Redox-Active Compounds To Utilize Electron Acceptors across a Wide Range of Redox Potentials. *Appl. Environ. Microbiol.* **2012,** 78, (19), 6987-6995.
267. Erickson, L. E.; Selga, S. E.; Viesturs, U. E., Application of mass and energy balance regularities to product formation. *Biotechnol. Bioeng.* **1978,** 20, (10), 1623-1638.
268. Rodriguez, B. A.; Stowers, C. C.; Pham, V.; Cox, B. M., The production of propionic acid, propanol and propylene via sugar fermentation: an industrial perspective on the progress, technical challenges and future outlook. *Green Chemistry* **2014**.
269. Liu, L.; Zhu, Y.; Li, J.; Wang, M.; Lee, P.; Du, G.; Chen, J., Microbial production of propionic acid from propionibacteria: current state, challenges and perspectives. *Crit. Rev. Biotechnol.* **2012,** 32, (4), 374-381.
270. Zhang, A.; Yang, S.-T., Propionic acid production from glycerol by metabolically engineered *Propionibacterium acidipropionici*. *Process Biochem.* **2009,** 44, (12), 1346-1351.
271. Zeng, A.-P.; Biebl, H., Bulk chemicals from biotechnology: the case of 1, 3-propanediol production and the new trends. In *Tools and Applications of Biochemical Engineering Science*, Springer: 2002; pp 239-259.
272. Nakamura, C. E.; Whited, G. M., Metabolic engineering for the microbial production of 1, 3-propanediol. *Curr. Opin. Biotechnol.* **2003,** 14, (5), 454-459.
273. Laffend, L. A.; Nagarajan, V.; Nakamura, C. E., Bioconversion of a fermentable carbon source to 1, 3-propanediol by a single microorganism. In Google Patents: 1997.
274. Nimje, V. R.; Chen, C.-Y.; Chen, C.-C.; Chen, H.-R.; Tseng, M.-J.; Jean, J.-S.; Chang, Y.-F., Glycerol degradation in single-chamber microbial fuel cells. *Bioresour. Technol.* **2011,** 102, (3), 2629-2634.
275. Alexandersson, E.; Saalbach, G.; Larsson, C.; Kjellbom, P., Arabidopsis plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. *Plant Cell Pysiol.* **2004,** 45, (11), 1543-1556.

276. Reiche, A.; Kirkwood, K. M., Comparison of *Escherichia coli* and anaerobic consortia derived from compost as anodic biocatalysts in a glycerol-oxidizing microbial fuel cell. *Bioresour. Technol.* **2012,** 123, (0), 318-323.
277. Rabaey, K.; Verstraete, W., Microbial fuel cells: novel biotechnology for energy generation. *Trends Biotechnol.* **2005,** 23, (6), 291-298.
278. Ji, X.-J.; Huang, H.; Ouyang, P.-K., Microbial 2, 3-butanediol production: a state-of-the-art review. *Biotechnol. Adv.* **2011,** 29, (3), 351-364.
279. Zeng, A.-P.; Sabra, W., Microbial production of diols as platform chemicals: recent progresses. *Curr. Opin. Biotechnol.* **2011,** 22, (6), 749-757.
280. Tompa, M.; Li, N.; Bailey, T. L.; Church, G. M.; De Moor, B.; Eskin, E.; Favorov, A. V.; Frith, M. C.; Fu, Y.; Kent, W. J., Assessing computational tools for the discovery of transcription factor binding sites. *Nat. Biotechnol.* **2005,** 23, (1), 137-144.
281. Koma, D.; Yamanaka, H.; Moriyoshi, K.; Sakai, K.; Masuda, T.; Sato, Y.; Toida, K.; Ohmoto, T., Production of p-Aminobenzoic acid by metabolically engineered *Escherichia coli*. *Biosci., Biotechnol., Biochem.* **2014**, (ahead-of-print), 1-8.
282. Blanchet, E.; Duquenne, F.; Rafrafi, Y.; Etcheverry, L.; Erable, B.; Bergel, A., Importance of the hydrogen route in up-scaling electrosynthesis for microbial CO_2 reduction. *E&E S* **2015,** 8, (12), 3731-3744.
283. Lorenz, P.; Eck, J., Metagenomics and industrial applications. *Nat. Rev. Microbiol.* **2005,** 3, (6), 510-516.
284. Lee, S. K.; Chou, H.; Ham, T. S.; Lee, T. S.; Keasling, J. D., Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. *Curr. Opin. Biotechnol.* **2008,** 19, (6), 556-563.
285. Weickert, M. J.; Doherty, D. H.; Best, E. A.; Olins, P. O., Optimization of heterologous protein production in *Escherichia coli*. *Curr. Opin. Biotechnol.* **1996,** 7, (5), 494-499.
286. Himmi, E.; Bories, A.; Boussaid, A.; Hassani, L., Propionic acid fermentation of glycerol and glucose by *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii ssp. shermanii*. *Appl. Microbiol. Biotechnol.* **2000,** 53, (4), 435-440.
287. Coral, J.; Karp, S. G.; de Souza Vandenberghe, L. P.; Parada, J. L.; Pandey, A.; Soccol, C. R., Batch fermentation model of propionic acid production by *Propionibacterium acidipropionici* in different carbon sources. *Appl. Biochem. Biotechnol.* **2008,** 151, (2-3), 333-341.
288. Maervoet, V. E.; Beauprez, J.; De Maeseneire, S. L.; Soetaert, W. K.; De Mey, M., *Citrobacter werkmanii*, a new candidate for the production of 1, 3-propanediol: strain selection and carbon source optimization. *Green Chemistry* **2012,** 14, (8), 2168-2178.
289. Abrini, J.; Naveau, H.; Nyns, E.-J., *Clostridium autoethanogenum*, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. *Arch. Microbiol.* **1994,** 161, (4), 345-351.
290. Köpke, M.; Mihalcea, C.; Bromley, J. C.; Simpson, S. D., Fermentative production of ethanol from carbon monoxide. *Curr. Opin. Biotechnol.* **2011,** 22, (3), 320-325.
291. Kopke, M.; Mihalcea, C.; Liew, F.; Tizard, J. H.; Ali, M. S.; Conolly, J. J.; Al-Sinawi, B.; Simpson, S. D., 2,3-butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas. *Appl Environ Microbiol* **2011,** 77, (15), 5467-75.
292. Bruno-Barcena, J. M.; Chinn, M. S.; Grunden, A. M., Genome Sequence of the Autotrophic Acetogen *Clostridium autoethanogenum* JA1-1 Strain DSM 10061, a Producer of Ethanol from Carbon Monoxide. *Genome Announcements* **2013,** 1, (4).
293. Harrington, T. D.; Mohamed, A.; Tran, V. N.; Biria, S.; Gargouri, M.; Park, J.-J.; Gang, D. R.; Beyenal, H., Neutral red-mediated microbial electrosynthesis by *Escherichia coli, Klebsiella pneumoniae,* and *Zymomonas mobilis*. *Bioresour. Technol.* **2015,** 195, 57-65.
294. Lovley, D. R.; Nevin, K. P., Electrobiocommodities: powering microbial production of fuels and commodity chemicals from carbon dioxide with electricity. *Curr. Opin. Biotechnol.* **2013**.
295. Marcellin, E.; Behrendorff, J. B.; Nagaraju, S.; DeTissera, S.; Segovia, S.; Palfreyman, R.; Daniell, J.; Licona-Cassani, C.; Quek, L.-e.; Speight, R.; Hodson, M. P.; Simpson, S. D.; Mitchell, W. P.; Kopke, M.; Nielsen, L. K., Low carbon fuels and commodity chemicals from waste gases - Systematic approach to understand energy metabolism in a model acetogen. *Green Chemistry* **2016**.

296. Daniell, J.; Köpke, M.; Simpson, S. D., Commercial biomass syngas fermentation. *Energies* **2012,** 5, (12), 5372-5417.
297. Bernhardt, P.; Chen, K.-I.; Sharpe, P., Transition metal complexes as mediator-titrants in protein redox potentiometry. *J. Biol. Inorg. Chem.* **2006,** 11, (7), 930-936.
298. Choi, O.; Kim, T.; Woo, H. M.; Um, Y., Electricity-driven metabolic shift through direct electron uptake by electroactive heterotroph Clostridium pasteurianum. *Scientific Reports* **2014,** 4, 6961.
299. Mock, J.; Zheng, Y.; Mueller, A. P.; Ly, S.; Tran, L.; Segovia, S.; Nagaraju, S.; Köpke, M.; Dürre, P.; Thauer, R. K., Energy conservation associated with ethanol formation from H2 and CO2 in Clostridium autoethanogenum involving electron bifurcation. *J. Bacteriol.* **2015,** 197, (18), 2965-2980.
300. Torres, C. I., On the importance of identifying, characterizing, and predicting fundamental phenomena towards microbial electrochemistry applications. *Curr. Opin. Biotechnol.* **2014,** 27, 107-114.
301. Nagarajan, H.; Sahin, M.; Nogales, J.; Latif, H.; Lovley, D. R.; Ebrahim, A.; Zengler, K., Characterizing acetogenic metabolism using a genome-scale metabolic reconstruction of Clostridium ljungdahlii. *Microb Cell Fact* **2013,** 12, 118.
302. Machado, D.; Herrgård, M. J., Co-evolution of strain design methods based on flux balance and elementary mode analysis. *Metabolic Engineering Communications* **2015,** 2, 85-92.
303. van Klinken, J. B.; van Dijk, K. W., FluxModeCalculator: an efficient tool for large-scale flux mode computation. *Bioinformatics* **2015**, btv742.
304. Fast, A. G.; Schmidt, E. D.; Jones, S. W.; Tracy, B. P., Acetogenic mixotrophy: novel options for yield improvement in biofuels and biochemicals production. *Curr. Opin. Biotechnol.* **2015,** 33, 60-72.
305. Ajinomoto, G. Feed-use Amino Acids Business. http://www.ajinomoto.com/en/ir/pdf/Feed-useAA-Oct2013.pdf
306. Becker, J.; Zelder, O.; Häfner, S.; Schröder, H.; Wittmann, C., From zero to hero—Design-based systems metabolic engineering of *Corynebacterium glutamicum* for L-lysine production. *Metab. Eng.* **2011,** 13, (2), 159-168.
307. Krömer, J. O.; Nunez-Bernal, D.; Averesch, N. J.; Hampe, J.; Varela, J.; Varela, C., Production of aromatics in *Saccharomyces cerevisiae*—A feasibility study. *J. Biotechnol.* **2013,** 163, (2), 184-193.
308. Jourdin, L.; Freguia, S.; Donose, B. C.; Chen, J.; Wallace, G. G.; Keller, J.; Flexer, V., A novel carbon nanotube modified scaffold as an efficient biocathode material for improved microbial electrosynthesis. *J. Mater. Chem.* **2014,** 2, (32), 13093-13102.
309. Chen, S.; He, G.; Liu, Q.; Harnisch, F.; Zhou, Y.; Chen, Y.; Hanif, M.; Wang, S.; Peng, X.; Hou, H., Layered corrugated electrode macrostructures boost microbial bioelectrocatalysis. *E&E S* **2012,** 5, (12), 9769-9772.
310. Bernhardt, P. V.; Jones, L. A., Electrochemistry of macrocyclic cobalt (III/II) hexaamines: electrocatalytic hydrogen evolution in aqueous solution. *Inorg. Chem.* **1999,** 38, (22), 5086-5090.

8 APPENDIX

8.1 ADDITIONAL INFORMATION TO THE METABOLIC MODELLING STUDY

The presented study in chapter 4.2 was published in BMC bioinformatics and the reader is referred to the online version of the article, which contains additional information as "*Additional file 1: Metabolic networks and theoretical yields*". This file provides detailed information about the studied *E. coli* carbon networks. The used stoichiometric reactions are given for the core network as well as for all production and electron transport pathways. Furthermore, all computed theoretical maximum yields for each substrate product combination and EET scenario are listed including maximum growth-coupled product yields and number of *efms*.

The file is downloadable from:

http://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-014-0410-2

8.2 ADDITIONAL INFORMATION FOR CALCULATIONS ON THE LYSINE EXAMPLE

The following section is explaining the underlying calculation for the example of electrified lysine production discussed in chapter 6.2.

Lysine production is assumed to be 50 000 t y^{-1} in 250 000 L batches. 120 g L^{-1} titer gives 30 t of lysine per batch. This means that 50 000 t y^{-1}/30 t = 1666 y^{-1} batches need to be done. Batch time is 30 h so 49 980 h fermentation time is needed. 8 760 h per year means that 5.7 vessels would need to run non-stop:

30 t per batch =>205 212mol_{lys} per batch

Scenario 1: Sucrose based bio-production

Theoretical yields of sugar based conventional as well as electrically enhanced fermentations were obtained from stoichiometric network analysis presented in this thesis chapter 4.2. Theoretical Yield: 0.62 g L-lysine per gram glucose. In many processes well

above 80% of theoretical yield is achieved. We assume 90%, which equates to 0.56 g lysine per gram glucose or 0.69 mol per mol_{hexose}.

297 408mol of glucose/batch; 148 704mol of sucrose/batch → 50.8 t of sucrose/batch

Scenario 2: Cathode driven bio-production

Theoretical Yield is $1mol_{lys}$/ mol_{hexose}. We again assume 90% of the theoretical yield achievable, 0.9 mol_{lys}/ mol_{hexose}

228 013mol of glucose/batch; 114 006mol of sucrose per batch → 39.0 t of sucrose per batch

Degree of reduction of glucose/fructose = 4.0 mol_{e^-}/mol_C;

Degree of reduction of lysine= 4.67 mol_{e^-}/mol_C

0.667 mol e^- needed per mol C, 6 C in lysine → 4mol electrons needed per mol lysine on top of what comes in via the substrate. In total 205 212×4 = 820848 mol electrons needed per batch.

(US: 38.75 c$US/ kg; EU: 45.85 c$US/ kg)

In the following **Table 20**, electric power prices are calculated based on average price of electricity in the United States of America (US), and in Europe (EU) as a function of used Voltage. The base kWh prices used for the calculations were obtained from the publications “Electric power monthly – Feb. 2014” from the US Department of Energy (0.0712 $US/kWh), and from the EU document “Energy prices and cost report” for the European commission, SWD(2014) 20 final/2 from 17/03/2014 (0.1642 $US/kWh). All of the prices are calculated in $US, using the €EU to $US conversion rate of 1.368 $US/€EU, obtained from Google in 22/05/2014. The raw bulk sugar prices in US and EU are used are used to calculate the price of sugar-derived electrons. The electron yield assumed was 140.2 mol of e^- per kg of sucrose. All of the prices are calculated in cents of $US (c$US), using the €EU to $US conversion rate of 1.368 $US/€EU, obtained in 22/05/2014. Sugar price in Europe was obtained from http://ec.europa.eu/agriculture/sugar/index_en.htm consulted in 22/05/2014, and sugar price in the US was obtained from a personal communication with L. K. NIELSEN, AIBN, UQ, Brisbane.

Table 20: Electricity and sugar prices in the US and EU.					
Power	1 kWh				
E_{cell}/ Vs	1	1.5	2	2.5	3
P/ A×h	1000	666.6667	500	400	333.333
P/ A×s	3600000	2400000	1800000	1440000	1200000
n/ mol_{e^-}	37.3115	24.8743	18.6558	14.9246	12.4372
US-price/ \$US/ mol_{e^-}	0.001908	0.002862	0.003817	0.004771	0.00572
EU-price/ \$US/mol_{e^-}	0.0044	0.0066	0.008799	0.010999	0.0132

	US-price		EU-price
c\$US/pound	17.58	c€/kg	33.52
c\$US/kg	38.756	c\$US/kg	45.855
c\$US/mol of e^-	0.276	c\$US/mol of e^-	0.327

Based on substrate consumption and assumed yield we calculate:

Table 21: Comparative costs on sucrose for normal and "electron-free" processes.		
	All bought in EU	All bought in US
Cost of sucrose	\$US	\$US
Normal process	23337	19685
Electron-free	17916	15112
Spare for electricity	5421	4573

One has to introduce in the reactors 820 848mol of electrons per batch:

Table 22: Total cost of electrons from electricity per batch.			
0.0044	\$US per mole of electrons (EU-price)	0.001908	\$US per mole of electrons (US-price)
3611	\$US cost of electrons per batch (EU-price)	1566	\$US cost of electrons per batch(US-price)

Savings: EU-price: 5421-3611 = \$US 1810 (30 Mio\$ in 10 years)

US-price: 4573-1566 = \$US 3007 (50 Mio\$ in 10 years).

1 666 batches per year for 10 years:

Saving in % (23337/(17916+3611)×100) -100 = **8.4% EU**

(19685/(15112+1566)×100)-100 = **18.03% US**

8.3 COVER PROFILE CHEMSUSCHEM 05/2015 (HAND DRAWN BY F. KRACKE)

The Front Cover picture of ChemSusChem issue 05/2015 (see **Figure 28**), drawn by co-author Frauke Kracke, was inspired by the concept of using electric energy and biomass for the production of fuels and chemicals. It depicts our present society based on fossil fuels, e.g., oil, which are converted into fuels and chemicals in chemical refineries, and a future greener world in which biomass is converted in electro-bioreactors using energy from renewable sources. The Concept article discusses current benefits and limitations of the technology and its potential to contribute to a more sustainable world. The article shows, for the first time, by the example of an established fermentation process, that there can be realistic economic incentives for using microbial electrochemical technologies for bio-production. However, it also addresses the many hurdles to be overcome before the depicted “green world” can become reality. More details can be found in the concept by HARNISCH et al.[83]

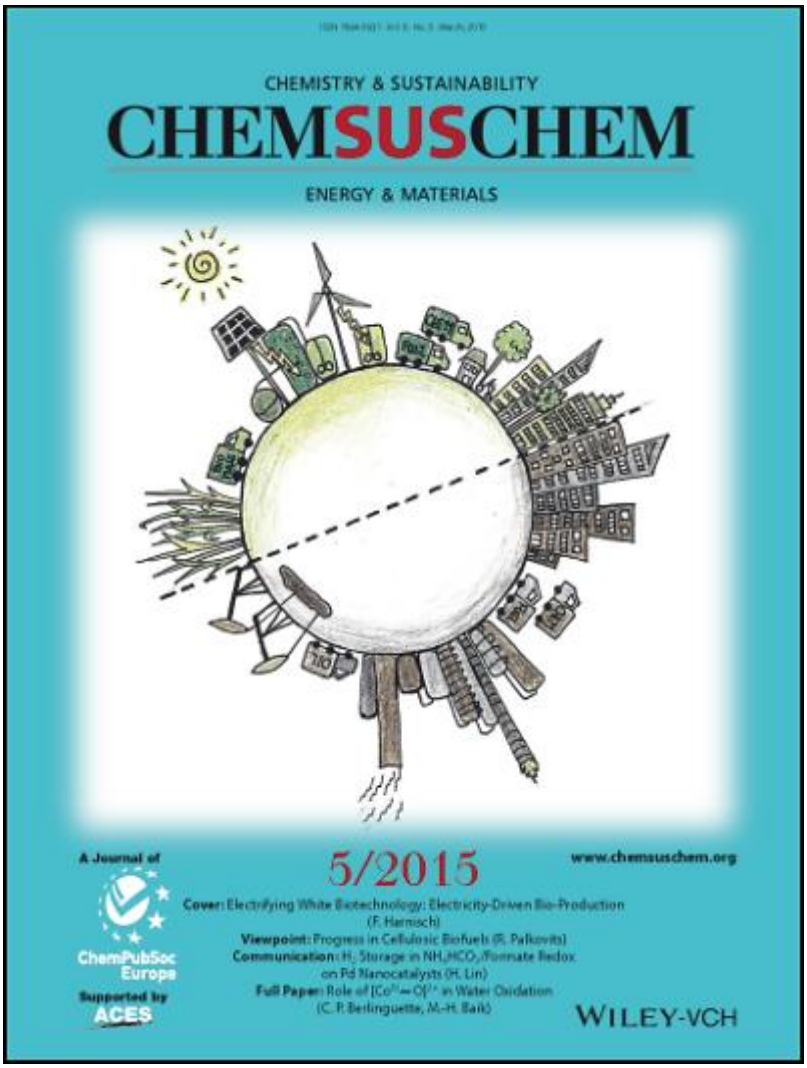


Figure 28: Cover profile ChemSusChem 05/2015.