The Pennsylvania State University

The Graduate School

Department of Civil and Environmental Engineering

HYDROGEN AND ELECTRICITY PRODUCTION FROM CELLULOSE AND MICROBIAL CHARACTERIZATION IN MICROBIAL FUEL CELLS

A Dissertation in

Environmental Engineering

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2008

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ABSTRACT

Cellulosic biomass, including waste products of agricultural and industrial activities, is one of the most unused and abundant renewable energy resources in the world. The direct production of hydrogen and electricity from cellulose offers the potential of integrating the treatment of cellulosic waste and the production of clean fuel and power resources.

The fermentation of cellulosic materials presents a sustainable means of biohydrogen production, but few studies supplied comparable data on cellulose conversion and hydrogen yield. Six mesophilic *Clostridium* species were quantitatively characterized in standardized batch experiments using MN301 cellulose, Avicel, and cellobiose. Results showed clostridia varied in H_2 production, substrate degradation, biomass accumulation, and fermentation product distribution. Of the species tested, *C. cellulolyticum* and *C. populeti* catalyzed the highest H_2 production with cellulose, with H_2 yields of 1.69 and 1.64 mol H_2/mol hexose on MN301 and 1.57 and 1.44 mol H_2/mol hexose on Avicel, respectively. This standardized comparison provides a quantitative baseline for improving H_2 production from cellulose through medium and process optimization and metabolic engineering.

Microbial fuel cells (MFCs) convert biodegradable materials into electricity, potentially contributing to an array of renewable energy production strategies tailored for specific applications. Compared with soluble substrates, cellulose is unique because it requires a microbial consortium that can both metabolize insoluble cellulose from suspension and reduce the insoluble anode. Since no known microorganisms can do both functions, the conversion of cellulosic biomass to electricity requires a synergistic

microbial community. Electricity was generated from cellulose in an MFC using a defined coculture of the cellulolytic fermenter *Clostridium cellulolyticum* and the electrochemically active *Geobacter sulfurreducens*. In fed-batch tests using two-chamber MFCs, the coculture achieved maximum power densities of 143 mW/m^2 (projected anode surface area) and 59.2 mW/m^2 from 1 g/L carboxymethyl cellulose (CMC) and MN301 cellulose, respectively. Neither pure culture alone produced electricity from these substrates. Cellulose conversion to electricity was also demonstrated using an uncharacterized mixed culture from activated sludge, and bioaugmentation was achieved by adding *Clostridium* to enhance cellulose degradation.

The microbial ecology of the defined coculture of *C. cellulolyticum* and *G. sulfurreducens* in cellulose-fed, two-chamber MFCs was further analyzed. Fluorescent *in situ* hybridization (FISH) and quantitative polymerase chain reaction (qPCR) showed that when particulate MN301 cellulose was used as the sole substrate, most *Clostridium* cells were found adhered to cellulose particles in suspension, while most *Geobacter* cells were attached to the electrode. By comparison, both bacteria resided in suspension and biofilm samples when soluble CMC was used. The biofilm in both systems were thin and patchy. This distinct function-related distribution of the bacteria suggests an opportunity to optimize reactor operation by settling cellulose and cellulose-adhered cellulolytic bacteria and decanting supernatant to extend cellulose hydrolysis and improve cellulose-electricity conversion.

Microorganisms that have extracellular electron transfer capability are not only important in MFCs, but also have broaden significance in biogeochemical cycles and bioremediation. There is emerging evidence that this capability is phylogenatically

widespread, yet the culture collection of these exoelectrogens is rather limited. Conventional dilution-to-extinction techniques for isolating such microorganisms are very labor and time intensive. A simple plate technique using an overlay of insoluble hydrous ferric oxide (HFO) as the electron acceptor was developed to isolate presumed dissimilatory metal reducers, which produce a clear zone in the HFO overlay. Using acetate plus lactate as electron donors, three pure cultures and one coculture were isolated from anaerobic sludge. All of these isolates reduced HFO in liquid culture and also showed electrochemical activity in MFCs, indicating this technique can serve as a surrogate for isolation of electrochemically active bacteria. This technique allowed an alternative way to isolate microorganisms that have extracellular electron transfer capabilities, allowing researchers to increase the available variety of microbial phenotypes.

TABLE OF CONTENTS

LIST OF FIGURES viii

LIST OF TABLES x

ACKNOWLEDGEMENTS xi

Chapter 1 Introduction 1

Bioenergy Production from Wastewater and Agricultural Waste 1
Research Summary and Organization of Dissertation 3
List of References 7

Chapter 2 Literature Review 11

The Potential of Cellulose in Renewable Energy Production 11
Hydrogen Production from Cellulose by Fermentation and Microbial Electrolysis Cells (MECs) 12
Electricity Production in Microbial Fuel Cells 14
Improvements and Challenges of MFCs 15
Electricity Production from Different Substrates and Inocula 16
Isolation of Exoelectrogenic Microorganisms 19
Microbial Ecology in MFCs 20
List of References 24

Chapter 3 Characterization of the Cellulolytic and Hydrogen-producing Activities of Six Mesophilic *Clostridium* Species 35

Abstract 35
Introduction 37
Materials and Methods 40
Results 44
Discussion 48
Acknowledgements 53
List of References 54

Chapter 4 Electricity Production from Cellulose in a Microbial Fuel Cell Using a Defined Binary Culture 65

Abstract 65
Introduction 67
Materials and Methods 69
Results 73
Discussion 78

Acknowledgements 81
List of References 82

Chapter 5 Electricity Production and Microbial Biofilm Characterization in Cellulose-fed Microbial Fuel Cells 91

Abstract 91
Introduction 92
Materials and Methods 93
Results and Discussion 98
Conclusions 101
Acknowledgements 102
List of References 103

Chapter 6 Isolation and Identification of Microorganisms Capable of Extracellular Electron Transfer Using a Hydrous Ferric Oxide (HFO) Plate Technique and Microbial Fuel Cells 111

Abstract 111
Introduction 113
Materials and Methods 114
Results 118
Discussion 120
Acknowledgements 123
List of References 124

LIST OF FIGURES

Figure **3-1**: Cumulative hydrogen production by six *Clostridium* strains on MN301 cellulose (A), Avicel (B) and cellobiose (C) (Values shown are averages of triplicate vials). Strains: *C. acetobutylicum* (○), *C. cellulolyticum* (●), *C. cellobioparum* (Δ), *C. celerecrescens* (▲), *C. populeti* (□), and *C. phytofermentans* (■)..... 61

Figure **3-2**: H_2 yield comparison of the six *Clostridium* species for each substrate. Strains: *C. acetobutylicum* (⬚), *C. cellulolyticum* (▥), *C. cellobioparum* (❑), *C. celerecrescens* (▤), *C. populeti* (■), and *C .phytofermentans* (⊠). 62

Figure **3-3**: Daily hydrogen production (●), substrate consumption (□), and biomass accumulation (Δ) for the species with the highest H_2 production on each substrate. (A: *C. cellulolyticum* on MN301 cellulose, B: *C. cellulolyticum* on Avicel, C: *C. acetobutylicum* on cellobiose)..... 63

Figure **3-4**: Maximum daily specific hydrogen production rates. Strains: *C. acetobutylicum* (⬚), *C. cellulolyticum* (▥), *C. cellobioparum* (❑), *C. celerecrescens* (▤), *C. populeti* (■), and *C .phytofermentans* (⊠). 64

Figure **4-1**: Electricity generation by (A) binary culture, (B) *C. cellulolyticum* (□) or *G. sulfurreducens* (Δ), and (C) mixed culture. 87

Figure **4-2**: Voltage (hollow symbols) and power density (solid symbols) vs. current density obtained by varying the external circuit resistance (50-50,000Ω). *G. sulfurreducens* in 12.3 mM acetate (Δ and ▲); binary culture in 1 g/L CMC (◊ and ♦); binary culture in 1 g/L MN301 (□ and ■) and after NaCl addition (○ and ●). 88

Figure **4-3**: Soluble metabolite concentrations (expressed as mg/L COD) for MFCs at the end of fed-batch cycles: Uncharacterized COD (❑), propionate (▦), butyrate (■), ethanol (⬚) and acetate (▧)..... 89

Figure **4-4**: SEM images of (A) coculture anode biofilm (5000X), (B) mixed-culture anode biofilm (5000X). 90

Figure **5-1**: Two-chamber microbial fuel cells used in this study. Anode chamber (left) was filled with growth medium, cathode chamber (right) was filled with buffered ferricyanide solution. 106

Figure **5-2**: Voltage (hollow symbols) and power density (solid symbols) vs. current density obtained by varying the external resistance (50-50,000Ω), 1 g/L CMC (● and ○) and 1 g/L MN301 (■ and □)..... 107

Figure **5-3**: FISH images from suspended samples: (A) CMC-fed MFC, and (B) MN301-fed MFC. Samples were hybridized with Cy3-labeled SRB385 probe targeting *Geobacter* and FITC-labeled EUB338 probe targeting both bacteria, resulting in yellow to orange *Geobacter* cells and green *Clostridium* cells. MN301 cellulose particles were stained with Congo red. Inset in panel B shows the fluorescent response of *Geobacter* cells. 108

Figure **5-4**: Confocal FISH images of biofilm growing on MFC anodes: (A) CMC-fed MFC, and (B) MN301-fed MFC. Anode biofilms were hybridized with Cy3-labeled SRB385 probe targeting *Geobacter* and FITC-labeled EUB338 probe targeting both *Clostridium* and *Geobacter*, resulting in yellow-orange *Geobacter* cells and green *Clostridium* cells. 109

Figure **5-5**: Comparison of gene copy numbers in four different conditions using real-time PCR. *C. cellulolyticum* (⊠); *G. sulfurreducens* (□). 110

Figure **6-1**: (A) Clear zones formed on a HFO plate. (B) A two chamber MFC used in electrochemical tests. 130

Figure **6-2**: Fe(II) generation by isolate 1(◆), isolate 2 (▲), isolate 3 (■), and co-culture (●) from 40 mM HFO in liquid media. 131

Figure **6-3**: Voltage (hollow symbols) and power density (solid symbols) vs. current density obtained by varying the external circuit resistance (10-5,000Ω) in (A) two chamber MFCs and (B) Single chamber MFCs with 0.5 mM cysteine. isolate 1(▽ and ▼), isolate 2 (Δ and ▲), isolate 3 (□ and ■), co-culture (○ and ●) and acclimated activated sludge (◊ and ♦). 132

LIST OF TABLES

Table **3-1**: Molar-based metabolites variation and carbon and electron recovery..... 60

Table **4-1**: Summary of substrate degradation and electron recovery 86

Table **6-1**: Phylogenetic analysis of isolates..... 129

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. John Regan for his guidance, encouragement and support during my PhD study. His precise style and enthusiasm for environmental biotechnology research will benefit me for life. I also like to thank Dr. Bruce Logan for mentoring me in hypothesis-driven research and independent thinking. I could not have asked for better professional role models.

I would like to thank Drs. Fred Cannon, Mary Ann Bruns, and Peggy Johnson for their help through this process and serving as committee members. I appreciate Drs. Matt Mench and Tony Huang for those invaluable discussions. My research was supported by grants from the U.S. Department of Agriculture, the Civil and Environmental Engineering Department, and the Penn State Materials Research Science and Engineering Center.

I would like to thank all my colleagues in Environmental Engineering, especially those in the Regan group and Logan group, for their help and company during this long journey. My special thanks go to David Jones, Peg Van Ornum, Judy Early and all staff for their help.

Finally I would like to thank my family. I could not accomplish this without their support. I am grateful to my wife, Ran Li, who always feeds me well and waits for me at home no matter how late. I thank my parents for supporting me all the time, even though I am half a world away. I thank my unborn son, because he is the reason I will keep going.

Chapter 1

Introduction

Bioenergy Production from Wastewater and Agricultural Waste

Worldwide concerns of energy depletion, global warming, and environmental pollution are driving environmental engineers to expand their responsibilities from pollution clean-up to sustainable development of energy and environmental systems. One example of this is bioenergy production from waste materials, which offers significant economic and environmental benefits because these feedstocks are essentially free and the energy produced offsets the energy deficit associated with conventional treatment process. The traditional strategy of wastewater treatment is very energy intensive, because it requires a considerable energy investment for aeration and sludge disposal. Every year, the U.S. uses approximately 57 TWh electricity for wastewater treatment, which accounts for 1.5% of our total electricity production (1). A sustainable perspective to municipal and agricultural waste treatment considers recovering the energy content of the organic matter while simultaneously achieving treatment objectives. It was estimated that the energy stored in wastewater is about the same amount of energy used for water infrastructure in the U.S., which means that it is possible we can make the water infrastructure self sufficient (1, 2).

Besides wastewater, cellulosic biomass is one of the most abundant and unused sources of renewable energy. The U.S. Departments of Agriculture and Energy estimate

the annual availability of 1.3 billion dry tons of biomass feedstock in the U.S., which could displace 30% or more of the country's present petroleum consumption (3). Cellulose is also a significant component in the annual production of 250 million tons municipal solid waste and 40 billion cubic meter wastewater (3, 4). Depending on the end-use application, cellulosic biomass could be converted to a variety of energy carriers such as ethanol (5), biodiesel (6), hydrogen (7, 8), and methane (9). Only recently has this versatility been extended to electricity (10-12).

As one promising alternative to fossil fuels, the hydrogen economy – an energy system based on hydrogen and electricity – is proposed as a future energy system. Containing the highest energy content per unit weight (143 GJ/tonne), H_2 offers a potential substitute for current transportation fuels, and it is also the only fuel whose oxidation products do not contain carbon dioxide and do not contribute to ozone depletion or acid rain (13). Moreover, H_2 can be produced biologically from cellulosic materials by cellulolytic bacteria, such as *Clostridium* spp., with the stoichiometric maximum yield of 4 mol of H_2 per mole hexose. Counting for the 10-30 billion tons of net primary production of terrestrial cellulose (10), the potential of a cellulose-derived hydrogen market is very promising.

Microorganisms not only can produce fuels such as hydrogen, ethanol, and methane, some also have been found to directly convert organic matter into electricity in devices known as microbial fuel cells (MFCs). In a typical MFC system, electrochemically active bacteria (exoelectrogens) at the anode catalyze the conversion of chemical energy stored in reduced compounds into electricity. Although this metabolic capability has been known for nearly a century (14), only recently has it been exploited for power production,

with several orders of magnitude power density increase in less than a decade of research (15). MFCs use bacteria as biocatalysts and have tremendous electron donor versatility, using virtually any biodegradable resource as substrates. Besides simple substrates such as glucose, acetate, and lactate (16-18), complex substrates such as municipal and industrial wastewaters (19, 20) and cellulose (10, 12, 21, 22) have already been used for electricity production. MFCs can also be configured to produce H_2 instead of electricity by applying a small voltage to reduce protons in an anaerobic cathode chamber (23-25), thereby providing an approach for high-yield H_2 production.

MFC-related systems represent a promising technology for renewable energy production. Recent improvements in architecture and materials have dramatically reduced physical and chemical constraints and improved MFC performance (1). With these advancements, improvements in our understanding of the microbial ecology in MFCs and the deliberate engineering of this information to increase system performance and substrate versatility are necessary for the realization of this potential.

Research Summary and Organization of Dissertation

My PhD research mainly addressed deriving hydrogen and electricity from cellulosic materials. This work included quantitative assessment of hydrogenic and electrogenic potential, molecular analysis of microbial ecology in cellulose-fed MFCs, isolation and characterization of exoelectrogenic bacteria, and assessment of biological constraints at the MFC anode. My dissertation is organized into separate chapters based on manuscripts that I have written during the course of my studies.

During the first year of my research, I characterized cellulose-derived H_2 production by six *Clostridium* species under standardized conditions. Since different clostridia vary in their metabolic pathways, proper natural stains can be characterized and selected for H_2 production, and further improvement can be developed. This work offers quantitative baseline information for these species and found *C. cellulolyticum* and *C. populeti* can be good candidates for metabolic engineering based on their cellulolytic and H_2-producing attributes. I performed the experiments, analyzed the data, and wrote the draft manuscript. Dr. Tom Ward contributed to experimental design and manuscript revision. This manuscript, which is in Chapter 3 of this dissertation, has been published in *Journal of Applied Microbiology* (7).

In my second year of research, I switched focus to cellulose-fed MFCs. I initially used activated sludge as inoculum and successfully produced electricity from cellulose, but there was no single bacterium available to complete this conversion. My hypothesis was that a simple binary culture of *Clostridium* and *Geobacter* could efficiently convert cellulose to electricity, because their coupled metabolisms include cellulose hydrolysis, anode reduction, and an overlap of fermentation products and suitable electron donors. This binary community would be favored thermodynamically over a hypothetical single bacterium, because a consortium can gain more energy than a single bacterium (26). The experimental results confirmed the hypothesis, with electricity produced by the binary culture but not by either pure culture. The unique contribution of this work is that it was the first time a defined coculture was used to directly convert cellulosic material into electricity. I also added *Clostridium* to sludge-inoculated reactors and achieved bioaugmentation. In this project, I designed and performed the experiments, analyzed the

data, and wrote the draft manuscript. Dr. Tom Ward contributed to manuscript revision. The binary culture work, which is presented in Chapter 4, has been published in *Environmental Science & Technology* (12). The mixed culture and bioaugmentation work was published in the proceedings of WEFTEC 2007 (27).

My third project focused on microbial ecology in cellulose-fed binary-culture MFCs, with the goals of using this information to subsequentially direct reactor operation and improve cellulose - power conversion. Cellulose-fed MFCs are unique because both electron donor and acceptor are insoluble. It was hypothesized that in the binary-culture system, *C. cellulolyticum* will mainly adhere on cellulose fibers in suspension, and *G. sulfurreducens* will directly attach to the anode for electron transfer. Our FISH and qPCR results clearly confirmed this hypothesis. This is the first time that molecular tools were used in MFC systems to evaluate the spatial distribution and heterogeneity of different functional groups. In this project, I designed and performed the experiments, analyzed the data, and wrote the draft manuscript. Lisa Steinberg performed qPCR analyses. The manuscript on this microbial ecology is presented in Chapter 5 and has been accepted by *Water Science & Technology* (4).

Exoelectrogenic microorganisms are capable of extracellular electron transfer, such as exhibited in dissimilatory metal reduction and electricity production in MFCs. Only limited cultures are currently available, mainly because conventional isolation techniques are either not appropriate for such microbes or time consuming. In my fourth project, I worked with Dr. Tom Ward on the development of a simple hydrous ferric oxide (HFO) plate technique to isolate presumed dissimilatory metal reducers, which produce clear zones in the HFO layer. Each of the isolates retrieved with this assay also produced

electricity in MFCs, indicating that this technique can also serve as a surrogate for isolation of electrochemically active bacteria. In this project, Dr. Tom Ward designed the HFO technique, recovered the isolates and binary culture, and confirmed biogenic ferrous production in liquid cultures. I completed the assessment of culture purity and phylogenetic identification, evaluated the isolates and binary culture in MFCs, and wrote the draft manuscript. This manuscript is finished (Chapter 6) and will be submitted soon.

I have also contributed to electrochemical characterization of MFC systems. Recent improvements in MFC system architecture dramatically relieved physical and chemical constraints by reducing internal resistance and increasing electrode surface area. However, with these advancements, we found the microbial kinetic constraints are becoming apparent (28). Working with Dr. Matt Mench and Dr. Rama Ramasamy in Mechanical Engineering at Penn State, I helped analyze MFC electrochemical behavior using electrochemical impedance spectroscopy (EIS). I also operated the MFCs and analyzed biofilm architecture using viability staining and confocal microscopy. We quantified and correlated the intrinsic resistance of MFCs with biofilm growth. We found that, in both two-chamber ferricyanide and single-chamber air-cathode systems, the anode kinetic resistance limited the power output, and the growth of anode biofilm decreased the anode polarization resistance and facilitated the kinetics of the electrochemical reactions. Our first paper has been published in *Bioengineering & Biotechnology* (28).

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Chapter 2

Literature Review

The Potential of Cellulose in Renewable Energy Production

Accounting for up to 60% by weight of the total biomass, cellulose is the most abundant and easy to degrade component of plant structural biomass and therefore is the crucial part for biomass-derived energy recovery. As a linear insoluble polymer, cellulose is composed of repeated units of D-glucose linked by β-1,4 glycosidic bonds. Through additional hydrogen bonds, the strands of cellulose are linked together, creating sheets of up to 10,000 glucose molecules and forming a crystalline structure (29). Although this unique structure is chemically homogeneous, the insoluble crystalline microfibrils are very hard to hydrolyze. No single enzyme is known to be able to hydrolyze native cellulose, and only a cluster of interacting enzymes or a multienzyme complex can degrade it (30). This unique structure means that cellulose-derived energy production requires a better understanding of the physiology and metabolism of cellulolytic microorganisms.

Microorganisms developed different strategies to degrade this relatively complex substrate. Aerobic microbes, like fungi, produce single but highly concentrated soluble enzyme components to hydrolyze the glycosidic bonds linking the glucose units (30). By comparison, most anaerobic cellulolytic bacteria like clostridia evolved a more elaborate way to digest cellulose - using an extracellular multi-enzyme complex – the so called cellulosome (31). The composition of different cellulosomes varies, but most of them are

cell protuberances that tightly bind to cellulose and thus mediate a close association between cells and their substrates so as to minimize diffusion losses of hydrolytic products-cellobiose and glucose, which are the monomers being used in cell compartments (2).

From a bioenergy prospective, anaerobic cellulose degradation shows great potential because anaerobes convert cellulose very efficiently into a large variety of metabolites such as acetate, ethanol, and hydrogen. As the depletion of fossil fuels becomes the main issue in the world, cellulose-derived hydrogen and ethanol have great prospect as biofuels. Moreover, most of the other fermentation products can be efficiently used by electrochemically active bacteria, and therefore, can be linked to biological electricity or hydrogen production. Consequently, deriving energy from cellulose offers the great potential of combining biomass disposal with energy production.

Hydrogen Production from Cellulose by Fermentation and Microbial Electrolysis Cells (MECs)

Hydrogen has been considered as a clean and efficient energy resource not only because it has the highest energy content per unit weight, but also owing to its environmentally friendly properties. Hydrogen is seen as a solution to two main problems in the world, providing an alternative transportation fuel and solving problems of pollution, because the only product from hydrogen oxidation in a fuel cell is water. Most commercial hydrogen is now produced from fossil fuels via chemical paths or water electrolysis, which are normally not economical or environmentally friendly (30). That's why biomass-derived biological hydrogen production from anaerobic fermentation and MECs has drawn much

research attention. Biological H_2 production from biomass is a carbon-neutral process, because it releases reassimilated CO_2 from autotrophic carbon fixation. Direct combustion of biomass is similarly carbon neutral, but biological conversion does not produce undesirable combustion products and is more suitable for wet biomass because it doesn't need additional energy input for pre-drying the substrate.

So far, the achieved fermentative H_2 yield from cellulose has been far below the theoretical maximum value of 4 mol H_2/mol hexose. Using heat-shocked sludge as an inoculum, Lay (32) observed a maximum H_2 yield of 0.35 mol/mol hexose occurred at initial 12.5 g/L cellulose concentration, while Logan et al. (33) only found a hydrogen yield of 0.005 mol H_2/mol hexose from cellulose. Using a pure culture of *Clostridium cellulolyticum*, Desvaux et al. (34) observed the H_2 yield decreased from 1.66 to 0.33 mol H_2/mol hexose when the initial cellulose concentration increased from 6.7 g/L to 29 g/L.

The theoretical H_2 yield can not be achieved experimentally because microbes also use some electrons to produce biomass, certain strains produce mixed soluble fermentation products that are more reduced than acetate, and also produced hydrogen may be reconsumed by uptake hydrogenases (35),. However, there is still a lot of potential to improve the fermentative hydrogen yield from cellulose. Although many clostridia have been reported to hydrolyze cellulose and produce hydrogen, few studies supplied quantitative data, and there are no comparable data available since the experimental conditions varied. Therefore, it would be desirable to characterize the capabilities of cellulolytic and H_2-producing bacteria under standard conditions, so the strain(s) with the best combination of properties can be identified, and essential information can be developed for further improvement.

H_2 can also be produced from cellulose using exoelectrogenic bacteria and a small applied voltage in an MEC. (See the following section for reactor details) (1, 23, 24). This approach is very promising because it can overcome the 4 mol H_2/mol hexose constraint of fermentation. By adding 0.6 V voltage, Cheng and Logan achieved 8.2 mol H_2/mol hexose from cellulose. When using acetate as the substrate, the main cellulose fermentation product, up to 99% of the theoretical H_2 yield was achieved based on its stoichiometric potential (4 mol H_2/mol acetate) (24).

Electricity Production in Microbial Fuel Cells

Microbial fuel cells present a promising technology for concurrent waste treatment and power generation. Using electrochemically active microorganisms (exoelectrogens) as biocatalysts, MFCs are bioelectrochemical reactors that can use any biodegradable resource as substrate and therefore have tremendous electron donor versatility. In a typical two-chamber system, anaerobic bacteria oxidize organic compounds in the anode chamber to acquire energy. Some of the electrons derived from this oxidation are transferred to the anode either by direct electron transfer from the bacteria or mediated by soluble electron shuttles, and then flow from the anode to the cathode through an external circuit, where they are harvested for direct electricity generation (MFCs) or used for hydrogen production (MECs). Protons produced from microbial oxidation pass through the ion-exchange membrane and combine with electrons and oxygen at the cathode surface to produce water (in an aerobic cathode) (1).

Improvements and Challenges of MFCs

The power output from MFCs has increased by six order of magnitude in less than a decade of research (36-38). This improvement can mainly be attributed to relieving physical and chemical constraints through architecture optimization. MFC power density (P) is inversely proportional to the total system resistance squared according to:

$$P = \frac{V^2 R_{ext}}{\left(R_{\text{int}} + R_{ext}\right)^2}$$

Since R_{ext} can be varied but R_{int} is fixed for a certain system, reducing internal resistance is the key to improving power production (39). From an early salt-bridge, two-chamber MFC to membrane-free, single-chamber cubic reactors with oxygen, R_{int} has decreased from around 20,000 Ω to less than 50 Ω, and the corresponding power density has increased from 2 mW/m^2 to more than 1000 mW/m^2 (38, 40, 41). Using the same cubic reactor system, several other strategies also showed significant success in improving power density by reducing R_{int}. These methods include reducing distance between electrodes (P increased from 720 mW/m^2 to 1210 mW/m^2) (42), increasing solution ionic strength and conductivity (P increased from 720 to 1330 mW/m^2) (12, 42), inserting a cloth separator between electrodes (P increased from 766 to 1120 mW/m^2) (37, 43), and changing the flow path (P increased from 423 to 1540 mW/m^2) (44).

Exoelectrogenic bacteria rely on the electrode for electron transfer, so it is very important to provide them good access for current generation. Many anode materials have been tested, including plain graphite (45), carbon paper (17), carbon cloth (46), reticulated vitreous carbon (RVC) (47), graphite granules (48), and activated carbon (49). A recent

development of graphite brush anodes provides an ideal solution for scaling up, because it has high specific surface area and an open structure to prevent fouling problems (38). Moreover, this makes the anode no longer affect power production, but instead brings up the challenge of a successful cathode development (1).

The effective and economical design of the cathode is becoming one of the greatest challenges for MFC application. Even though ferricyanide or permanganate can give a higher cathode open circuit potential, oxygen is considered as the electron acceptor for eventual MFC applications (1, 50, 51). However, the tri-phase reaction among oxygen gas, solid catalyst, and liquid electrolyte makes this quite difficult. Because of a low oxygen solubility in water, a direct air cathode is much more effective and energy efficient than an aqueous air cathode (41). Although a recent 3D design of a tubular electrode has the potential of scalability, more tests are needed to reduce the internal resistance and increase its stability (52). Moreover, although Pt catalyst has been widely used in lab-scale experiments, the high cost of Pt is driving the search for less expensive and efficient alternatives. Other metal catalysts such as Co-tetra-methyl phenylporphyrin (CoTMPP) have been tested and show comparable performance as Pt (53), but a more economical way might be using bacteria as biocatalysts, since they are free and sustainable (54, 55).

Electricity Production from Different Substrates and Inocula

With tremendous electron donor versatility, MFCs have been used for power production from many different substrates. Besides simple sugars and derivatives (17, 45), many complex waste materials have also been utilized, such as different wastewaters (19, 56),

starch (57), protein (58), biomass hydrolysate (11), and recently cellulose and landfill leachates (10, 12, 21, 59). However, the power output highly depends on the degradability of the substrate, the architecture of reactor, and the active microbial community. Higher power was usually achieved from simple substrates, while much lower electricity was found from recalcitrant wastes. For example, by using a 4-cm cubic, air-cathode reactor and a sludge inoculum, the maximum power density achieved from acetate, brewery wastewater, and swine wastewater was 766, 205, and 225 mW/m^2 (projected anode area), respectively (19, 43, 60).

Results from different studies also reveal a very broad diversity of the microbial community, indicating the variety of inocula also affects power output (36). While MFCs using marine sediments and anaerobic sludge showed the enrichment of *Deltaproteobacteria* of the family *Geobacteraceae* (61, 62), another MFC inoculated with marine sediments and fed with cysteine showed the community was dominated by *Gammaproteobacteria* with the main species of *Shewanella affinis* (58). Inoculation of an MFC with river water led to the enrichment of *Betaproteobacteria*, while inoculation with wastewater resulted in the dominance of *Alphaproteobacteria* (63).

Among these bacteria, *Geobacter, Shewanella, Pseudomonas*, and *Rhodoferax* are the main isolates whose electrochemical activities have been widely studied (62, 64-66). Although these bacteria have been found to develop various strategies to conduct extracellular electron transfer, all of them can only use easily degraded organic substrates, such as glucose, acetate, or lactate as electron donors. Experiments indicated that electrons derived from the oxidation of organic matter may be nearly fully recovered as electricity in these pure culture MFCs, showing 80-96% coulombic efficiency (16, 64). However, they

hardly can adapt to the natural environment efficiently, because the substrates they need are not directly prevalent naturally and have to be produced by other metabolisms such as fermentation. On the other hand, even though some fermentative bacteria such as *Clostridium butyricum* have been isolated from MFCs, they cannot be a strategy for efficient power production either, because most of the electrons remain in fermentation products that do not readily react with electrodes without anaerobic respiration (67). Although it is possible that there are bacteria that can completely oxidize complex polysaccharides such as cellulose or starch to carbon dioxide and release electrons to electrodes, they cannot compete with the consortium of fermenters and acid-oxidizing, electricity producing bacteria owing to thermodynamic considerations. The thermodynamic rationale is not the amount of energy available per mole of electron donor metabolized, but rather is the amount of energy released per electron transferred (68). This is consistent with the finding that the majority of glucose was fermented in Fe(III)-reducing sediments instead of being directly oxidized to CO_2 with Fe(III) oxide as the electron acceptor (26). *Rhodoferax ferrireducens* is a strain that was isolated from subsurface sediments. Unlike most other exoelectrogenic bacteria, which only can use fatty acids and hydrogen, it can oxidized sugars such as glucose, sucrose, and lactose to CO_2 with over 80% coulombic efficiency in an MFC (64). Although *R. ferrireducens* was also observed to form a biofilm on an electrode surface like *Geobacter* and produced power for long periods of time, it has not yet been identified on sediment MFCs. This further supports the consideration above that a single species cannot compete with the combination of fermenters and fatty acid-oxidizing electrochemically active bacteria in nature (69).

Therefore, the best strategy to derive electricity from complex organic compounds, including those found in most wastes and biomass, requires the cooperation of a consortium of polymer-degrading fermentative bacteria and electrochemically active microorganisms. The fermenters break down the complex organic matter such as cellulose, starch, and proteins into fermentable sugars and amino acids and further into fatty acids and solvents, then the exoelectrogens oxidize the fermentation products to carbon dioxide, with the anode serving as the electron acceptor. Taking insoluble cellulose as an example, bacterial consortium from rumen fluid (10), rice paddy field soil (22) and activated sludge (12) have all been used to convert cellulose into electricity.

Isolation of Exoelectrogenic Microorganisms

As noted before, exoelectrogens are microbes that have extracellular electron transfer capability and can reduce solid electron acceptors, such as metals and electrodes (1). Exoelectrogens are ubiquitous in the environment and play a key role in biogeochemical cycles through mineral transformation and carbon oxidation, and they also contribute to biocorrosion and bioremediation (70). Most known exoelectrogens are dissimilatory metal -reducing bacteria (DMRB), such as *Geobacter* and *Shewanella*, and all natural strains of these bacteria also show electrochemical activity in MFCs (16, 65, 71). However, a recent study showed *Pelobacter carbinolicus*, a dissimilatory iron reducer, was unable to produce electricity in MFCs (72). Conversely, a newly isolated *Ochrobactrum anthropi* YZ-1 was found produce electricity from a variety of substrates, but could not reduce hydrous Fe (III)

oxide (73). This indicates the mechanisms for extracellular electron transfer to metal oxides and fuel cell anodes are closely related, but may not be the same.

The special metabolism and application potential of exoelectrogens have attracted considerable research interest, but only a small fraction of them have been cultured and characterized, mainly because the conventional serial dilution-to-extinction techniques for isolating such microorganisms are not directly connected with the function and very time consuming (74, 75). The Hungate roll tube method provides a direct way to isolate this class of microbes, but the narrow necks of the tubes render isolation and sub-culturing of the tiny colonies quite difficult (76-78). A recent U-tube MFC was used for specifically isolating electrochemically active bacteria (73), but few simple methods are available to specifically isolate dissimilatory metal reducers.

Microbial Ecology in MFCs

Significant research attention has been focused on understanding the microbial ecology in MFC systems. The uniqueness of this system is not only related to the special extracellular electron transfer to solid electron acceptors (anodes) or from electron donors (cathodes), but also comes from how different functional groups interact with each other and form a robust microbial ecological structure.

Extracellular electron transfer has been studied for many years, but how exoelectrogens interact with the electrode is still in debate (69, 79). Generally, it is hypothesized that bacteria can either transfer electrons through immobilized structures or using mobile electron shuttles (69). Some exoelectrogens including *Geobacter* and

Shewanella species establish a direct contact strategy for efficient electron transfer. It has been found that some c-type cytochromes and other outer-membrane proteins were required when *Geobacter* uses Fe (III) or electrodes as an electron acceptor (80). But those proteins are not always sufficient, because *Geobacter* also produced pilus-like nanowires for direct attachment (81). Likewise, *Shewanella oneidensis* MR-1 also produces conductive nanowires, but those pili are longer and arrayed in bundles (82).
By comparison, other bacteria can produce and use soluble redox mediators as an indirect transport system. For example, *Pseudomonas* species can produce phenazines as extracellular electron shuttles (65), and many other bacteria can use externally provided mediators, such as neutral red, AQDS, and some humics (83, 84) . However, since the production and utilization of shuttles require a lot of energy, it has been considered less favorable than direct electron transfer.

The biofilm on an MFC anode is different from conventional biofilm systems. Not only do bacteria need exposure to the bulk solution for electron donors, they also need direct access to the anode for electron release. This creates a duality between bacteria on the bottom of the biofilm and those at the top, because the former group can transfer electrons directly to the anode but lacks substrate availability, while the latter has enough electron donors but is limited in access to the anode. Unlike in traditional chemical fuel cells with metal catalysts or biofuel cells with enzyme catalysts, live bacteria in the anode biofilm are the catalysts for MFCs, so their viability and stability directly determine system performance.

Recent studies showed the microbial biofilm on the MFC anode constraints system kinetics in both two-chamber and single-bottle systems (28 and unpublished data). The

growth of the microbial biofilm was found to decrease the anode polarization resistance by 50% and facilitate the kinetics of the electrochemical reactions. Further analysis showed that during biofilm maturation, the anode polarization resistance was stabilized but the charge transfer resistance on air-cathode (with biofilm) increased. Moreover, biofilm density and morphology changed over time, with denser and more filamentous structures accumulated around carbon fibers. Similar trends were found in continuous running stack MFCs. The diversity of the microbial community decreased, accompanied by a decrease of internal resistance (from 6.5 to 3.9 Ω) and increase of power output (from 73 to 275 W/m^3) (48). This continuous community shift have been confirmed by many other studies (15, 61), and one interesting finding is that some non-electricity producing groups, such as gram positive bacteria, were always found in the system. This demonstrates that an electrochemically competent microbial architecture requires the community succession of different functional groups. This is more apparent when complex substrates were used. However, little is known about the structure of the anode biofilm, the interaction among different groups, and how such information directs a better operation strategy.

As a whole cell technique, FISH is ideally suited to study the architecture of the anode biofilm. This molecular technique allows the *in situ* visualization of target organisms in mixed cultures through the selective hybridization of a fluorescently labeled oligonucleotide probe to the ribosomal RNA of the target cells (85). Prior to the publication of Chapter 5, no study has been reported using FISH to analyze the spatial distribution of exoelectrogens and fermenters in MFCs. The regular procedure of FISH includes the following steps: sample fixation, sample preparation, hybridization with probes, washing off unbound probes, mounting, visualization, and documentation of

results (85). The specificity of the hybridization is achieved by designing a probe sequence that exclusively complements the target organism, and by optimizing the hybridization conditions through the addition of variable amounts of nucleic acid denaturant (86). For analyzing optically thick samples such as multilayer biofilms on an anode, a relatively smooth attachment surface is preferred and confocal scanning laser microscopy (CSLM) can be used in conjunction with FISH to generate optical sections (87).

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Chapter 3

Characterization of the Cellulolytic and Hydrogen-Producing Activities of Six Mesophilic *Clostridium* Species

Abstract

Aims: To characterize cellulolytic, hydrogen-producing clostridia on a comparable basis.

Methods and Results: H_2 production from cellulose by six mesophilic clostridia was characterized in standardized batch experiments using MN301 cellulose, Avicel, and cellobiose. Daily H_2 production, substrate degradation, and biomass production and the end-point distribution of soluble fermentation products varied with species and substrates. All species produced a significant amount of H_2 from cellobiose, with *Clostridium acetobutylicum* achieving the highest H_2 yield of 2.3 mol H_2 mol^{-1} hexose, but it did not degrade cellulose. *C. cellulolyticum* and *C. populeti* catalyzed the highest H_2 production from cellulose, with yields of 1.7 and 1.6 mol H_2 mol^{-1} hexose from MN301 and 1.6 and 1.4 mol H_2 mol^{-1} hexose from Avicel, respectively. These species also achieved 25-100% higher H_2 production rates from cellulose than the other species.

Conclusions: These cellulolytic, hydrogen-producing clostridia varied in H_2 production, with *C. cellulolyticum* and *C. populeti* achieving the highest H_2 yields and cellulose degradation.

Significance and Impact of the Study: The fermentation of cellulosic materials presents a means of H_2 production from renewable resources. This standardized comparison provides

a quantitative baseline for improving H_2 production from cellulose through medium and process optimization and metabolic engineering.

Key words: clostridia, cellulose, fermentation, hydrogen, biomass

Material presented in this chapter was modified from the following paper:

Ren, Z., Ward, TW., Logan, BE., and Regan, JM. (2007). Characterization of the cellulolytic and hydrogen-producing activities of six mesophilic *Clostridium* species. *J. Appl. Microbiol.* 2007, 103(6), 2258-66.

Introduction

To address the global concerns of energy-related environmental pollution, hydrogen has been proposed as a clean and efficient energy carrier of the future. Containing the highest energy content per unit weight (143 GJ $tonne^{-1}$), it offers a potential substitute for current transportation fuels, and it is also the only fuel whose direct oxidation products do not contain carbon dioxide and do not contribute to ozone depletion or acid rain (1). Moreover, H_2 can be produced biologically from renewable resources, including biomass, which is environmentally sustainable and can reduce dependence on fossil fuels (2, 3). Biological H_2 production from biomass is a carbon-neutral process because it releases reassimilated CO_2 from autotrophic carbon fixation, and unlike direct combustion of biomass, biological conversion does not release undesirable combustion products and does not need additional energy input for pre-drying the substrate.

Cellulose, which accounts for 30-50% by weight of biomass, is the most abundant biopolymer on earth. Approximately 30 billion tons of terrestrial cellulose is produced each year (4). The quantity and availability of cellulose make it a major renewable resource (5), and the fermentation of its glucose subunits to H_2 offers one means of combining biomass degradation with energy production. The current bottleneck of this utilization is the hydrolysis of cellulose, and considerable research has been focused on accomplishing this efficiently through physicochemical techniques such as steam explosion and dilute-acid pretreatment, as well as enzymatic hydrolysis (6, 7).

There are many microbial consortia capable of both cellulose hydrolysis and H_2 production, such as ruminal and soil communities, and certain members of the genus *Clostridium* possess both capabilities and are often found dominant in cellulolytic, H_2-producing reactor systems (8-10). Their use could significantly reduce physicochemical or enzymatic pretreatment costs associated with fermentative H_2 production from cellulose. The maximum theoretical yield of H_2 from such a fermentation is 4 mol H_2 mol^{-1} hexose, with the corresponding production of acetate and carbon dioxide. However, this theoretical yield cannot be achieved experimentally because microbes use some electrons to produce more biomass, certain strains produce additional soluble fermentation products that are more reduced than acetate, or the H_2 produced may be reconsumed by uptake hydrogenases of the H_2 producer or other community members in a mixed culture (11). Strategies to address this latter H_2 loss have been demonstrated that apply various selective pressures to repress the activities of H_2 consumers, such as low pH, short retention time, and heat or alkaline treatment (12, 13).

Researchers have tested various inocula for H_2 production from cellulose, but the H_2 yields were generally very low. For example, using an uncharacterized, heat-shocked soil inoculum, Logan et al. (14) found the H_2 yield from cellulose was very low (0.005 mol H_2 mol^{-1} hexose added) compared to glucose (0.92 mol H_2 mol^{-1} hexose) due to poor cellulose hydrolysis. Lay (15) observed a maximum H_2 yield of 0.35 mol mol^{-1} hexose, which occurred at a cellulose concentration of 12.5 g l^{-1} when inoculated with heat-shocked sludge. Using microcrystalline cellulose, Miller and Wolin (16) reported a H_2 yield of 0.007 mol H_2 mol^{-1} hexose added with a pure culture of the ruminal bacterium *Ruminococcus albus*, and a very small amount of H_2 when coupling with a H_2-consuming

acetogen. With *C. cellulolyticum* and MN301 cellulose, Desvaux et al (17) observed that the H_2 yield and the extent of cellulose degradation were highly dependent on the initial cellulose concentration, with the H_2 yield first increasing to 1.66 mol H_2 mol^{-1} hexose at an initial hexose concentration of 44.1 mmol l^{-1}, and then decreasing to 0.33 mol H_2 mol^{-1} hexose at an initial hexose concentration of 179.6 mmol l^{-1}. The degree of cellulose degradation continuously decreased with increasing substrate concentration, from more than 85% at the low substrate concentration down to 45% at high substrate concentration. There are numerous other reports of isolated clostridia that hydrolyze cellulose and produce H_2 (18, 19), but quantitative data collected from comparable experimental conditions are lacking.

The objective of this research was to quantify H_2 production from cellulose for several selected *Clostridium* species. There has been considerable research on the characterization and genetic manipulation of cellulolytic clostridia for enhanced solventogenesis, but this has not been explored for H_2 production. A matrix of batch experiments was performed using six species and three types of cellulosic substrates, with daily monitoring of H_2, substrate, and biomass, and end-point monitoring of soluble fermentation products. These data provide a comparable characterization of the cellulolytic and hydrogen-producing capabilities of these strains, which can serve as a baseline for improving cellulose utilization and H_2 production efficiency through strain selection, medium and process optimization, and potentially metabolic engineering.

Materials and Methods

Cultures and media

Six mesophilic species of *Clostridium* were selected to evaluate cellulose degradation and H_2 production. These species included *C. acetobutylicum* (ATCC 824), *C. cellulolyticum* (ATCC 35319), *C. phytofermentans* (ATCC 700394), *C. cellobioparum* (DSM 1351), *C. celerecrescens* (DSM 5628), and *C. populeti* (DSM 5832). Strain selection was based on obtaining physiologic variety and including species on which extensive background research has been performed, with strains having partially or fully sequenced genomes preferred to facilitate subsequent genetic analyses and modification. The optimum reported growth temperatures for these species ranged from 30-37ºC. *C. acetobutylicum* was included as a negative control because it is a well characterized species that does not grow on cellulose (20). Strains were purchased from ATCC (Manassas, VA, USA) or DSMZ (Braunschweig, Germany), cultured in CM3 medium (21) with modifications as detailed below, and preserved as frozen stocks at -80^{o}C in 15% glycerol. The components of modified CM3 medium (per liter of deionized water) were 5 g cellobiose, 1.3 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 2.9 g $K_2HPO_4 \cdot 3H_2O$, 0.2 g $MgCl_2 \cdot 6H_2O$, 2.5 mg $FeSO_4 \cdot 7H_2O$, 0.075 g $CaCl_2 \cdot 2H_2O$, 0.5 g L-cysteine, 1 mg resazurin, 1 ml mineral solution, and 2 g yeast extract. Mineral solution contained (per liter of deionized water) 1.5 g $FeCl_2 \cdot 4H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2 \cdot 4H_2O$, 36 mg H_3BO_3, 190 mg $CoCl_2 \cdot 6H_2O$, 2.0 mg $CuCl_2 \cdot 2H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, and 36 mg $Na_2MoO_4 \cdot 2H_2O$. The medium was buffered with 0.03 mol l^{-1} 2-(N-morpholino) ethanesulfonic acid monohydrate (MES) and

adjusted to pH 6.5 using 1 mol l^{-1} NaOH, and then was made anaerobic by sparging with nitrogen gas following the technique of Oh et al (12).

Before each batch experiment, aliquots of the frozen stocks were cultured in medium containing cellobiose and used as inocula when the cultures reached exponential growth conditions. Modified CM3 medium was also used for the batch experiments, with cellobiose (the primary soluble product of cellulose hydrolysis) used as a substrate control for H_2 production without the requirement for cellulolytic activity, and equal-mass replacements of cellobiose with MN301 (a combination of amorphous and microcrystalline cellulose; Macherey-Nagel, Duren, Germany) or Avicel (microcrystalline cellulose; PH 105, gift from FMC Corporation, Philadelphia, PA, USA) to examine H_2 production from cellulosic substrates.

Batch culture experiments

Batch H_2-production experiments were conducted with each strain using a large headspace, intermittent gas-release method (22). This is a modification of the Owen method (23), using a small liquid volume relative to the headspace volume to reduce inhibition caused by H_2 accumulation (11) without the need for continuous gas release and monitoring by respirometry (14). In an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI, USA), 120 ml serum bottles (Wheaton Scientific, Millville, NJ, USA) were each filled with 18 ml anaerobic modified CM3 medium (without the growth substrate) and sealed with a butyl rubber stopper (Bellco Glass, Vineland, NJ, USA) and aluminum seal. Concentrated carbohydrate stocks (10×) were prepared similarly in

separate bottles. After autoclaving all the bottles for 15 min, 2 ml of the carbohydrates were transferred aseptically into respective reaction vessels to give a final concentration of 5 g l^{-1}. Bottles were inoculated with the respective pure culture, adjusting the inoculum volume based on OD_{600} values to introduce approximately 2×10^8 cells into each bottle. The batch experiments were performed at 35°C in a shaker with agitation at 100 rpm. Controls without inocula for each substrate (abiotic) and without carbohydrate for each clostridial species (biotic) were also included. All tests (except controls) were run in triplicate with results averaged.

Analyses

Gas production volume was measured and released daily using glass syringes (5-50 ml). H_2 concentration was measured by injecting 0.1 ml of headspace gas using a gastight syringe (Hamilton, Reno, NV, USA) into a gas chromatograph (Model 310; SRI Instruments, Torrence, CA, USA) equipped with a thermal conductivity detector and a molecular sieve column (Alltech Molesieve 5A 80/100 6×1/8×0.085; Alltech Associates Inc., Deerfield, IL, USA) with argon as the carrier gas. Carbon dioxide was analyzed similarly except that a different molecular sieve column (Alltech Porapak Q 80/100 6×1/8×0.085) was used with helium as the carrier gas (12).

Biomass was determined by cellular protein measurement. Samples (0.2 ml) were centrifuged (5,900 × g, 5 min), washed using phosphate-buffered saline (130 mmol l^{-1} NaCl, 10 mmol l^{-1} Na_2HPO_4, pH 7.2), and resuspended in 0.1 ml of 0.2 mol l^{-1} NaOH solution. After another equivalent centrifugation, protein in the supernatant was quantified

spectrophotometrically at 750 nm using the DC protein assay (Bio-Rad Laboratories, Herculues, CA, USA), with bovine gamma globulin as a standard. The remaining pellets not dissolved by NaOH in the MN301 and Avicel experiments were stored at -20°C for subsequent cellulose analysis. Residual cellulose after the protein extraction was then solubilized in 67% sulfuric acid for 1 hour at 30ºC as described by Updegraff (24) and quantified using the phenol-sulfuric acid method for sugars with glucose as the standard (25). Standard curves were also performed for cellobiose, MN301 cellulose, and Avicel using these assay conditions and they were found identical. Cellobiose concentration was determined similarly after filtration of the NaOH supernatant through a sterile 0.45 μm syringe filter.

For metabolite analysis, samples were centrifuged and the supernatant was filtered through 0.45 μm membranes and stored at -20°C. The concentrations of organic acids (formate, acetate, propionate, and butyrate) and solvents (acetone, methanol, ethanol, n-propanol, and butanol) were determined by gas chromatography (Agilent 6890N; Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector and a fused silica capillary column (DB-FFAP) with helium as the carrier gas at a 38.8 ml min^{-1} flow rate.

Culture purity determination

At the end of the experiment, culture purity in each bottle was tested by ribosomal intergenic spacer analysis (RISA) (26). A final sample was withdrawn from each bottle and centrifuged to obtain cell pellets. DNA was extracted and purified from pellets using

the DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocol. Purified genomic DNA was PCR amplified using the eubacterial primer set 926f (16S rDNA, 5'-AAACTYAAAKGAATTGACGG-3') and 115r (23S rDNA, 5'-GGGTTBCCCCATTCRG-3') (27). The thermal profile involved an initial 2 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 56°C, and 1.5 min at 72°C; and a 5-min final extension at 72°C. The PCR products were analyzed by agarose gel electrophoresis.

Statistical analysis

Mean H2 yield values for each substrate were analyzed by one-way analysis of variance (ANOVA) and Tukey's test at the 5% level of significance (28).

Results

H_2 production

With MN301 cellulose, *C. cellulolyticum* produced the most H_2 (15.7 ± 1.2 ml after 9 d) (Fig. 3-1A). The corresponding H_2 yield was 1.7 ± 0.1 mol H_2 mol^{-1} hexose consumed (Fig. 3-2). By comparison, *C. populeti* produced 15.2 ± 1.1 ml H_2 in 8 d (Fig. 3-1A), with a corresponding yield of 1.6 ± 0.1 mol H_2 mol^{-1} hexose consumed (Fig. 3-2). The H_2 yields of *C. cellulolyticum* and *C. populeti* on MN301 were higher than the other four species ($P < 0.05$), and there was no significant difference between these two species within a 95% confidence interval. *C. celerecrescens* produced 20% less H_2 volume on MN301 than *C. cellulolyticum* (13% less on a yield basis). *C. cellobioparum* and *C.*

phytofermentans grew slowly and produced considerably less H_2 (41% and 38% less H_2 volume, respectively, than *C. cellulolyticum*) (Fig. 3-1A). As expected, *C. acetobutylicum* did not degrade cellulose; the negligible H_2 volume produced by *C. acetobutylicum* can be attributed to cellobiose carryover with the inoculum.

The trends with Avicel were similar to MN301, but the amounts of gas produced were smaller and the lag times were longer (Fig. 3-1B). *C. cellulolyticum* again produced the highest H_2 volume (13.9 ± 0.9 ml) and yield (1.6 ± 0.1 mol H_2 mol^{-1} hexose consumed) (Fig. 3-2). *C. populeti* produced a comparable amount of H_2 (13.2 ± 0.4 ml), but the yield was lower (1.4 ± 0.1 mol mol^{-1}) due to greater Avicel degradation. As with MN301, the H_2 yields of *C. cellulolyticum* and *C. populeti* on Avicel were both higher than the other four species ($P < 0.05$), and there was no significant difference between these two species. The yields of H_2 from Avicel with *C. cellobioparum*, *C. celerecrescens*, and *C. phytofermentans* were 29, 21, and 38% less than *C. cellulolyticum*, respectively (Fig. 3-2). Again, *C. acetobutylicum* did not produce gas from Avicel.

The pattern of H_2 production from cellobiose was quite different (Fig. 3-1C). Cellobiose is a soluble hydrolysis product of cellulose, and all the bacteria except *C. phytofermentans* completed gas production in 2 d, accompanied by more than 93% cellobiose consumption. The highest H_2 volume (26.5 ± 2.1 ml) and yield (2.3 ± 0.5 mol mol^{-1}) were achieved by *C. acetobutylicum* ($P < 0.05$). Other strains had H_2 yields between 1.6 and 1.9 mol H_2 mol^{-1} hexose consumed using cellobiose (Fig. 3-2). *C. phytofermentans* showed a longer lag phase and gradual H_2 production, and the final volume of H_2 was only 18.0 ml coupled with 88% consumption of cellobiose.

There was no measurable H_2 production in the uninoculated bottles containing carbohydrates or in the bottles lacking added carbohydrates, even though they contained yeast extract and a small amount of medium carryover from the inoculum.

Substrate degradation and biomass accumulation

In addition to gas production, substrate degradation and biomass accumulation were measured daily (Fig. 3-3 for the species with the highest H_2 yield obtained for each substrate). The consumption of substrate and production of biomass coincided with the production of H_2. Compared to over 90% (4.5 g l^{-1}) cellobiose consumption in 2 days (Fig. 3-3C), MN301 and Avicel degradation was slower and less complete, with *C. cellulolyticum* converting 56% (2.8 g l^{-1}, Fig. 3-3A) and 46% (2.3 g l^{-1}, Fig. 3-3B), respectively, after approximately 10 days. *C. populeti* hydrolyzed the greatest percentage of cellulose, with the conversion of 62% (3.1 g l^{-1}) MN301 and 52% (2.6 g l^{-1}) Avicel. Microscopic observations showed that most cells were adhering to cellulose fibers during the exponential growth phase, while most cells were present in the supernatant at the end of the experiment, suggesting they were no longer participating in efficient cellulolysis.

The maximum specific daily H_2 production rates, normalized by protein measurements, were calculated for each strain and each substrate (Fig. 3-4). Strains with higher H_2 yields also showed faster specific H_2 production. Hydrogen production rates from cellulose varied between 0.74 to 2.06 ml H_2 mg^{-1} protein day^{-1}, which was much lower than from cellobiose (2.41 -3.14 ml H_2 mg^{-1} protein day^{-1}). However, the molar growth yields normalized by substrate consumed were similar, around 32 ± 5.4 g cells mol^{-}

^{1} hexose, with *C. populeti* usually on the high side (35.6-37.1 g cells mol^{-1} hexose), and *C. cellobioparum* commonly low (25.4-28.9 g cells mol^{-1} hexose).

Fermentation products and carbon and electron recovery

In addition to gaseous metabolites and biomass, the fermentation of carbohydrates generates various soluble products, including volatile fatty acids and solvents. The distribution of fermentation products varied for the different strains, but the major products from each strain were generally similar for all three substrates (Table 3-1). Acetate was the only soluble product common to all six species. *C. acetobutylicum*, which grew only on cellobiose, also produced butyrate and a small amount of ethanol (2.5% of the degraded substrate as carbon [notation throughout text]). *C. cellulolyticum* produced acetate as well as an average of 5 times more ethanol than *C. acetobutylicum* and a small amount of n-propanol (1.7-2.1%). *C. cellobioparum* produced primarily acetate, with a similar amount of ethanol as *C. cellulolyticum* (on average 12%) and a slight amount of butyrate. *C. celerecrescens* was the only strain that produced acetone, although only a small amount (0.6-1.5%); the major products were ethanol, acetate, and butyrate. The fermentation product pattern of *C. populeti* was unique in that this strain produced more butyrate than the other species, especially from cellulose, and it did not produce detectable ethanol. *C. phytofermentans* produced acetate, a significant amount of ethanol (up to 23%), and formate (1.3-1.4%).

The carbon recovery ranged from 72.2% to 89.3%. This was calculated based on the measured production of biomass, measured CO_2 gas in the headspace, dissolved CO_2,

and soluble fermentation products compared to the carbon associated with substrate utilization. There were some potential carbon fluxes that were not measured in this experiment, such as lactate and extracellular pyruvate. The dissolved fraction of the produced CO_2 was calculated based on pH, Henry's law, and the Henderson-Hasselbach equation. Results showed that the dissolved CO_2 was less than 10% of the observed headspace CO_2. The percentage of carbon assimilated into biomass, assuming a protein content of 55% and a cell formula of $C_4H_7O_2N$ (29), was approximately 20% for each of the strains, meaning that growth yields were comparable, as discussed above. The electron (redox) recovery ranged from 70-89%, accounting for electrons ending up in H_2, measured soluble products, and biomass.

Confirmation of culture purity

After each experiment, RISA fingerprinting (26) was used to assess the purity of the final cultures. Gels showed identical band patterns in the initial and final samples for each species (data not shown), indicating no contamination at detectable levels, which has been estimated to be 1% of the total DNA.

Discussion

The amount of H_2 produced from sugar fermentation depends on the catabolic pathways used by a bacterium (Eq. 1-3). H_2 production is mainly coupled with the acetate and butyrate production pathways in *C. acetobutylicum* (30, 31), with a greater H_2 molar

yield associated with acetate than butyrate because the latter requires additional reducing equivalents. Stoichiometry alone reveals that H_2 production is not coupled directly with ethanol production.

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2\ C_2H_4O_2 + 4\ H_2 + 2\ CO_2 \quad (1)$$

$$C_6H_{12}O_6 \longrightarrow C_4H_8O_2 + 2\ H_2 + 2\ CO_2 \quad (2)$$

$$C_6H_{12}O_6 \longrightarrow 2\ C_2H_6O + 2\ CO_2 \quad (3)$$

Therefore, the theoretical maximum H_2 yield of 4 mol H_2 mol^{-1} hexose would be achieved with the production of only acetate, and reduced H_2 yields would occur as the distribution of fermentation products shifts toward butyrate and ethanol. This is an important consideration in the selection of strains and the engineered redirection of metabolic flux toward increased H_2 yields. The acetate produced through this fermentation process can also be converted to H_2 using a newly developed microbial fuel cell-based process (32). By adding a small applied voltage (~ 250 mV) with an energy equivalence of approximately 0.5 mol H_2 to a completely anaerobic system, it is possible to convert acetate to hydrogen at yields of up to ~ 3 mol-H_2 per mol-acetate, thereby achieving a net overall increase of five moles of H_2 produced per hexose equivalent.

Of the strains tested in this study, *C. cellulolyticum* and *C. populeti* achieved higher H_2 production and yield on the cellulosic substrates than the other three cellulose-degrading strains, while *C. phytofermentans* showed the lowest H_2 production. These findings are consistent with the stoichiometry mentioned above, because *C. cellulolyticum* produced the highest percentage of acetate (30 ± 4.1%), *C. populeti* generated the most butyrate (29 ± 5.8%), and *C. phytofermentans* produced the most ethanol (21 ± 2.1%). These results are also consistent with other studies of fermentation products. Desvaux (33)

found acetate was the main metabolite (39-58%) coupled with a large amount of H_2 when analyzing carbon flux distribution of *C. cellulolyticum*. Warnick et al (34) found 58% of the metabolites were ethanol when feeding *C. phytofermentans* with cellobiose, but no H_2 data were provided. This present study did not explore variability in cellulose hydrolysis and H_2 production among strains within clostridial species. It is possible that there are other strains with favorable properties beyond those tested here. Moreover, the performance of each strain might differ with other cellulose types. For example, it was demonstrated with *C. lentocellum* that the acetate to ethanol ratio increased when crude biomass was used instead of pure cellulose, but no H_2 data were reported (6, 35).

Cellulosome efficiency also affects the cellulose conversion process, and strains with high conversion kinetics allow smaller reactors with shorter retention times. Most strains produced 70-80% of the total H_2 in 4-5 d on cellulose, but *C. phytofermentans* produced H_2 at a constant rate for 12 d. On this basis, as well as H_2 yield, *C. cellulolyticum* and *C. populeti* are the two best wild type strains for hydrogen production (Figs. 3-2 and 3-4). *C. populeti* initially produced H_2 faster than *C. cellulolyticum* from MN301, potentially because its cellulosome was synthesized more rapidly or was more efficient in adhesion.

Another reason for differing performances of the species may be due to the uniform experimental conditions. Although we attempted to address all of the nutritional requirements of each strain in the medium design, it is possible that the consensus medium was suboptimal for some of the species. *C. cellulovorans* (DSM3052) was initially selected for inclusion in the experiment, but it failed to grow in the consensus medium and was removed from the study. Since the original report for this species (36) states that yeast

extract can be substituted for the more commonly used trace mineral and vitamin components, we have no satisfactory explanation for its failure to grow.

As mentioned above, the extent of cellulose degradation was incomplete in these experiments, with approximately half of the cellulose hydrolyzed and no further growth observed after 12 d. We attribute the lag phase for cellulose degradation observed in these experiments to the synthesis and assembly of the multi-enzyme cellulosome complex (37), given that the culturing in cellobiose does not require cellulosome activity. After this lag, the pH in the bottles dropped to approximately 5.6 due to the accumulation of acidic metabolites, potentially contributing to limitation of cell growth. The inhibition of growth and H_2 production may also have been caused by the depletion of a particular nutrient from the culture medium (38) or inefficiently regulated carbon flow that led to an accumulation of inhibitory intracellular compounds in the cells (39), since other studies showed that reinoculation with new cells led to further cellulose degradation (17). To address these limitations, running reactors in continuous mode and increasing the buffer capacity were reported as successful strategies to improve the degree of cellulose degradation (40). The decline in cellulose hydrolysis rate and the significant amount of residual cellulose also may have been due to a more recalcitrant fraction of the initial cellulosic substrates and fewer new adherence sites on the cellulose fibers such that cells in the supernatant could not participate in the degradation of the residual cellulose (17). This is consistent with the higher H_2 production obtained from MN301 cellulose than from Avicel, presumably because it was harder to hydrolyze the crystalline material more prevalent in Avicel.

Metabolic engineering provides an opportunity to improve H_2 production from cellulose. One strategy would be to start with an efficient cellulose-degrading strain and

improve the hydrogen-producing enzymatic machinery. An analogous approach was used by Guedon et al (41) to enhance ethanol production from cellulose. They introduced *pdc* and *adhII* genes, organized as an artificial operon, into *C. cellulolyticum* to eliminate excess intracellular pyruvate accumulation and redirect the excess pyruvate toward ethanol production. An alternative approach would involve the use of *C. acetobutylicum*, a non-cellulolytic clostridia, because it had a significantly higher H_2 yield from cellobiose than the other strains (Fig. 3-2). Genome sequencing has shown that *C. acetobutylicum* apparently possesses a complete cellulosomal gene cluster, but the genes do not allow the cells to grow on cellulose although several of them are expressed. It was speculated that this lack of growth on cellulose could be due to the absence or inactivity of the major catalytic components of the cellulosome, such as Cel48F and Cel9E homologues (42, 43). Analysis of the genomic sequence of *C. acetobutylicum* to identify the defect(s), followed by introduction of functional copies of the defective gene(s), or introduction of a cellulosomal operon from an active cellulose-degrading strain, offer potential solutions to this problem with *C. acetobutylicum*. Thus, multiple opportunities exist for metabolic optimization through the integration of a high hydrogen-yielding central metabolism and an efficient cellulolytic activity.

To exploit the enormous potential of metabolic engineering for redirecting electron flux to H_2 production from cellulose, it is essential to identify the best target species. Higher H_2 yields and rates from cellulose and higher cellulose degradation are important factors. *C. cellulolyticum* and *C. populeti* showed significantly better performance than the other three cellulose-degrading strains in all of these criteria, indicating they would serve as good parent strains for research on biological hydrogen production from cellulose.

Acknowledgements

This work was supported by a grant from the United States Department of Agriculture (Grant No. 68-3A75-3-150). The authors thank David Jones for help with the measurements of soluble fermentation products.

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Table **3-1**: Molar-based metabolites variation and carbon and electron recovery *.

Clostridium species	Substrate	Electron Recovery (%)	Carbon Recovery (%)	Degraded substrate	H_2	Biomass	CO_2‡	Acetone	Ethanol	n-Propanol	Acetate	Formate	Butyrate
acetobutylicum	MN301	ND†	ND	ND	<0.1	<0.1	ND	ND	ND	ND	ND	ND	ND
	Avicel	ND	ND	ND	<0.1	<0.1	ND	ND	ND	ND	ND	ND	ND
	Cellobiose	89	89	23.1	52.3	9.5	26.8	0.1	1.7	<0.1	14.2	<0.1	6.4
cellulolyticum	MN301	82	82	18.3	31.0	6.1	19.8	<0.1	4.1	0.8	17.2	<0.1	<0.1
	Avicel	82	80	17.4	27.4	6.2	17.9	<0.1	5.2	0.6	14.5	<0.1	<0.1
	Cellobiose	80	77	22.3	40.6	6.2	24.8	<0.1	8.2	<0.1	18.6	0.2	<0.1
cellobioparum	MN301	72	76	12.8	18.6	4.2	15.0	<0.1	2.6	<0.1	8.9	<0.1	0.8
	Avicel	70	73	13.9	15.8	4.1	15.4	<0.1	4.8	<0.1	7.8	<0.1	1.0
	Cellobiose	81	78	21.8	42.3	6.7	26.0	<0.1	7.8	<0.1	15.9	<0.1	0.6
celerecrescens	MN301	74	74	16.7	25.1	5.7	17.2	0.3	5.0	<0.1	8.5	<0.1	1.5
	Avicel	77	79	15.6	19.9	4.9	17.4	0.2	5.7	<0.1	7.2	<0.1	2.6
	Cellobiose	77	76	23.7	38.0	5.5	26.9	0.7	7.9	<0.1	16.6	<0.1	2.0
populeti	MN301	76	82	18.4	30.0	6.1	21.4	<0.1	<0.1	<0.1	6.5	<0.1	7.8
	Avicel	74	82	18.1	26.2	6.1	22.1	<0.1	<0.1	<0.1	3.3	<0.1	9.1
	Cellobiose	79	83	23.8	45.3	7.5	27.8	<0.1	<0.1	<0.1	16.5	<0.1	6.7
phytofermentans	MN301	83	83	13.9	19.3	4.9	14.7	<0.1	6.1	<0.1	10.6	1.2	<0.1
	Avicel	73	72	14.1	14.2	4.0	12.9	<0.1	7.3	<0.1	8.0	1.1	<0.1
	Cellobiose	80	77	22.6	35.6	5.9	27.1	<0.1	13.6	<0.1	13.2	<0.1	<0.1

* Unless noted, the units for all data are mmol l^{-1}.

† ND: Not determined

‡ CO_2 is the sum of headspace CO_2 and dissolved CO_2 (all species).

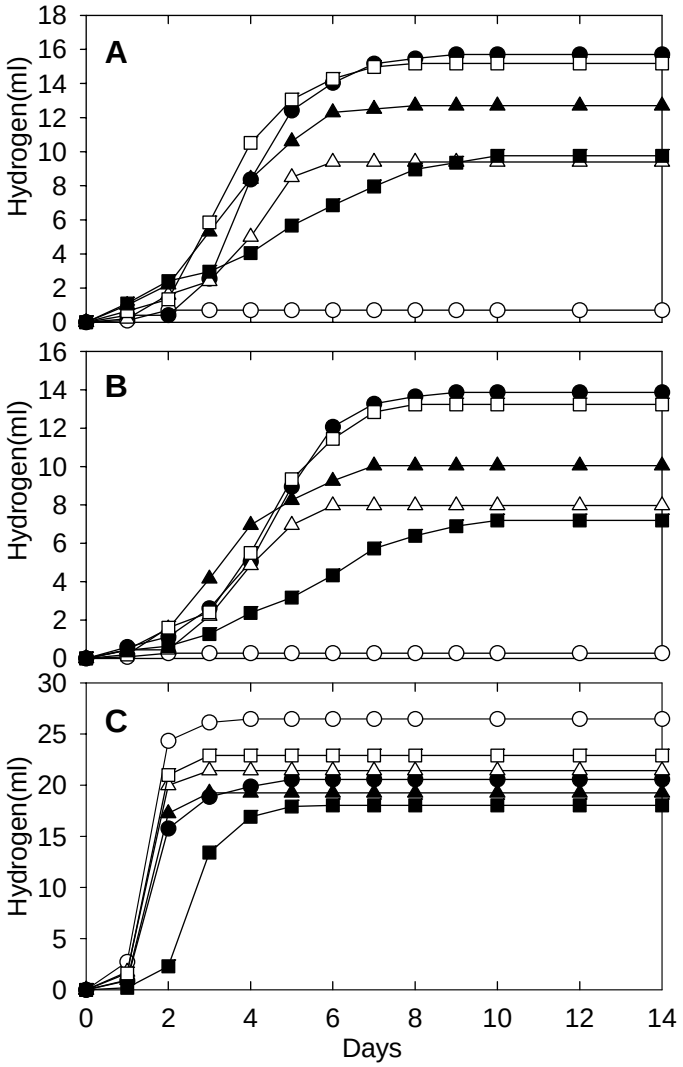

Figure **3-1**: Cumulative hydrogen production by six *Clostridium* strains on MN301 cellulose (A), Avicel (B) and cellobiose (C) (Values shown are averages of triplicate vials). Strains: *C. acetobutylicum* (○), *C. cellulolyticum* (●), *C. cellobioparum* (Δ), *C. celerecrescens* (▲), *C. populeti* (□), and *C. phytofermentans* (■).

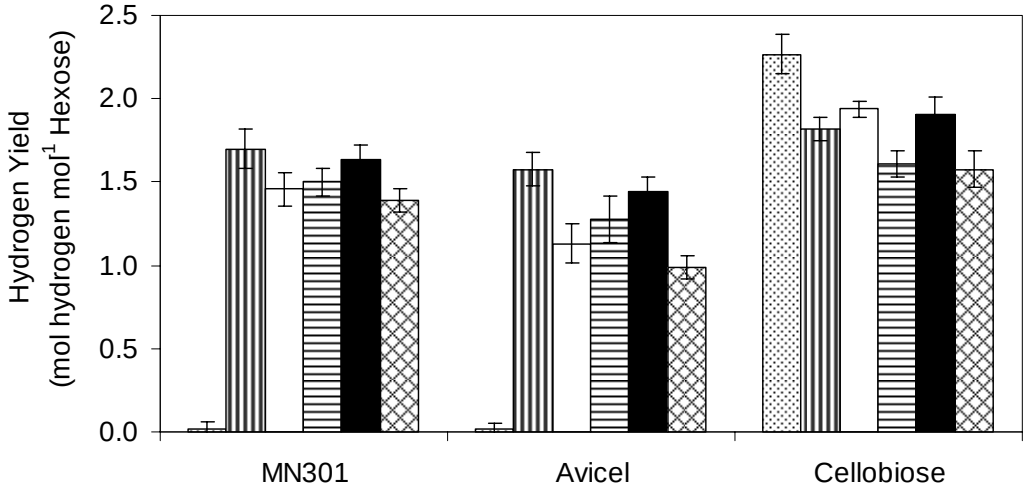

Figure **3-2**: H_2 yield comparison of the six Clostridium species for each substrate. Strains: *C. acetobutylicum* (⬚), *C. cellulolyticum* (▥), *C. cellobioparum* (□), *C. celerecrescens* (▤), *C. populeti* (■), and *C .phytofermentans* (▩).

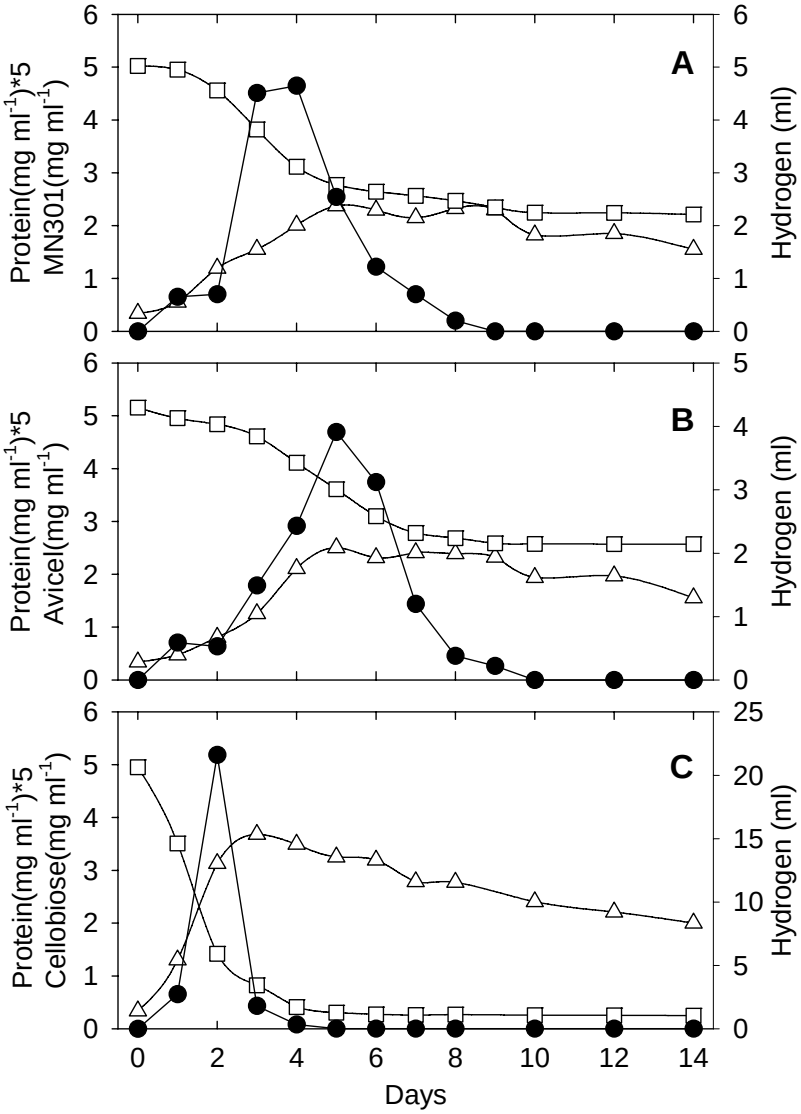

Figure **3-3**: Daily hydrogen production (●), substrate consumption (□), and biomass accumulation (Δ) for the species with the highest H_2 production on each substrate. (A: *C. cellulolyticum* on MN301 cellulose, B: *C. cellulolyticum* on Avicel, C: *C. acetobutylicum* on cellobiose)

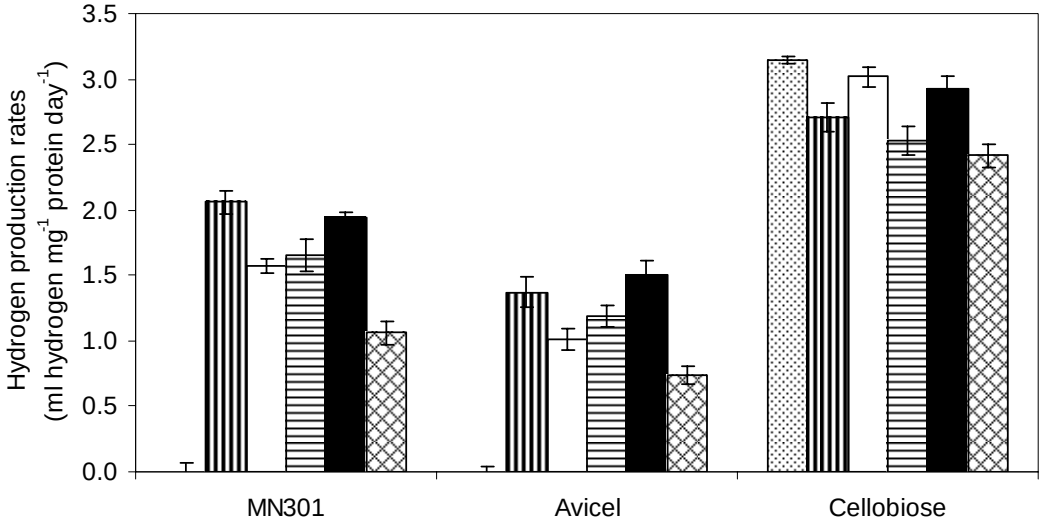

Figure **3-4**: Maximum daily specific hydrogen production rates. Strains: *C. acetobutylicum* (⬚), *C. cellulolyticum* (▥), *C. cellobioparum* (□), *C. celerecrescens* (▤), *C. populeti* (■), and *C .phytofermentans* (▩).

Chapter 4

Electricity Production from Cellulose in a Microbial Fuel Cell Using a Defined Binary Culture

Abstract

Microbial fuel cells (MFCs) convert biodegradable materials into electricity, potentially contributing to an array of renewable energy production strategies tailored for specific applications. Since there are no known microorganisms that can both metabolize cellulose and transfer electrons to solid extracellular substrates, the conversion of cellulosic biomass to electricity requires a synergistic microbial community that uses an insoluble electron donor (cellulose) and electron acceptor (anode). Electricity was generated from cellulose in an MFC using a defined coculture of the cellulolytic fermenter *Clostridium cellulolyticum* and the electrochemically active *Geobacter sulfurreducens*. In fed-batch tests using two-chamber MFCs with ferricyanide as the catholyte, the coculture achieved maximum power densities of 143 mW/m^2 (projected anode surface area) and 59.2 mW/m^2 from 1 g/L carboxymethyl cellulose (CMC) and MN301 cellulose, respectively. Neither pure culture alone produced electricity from these substrates. The coculture increased CMC degradation from 42% to 64% compared to a pure *C. cellulolyticum* culture. COD removal using CMC and MN301 was 38 and 27% respectively, with corresponding coulombic efficiencies of 47 and 39%. Hydrogen, acetate, and ethanol were the main residual metabolites of the binary culture. Cellulose

conversion to electricity was also demonstrated using an uncharacterized mixed culture from activated sludge with an aerobic aqueous cathode.

Keywords: Cellulose, Electricity, Microbial fuel cell, *Clostridium*, *Geobacter*

Material presented in this chapter was modified from the following paper:

Ren, Z., Ward, TW., and Regan, JM. Electricity production from cellulose in a microbial fuel cell using a defined binary culture. *Environ. Sci. Technol*. 2007, 41(13); 4781- 86.

Introduction

The concerns of global fossil fuel depletion and environmental pollution from fossil fuel combustion are driving the search for carbon-neutral, renewable energy alternatives. As one of the most abundant renewable resources, cellulosic biomass, including waste products of agricultural and industrial activities, is particularly attractive in this context because of its relatively low cost and plentiful supply (1, 2). The U.S. Departments of Agriculture and Energy estimate the annual availability of 1.3 billion dry tons of biomass feedstock in the U.S., which could displace 30% or more of the country's present petroleum consumption (3). Depending on the end-use application, this biomass could be converted to a variety of energy carriers such as ethanol (4), biodiesel (5), and hydrogen (6), as well as to electricity indirectly derived from cellulose by coupling cellulolytic, fermentative hydrogen production with the catalytic oxidation of hydrogen at a fuel cell anode (7). This latter strategy is restricted by the theoretical fermentative hydrogen yield of 4 mol H_2/mol hexose, with the balance of electrons appearing in soluble fermentation products and cells.

Using electrochemically active microorganisms as biocatalysts, microbial fuel cells (MFCs) are bioelectrochemical reactors that convert organic material directly into electricity (8). Unlike chemical or enzyme-based fuel cells, which are tailored to oxidize specific electron donors, MFCs have tremendous electron donor versatility. This includes simple substrates such as glucose, acetate, and lactate (9-11); complex substrates such as municipal and industrial wastewaters (12, 13); and even steam-exploded corn

stover hydrolysate (14). MFCs can also be configured to produce hydrogen instead of electricity using an anaerobic cathode and a small applied voltage to reduce protons in the cathode chamber (15), thereby providing an approach for overcoming the 4 mol H_2/mol hexose constraint of fermentation.

Although many types of bacteria have been found to be electrochemically active, none of them show cellulolytic activity, but rather require products of cellulose hydrolysis and fermentation as electron donors (9, 16-18). With the lack of an isolated microbe that can both hydrolyze cellulose and reduce extracellular electron acceptors, one strategy to directly produce electricity from cellulosic biomass, without the expense of chemical catalysts and overcoming the loss of electrons to soluble fermentation products, would involve a synergistic consortium of polymer-degrading, fermentative microorganisms and fermentation product-utilizing, electrochemically active microorganisms. *Clostridium cellulolyticum* degrades cellulose effectively, with the main fermentation products being acetate, ethanol, and hydrogen (19, 20). This makes it a good match with *Geobacter sulfurreducens*, an electrochemically active bacterium that can oxidize most of these fermentation products (21) and use an electrode as an electron acceptor (9). In this study, a defined binary culture of *C. cellulolyticum* and *G. sulfurreducens* was tested in MFCs for *in situ* electricity production directly from cellulose. A mixed culture from activated sludge was also used to test the capability of natural inocula for cellulose-electricity conversion.

Materials and Methods

Cultures and Media

G. sulfurreducens (ATCC 51573) and *C. cellulolyticum* (ATCC 35319) frozen stock cultures were cultured anaerobically in Balch tubes. The media used for the two strains had identical components except for the electron donor and electron acceptor. Both media contained (per liter) 1.05 g NH_4Cl, 1.5 g KH_2PO_4, 2.9 g $K_2HPO_4 \cdot 3H_2O$, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.075 g $CaCl_2 \cdot 2H_2O$, 10 ml trace mineral mix, and 10 ml vitamin mix (22). In addition, *Geobacter* medium contained (per liter) 1.64 g sodium acetate as electron donor and 13.7 g ferric citrate as electron acceptor. *Clostridium* medium contained (per liter) 2 g sodium carboxymethyl cellulose (CMC, MP Biomedicals, Inc.) and 0.5 g yeast extract. The media were adjusted to pH 6.8 and flushed with N_2-CO_2 (80:20) for *Geobacter* and N_2 (100%) for *Clostridium* before distributing and autoclaving in sealed vials. Activated sludge was obtained from the Pennsylvania State University Wastewater Treatment Plant and was stored at 4°C until inoculation into MFCs.

MFC Construction and Operation

Two-chamber MFCs (23) were used in this study, despite their characteristically high internal resistance, because they allow the maintenance of strictly anaerobic conditions in the anode chamber. Reactors were constructed from two medium bottles (310 ml capacity, VWR Inc.) joined by a glass bridge with a proton exchange membrane (Nafion™ 117, Dupont Co.) clamped between the two glass tubes. The anode chamber was sealed with a black rubber stopper in which a 10 mm diameter hole was drilled and

then sealed by a butyl septum stopper to make a gas-tight seal. Anode chambers were filled anaerobically with the medium described above but with 0.1 g/L yeast extract and containing either acetate (without ferric citrate), CMC (soluble; substitution degree 0.65-0.90 carboxymethyl groups per 10 anhydroglucose units), or MN301 cellulose (insoluble; a combination of amorphous and microcrystalline cellulose; Macherey-Nagel, Duren, Germany) and mixed with magnetic stir bars. Cathode chambers were filled with 100 mM $K_3Fe(CN)_6$ solution in 100 mM KH_2PO_4 buffer (pH 7) for the pure- and binary-culture tests. For the mixed-culture MFC, the cathode chamber contained the 100 mM phosphate buffer into which air was continuously sparged to supply oxygen as the electron acceptor. Graphite plates (15.2-cm^2 surface area) were used as anodes. Plain carbon paper and carbon paper with Pt catalyst (0.35 mg/cm^2; 10% Pt) on one side were used as cathodes in the defined culture systems and mixed-culture system, respectively. The electrodes were connected to a 1000 Ω external circuit using copper wires. A reference electrode (RE-5B, Bioanalytical Systems, Inc) was introduced into the anode chamber by embedding it through the stopper. All MFCs were operated in fed-batch mode at 30°C, and sterile medium and $K_3Fe(CN)_6$ were completely replaced when the voltage dropped below 40 mV. Each batch condition was repeated at least three times.

Inoculation and Acclimation

Sterilized anaerobic MFC anode chambers were inoculated with stationary-phase resuscitated pure cultures. *G. sulfurreducens* (5 ml) was transferred into MFC-1 containing 8 mM sodium acetate as the electron donor and the anode as the sole electron

accepter. Two other MFCs (MFC-2 and MFC-3) containing 2 g/L CMC as substrate were inoculated with 5 ml *C. cellulolyticum*. When the voltage of MFC-1 stabilized at approximately 430 mV after 62 hours, the anode of MFC-2 was replaced with the anode of MFC-1, which had been colonized by *G. sulfurreducens*. A new sterile graphite electrode was then inserted into MFC-1. Therefore, MFC-1 contained pure *Geobacter*, MFC-2 contained a coculture of *Geobacter* and *Clostridium*, and MFC-3 contained pure *Clostridium*. For the uncharacterized mixed culture MFC, 2 ml of activated sludge was transferred into an anode chamber containing either 2 g/L CMC or MN301 as substrate. All transfers were performed aseptically in an anaerobic chamber to prevent oxygen intrusion.

Analyses

Voltage (V) was continuously monitored using a data acquisition system (ADC22, Pico Technology, Ltd.) and periodically confirmed with a multimeter. Polarization data was collected by changing the external resistance using a variable resistor box during the stable power production stage of each batch experiment. The calculations of power density and coulombic efficiency (i.e., the fraction of electrons removed from the electron donor that are recovered as current through the external circuit) were performed according to Cheng et al (22). CMC concentration was determined using the phenol-sulfuric acid method for sugars after filtering samples through 0.2 μm PES membrane filters (Pall Corp.) to remove cells. Biomass binding to MN301 was removed by centrifugation at 5,900 × g for 5 minutes and washed using phosphate-buffered saline

(130 mM NaCl, 10 mM Na_2HPO_4, pH 7.2), and resuspended in 0.2 M NaOH solution. After another equivalent centrifugation, the remaining MN301 cellulose pellets (not dissolved by NaOH as is the biomass) were analyzed using the phenol-sulfuric acid method for sugars (19). COD was measured using a colorimetric assay (HACH Co.) after the removal of biomass (24), by filtration for CMC-fed reactors, and for MN301 fed reactors on both the filtrate and the solid after filtration and NaOH lysis and washing. For soluble metabolite analysis, samples were centrifuged and the supernatant was filtered through 0.2 μm membranes and stored at -20°C. The concentrations of organic acids and solvents were determined by gas chromatography (Varian Star 3400) with a flame ionization detector as previously reported (24). The anode headspace gas composition was analyzed for H_2, CH_4, CO_2 and N_2 by gas chromatography (model 310; SRI Instruments) equipped with a thermal conductivity detector as previously reported (24). The internal resistance of the MFCs was measured by electrochemical impedance spectroscopy using a PC4/750 potentiostat (Gamry Ins.) with the anode as the working electrode and the cathode as the counter electrode (22). Conductivity was measured using a conductivity meter (Oaklon).

Scanning electron microscopy (SEM) was used to examine the anode biofilm and suspended microorganisms. Samples were prepared by 2% glutaraldehyde fixation. Biofilm and filtered solution samples were then prepared for SEM observation following the technique of Liu et al (25), and the SEM (JSM 4500, JEOL) was operated at 20 keV.

Results

Electricity Generation by the Binary Culture

Upon transferring the anode from MFC-1 into MFC-2, the power density rapidly increased to 151 mW/m^2 (projected anode surface area, throughout) (Figure 4-1A) as *G. sulfurreducens* oxidized the CMC fermentation products of *C. cellulolyticum*. After attaining this level, the power density slowly declined, but electricity was generated for 24 days. When the anode solution was replaced with new medium containing 1 g/L CMC, power production resumed quickly even though no pre-fermentation by *C. cellulolyticum* occurred, and the maximum power density of 143 mW/m^2 was comparable to the initial batch. After five repeated cycles (complete data not shown), 1 g/L MN301 cellulose was used as the substrate instead of CMC. The maximum power density achieved from MN301 was 59.2 mW/m^2 after 3 days, and the power density dropped slowly to 0.76 mW/m^2 over the next 20 days (Figure 4-1A). Comparable results were obtained with three repeated MN301 additions.

For the pure-culture MFCs, as expected, *G. sulfurreducens* produced power quickly when fed acetate in MFC-1 (Figure 4-1B) (9). However, when the substrate was changed to CMC, power was not produced. Moreover, no cellulose degradation occurred in that cycle, indicating CMC was not degraded by *G. sulfurreducens*. When acetate was added back into MFC-1 after a period of 193 hrs with only CMC, power production resumed very quickly, indicating that the cells were still alive. In MFC-3, *C. cellulolyticum* did not show power production from either CMC or cellobiose (Figure 4-1B), even though it did degrade these substrates. Very small amounts of power (max.

1.16 mW/m^2) of short duration were detected immediately following each medium transfer, but this was presumably not due to microbial activity.

To compare electron recovery and power production between the *Geobacter* pure culture and the binary culture, new medium containing 12.3 mM acetate was fed to the *G. sulfurreducens* MFC (MFC-1) to provide the same electron equivalence (98.4 mM) as the cellulose-fed coculture system, assuming acetate was the only soluble fermentation metabolite produced from the cellulose (2 mol acetate/mol hexose). The differences in power production across a range of current densities were obtained by varying the external resistance (50-50000 Ω) (Figure 4-2). The power density achieved by *G. sulfurreducens* with acetate was comparable to that from the CMC-fed binary culture, with maximum power densities of 154 ± 4.1 mW/m^2 and 143 ± 7.2 mW/m^2, respectively. The power density achieved from the MN301-fed binary culture was lower, with a maximum value of 59.2 ± 3.5 mW/m^2. When MN301 was used instead of CMC, the internal resistance of the system increased from 770 Ω to 890 Ω and the conductivity of the anode solution decreased from 7.43 ms/cm to 5.81 ms/cm. After adding 3 g/L NaCl to the MN301 medium, the solution conductivity increased from 6.08 ms/cm to 11.34 ms/cm, but the maximum power density only increased to 67.9 mW/m^2 (Figure 4-2), indicating that decreased solution conductivity did not explain the significant drop in power density with MN301.

When fresh cultures of *G. sulfurreducens* and *C. cellulolyticum* were inoculated together into new medium with a sterile anode, a longer lag time (42 h) was observed and gradual power generation (max. 91.6 mW/m^2) was detected, but when the anode solution was replaced with fresh CMC medium, power generation resumed quickly (max. 134

mW/m^2) (data not shown), which indicated that the pre-fermentation and anode pre-colonization (MFC-2) were helpful for startup but not required in a cellulose-fed MFC.

Electricity Generation by a Mixed Culture

With 2 g/L CMC as the substrate, the uncharacterized mixed culture generated electricity after a lag time of 51 hours following inoculation, but the maximum power density was only 14.9 mW/m^2. The power density increased after subsequent medium changes, presumably indicating the enrichment of functionally relevant community members (Figure 4-1C). The maximum power density achieved by the mixed culture after three medium changes was 42.2 ± 6.1 mW/m^2 on CMC and 33.7 ± 4.9 mW/m^2 on MN301. However, a lower cathode potential due to the use of aqueous oxygen instead of ferricyanide precludes a direct comparison between the mixed- and pure-culture systems.

Cellulose Degradation and Coulombic Efficiency

Cellulose degradation, COD removal, and coulombic efficiency for the various MFC conditions are compared in Table 4-1, based on analyses of samples from the end of batch cycles. *G. sulfurreducens* consumed 76 ± 3% of the COD when using 12.3 mM acetate as the electron donor, and directed approximately half of these electrons to anode reduction. For the 1 g/L CMC tests, 42 ± 5% of the sugars present in CMC were metabolized in the pure *C. cellulolyticum* system, and most of the COD of those sugars remained in solution as other metabolites, resulting in a COD removal of only 16 ± 5%.

With no current produced in this system, the COD loss was presumably attributable to biomass generation, hydrogen production, and neutral metabolites diffusing to the cathode chamber. The coculture degraded 64 ± 4% of the CMC in 16 days, which was 52% more than the pure culture, showing relief of product inhibition through their consumption by *Geobacter*. The corresponding COD removal by the coculture using CMC was 38 ± 3%, with electricity production accounting for nearly half of these electrons and most of the other degraded CMC recovered as residual soluble fermentation products as described below. The coculture degraded 49 ± 6% of the MN301, which was less than observed with CMC, but still higher than the CMC-fed *C. cellulolyticum* pure culture. This MN301-fed system had a similar degree of COD loss relative to the extent of cellulose degradation (55%) as the CMC reactor, but a slightly lower coulombic efficiency.

The sludge inoculum showed a similar amount of degradation with both CMC and MN301 (41 ± 5% and 40 ± 7%, respectively). Only a quarter of the electrons removed from solution were transferred to the anode. The coulombic efficiencies with the mixed culture were lower than with the binary culture presumably due to oxygen diffusion from the cathode and non-productive metabolisms such as methanogenic activity. The final pH ranged from 5.2-5.9 for the various conditions, including binary- and mixed-culture systems.

Metabolite Variation

C. cellulolyticum fermented cellulose primarily into acetate, ethanol, and hydrogen as expected (20), with acetate plus ethanol accounting for nearly 90% of the soluble COD products (Figure 4-3). *Geobacter* consumed some of these as electron donors in conjunction with anode reduction. Hydrogen comprised approximately 50% of the headspace in both cases on day one and declined slowly until it reached 15% in the headspace of the coculture reactor and 29% in the *Clostridium* reactor. By comparison, the metabolites in the mixed-culture

MFC were more diverse, with acetate, butyrate, propionate, and ethanol as the main components (Figure 4-3). Their total residual concentrations were less than in the coculture system. There was no hydrogen detected in the headspace of the mixed-culture system, and the methane proportion was greater than 70%.

Anode Biofilms

Scanning electron micrographs taken of the anode biofilms showed various biofilm morphologies for different communities (Figure 4-4). The binary culture formed tight microcolony structures, while the mixed culture from activated sludge showed heterogeneous cell morphologies and a looser biofilm structure. Lower magnification images showed that the coculture biofilm had patchy coverage of the anode, while the mixed culture formed a more uniform biofilm over the surface of the graphite plate.

Discussion

The binary culture of *C. cellulolyticum* and *G. sulfurreducens* converted cellulose into electricity, without enzymatic pretreatment or an exogenous catalyst. In this defined system, *C. cellulolyticum* fermented cellulose mainly into acetate, ethanol, hydrogen, and carbon dioxide, and *G. sulfurreducens* transferred electrons from some of these fermentation products to the anode via anaerobic respiration. Since no known microbial strain performs this complete conversion by itself, this complementary pairing of cellulolytic and exoelectrogenic activities is currently necessary for electricity production from cellulose. It has been proposed that this coupling of fermentation with anaerobic respiration has a thermodynamic advantage over complete anaerobic oxidation of a fermentable substrate by a single microbe, based on the energy yield per electron transferred in the respective reactions (18, 26). This is consistent with the finding that the majority of glucose was fermented in Fe(III)-reducing sediments instead of being directly oxidized to CO_2 with Fe(III) oxide as the electron acceptor (27), even though a glucose-oxidizing iron reducer has subsequently been isolated from this environment (17). Therefore, a consortium of cellulolytic fermenters and electrochemically active bacteria might have a competitive advantage over a hypothetical cellulolytic exoelectrogen.

The maximum power density achieved by the coculture with CMC (143 mW/m^2) was comparable to the power output using acetate as the electron donor for *G. sulfurreducens* (154 mW/m^2). These results were higher than a similar two-chamber system (75 mW/m^2) that used 20 mM acetate, a mixed-culture inoculum, and ferricyanide as a catholyte (28). However, the MN301-fed coculture system produced less than half

this power density, for reasons other than MN301 effects on solution conductivity. The chemical functionalization of CMC makes it soluble and more accessible for degradation than native cellulose. Amorphous and microcrystalline MN301 is more similar to native cellulose, and the lower rate of MN301 hydrolysis and fermentation by *C. cellulolyticum* contributed to the lower power density with this substrate. With both cellulosic substrates, COD removal was less than cellulose degradation because *G. sulfurreducens* did not use all of the available fermentation products by the time power production decreased in each batch cycle, as confirmed by the residual acetate and ethanol concentrations. A likely explanation for this is that the final pH usually dropped to approximately 5.2 due to the acidic products of fermentation, which may have inhibited the activity of *G. sulfurreducens*. This was supported by a control experiment, showing that power production resumed quickly from a non-productive pH 5.2 reactor after an injection of sterilized NaOH, which increased the pH to 7.0. Since the buffer capacity of the medium was designed based on common *Clostridium* media, *Geobacter* probably could not tolerate such a range of pH variation.

The activated sludge inoculum also generated electricity from cellulose. However, the different experimentally measured cathode open-circuit potentials (+427 mV vs normal hydrogen electrode (NHE) for aqueous oxygen and +577 mV vs NHE for ferricyanide) and different overpotential losses associated with aerobic and ferricyanide cathodes preclude a direct comparison of power density between these experiments. Ferricyanide was used as the cathodic electron acceptor in the defined culture experiments (29, 30), which enabled oxygen-free operation of the cathode compartment. This was critical because both species used in these tests are strict anaerobes. Based on

previous experience, *G. sulfurreducens* can tolerate a slight amount of oxygen once acclimated to the anode, but it was difficult to initiate the growth of either *G. sulfurreducens* or *C. cellulolyticum* in MFCs using aqueous oxygen as the cathodic electron acceptor, unless an oxygen scavenger such as cysteine is added to the anode chamber. However, cysteine can also be used as an electron donor for electricity production (23). In contrast, aqueous air cathodes, which are more practical for eventual MFC applications, were used in the mixed-culture fuel cell because microaerophilic and/or facultative bacteria would be present and could grow on the PE membrane, scavenge oxygen, and reduce its diffusion into the anode chamber. The coulombic efficiency was also lower in the mixed culture than in the binary culture, presumably due to competing metabolisms and oxygen diffusion through the membrane. The presence of methane in the headspace showed that methanogenesis was a significant competitive process to extracellular electron transfer. However, the results of these tests showed that natural consortia can also achieve cellulose-electricity conversion, but more efficient cultures would accelerate this process.

SEM pictures showed different biofilm architectures between the defined binary culture and the undefined mixed culture. While the performance data indicate that *C. cellulolyticum* remained in the binary-culture system at sufficient density to rapidly produce metabolites for *Geobacter* growth, the SEM pictures do not reveal the specific localization of the two functional species. More specific identification tools, such as fluorescent *in situ* hybridization, may be needed to ascertain the detail of the interaction between the two strains.

This research demonstrates the use of whole cells as biocatalysts for cellulose-derived electricity production, and it shows promise as a new method to accomplish both waste biomass treatment and electricity generation. Optimizing the reactor configuration, especially for particulate substrates, is necessary for the further development of cellulose-derived electricity production. In addition, running reactors in continuous mode and increasing the buffer capacity should increase power density, cellulose degradation, and electron recovery (31). Higher power is anticipated if physical and chemical constraints of the reactors are addressed (32). Furthermore, this MFC system employs a unique microbial ecology in which both electron donor and acceptor are insoluble. It should be very helpful for studying the microbial communities and improving our understanding of how bacteria degrade solid substrates and how they transfer electrons to solid electron acceptors.

Acknowledgements

This work was supported by a grant from the U. S. Department of Agriculture (No.68-3A75-3-150). The authors thank Dr. Shaoan Cheng for his valuable assistance and David Jones for the measurements of soluble fermentation products.

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Table **4-1**: Summary of substrate degradation and electron recovery

Parameter	***Geobacter* (Acetate)**	***Clostridium* (CMC)**	**Coculture (CMC)**	**Coculture (MN301)**	**Sludge (CMC)**	**Sludge (MN301)**
COD degradation* (%)	76 ± 3	16 ± 5	38 ± 3	27 ± 4	32 ± 4	34 ± 7
Cellulose degradation (%)	NA	42 ± 5	64 ± 4	49 ± 6	41 ± 5	40 ± 7
Coulombic efficiency† (%)	51 ± 4	0	47 ± 4	39 ± 6	27 ± 4	22 ± 5

* All COD were measured after removal of biomass.

† Based on COD removed

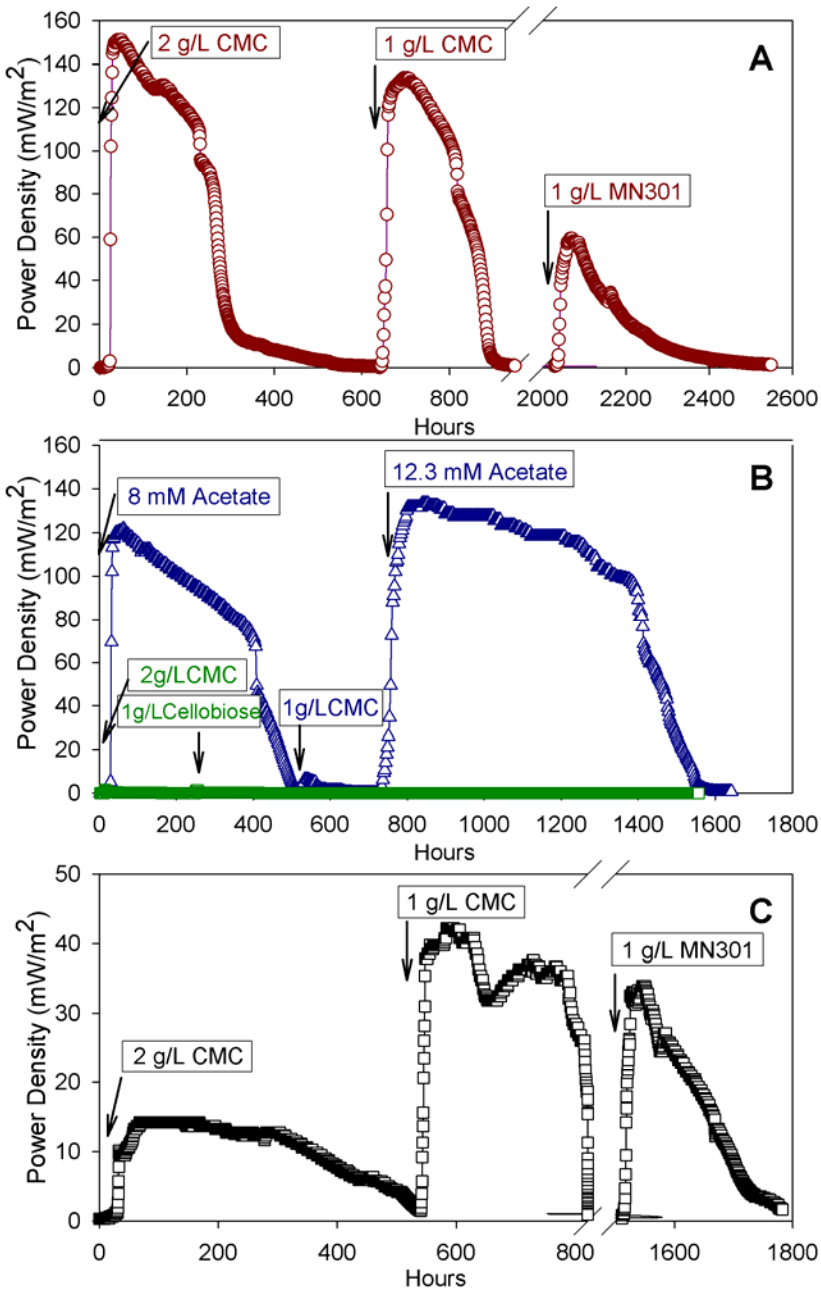

Figure **4-1**: Electricity generation by (A) binary culture, (B) *C. cellulolyticum* (□) or *G. sulfurreducens* (Δ), and (C) mixed culture.

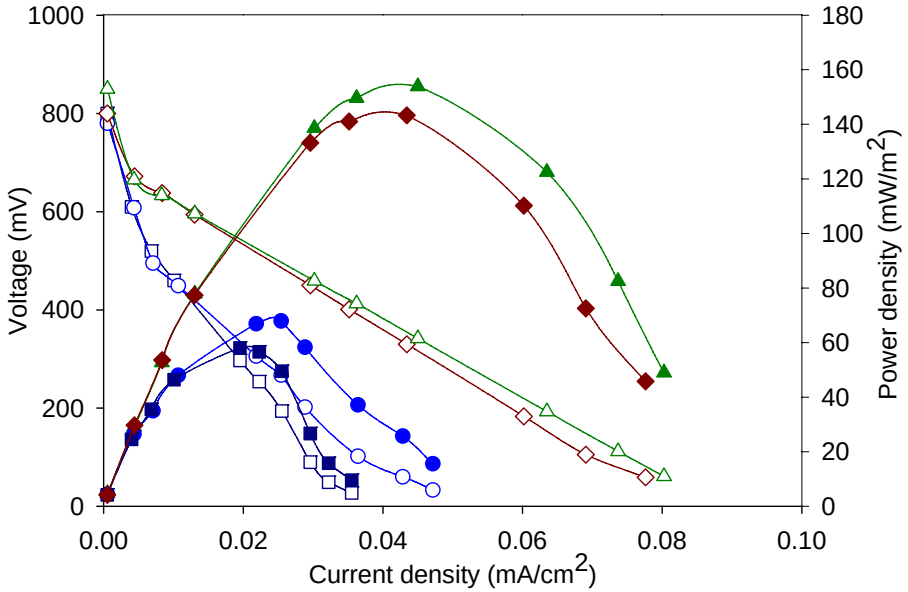

Figure **4-2**: Voltage (hollow symbols) and power density (solid symbols) vs. current density obtained by varying the external circuit resistance (50-50,000Ω). *G. sulfurreducens* in 12.3 mM acetate (Δ and ▲); binary culture in 1 g/L CMC (◊ and ♦); binary culture in 1 g/L MN301 (□ and ■) and after NaCl addition (○ and ●).

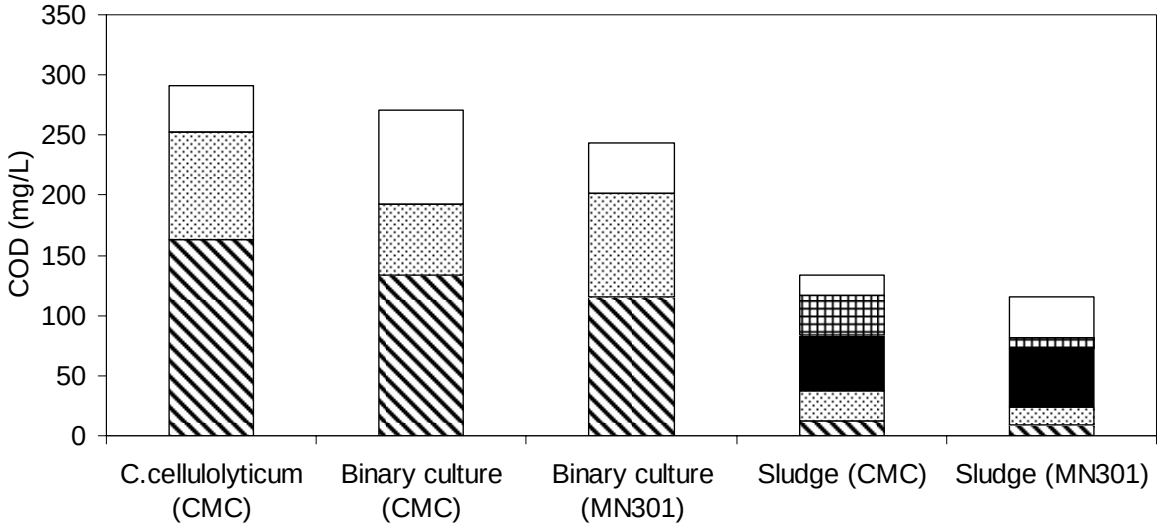

Figure **4-3**: Soluble metabolite concentrations (expressed as mg/L COD) for MFCs at the end of fed-batch cycles: Uncharacterized COD (□), propionate (▦), butyrate (■), ethanol (⬚) and acetate (▨).

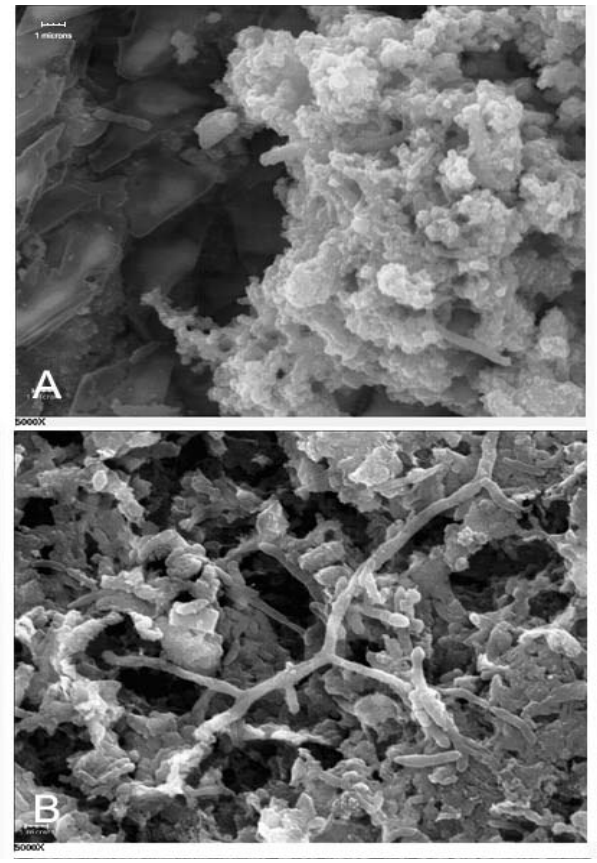

Figure **4-4**: SEM images of (A) coculture anode biofilm (5000X), (B) mixed-culture anode biofilm (5000X).

Chapter 5

Electricity Production and Microbial Biofilm Characterization in Cellulose-Fed Microbial Fuel Cells

Abstract

Converting biodegradable materials into electricity, microbial fuel cells (MFCs) present a promising technology for renewable energy production in specific applications. Unlike typical soluble substrates that have been used as electron donors in MFC studies, cellulose is unique because it requires a microbial consortium that can metabolize both an insoluble electron donor (cellulose) and electron acceptor (electrode). In this study, electricity generation and the microbial ecology of cellulose-fed MFCs were analyzed using a defined co-culture of *Clostridium cellulolyticum* and *Geobacter sulfurreducens*. Fluorescent *in situ* hybridization and quantitative PCR showed that when particulate MN301 cellulose was used as sole substrate, most *Clostridium* cells were found adhered to cellulose particles in suspension, while most *Geobacter* cells were attached to the electrode. By comparison, both bacteria resided in suspension and biofilm samples when soluble carboxymethyl cellulose was used. This distinct function-related distribution of the bacteria suggests an opportunity to optimize reactor operation by settling cellulose and decanting supernatant to extend cellulose hydrolysis and improve cellulose-electricity conversion.

Material presented in this chapter is modified from the following paper:

Ren, Z., Steinberg, LM., and Regan, JM. Electricity production and microbial biofilm characterization in cellulose-fed microbial fuel cells. *Water Sci. Technol.* 2008, In print.

Introduction

The finite resource of fossil fuels and environmental pollution derived from their use are driving the search for renewable and clean energy alternatives. This replacement of fossil fuels will require the use of myriad energy sources and energy carriers suited to meet different end uses. As the most abundant biopolymer on earth, cellulose is a promising renewable resource for sustainable energy production. In the United States alone, besides 65-90 billion dry tones of standing cellulosic vegetation, cellulose is also a significant component in the annual production of 250 million tons municipal solid wastes and 40 billion cubic meter wastewater (1). Cellulose can be converted to a variety of energy carriers such as ethanol (2), biodiesel (3), and hydrogen (4), but the treatment and energy recovery from cellulose in wastewater is very difficult because of its recalcitrance to biological treatment and low energy density.

Representing an alternative method of renewable energy recovery, the direct conversion of organic waste to electricity using a microbial fuel cell (MFC) or hydrogen using a microbial electrolysis cell (MEC) offers the potential of clean and distributed energy production (5). MFCs use electrochemically active microorganisms as biocatalysts and have tremendous electron donor versatility, using virtually any biodegradable resource as substrates. Only recently has this versatility been extended to cellulose (6-9). Because there are no known microorganisms that can both use cellulose

as an electron donor and transfer electrons to the anode as an electron acceptor, the conversion of cellulosic wastes to electricity requires a synergistic consortium. Ren et al. (6) employed a defined co-culture of polymer-degrading, fermentative *Clostridium cellulolyticum* and fermentation product-utilizing, electrochemically active *Geobacter sulfurreducens* to synergistically convert cellulose into electricity in an MFC. The maximum power density using a two-chamber MFC with a ferricyanide catholyte was 143 mW/m^2 from soluble carboxymethyl cellulose (CMC) and 59.2 mW/m^2 from insoluble MN301 cellulose.

The coupling of insoluble electron donor and acceptor substrates, combined with discrete community members that can metabolize each substrate, exerts a unique ecological pressure presumably with the cellulose degrader adhering to cellulose particles and the anode reducer forming a biofilm on the electrode surface. Further analyses of bacterial distribution and operational implications are needed to improve the performance of cellulose-fed MFCs. In this study, the same binary culture of *C. cellulolyticum* and *G. sulfurreducens* was used in cellulose-fed, two-chamber MFCs, and the bacterial distribution and anode biofilm architecture were investigated.

Materials and Methods

MFC inoculum, construction, and operation

Two-chamber MFCs as previously described were used in this study (6). Two medium bottles (310 ml capacity, VWR Inc.) were separated by a proton exchange membrane

(Nafion^{TM} 117, Dupont Co.) (Figure 5-1). The anode chamber was sealed with a stopper and bottle cap to keep it free of oxygen. *G. sulfurreducens* (ATCC 51573) and *C. cellulolyticum* (ATCC 35319) cultures were inoculated into the anode chambers with medium containing (per liter) 1.05 g NH_4Cl, 0.1 g KCl, 4.90 g $NaH_2PO_4 \cdot H_2O$, 9.15 g Na_2HPO_4, 0.2 g yeast extract, 2.5g $NaHCO_3$, 10 ml trace mineral mix, and 10 ml vitamin mix (10). For electron donor, 1 g/L sodium CMC or MN301 was used in different reactors. Graphite plates (16 cm^2 surface area, equally fluted into 4 divisions) were used as anodes. Cathode chambers were filled with 100 mM $K_3Fe(CN)_6$ solution in 100 mM KH_2PO_4 buffer (pH 7.0) and exposed to air. Plain carbon paper (same projected surface area as anodes) was used for cathodes. The electrodes were connected through an external circuit by a copper wire. All exposed surfaces of the wire were coated with nonconductive epoxy. MFCs were operated in fed-batch mode in a 30°C room, and medium and $K_3Fe(CN)_6$ were replaced when the voltage dropped below ~ 40 mV.

Analyses

MFC voltage (V) was continuously monitored using a data acquisition system (ADC22, Pico Technology, Ltd.) under a fixed load of 1000 Ω. Polarization measurements were conducted during the stable power production stage of each batch. Power (P) was calculated as $P = V^2/R$ (where R is the external circuit resistance) and normalized by the surface area of the anode to give power density (10).

FISH procedure

Suspension and anode samples were collected for fluorescent *in situ* hybridization (FISH) analyses after attaining stable power generation (3 to 4 batches of operation). MFCs were disassembled in an anaerobic chamber, and samples were fixed in 4% paraformaldehyde for 8 hours at 4°C. After fixation, sections of graphite anode were mounted onto glass slides using silicon adhesive and surrounded by an equal height of support to allow a coverslip to rest above the biofilm. Suspension samples were concentrated by centrifuge, resuspended in PBS, and spotted onto Teflon-coated slides (11, 12). Following the dehydration of samples in a gradient of 50, 80, and 95% ethanol for 3 min each, hybridizations with oligonucleotide probes were performed in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 0.01% SDS, 30% formamide, pH 7.2) at 46°C for 2 hours. After hybridization, the samples were washed (71 mM NaCl, 20 mM Tris-HCl, 0.01% SDS, pH 7.2) at 48°C for 20 min, rinsed with deionized ice-cold water, air dried, and mounted in DABCO to reduce photo-bleaching. Two oligonucleotide probes were used: modified SRB385 (5'-CGGCGTYGCTGCGTCAGG-3') labeled with Cy3 to target *G. sulfurreducens*, and EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') labeled with FITC (13) to target both members of the binary community. After the FISH procedure, some samples were counterstained using 4'-6-diamidino-2-phenylindole (DAPI) or SYTO-9. In some MN301 samples, a 1% solution of Congo red was added to the fixed samples on the slides for 5 min before the dehydration step to stain cellulose particles (14, 15). Visualization of fluorescently stained cells was performed on a Zeiss Axiophot epifluorescent microscope equipped with a mercury lamp and an Olympus Fluoview

1000 Confocal Laser Scanning Microscope (CLSM; Olympus America Inc., Melville, NY) equipped with 3 lasers (peaks at 488, 543, and 633 nm). The three-dimensional anode biofilm architecture was scanned and displayed as ortho view. Images were analyzed by Photoshop and FV10-ASW software.

Real-time PCR

Primers were designed to quantify *C. cellulolyticum* and *G. sulfurreducens* abundance in MFC suspensions and anode biofilms. The primer pair targeting *C. cellulolyticum* and other members of *Clostridium* Group III consisted of forward primer Clos956f (5'-CCTTCTGTGCCGGAGTTAACA-3') and reverse primer Clos1428r (5'-CCCACMA TCTGAACTGGGACTAT-3'), which generated a fragment of approximately 470 bp. The primer pair targeting *G. sulfurreducens* and other closely related *Geobacter* consisted of forward primer Geo587f (5'-GGGAGGGAAGAAATGATTG -3') and reverse primer Geo978r (5'-GGGCTCAATACCCGCCAACA-3'), which generated a fragment of approximately 400 bp. Primer sequences were checked for specificity using the Probe Match function of the Ribosomal Database Project II release 9 (http://rdp.cme.msu.edu/) and BLAST function of GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html), and they were checked for appropriate melting temperature and possible secondary structures using NetPrimer (http://www.premierbiosoft.com/netprimer/index.html). Experimental primer verification was performed with DNA extracted from cultures of *C. cellulolyticum* and *G. sulfurreducens*. Polymerase chain reaction (PCR) conditions consisted of an initial denaturation at 95°C for 3 min followed by 30 cycles of

denaturation at 95°C for 30s, annealing at 54°C for 45s, and extension at 72°C for 30s, with a final extension at 72°C for 7 min. PCR products were run on a 1% agarose gel at 15 V/cm for 25 min to confirm appropriate size and no non-specific amplification. Fragments from this PCR were ligated into vector pCR2.1 and cloned into *E. coli* Top10 cells using a TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Blue-white screening was used to determine clones containing the PCR fragments. Positive clones for each PCR product were grown overnight at 37°C in LB broth containing 100 μg/mL ampicillin. Plasmids containing the PCR fragment insert were purified using a QIAprep Miniprep Kit (QIAGEN, Valencia, CA). Plasmid was quantified at 260 nm on a spectrophotometer, and stock solutions of 10 ng/μl were made in 10 mM Tris-HCl, pH 8.5. Standard curves for real-time quantitative PCR (qPCR) were prepared in 10 mM Tris-HCl, pH 8.5 as 10-fold dilutions from 1 ng/μl to 0.002 pg/μl. Quantitative PCR was performed on a Bio-Rad iCycler using 96-well polypropylene plates and optical sealing tape (Bio-Rad, Hercules, CA). Standards were run in duplicate and samples from the MFCs were run in triplicate. Total reaction volumes of 20 μl contained final concentrations of 1X PCR buffer and 0.03 U/μl Taq polymerase (USB, Cleveland, OH), 2.5 mM $MgCl_2$, 0.25 μM each primer, 0.5 M Betaine, 10 nM fluoroscein (Bio-Rad, Hercules, CA), a 150,000x dilution of SYBR Green (Molecular Probes, Invitrogen, Carlsbad, CA), and 2 μl of template DNA. Reaction conditions were the same as already described except that 40 cycles of PCR were run and each cycle contained a 10 s step at 85°C inserted after extension for image capture. This step was necessary to eliminate fluorescence caused by the formation of primer dimers during the PCR.

Results and Discussion

Power production

Cultures of *G. sulfurreducens* and *C. cellulolyticum* were added concurrently to an MFC. Previous data showed that pre-acclimation of a *G. sulfurreducens* anode biofilm reduced the lag time relative to a concurrently inoculated system, but eventually both inoculation strategies produced comparable maximum power densities after reaching stable operation (6). After four batches of operation, the voltage of the CMC-fed reactor was stable at approximately 470 mV (1000 Ω resistance), while the voltage of the MN301-fed reactor was stable at approximately 350 mV. Power density curves showed maximum power densities of 153 mW/m^2 for CMC and 83 mW/m^2 for MN301, respectively (Figure 5-2). These maxima were slightly higher than reported for previous experiments with this binary culture because a higher buffer capacity was used in this study to maintain a stable pH. Lower electricity generation was still observed from MN301 relative to CMC, presumably due to the lower rate of MN301 hydrolysis by *C. cellulolyticum*. Refer to Ren et al. (6) for end-product variation and cellulose consumption data for similarly operated reactors.

FISH analysis of suspension and biofilm samples

Two fluorescently labeled oligonucleotide probes were applied to the samples, modified SRB385 to specifically target *G. sulfurreducens* and EUB338 targeting both bacteria. FISH images of MFC suspension samples showed *C. cellulolyticum* cells were the

dominant community member in suspension in both CMC- and MN301-fed reactors (Figure 5-3). In the CMC-fed reactor, *Clostridium* aggregates were observed in suspension, with individual *Geobacter* cells at a much lower cell density. In the MN301-fed reactor, many of the *Clostridium* cells were attached to the surface of cellulose particles, and very few *Geobacter* cells could be found in suspension. CLSM was used to analyze the architecture of the anode biofilms. It was found that both *Clostridium* and *Geobacter* resided on CMC-fed anode biofilms and they tended to form aggregates instead of evenly colonizing the whole anode area (Figure 5-4). In areas of biofilm coverage, the thickness of the biofilm was approximately 25 μm, with no clear stratification of the two bacteria in the thin biofilm with soluble cellulose. Only *Geobacter* cells were found in the biofilm of MN301-fed reactors, and the cells were quite scarce on the electrode compared to the CMC biofilm. Counterstaining of cells with DAPI or SYTO-9 showed a correspondence with the EUB338 probe fluorescence, indicating successful hybridization.

Real-time PCR analyses

The FISH results were complemented with quantitative PCR analyses to separately quantify *C. cellulolyticum* and *G. sulfurreducens* abundance. The results showed cell concentrations of *Clostridium* in suspension were 21 and 370 times higher than *Geobacter* in CMC and MN301 reactors, respectively (Figure 5-5). This is consistent with the qualitative FISH observations, in which *G. sulfurreducens* cells were rarely visualized in the MN301 suspension samples. Additionally, there was a five thousand

times higher *Clostridium* density in CMC biofilm than in MN301 biofilm, while 25 times more *Geobacter* were found attached on the anode in the MN301 reactor than in the CMC reactor. These quantitative data were consistent with FISH observations, suggesting a distinct function-related distribution of the two bacteria. When soluble substrate was used, both bacteria resided in bulk solution as well as biofilms, but when particulate cellulose was used, much more *Clostridium* cells were found attached on cellulose in suspension than in biofilm, while more *Geobacter* were adhered on the biofilm than in bulk solution. Based on standard curves, the linear dynamic range for qPCR was seven orders of magnitude, from 4.15×10^8 to 415 target gene copies/reaction. The reaction efficiency ranged between 90-100%, with a Y-intercept of 38-40 and R^2 of greater than 0.99 for all standard curves. No-template controls did not show amplification.

C. cellulolyticum does not need the anode for cellulose fermentation, but adhesion of these cells to cellulose is required for rapid and efficient cellulose hydrolysis and soluble cellodextrin utilization (4, 16). Therefore, it is understandable that most *Clostridium* were found adhered to MN301 cellulose particles and very few cells were found in the anode biofilm, presumably growing on the soluble MN301 hydrolysis products generated by suspended cells. Contrarily, *C. cellulolyticum* would be expected to metabolize CMC both in suspension and on the surface of electrode, since this is already a soluble form of cellulose. For *G. sulfurreducens*, which used the electrode as the sole electron acceptor in these systems, cellular attachment to the anode was apparently preferential for electron transfer. That almost all *Geobacter* cells were found attached to the electrode is consistent with the findings of other studies (17). The limited number of *Geobacter* found in suspension may have been due to the detachment of

biofilm-associated *Geobacter* by continuous stirring or sample collection. There was no clear stratification of the two bacteria found within the anode biofilm. The distinct roles of the two bacteria in this unique system with insoluble electron donor and acceptor led to a function-related distribution, with *Clostridium* hydrolyzing and fermenting cellulose primarily in suspension and *Geobacter* preferentially attaching to the anode to transfer electrons. Coupled with the consideration that cellulose hydrolysis is the rate-limiting factor for power generation from cellulose, an improvement in reactor performance might be realized in a system with settling and decanting.

Conclusions

Using two-chamber MFCs, cellulose was directly converted into electricity by a co-culture of *C. cellulolyticum* and *G. sulfurreducens*. FISH and quantitative PCR analyses showed a consistent function-related bacterial distribution, with most *Geobacter* attached to the anode surface and most *Clostridium* adhered to MN301 cellulose particles. When soluble cellulose (i.e., CMC) was used, a more even distribution was observed. This study revealed a unique ecology system in MFCs, and further study is needed to correlate biocatalyst density with reactor performance for system optimization and to explore the effectiveness of settling and decant at retaining a higher cellulolytic cell density for enhanced kinetics and extent of cellulose conversion.

Acknowledgements

This research was supported by grant 68-3A75-3-150 from the Natural Resources Conservation Service of the United States Department of Agriculture.

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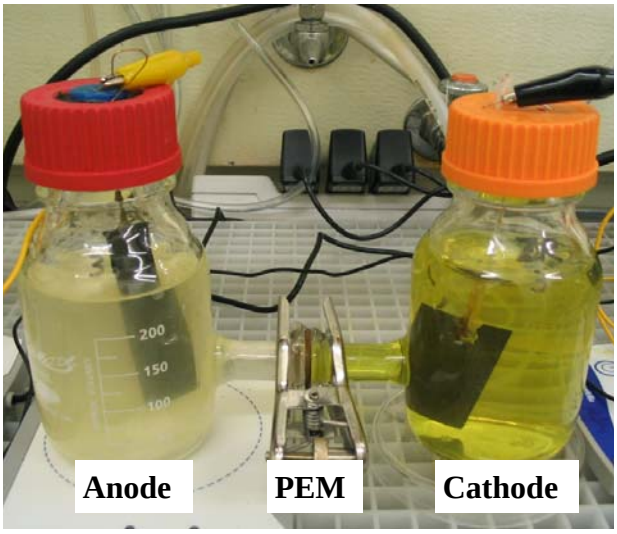

Figure **5-1**: Two-chamber microbial fuel cells used in this study. Anode chamber (left) was filled with growth medium, cathode chamber (right) was filled with buffered ferricyanide solution.

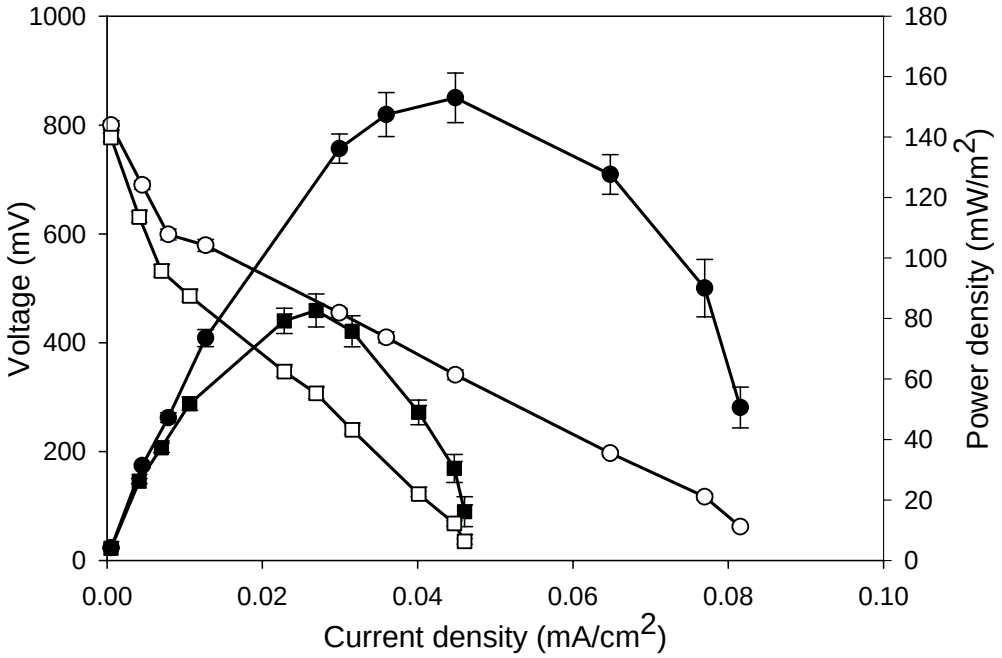

Figure **5-2**: Voltage (hollow symbols) and power density (solid symbols) vs. current density obtained by varying the external resistance (50-50,000Ω), 1 g/L CMC (● and ○) and 1 g/L MN301 (■ and □).

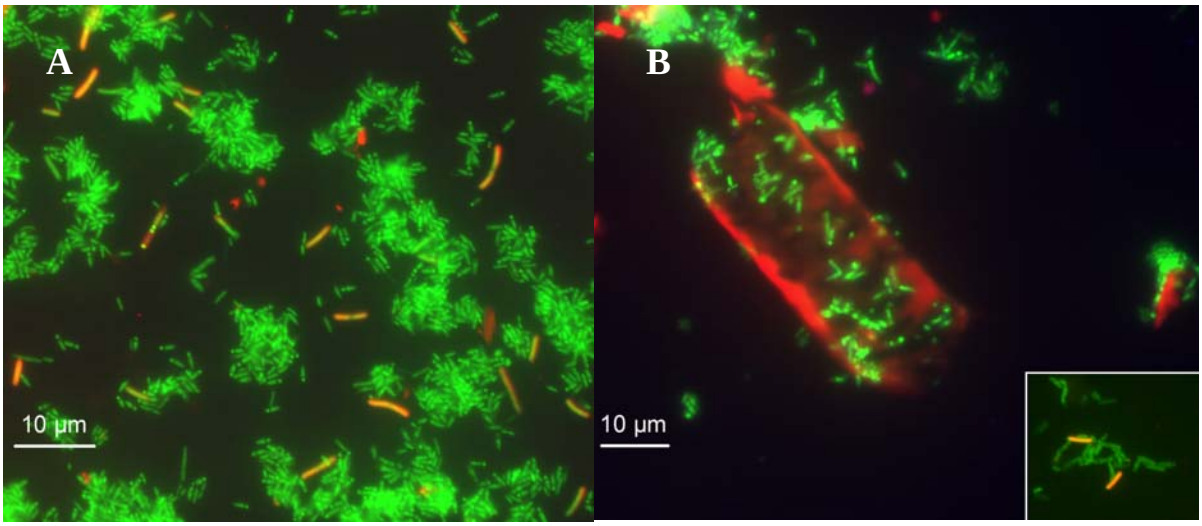

Figure **5-3**: FISH images from suspended samples: (A) CMC-fed MFC, and (B) MN301-fed MFC. Samples were hybridized with Cy3-labeled SRB385 probe targeting *Geobacter* and FITC-labeled EUB338 probe targeting both bacteria, resulting in yellow to orange *Geobacter* cells and green *Clostridium* cells. MN301 cellulose particles were stained with Congo red. Inset in panel B shows the fluorescent response of *Geobacter* cells.

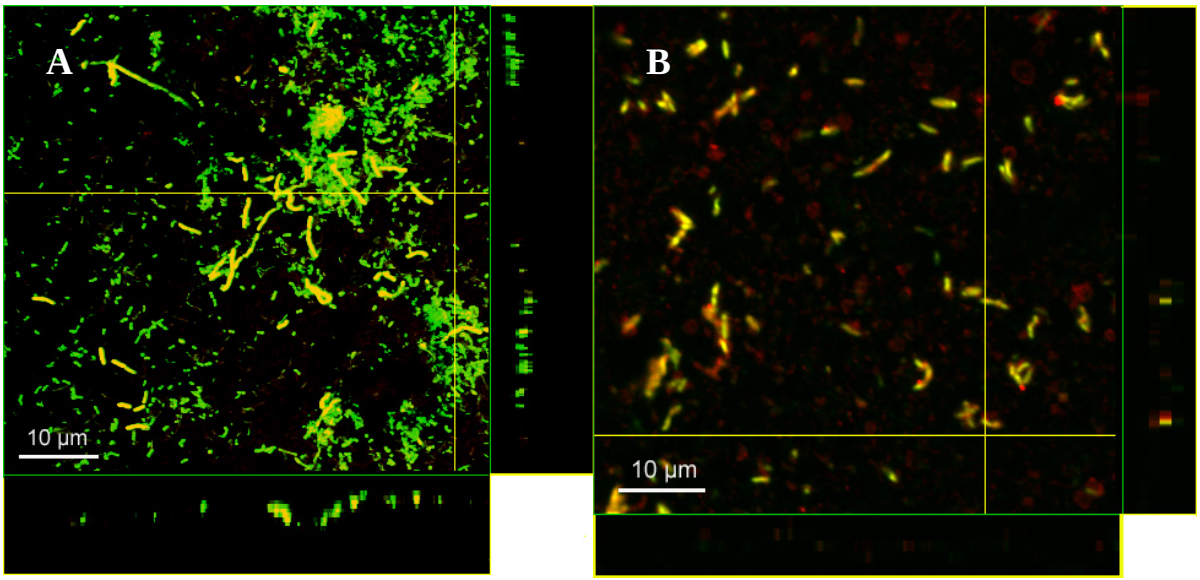

Figure **5-4**: Confocal FISH images of biofilm growing on MFC anodes: (A) CMC-fed MFC, and (B) MN301-fed MFC. Anode biofilms were hybridized with Cy3-labeled SRB385 probe targeting *Geobacter* and FITC-labeled EUB338 probe targeting both *Clostridium* and *Geobacter*, resulting in yellow-orange *Geobacter* cells and green *Clostridium* cells.

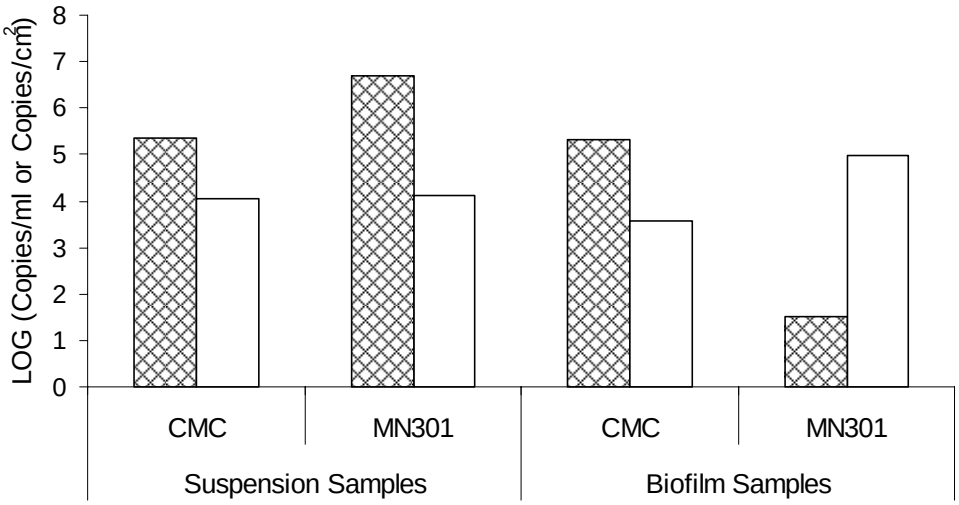

Figure **5-5**: Comparison of gene copy numbers in four different conditions using real-time PCR. *C. cellulolyticum* (⊠); *G. sulfurreducens* (□).

Chapter 6

Isolation and Identification of Microorganisms Capable of Extracellular Electron Transfer Using a Hydrous Ferric Oxide (HFO) Plate Technique and Microbial Fuel Cells

Abstract

Microorganisms that have extracellular electron transfer capability are important in biogeochemical cycles and have significant potential for bioenergy production and bioremediation. Conventional dilution-to-extinction techniques for isolating such microorganisms are very labor and time intensive. A simple plate technique using an overlay of insoluble hydrous ferric oxide (HFO) as the electron acceptor was developed to isolate presumed dissimilatory metal reducers, which produce clear zones in the HFO layer. Iron reduction was confirmed by transferring clear zone cores to liquid medium with HFO and measuring ferrous production. Using acetate plus lactate as electron donors, three pure cultures and one co-culture were isolated from the anaerobic sludge of a sewage treatment plant using two cycles of HFO plating and amplification in liquid medium. Based on 16S rDNA sequence, these bacteria were indentified as *Firmicutes*, with the exception of one member of the co-culture that was similar to the deltaproteobacterium *Desulvovibrio intestinalis*. All of these isolates reduced significant amounts of HFO in liquid culture and also showed electrochemical activity in microbial fuel cells (MFCs), indicating this technique can serve as a surrogate for isolation of electrochemically active bacteria. In two-chamber MFCs with ferricyanide as a catholyte,

the co-culture and a *Clostridium*-like isolate produced approximately two-thirds of the power density produced by an MFC-acclimated mixed culture control. In single-chamber, air-cathode MFCs, the cultures did not produce electricity unless L-cysteine was added to the anode medium. Under this condition, a *Clostridium*-like isolate produced 89% of the maximum power density of the mixed-culture control. This HFO plate technique allowed an alternative way to isolate microorganisms that have extracellular electron transfer capabilities, allowing researchers to increase the available variety of microbial phenotypes.

Introduction

Microorganisms that can transfer electrons to solid extracellular electron acceptors, such as Fe(III) and Mn(IV) minerals, are common in anaerobic environments. Coupling anaerobic oxidation of carbon compounds to metal reduction, these microbes are important in organic matter degradation and nutrient cycling in submerged soils and freshwater and marine sediments (1-3). In addition, dissimilatory metal reducing microorganisms have significant potential for the bioremediation of subsurface environments contaminated by petroleum, toxic xenobiotic organic compounds, landfill leachates, and soluble toxic metal and radioactive wastes (4, 5). Recently, this capability has also been used to harvest electricity from waste organic matter and aquatic sediments using microbial fuel cells (MFCs) in which the anode acts as an electron acceptor (6, 7).

The special metabolism and application potential of this class of microorganisms have attracted considerable research interest, but only a small fraction of them have been cultured and characterized, because the conventional serial dilution techniques for isolating such microorganisms are very time and labor intensive (8, 9). The Hungate roll tube method is a related isolation technique, but the narrow necks of the tubes render isolation and subculturing of the tiny colonies difficult and impractical (10, 11). Using the dilution-to-extinction method and poorly crystalline Fe(III) oxide as an electron acceptor, Holmes et al (12), isolated the exoelectrogenic *Geopsychrobacter electrodiphilus* from a marine sediment fuel cell. Kostka and Nealson (13) developed a

strategy for directly isolating manganese reducers, using a MnO_2 overlay on a soft agar plate in which clearing zones develop around single colonies.

The method of Kostka and Nealson was herein adapted for the isolation of iron reducers, to simplify the isolation process and get more isolates with extracellular electron transfer capability, by using an overlay of red hydrous ferric oxide (HFO) as the electron acceptor. HFO represents a class of minerals that form from the weathering of minerals that contain iron (Fe) and hydroxides (OH^-), but not water (14). They are poorly crystalline, highly porous, and have large surface areas. In natural environments, HFO has been suggested as a principal form of Fe (III) oxide which is reduced by dissimilatory metal-reducing bacteria (15, 16). Using an overlay of HFO as the sole terminal electron acceptor, this plate technique allows direct visualization of iron reduction by individual colonies. Moreover, this technique is an appropriate surrogate for isolating electrochemically active bacteria with this phenotype, since all of the final candidates which reduced iron in liquid growth media showed electrochemical activity in microbial fuel cells.

Materials and Methods

The plating technique

The agar layer of the HFO plates contained, per liter, 0.8 g sodium acetate, 0.9 g lactic acid, 2.4 g K_2HPO_4, 1.4 g KH_2PO_4, 1.0 g NH_4Cl, 0.15 g $CaCl_2$, 25 mg $MgSO_4$.$7H_2O$, 2.9 g NaCl, 2.0 g yeast extract, and 15 g agar (pH 7.0). Medium was autoclaved for 30 min.

(17, 13) and then distributed to Petri dishes in air, the surfaces of the molten agar were flamed to burst bubbles and provide a flat surface, and after hardening, the plates were stored at room temperature in an anaerobic chamber for 5-7 days to allow oxygen to diffuse away and the plates to dry out.

Highly amorphous and porous hydrous ferric oxide was prepared by neutralizing a 1M $FeCl_3$ solution with 10M NaOH to pH 7.0 with stirring, followed by repeated centrifugation and washing with deionized water to remove sodium chloride (15, 18, 19). The dark red HFO slurry was then stored in an anaerobic chamber with a loosened cap for several days prior to use.

The activated sludge inoculum was obtained from the secondary sedimentation tank of the Pennsylvania State University Wastewater Treatment Plant. In an anaerobic chamber, a dilution series (1:1 to 1:100) of sludge in buffer solution, containing the same salts as the medium, was made and then aliquots were mixed with HFO slurry in microcentrifuge tubes. The slurry was spread evenly on the agar plates, allowed to harden for a day right side up, and incubated upside down in the anaerobic chamber at room temperature. Microorganisms that were able to use the insoluble electron acceptor reduced the Iron III and formed clear zones in the HFO layer after several days.

Growth in liquid medium

In order to further confirm iron reduction and amplify cell numbers, growth in liquid medium simulating the plate culture conditions was performed. The clear zones were cored using short, sterile glass tubes, and the cores were vortexed with buffer which was

then transferred into sealed, sterilized Hungate tubes containing liquid medium with the same components as the plates (except agar) and 40 mM HFO, and incubated anaerobically with shaking at room temperature. Reduced iron produced was quantified using a modification of the typical spectrophotometric assay. Homogeneous culture sample (50 μL) was added into 2.5 mL of 0.5N HCl and incubated for 15 minutes at room temperature, followed by adding 50 μL of this HCl solution to 2.5 mL of 50 mM HEPES (pH 7.0) buffer containing 1 mg/mL Ferrozine. Fe(II) was determined by measuring the optical density of the ferrous-ferrozine complex at 562 nm (20) after 10 minutes at room temperature. Fe(II) standards were prepared using ferrous sulfate.

Determination of culture purity and phylogenetic analysis

When significant iron reduction was achieved during growth in liquid medium, culture purity/homogeneity in each tube was tested by ribosomal intergenic spacer analysis (RISA) as previously described (21, 22). DNA was extracted and purified from the cultures using the PowerSoil™ DNA isolation kit (MoBio) following their instructions and PCR amplified using the eubacterial primer set 926f and 115r with the thermal profile of Ren et al (22). The PCR products were analyzed by agarose gel electrophoresis.

Plate isolation and liquid medium amplification were repeated twice, after which a single, major gel band was obtained for some of the isolates. PCR products were ligated into a plasmid vector (TOPO TA, Promega) and introduced into competent *E.coli* cells. Plasmids were isolated from individual colonies (Qiagen kit) and the inserts were sequenced in both directions using M13 universal - M13 reverse primers as well as 926f

– 115r primers (ABI Hitachi 3730XL analyzer) (21). Eight clones were sequenced from each PCR reaction and the sequence editing and alignment were conducted using the MEGA 3.1 program (23). The closest relatives of the retrieved sequences were identified using the BLAST search in GenBank (24).

Electrochemical activity tests

After isolates were sequenced and identified, they were inoculated into microbial fuel cells (MFCs) for electrochemical activity tests. The isolates were firstly inoculated into two chamber MFCs to maintain strictly anaerobic conditions. Two cylindrical lexan chambers (2 cm long by 3 cm in diameter, each with a volume of 14 mL) were screwed together but separated by a proton exchange membrane (PEM, Nafion^{TM} 117, Dupont Co.). A carbon cloth anode and plain carbon paper cathode were clamped on the outside end of each chamber, respectively. Anode chambers were filled with the growth medium without HFO, while cathode chambers were filled with 100 mM $K_3Fe(CN)_6$ solution in 100 mM KH_2PO_4 buffer (pH 7.0) (17). (Figure 6-1B). For some experiments, single chamber MFCs were assembled by removing the PEM and the cathode chamber and replacing the carbon paper cathode with a platinum air cathode coated with diffusion layers as previously described (25), to test the ability of the isolates to function at low levels of oxygen. An acclimated sludge inoculum from other MFCs was used as a positive control under both conditions. Voltage (V) was continuously monitored using a data acquisition system (ADC22, Pico Technology, Ltd.) and polarization data was collected by changing the external resistance using a variable resistor box during the

stable power production stage. All MFCs were operated in fed-batch mode at 30°C until voltage dropped to below 30 mV, and each such cycle was repeated at least five times.

Results

Isolate characterization

Various numbers of clear zones were formed after 5-8 days depending on the inoculum and dilution (Figure 6-1A). Twelve clear zones were picked from plates inoculated with various dilutions of sludge and transferred into liquid medium. Six of these showed significant iron reduction with the quantitative assay, also turning the reddish HFO medium a lighter greenish-yellow. After a second round of plating various dilutions of these initial tubes, coring, and amplification in liquid including the Iron II assay, eight isolates which produced significant Iron II were analyzed by RISA fingerprinting. Sequencing results showed that three of these cultures were pure (all 8 clones analyzed yielded the same sequence), while one is a co-culture of two different bacteria (Table 6-1).

For the five sequences from these cultures, isolates 1, 3 and one member of the coculture showed greater than 98% similarity to known, cultured bacteria, while the best matches for the other two were sequences from uncultured bacteria. Although the closest match to a cultured bacterium of these two are also listed in Table 6-1, their similarities are below 96%, meaning that the isolates and the closest cultured bacteria were quite

distinct from each other (26). Most of isolates are *Firmicutes*, while one culture is in the *Delta-proteobacteria*.

HFO reduction in liquid medium

The Fe (II) generated by HFO reduction by these isolates is shown in Figure 6-2. The coculture showed a higher degree of iron reduction than the three pure isolates and reduced 63% of the available HFO in 13 days. Isolate 1 showed a high iron reduction rate for the first 7 days but produced very little additional Fe (II) during the following 7 days, which meant that it reduced the least amount of HFO totally. Isolates 2 and 3 showed continuous iron reduction and reduced 14.2 mM (36%) and 19.6 mM (49%) of the HFO, respectively.

Electricity generation in MFCs

All of the cultures tested showed rapid electricity generation after being inoculated into two chamber (strictly anaerobic) fuel cells. During stable stages, the voltage increased rapidly after each medium transfer and reached the peak value within 3-5 hours then dropped slowly to approximately 30 mV over the next 36-48 hours, at which point the next medium change was performed (data not shown). The differences in power density across a range of current densities for the cultures were obtained by varying the external resistance (10-10000 Ω) (Figure 6-3A). The co-culture and isolate 2 showed comparable maximum power densities (103.2 mW/m^2 (projected anode surface area, throughout) and

93.8 mW/m^2, respectively). Isolate 3 produced the lowest power (45.9 mW/m^2) while isolate 2 was intermediate (66.7 mW/m^2). However, the acclimated sludge control showed a higher power density than any of the individual cultures (152.3 mW/m^2), presumably indicating that an acclimated mixed culture may have an advantage by working together.

When the isolates were transferred to single chamber MFCs, which used oxygen as the terminal electron acceptor, none of them generated voltage within 14 days (3 medium changes), except the acclimated sludge control. But when 0.5 mM cysteine was added to the medium as an oxygen scavenger using the same reactor conditions, all of the isolates showed rapid electricity production (Fig 6-3B). This shows that all of the isolates are obligate anaerobic bacteria and quite sensitive to oxygen. The acclimated sludge and isolate 2 showed much higher maximum power densities (121.7 mW/m^2 and 102.0 mW/m^2, respectively) than the other three cultures under these conditions. The lower power densities of the other three may indicate that they are more sensitive to oxygen than isolate 2.

Discussion

A plate assay has been developed in this research employing HFO as a selective agent for isolating microorganisms that have extracellular electron transfer capability. This technique is a modification of the agar overlay technique using MnO_2 developed by Kostka and Nealson (13), which is easy to perform and more practical than traditional dilution-to-extinction techniques. This method possesses electron acceptor versatility

because many other insoluble forms of oxidized elements, including metals such as Mn(IV) and U(VI) and elemental sulfur could presumably be used as electron acceptors in place of HFO. HFO reduction can apparently be used as a surrogate for electricity production because all of the isolates from HFO plates which reduced iron III during liquid growth were also found to be electrochemically active, which offers a new way to isolate exoelectrogens with this phenotype.

Because the development of this assay is based on the presumption that the microorganisms require contact with the insoluble electron acceptors (solid metal oxides or electrodes), it prohibits the possibility of using mediators during electron transfer. The microbes formed clear zones (plaques) in the HFO layer and usually not visible colonies. The isolates were presumably attached to the anode surface in the MFCs, because the voltage increased rapidly at the beginning of each cycle even though the medium was completely changed every cycle. However, the rates of iron reduction and anode reduction are not directly correlated. Isolate 3 generated much more Iron II from HFO in liquid medium than the other isolates, but produced the least amount of power in MFCs, while isolate 2 had a relatively low rate of Iron II production but generated more power than the other pure isolates in the single chamber MFC. Since the liquid medium and MFC contained the same nutrients except for the electron acceptor, our only explanation involves the difference of respiratory mechanism of the bacteria in reducing metal oxides versus electrodes. These observations further reinforce the conclusion that, while closely related, the phenomena of reduction of solid extracellular iron (III) and the transfer of electrons to MFC anodes differ in the details and potentially in the gene products utilized (27, 28).

Not all of the clear zones picked from the HFO plates generated Iron II at a significant rate in liquid medium. The HFO plates were incubated in an anaerobic chamber which averaged approximately 2% hydrogen, and this gas is a potential electron donor. There may have been some bacteria that used hydrogen to reduce iron and form clear zones. But they could not reduce significant amounts of iron in the anaerobic Hungate tubes, because the small amount of hydrogen in these tubes would have been very rapidly used up.

All of the cultures which produced significant amounts of reduced iron during liquid growth showed electrochemical activity in two chamber MFCs, but none of them produced electricity in single chamber MFCs using an air cathode, unless cysteine was added as an oxygen scavenger. This indicates that all the isolates are strictly anaerobic bacteria, but they showed different oxygen tolerance, which represented as different electricity production in single chamber MFCs.

Two of the best matches for the isolates are sulfate reducing bacteria (SRB), while two of isolates show closest homology to *Clostridium* spp. It is not surprising that SRBs were isolated using this technique, because they are a predominant group performing anaerobic respiration and several of them have been shown to have extracellular electron transfer capability (29-32). For the isolates related to *Clostridium*, the similarity to cultured species was rather low, probably indicating a new species (possibly a new genus) (26), and further characterization of these isolates is in progress.

The HFO plate technique offers a new way to isolate microorganisms that have extracellular electron transfer capability. This should allow researchers to expand the variety of microbial phenotypes available for dissimilatory metal-reducing bacteria and

electrochemically active bacteria, thus facilitating studies of biogeochemical cycles, bio-energy production and bioremediation. Using this technique, a wide variety of electron donor and acceptor combinations can readily be investigated, and many more unique isolates are expected to be discovered.

Acknowledgements

This work was supported by grants from the U. S. Department of Agriculture and Air Force Office of Scientific Research . We thank Dr. John Senko for many valuable discussions.

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Table **6-1**: Phylogenetic analysis of isolates

Isolate number	**Highest homology**	**% similarity**	**No. of identical base pairs**	**Taxon**
1	*Desulfotomaculum guttoideum* (Y11568)	99	571/572	*Firmicutes*
2	Uncultured bacterium (AJ488093)	95	552/578	*Bacteria*
	Clostridium orbiscindens (AY730665) (Closest cultured organism)	92	523/565	*Firmicutes*
3	*Eubacterium aggregans* (AF073898)	98	626/635	*Firmicutes*
Co-culture*	*Desulfovibrio intestinalis* (AJ295680)	99	422/424	*Delta-proteobacteria*
	Uncultured bacterium (DQ011255)	99	502/507	*Bacteria*
	Clostridium sticklandii (L04167) (Closest cultured organism)	96	567/587	*Firmicutes*

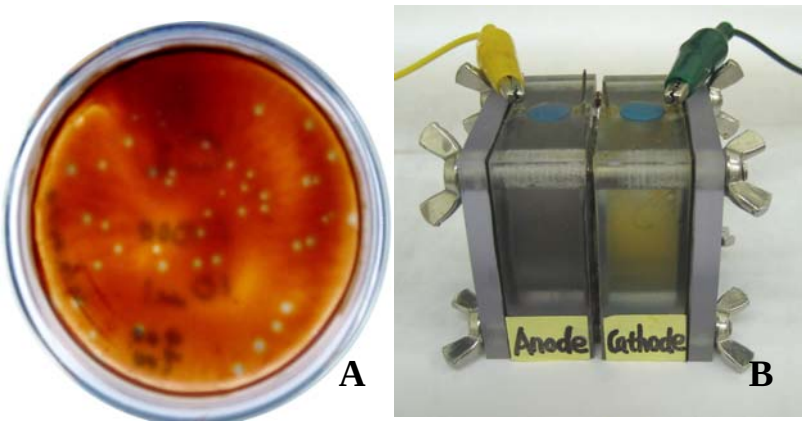

Figure **6-1**: (A) Clear zones formed on a HFO plate. (B) A two chamber MFC used in electrochemical tests.

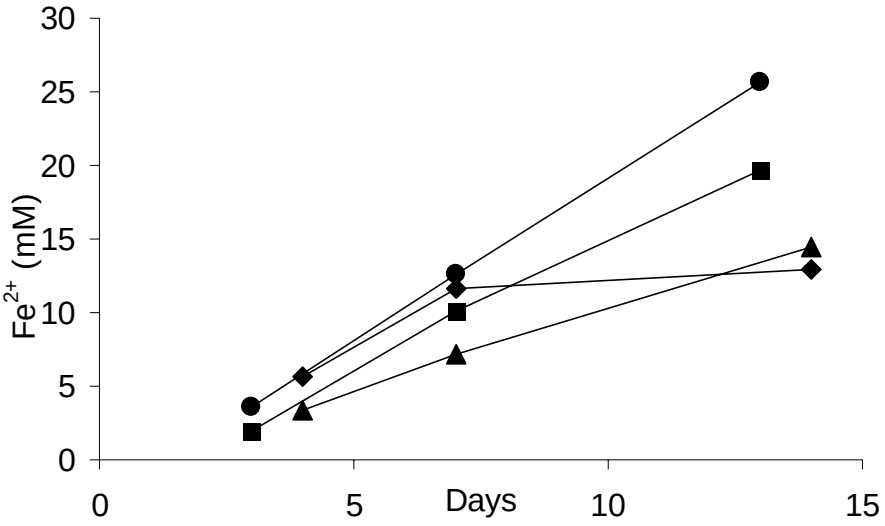

Figure **6-2**: Fe(II) generation by isolate 1(◆), isolate 2 (▲), isolate 3 (■), and co-culture (●) from 40 mM HFO in liquid media.

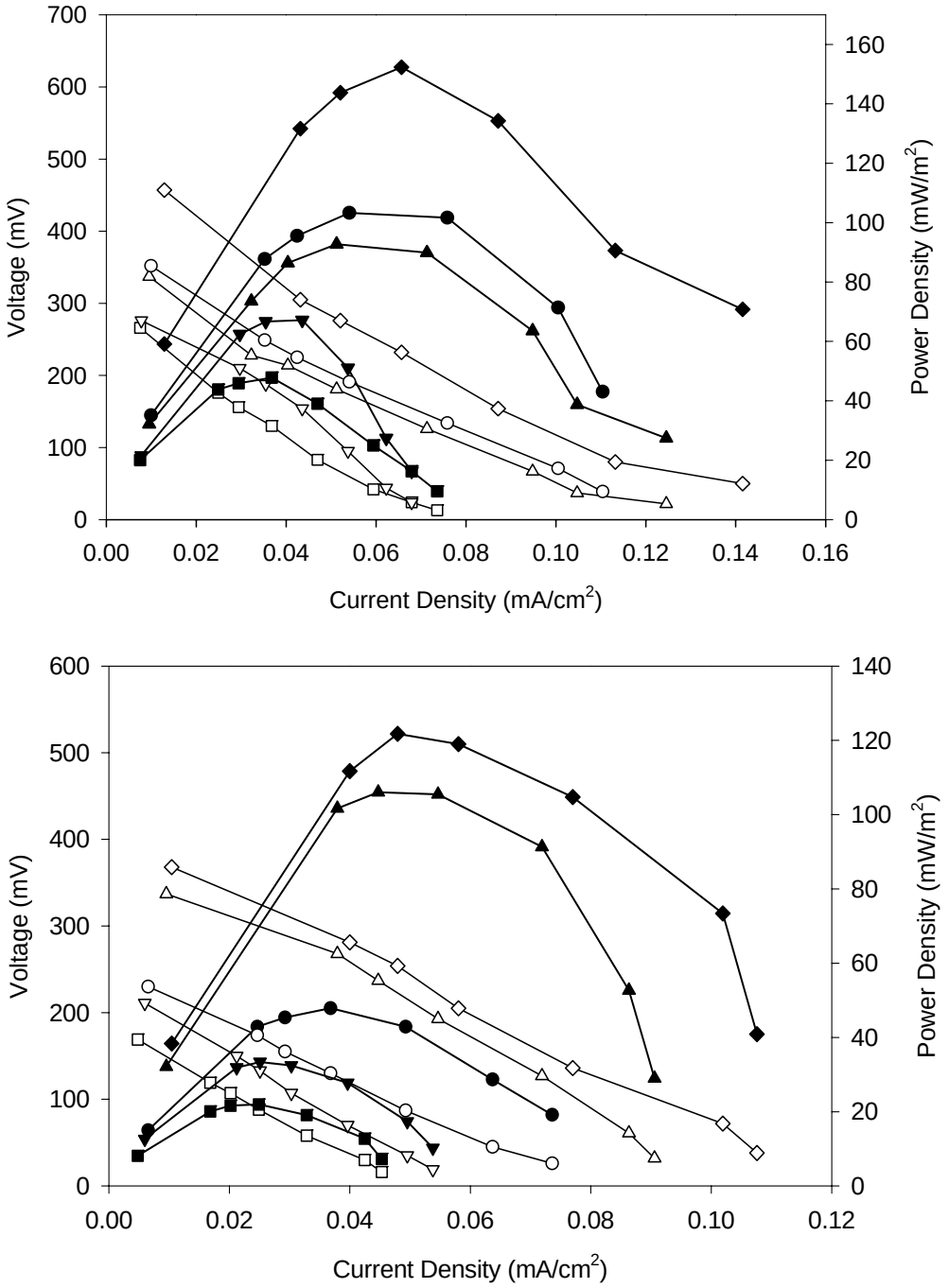

Figure **6-3**: Voltage (hollow symbols) and power density (solid symbols) vs. current density obtained by varying the external circuit resistance (10-5,000Ω) in (A) two chamber MFCs and (B) Single chamber MFCs with 0.5 mM cysteine. isolate 1(▽ and ▼), isolate 2 (Δ and ▲), isolate 3 (□ and ■), co-culture (○ and ●) and acclimated activated sludge (◊ and ♦).

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Selected Publications

Ren, Z., Ward, TW., and Regan, JM. (2007). Electricity production from cellulose in a microbial fuel cell using a defined binary culture. *Environ. Sci. Technol.* 41(13); 4781- 86.

Ren, Z., Ward, TW., Logan, BE., and Regan, JM. (2007). Characterization of the cellulolytic and hydrogen-producing activities of six mesophilic *Clostridium* species. *J. Appl. Microbiol.* 103(6); 2258-66.

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Ren, Z., Qi, Y., and Li, R. (2002). Nitrogen removal by biological pre-treatment in water plant. *Urban Environment & Urban Ecology.* 15(3); 56-58.

Manuscripts in Preparation

Isolation and characterization of microorganisms capable of extracellular electron transfer using a hydrous ferric oxide (HFO) plate assay and microbial fuel cells.

Evolution of biofilm catalyst density, viability and electrochemical response in single chamber microbial fuel cells.