MICROBIAL BIOPROCESSING: FROM NATURE TO INDUSTRY

By

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ABSTRACT

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Microorganisms play an essential role in the processing of plant-derived matter in nature and in industrial settings. Decomposition of plant matter in nature is a key process in the feedback between soils and the atmosphere and an important parameter to incorporate into climate models to increase their predictive value. The mechanisms that enable decomposers to process plant matter in nature are not known. To gain insights, I investigated the response of *Cellulomonas* decomposers to nitrogen disturbances such as those caused by the addition of fertilizers or atmospheric deposition of greenhouse gases. These decomposers adapt to low nitrogen by specifically colonizing plant-derived substrates as biofilms and sequestering significant amounts of carbon in the biofilm matrix. The biofilm strategy enabled cells to be closer to the substrate and to degrade it more efficiently despite the low availability of nitrogen sources. The process is reversible and potentially manageable, thus showing promise for its use as a carbon remediation technology to mitigate the accumulation of greenhouse gases.

Knowledge gained from studying *Cellulomonas uda* can be utilized in industry as interest in cellulolytic microorganisms has increased in recent years due to their role in bioethanol production. To minimize the cost of bioethanol, agricultural residues and dedicated bioenergy crops are preferable as substrates instead of starch and sugar. These lignocellulosic substrates are more recalcitrant and require a chemical

pretreatment step plus an additional step of enzymatic hydrolysis to make them fermentable. Consolidated bioprocessing (CBP), i.e. a combined platform that catalyzes the breakdown and fermentation of cellulose in one single step, is a cost-effective approach to producing ethanol from lignocellulosic substrates. *C. uda* is an attractive candidate for industrial consolidated bioprocessing of lignocellulose. The activities of this organism is limited, however, by the accumulation of ethanol and fermentation byproducts, which in nature are rapidly removed by other organisms. Our lab has developed a platform for the bioprocessing of chemically-pretreated agricultural residues such as corn stover by *C. uda* and *Geobacter sulfurreducens* into ethanol and biohydrogen using a microbial electrochemical cell (MEC). As predicted, nitrogen supplementation prevented the accumulation of carbon as a curdlan biofilm matrix and resulted in 2-fold increases in the energy recoveries from the fermentation of Ammonia Fiber Expansion pretreated corn stover (AFEX-CS). Improving culture conditions and developing a faster fermenting strain led to a 12-fold increase in ethanol productivity.

Another bioprocessing scheme of industrial significance is the generation of value-added co-products from glycerol, the major waste product in the production of biodiesel. Harnessing the glycerin waste stream to produce value-added products will diminish the cost and waste of biodiesel production. We identified a glycerol-fermenting bacterium (*Clostridium cellobioparum*) that converts glycerol into ethanol at high rates and generates waste fermentation byproducts that are converted into hydrogen in *Geobacter*-driven MECs. This scheme shows promise as a wastewater treatment method as optimization of the platform resulted in glycerol consumption of 50g/L.

DEDICATION

This dissertation is dedicated to “family” in every sense of the word. In particular to my family back home in Georgia. They are an amazing support system and have listened tirelessly to both the good and the bad over the years. I would also like to dedicate this to my Michigan family, the friends and labmates who helped me through the tough times and laughed with me through the good times. Lastly, to Tucker, who makes me laugh every day.

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LIST OF SYMBOLS AND ABBREVIATIONS

AA3, allyl alcohol 10^{-3} M

AA7, allyl alcohol 10^{-7} M

AFEX, Ammonia Fiber Expansion

AFEX-CS, Ammonia Fiber Expansion pretreated corn stover

BCA, Bicinchoninic Acid

BSA, Bovine Serum Albumin

BES, bioelectrochemical system

CB, cellobiose

CBP, consolidated bioprocessing

CFU, colony forming units

CLSM, confocal laser scanning microscopy

CMC, carboxymethylcellulose

CMCase, carboxymethylcellulase

DMSO, dimethyl sulfoxide

DNS, 3,5-dinitrosalicylic acid

EPS, exopolysaccharide

FAEE, fatty acid ethyl esters

FAME, fatty acid methyl esters

GC, gas chromatography

HPLC, high pressure liquid chromatography

MEC, microbial electrolysis cell

NAG, *N*-acetyl glucosamine

OD, optical density

RBB, Remazol Brilliant Blue

RPM, rotations per minute

SEM, scanning electron microscopy

TEM, tunneling electron microscopy

WT, wild-type

Chapter 1

A history and applications of industrial microbiology

1.1 Industrial Microbiology: An Understanding

Industrial biotechnology is the use of biological methods applied to industrial processes, which can be used to produce chemicals, materials, and energy, often in a beneficial manner. Biotechnology has the potential to benefit the environment through reduction in greenhouse gas emissions and reduction in harmful waste generations. It can also help the economy through stimulation of agricultural markets, creation of products with improved process rates and efficiencies, and lower energy costs (52).

The main proponent of change leading to the benefits of biotechnology is the use of renewable resources. Biotechnology provides a mechanism to move away from fossil fuels and rely instead upon renewable substrates. Industry originally relied on renewables prior to the industrial revolution when petrochemical processes became dominant. Over the past several decades, which included the oil crisis in the 1970's and continually rising oil prices, it has become increasingly clear that non-renewable resources are just that, a finite resource on which dependency cannot be sustained. Despite this realization, market costs drive the demand for sustainable products. While benefits to the environment and diminished generation of waste and harmful byproducts are attractive advantages, production costs will always play a role in the choice between sustainability and economics. The successful biotechnology advances will be those that combine environmental and economic advantages (52).

Microorganisms play an important role in industrial biotechnology as they catalyze natural processes of interest, but their natural environments do not select for optimum product formation. Adjusting conditions and genetic material can improve these bioprocesses and allow biotechnologists a quick and efficient way to make

sustainable and profitable products (52). Microorganisms have been used in several different industries over the years including food, agriculture, chemical and pharmaceutical applications. The advantages of using microorganisms over chemical pathways include opting for an environmentally friendly system by limiting use of harsh reagents (heavy metals, strong acids and bases, solvents) and harnessing native abilities evolved for specificity and efficiency (enzyme complexes) (13).

1.2 Industrial Microbiology: A History

Before the actual discovery of microorganisms, they played a major role in human lives. Microorganisms allowed the preservation of food, production of beverages, cheese, bread, pickled food, and vinegar, and all these processes were possible without knowledge of the root of the process. Fermentations by yeast go back to 7000 B.C. with the first recording of beer in Babylonia. Egyptian records show use of yeast to make leavened bread in 4000 B.C., and the Assyrians were making wine in 3500 B.C. (12) (Fig. 1.1).

The first piece to the puzzle began in the 17th century, when Antonie van Leeuwenhoek used a microscope and first described microorganisms that he called “animalcules” (Fig. 1.1) Then, in the early nineteenth century, three independent scientists (Charles Cagniard, Theodor Schwann, and Friedrich Traugott Kützing) connected microorganisms to the phenomenon of fermentation, but opposition remained. Some still believed it to be a strictly chemical reaction. Finally, the negation of spontaneous generation in the late nineteenth century by Louis Pasteur allowed the advancement of microbiology. The combination of this discovery and the discovery that

distinct microorganisms exhibited different characteristics (e.g. disease symptoms, fermentation products) had major impacts on the medical field as well as understanding other processes like fermentation.

In 1879, Robert Koch determined that bacteria cause disease. This provided direction for the fight against disease and the search began for compounds toxic to bacteria that do not harm the host. Finally in 1927, Alexander Flemming discovered Penicillin and observed cell extracts of mold that would inhibit the growth and lyse bacteria but did not harm animals (Fig. 1.1). However, this discovery was limited by native production rates and activity of the drug. During WWII, soldiers were developing bacterial infections in their wounds, and the need for antibiotics became increasingly important. This began strain improvements via mutations resulting in successful development of high-production strains. Penicillin was followed by the discovery of more antibiotics from other yeast and bacteria, specifically from Actinomycetes, including cephalosporins, streptomycin, tetracycline, chloramphenicol, vancomycin, and many more (12). While the discovery of bacteria being the root of disease and the subsequent creation of the antibiotics industry makes up a major sector of bioindustry, other products were of interest as well.

In the late 19th century, as microbes were first being understood, another breakthrough occurred, the discovery of enzymes. In 1877, Moritz Traube was the first to describe enzymes, what he called “protein-like catalysts.” Two decades later, Eduard Buchner founded the field of Biochemistry by catalyzing a reaction with cell free extracts by converting sucrose to ethanol with yeast cell lysates. In the early 20th century, primary metabolites, such as amino acids, vitamins, flavor nucleotides, organic acids,

and alcohols, were produced at industrial scale. For example, citric acid, acetic acid, and vitamin C all became industrial products via microbial fermentation. As the century moved on amino acids, and more vitamins and organic acids also became commercially available (12) (Fig. 1.1)

Then, in the 1970s, an energy crisis turned the focus back to ethanol, but this time for energy instead of food and beverages (Fig. 1.1). The crisis was short-lived, and bioenergy lost focus when oil prices dropped, and the technology was not advanced enough for industry (12). Then, in 2007, Congress passed the Energy Independence and Security Act (Fig. 1.1) which required 36 billion gallons of ethanol to be produced per year by 2022, and 21 billion gallons to be specifically “advanced biofuels” such as cellulosic ethanol (11). Today, we once again have high oil prices, renewable resources are cheap, and a renewed interest in alternative fuels has resumed. The biofuel industry is a perfect example of purposing microorganisms for yielding valuable products (13).

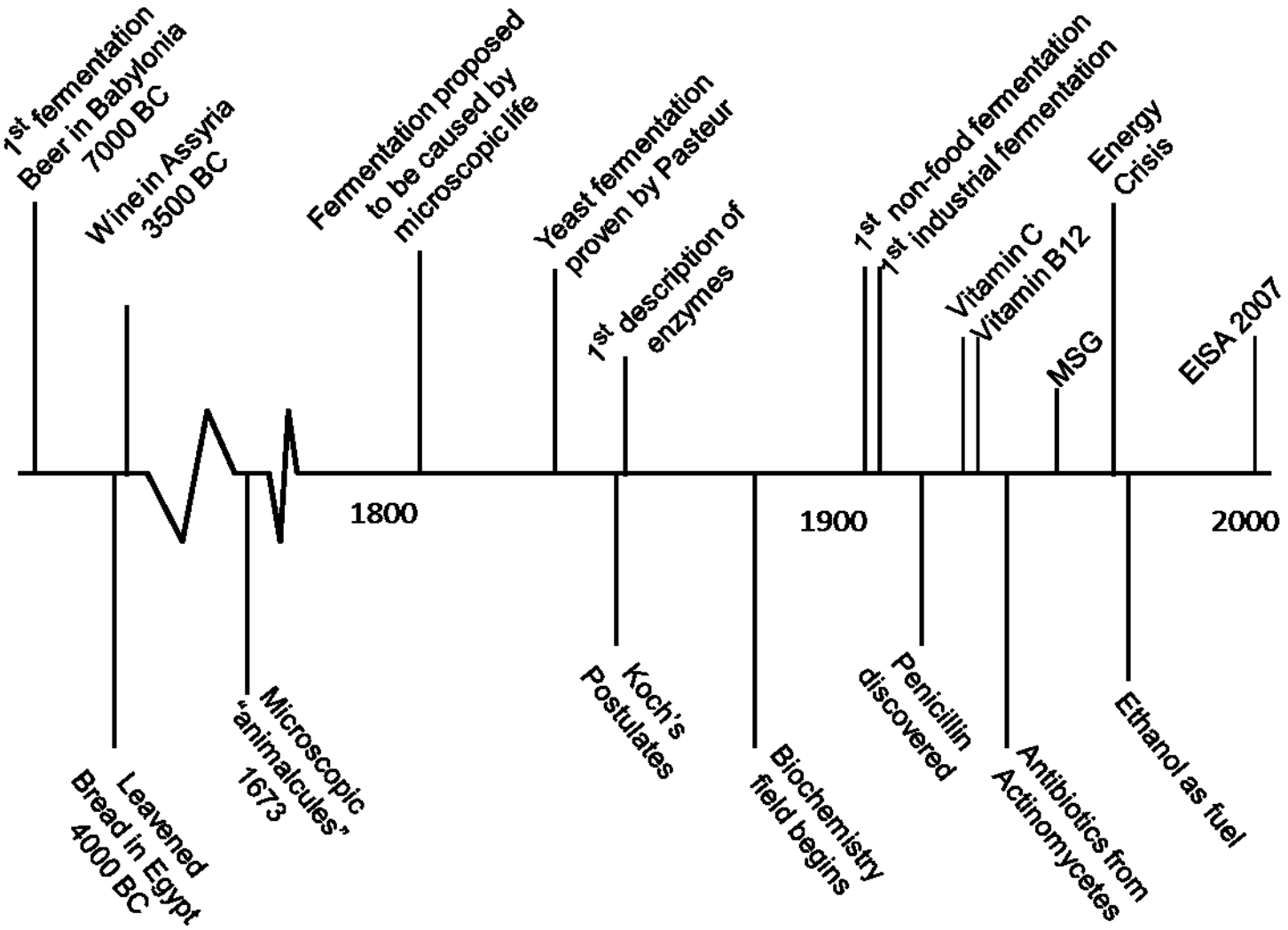

Figure 1.1: Timeline of the history of industrial microbiology

1.3 Biofuels

1.3.1 Overview

Other types of renewable energy also exist including solar, wind, waves, tidal flows, and nuclear fusion (18), but replacing liquid transportation fuel is essential as 60% of our oil consumption goes towards transportation needs (2). Developing biofuels derived from renewable sources to replace petrodiesel will benefit the economy by reducing imports and boosting the domestic agriculture industry. Decreasing our reliance on foreign oil will improve national security and constrain oil prices. Additionally, cleaner emissions will result in improved environmental conditions (55, 56).

When comparing liquid transportation fuels, several factors are important to consider. Octane and cetane numbers describe the ignition quality of the fuel with high numbers being favorable. Energy content of fuels will affect torque, acceleration, and miles per gallon. Additionally, emissions quality will determine the environmental impact of the fuel (55, 56).

There are two main types of transportation fuel, gasoline and diesel. Gasoline contains hydrocarbons 4-12 carbons long and has an octane number range of 91-99. Alternatives to gasoline include ethanol and butanol. Ethanol has a favorable octane number of 129 but an energy content at 70% of traditional gasoline. Ethanol, especially when derived from cellulosic resources, has the potential to reduce greenhouse gas emissions (17) with some estimates as high as 60-90% reductions compared to conventional gasoline (9). Limitations in ethanol as a biofuel include low vapor pressure limiting ease of combustion especially at low temperature (56) and its corrosive nature preventing use in pure form in the existing infrastructure (13).

Butanol, which can actually replace either gasoline or biodiesel, has two more carbons per molecule than ethanol, therefore, a higher energy content than ethanol by 40% but a lower octane number (96) although it is within the range of gasoline (32). Butanol also exhibits reduced emissions (26). Use of butanol as an alternative fuel is mostly limited by low product yields (26).

Diesel contains hydrocarbons ranging from 9-23 carbons long whereas its biofuel alternative, biodiesel, ranges from 12-22 resulting in similar energy content and cetane numbers (32). Biodiesel has reduced emissions compared to diesel in terms of carbon monoxide and particulates, but actually releases more nitrogen oxides due to higher rates of oxidation (15). The major limitation of biodiesel is poor cold flow properties (15, 47).

As ethanol is the major renewable fuel currently used in the United States, and biodiesel is preferred in Europe (18), I will concentrate on these two alternative fuels.

1.3.2 Ethanol

While the Energy Independence Act of 2007 has renewed interest in ethanol as a biofuel, it is not a new idea. Ethanol was used as fuel in original ignition engines in the late 19^{th} century and early 20^{th} century (1). Ethanol dominates the current biofuel market as 67 billion literes were produced in 2008 (40) with most of that production coming from Brazil, the United States, and Asia (9). Currently, ethanol is mostly derived from sugar and starch substrates fermented by yeast and bacteria (1), but it has the potential to be produced from low cost feedstocks (60).

Under optimistic conditions, ethanol could penetrate 52% of the fuel market by 2030. This would depend on the country adopting flex fuel cars that could run on 85% ethanol blends. Otherwise, penetration could only ever reach 15%, as non-flex fuel cars can only use gasoline blends up to 15% ethanol (56).

1.3.3 Biodiesel

Using plant oils as a fuel source goes back to the late 19th century. The first scientists to use transesterification of plant oils to produce biodiesel were E. Duffy and J. Patrick in 1853, but it was Rudolf Diesel who first tested it as a fuel in a car in 1893. Furthermore, after a demonstration at the International Exhibition in Paris using peanut oil to run a diesel engine, R. Diesel intuitively asserted “Usage of plant oil as fuel for cars may seem insignificant nowadays but such oils, with time, may become as important as coal and oil products now” (18). The credit for the first production of biodiesel as we know it today goes to G. Chavanne who patented the process while at the University of Brussels using palm oil in 1937 (30).

From 1998 to 2008 the biodiesel industry exhibited incredible growth both in the US and overseas. Domestically, biodiesel production increased 350-fold from 2 million to 700 million gallons a year. The European Union saw a 33-fold increase in production from 475 thousand tons to 16 million tons (18).

The big picture benefits of biodiesel result from being a renewable energy source (reduced carbon footprint, renewable feedstock supply), cleaner emissions, and expansion of agricultural markets and economy. The fuel properties of biodiesel show promise, but are still limited in some ways. Biodiesel compared to diesel has a higher

flash point, greater lubricity, and can mix with petrodiesel in any concentration. Unfortunately, cold fuel properties limit the usability of this fuel presently. Cloud point and pour point at freezing temperatures limit the use of this fuel during winter months in some climates. If the cold fuel properties are addressed and oil yields increase (via plant genetic manipulation or new and improved feedstocks), biodiesel could become economically feasible. At present, biodiesel market penetration will only reach 6% by 2030 (55).

1.4 Pretreatment technologies as the first step in biomass conversion to ethanol

Conversion of lignocellulose to ethanol faces several challenges, the first of which is the natural recalcitrance of this substrate. The substrate complexity and inherent resistance to degradation requires pretreatment prior to biological conversion to ethanol (53). Lignocellulose is composed of lignin, cellulose, hemicellulose and small amounts of protein, pectin, extractives, and ash. Composition and construction of these components vary by plant type. Lignin serves as a barrier of the plant cell wall to microbial degradation (28). The goal of pretreatment is to break the lignin seal and decrease crystallinity of cellulose in order to increase accessibility of carbohydrate polymers for enzymatic hydrolysis and eventually fermentation (39, 57). The pretreatment process should minimize energy consumption and chemical and mechanical usage, be scalable to industry, and require little input via enzymes and chemicals post-pretreatment and before fermentation.

Current technologies do not reach all these goals, but trade-offs can be made to optimize methods (28). There are two main classes of pretreatment technologies

utilized in industry, physical and chemical pretreatment methods. Physical pretreatments exploit particle size and mechanical stress to reduce crystallinity. Chemical pretreatments use acids and bases in varying methodologies to accomplish this goal (53).

Acid pretreatments use high temperature and pressure to target and hydrolyze hemicellulose and lignin, resulting in a solubilized fraction containing hemicellulosic sugars and degradation products (53). Steam pretreatment and acid pretreatments utilize high temperature and sometimes pressure with their catalysts (water or acid) to disrupt cellulose crystallinity. Lignin and hemicellulose are solubilized while cellulose microfibrils remain mostly intact. The resultant product is a slurry of semi-degraded lignocellulose (28).

Alkaline treatments target acetyl groups and ester linkages connecting the lignin to the cellulose and hemicellulose. Hemicellulose is not degraded so additional enzymes are needed downstream, but fewer degradation products occur (53). Alkaline pretreatments aim to solubilize lignin while hemicellulose and cellulose remain solid. Ammonia Fiber Expansion (AFEX) is a physicochemical pretreatment that uses moderate temperature and high pressure. The ammonia hydroxide targets the acetyl groups and ester linkages of lignocellulose (53). The ammonia is evaporated and recovered, resulting in a dry product with low concentrations of inhibitors (28). Ammonia pretreatment yields optimal sugars by depolymerizing lignin and cleaving the lignin carbohydrate linkages. Ammonia costs play a major role in the economy of this technology, but as ammonia can be recovered and reused, costs are minimized (39).

The resulting substrate is decrystallized cellulose with solubilized lignin and increased access for hydrolysis (3).

1.5 Native Microbial Bioprocessors

1.5.1 Microbial bioprocessing in Nature

Microorganisms play a central role in the cycling of carbon in terrestrial environments (51). Cellulose degraders contribute to ca. 90% of global carbon cycling of plant-derived cellulose (19) as complex microbial consortia composed of cellulolytic and saccharolytic microorganisms degrade and ferment cellulose anaerobically to CO_2, H_2, organic acids, alcohols, and fatty acids (33). In the absence of inorganic electron acceptors such as nitrate, Mn(IV), Fe(III) and sulfate, the products of cellulose fermentation serve as growth substrates for other microorganisms, such as methanogens and homoacetogens, which complete the C cycle by producing CO_2 and CH_4 (33).

Microorganisms naturally produce a wide-range of valuable products that can be harnessed in industry including organic acids, chemicals, vitamins, antibiotics, pharmaceuticals, and biofuels. While these native organisms tend to grow slowly and have low product yields, they maintain several advantages as industrial candidates. Biological conversions have innate synthetic pathways to desirable products with high specificity for their substrates and often eliminate the need for environmentally harmful compounds such as heavy metals and solvents (13). Harnessing these natural processes could allow for energy recovery from biomass waste and residue from the agricultural sector as well as other industries (45).

1.5.2 Microbial bioprocessing in Industry: Bioconversion to Ethanol

To convert biomass to ethanol, pretreated substrates must first be hydrolyzed to fermentable sugars. Cellulose is hydrolyzed by cellobiohydrolases (exoglucanases) that cleave cellobiose off the ends of cellulose chains, endoglucanases that hydrolyze internal bonds along the chain, and β-glucosidase that splits cellobiose into glucose units. Since hemicellulose is composed of a variety of hexose and pentose sugars, various other enzymes are required such as xylanases, mannases, and galactosidases depending on the biomass composition (28, 42).

Two conversion methods utilizing enzyme cocktails are separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) (59). SHF separates the two major processes of lignocellulose conversion to ethanol. First optimal conditions for enzymatic hydrolysis are employed to convert pretreated lignocellulose into monomeric sugars. SSF combines the two processes in a single reactor by combining the enzymes and microbial catalyst and reducing costs (59).

A more promising and cost-effective approach, consolidated bioprocessing (CBP), combines degradation of cellulose and conversion of sugars to ethanol into one step using a single microorganism capable of production of saccharolytic enzymes and fermentation of enzymatically-derived hexose and pentose sugars (9, 37). This process should lower the cost and increase the efficiency of bioethanol production from lignocellulosic substrates. The potential of the process stems from enzyme-microbe synergy due to complex cellulase systems. Two main strategies are employed to develop a CBP microorganism. The native cellulolytic approach utilizes natural saccharolytic microorganisms that can be metabolically engineered to improve product

yields. The recombinant cellulolytic strategy employs ethanologens and engineers them to incorporate cellulase systems into their genomes (37).

Plants use lignin in their cell walls to protect themselves, but microbial interactions in nature are still quite common. Sometimes these interactions are negative which is the case with pathogens, but they can also be positive as with symbionts. Also, microbes play a major role in carbon cycling (saprotrophs) which shows that microbes have found a way around the plants native defense (20). This illustrates the potential of native cellulolytic organisms for use in ethanol production from lignocellulosic biomass. Cellulose is the most abundant polysaccharide on Earth, and many organisms are involved in the conversion of this substrate into soluble sugars. The complex nature of lignocellulose requires an intricate system of enzymes to efficiently degrade the polymers. Certain bacterial species form complex cellulolytic complexes called cellulosomes where catalytic enzymes organize amongst ‘scaffold’ and ‘dockerin’ proteins and use ‘cohesion’ domains to bind to the substrate (5). Cellulosomes are multifunctional enzyme complexes that form protuberances on the outside of the cell and mediate cell-substrate contact, but can also maintain activity once released into the environment (6).

1.5.3 Cellulomonads

Cellulomonas spp. play a critical role in decomposition in many terrestrial ecosystems as they are the only bacteria known to decompose plant litter aerobically and anaerobically. Their versatile metabolism is due to their unique ability to produce free non-complexed enzymes, which are the major enzymatic strategy for aerobic

decomposition, as well as enzyme complexes or cellulosomes, analogous to those produced by anaerobic decomposers (10, 38). The main habitat of this genus is soil, but they are often found in other cellulose enriched environments such as rumen, activated sluge, and sugar fields (54). Cellulomonads were of original interest to industry as efficient producers of single cell protein for use as a food additive. *Cellulomonas* spp. were used to digest agricultiral residues like sugar cane bagasse and rice straw to produce single cell protein (25, 29, 46, 54).

Cellulomonas strains have a history in co-cultivation methods for improved performance or value-added products. To improve biomass production for single cell protein production, *Cellulomonas* strains were co-cultivated with cellobiose-consuming yeast to minimize feedback inhibition of enzymatic activity in the presence of cellobiose (24). This method was also utilized in cocultures with *Candida utilis* to minimize the need for yeast extract or vitamin additions (31). These co-culture methods also extended to creating value-added products from waste material. A *Cellulomonas* strain was co-cultured with *Desulfovibrio vulgaris* to produce methane from cellulose as the *Cellulomonas* strain converted cellulose and xylan into formate and ethanol, which were then converted by *D. vulgaris* into methane (21). Co-cultivation with nitrogen-fixers on cellulose of wheat straw also resulted in energy-yielding products (22, 23). More recently, a consortia of *C. uda* and *Geobacter sulfurreducens* was shown to convert pretreated agricultural residue to ethanol and hydrogen. The ability of Cellulomonads to produce valuable products combined with their native cellulolytic abilities make them ideal candidates for consolidated bioprocessing.

1.5.4 Microbial bioprocessing in industry: Wastewater treatment

Anaerobic digestion is a method of using a complex mixture of anaerobic microorganisms to degrade organic matter in industrial wastewater and solid wastes to reduce contaminants and release energy via biomethanation (36). The pulp and paper mill industry for example, began using anaerobic wastewater treatment in the 1970s (44). Various other industries have also employed similar methods including textile industries for the degradation of color effluents (49), fisheries (41) as well as distilleries and the food industry (36).

Of particular interest is the crude glycerol wastewater of the biodiesel industry. As the biodiesel market has expanded, the market for glycerol as a value-added product has been saturated resulting in economic and environmental liabilities associated with the wastewater (16, 27). Bioconversion of crude glycerol to valuable products could prove an effective method of wastewater treatment for the biodiesel industry. Ethanol is of particular interest as it can be used as a feedstock in the transesterification process used to convert triacylglycerides to biodiesel (8). Potential of the treatment of biodiesel wastewater has been explored in particular for the production of ethanol and/or hydrogen as value-added products (48, 61).

1.5.5 Clostridia

The *Clostridium* genus encompasses a broad range of gram-positive, rod-shaped, endospore forming bacteria making up the 2nd largest bacterial genus. The group of organisms (organized into 19 clusters) is often associated with disease-causing bacteria and toxins, but these organisms also are filled with potential for biotechnological

applications. Of particular interest to the bioenergy industry are cellulolytic Clostridia. These organisms can be found in environments rich in decaying plant matter including soil, sediment, compost, and gastrointestinal tracts of herbivorous and wood-eating organisms. Some Clostridia are generalists and can utilize a wide range of sugar substrates, while others are specialists and require certain carbon sources (14).

Cellulolytic *Clostridium spp*. are well known for their robustness in industrial fermentations, including the fermentation of glycerol (the major waste product of biodiesel plants) into value-added products (7). Fermentative products of interest from *Clostridium* spp. include ethanol (1, 37), butanol (1, 62), acetone (1), 1,3-propanediol (4, 50, 62), and hydrogen (43).

C. cellobioparum, a member of Cluster III (cellulolytic, nitrogen-fixing) of Clostridia, is a generalist able to use a wide range of substrates to produce acetate, ethanol, formate, lactate, and hydrogen (14). *C. cellobioparum* was isolated from the cow rumen. Other members of Cluster III include *C. hungatei*, *C. thermocellum*, *C. termitidis*, *C. celluloyticum*, *C. papyrosolvens* (34, 58). This clade was recently reclassified as *Ruminococcaceae* (35)

1.6 Summary

Use of microbes in industry has a long history impacting major industries including food, medicine, and chemicals. Recently, microbial biotechnology in regards to biofuels has become increasingly important. With a large abundance of carbon energy available in lignocellulosic biomass, understanding microbial bioprocesses can lead to the use of such organisms in industry.

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Chapter 2

Reversible control of biofilm formation by *Cellulomonas* spp. in response to nitrogen availability

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The work in this chapter builds upon and includes some results from:

Reguera, G. 2001. Chitin Degradation by the Facultatively Aerobic Cellulolytic Bacterium *Cellulomonas uda*. Dissertation. University of Massachusetts Amherst.

2.1 SUMMARY

The microbial degradation of cellulose contributes greatly to the cycling of carbon in terrestrial environments and feedbacks to the atmosphere, a process that is highly responsive to nitrogen inputs. Yet how key groups of cellulolytic microorganisms adaptively respond to the global conditions of nitrogen limitation and/or anthropogenic or climate nitrogen inputs is poorly understood. The actinobacterial genus *Cellulomonas* is of special interest because it incorporates the only species known to degrade cellulose aerobically and anaerobically. Furthermore, despite their inability to fix nitrogen, they are active decomposers in nitrogen-limited environments. Here we show that nitrogen limitation induced biofilm formation in *Cellulomonas* spp., a process that was coupled to carbon sequestration and storage in a curdlan-type biofilm matrix. The response was reversible and the curdlan matrix was solubilized and used as a carbon and energy source for biofilm dispersal once nitrogen sources became available. The biofilms attached strongly to cellulosic surfaces and, despite the growth limitation, produced cellulases and degraded cellulose more efficiently. The results show that biofilm formation is a competitive strategy for carbon and nitrogen acquisition and provide valuable insights linking nitrogen inputs to carbon sequestration and remobilization in terrestrial environments.

2.2 INTRODUCTION

Microorganisms play a central role in the cycling of carbon in terrestrial environments and the biogenesis of greenhouse gases such as CO_2, CH_4, and N_2O (50). However, the magnitude of these responses is still unclear, due in part to our limited understanding of the mechanisms that enable microorganisms to adjust their physiology

to changes in their ecosystem that have the potential to affect climate (2). Plant-derived cellulose is the most abundant terrestrial C input and accounts for nearly 50% of all the C sequestered by plant biomass during photosynthesis annually (33). As a result, cellulose degraders contribute to the cycling of ca. 90% of the global plant production that enters soils and sediments as dead organic matter (14) and to the release of approximately half of the 120 billions of C fixed through photosynthesis every year (50). The aerobic decomposition of cellulose is restricted to a top thin crust of soils (33) and is mostly driven by cellulolytic fungi and actinobacteria (35), which primarily mineralize cellulose to CO_2. Approximately 5-10% of the cellulose is degraded in anaerobic environments, with highest activities occurring in proximity to the surface of soils, composts, and fresh water sediments (31, 33). Complex microbial consortia composed of cellulolytic and saccharolytic microorganisms degrade and ferment cellulose anaerobically to CO_2, H_2, organic acids, alcohols, and fatty acids (31). In the absence of inorganic electron acceptors such as nitrate, Mn(IV), Fe(III) and sulfate, the products of cellulose fermentation serve as growth substrates for other microorganisms, such as methanogens and homoacetogens, which complete the C cycle by producing CO_2 and CH_4 (31). The sediments in natural wetlands alone contribute with 20 to 40% of the global CH_4 budget (13, 17) and weather- and climate-induced fluctuations in these ecosystems account for 90% of the variability of global CH_4 emissions (47). Thus, the activity of aerobic and anaerobic cellulolytic microorganisms contributes greatly to terrestrial C cycling and climate feedbacks as a direct source of CO_2 or, indirectly, of

CH_4.

The activity of cellulose degraders is intimately connected to the availability of N sources (22, 46). N limitation is globally distributed (28) and can be exacerbated by C supplementation, such as that resulting from the incorporation of cellulose derived from crop residues into soils, which can progressively limit decomposition (16) and C-use efficiency (11, 18). By contrast, increased N inputs in soils, such as from atmospheric deposition, fertilizers, pollution, and plants associated with N_2-fixing symbionts, can affect the enzymatic activity and diversity of the heterotrophic community (38, 56, 61) and enhance decomposition and C release to the atmosphere (4). Climate also affects plant diversity and, therefore, litter quality and its N content (14), which may impact cellulose degradation. Not surprisingly, bacterial community structure is highly responsive to disruption of the natural C:N ratio (43). However, it is difficult to predict the functional implications of these responses without knowledge of how cellulose degraders respond to C:N disturbances (50).

According to ecological stoichiometry theory, bacteria are under great selective pressure to adapt to imbalances in substrate C:N in order to maintain a constant C:N ratio inside the cell (54). This is especially challenging for cellulose degraders, which inhabit environments with a high C:N ratio. Because of this, it has been proposed that N limitation enriches for free-living, N_2-fixing decomposers (32). This is supported by the wide distribution of nitrogenases among microbial lineages (7) and the ability of N_2-fixing, clostridial decomposers to couple the anaerobic degradation of cellulose to N_2 fixation (32). However, most cellulolytic organisms cannot fix N_2 and require a source of

combined N to grow (7). Furthermore, the presence of O_2 limits N_2 fixation (10) and, therefore, the availability of combined N sources from N_2 fixers in the aerobic zones of soils or in areas prone to O_2 intrusions, where most of the cellulose is degraded (31, 33, 35). This suggests that adaptive responses other than N_2 fixation drive decomposition.

To gain insights into these adaptive responses, we focused our investigations on the genus *Cellulomonas*, which comprises the only microorganisms known to degrade cellulose aerobically and anaerobically (35). *Cellulomonas* spp. produce non-complexed and complexed (or cellulosomes) cellulase enzyme systems (8, 27, 57), which are enzymatic strategies linked to the predominance of cellulolytic microorganisms in either aerobic (non-complexed) or anaerobic (complexed) habitats (35). They are not known to fix N_2 (53) or to contain nitrogenase genes (1), yet they are ubiquitous and can be abundant in terrestrial ecosystems where cellulose degradation is a significant process (3, 35, 42, 48). Here we show that *Cellulomonas* spp. undergo a physiological shift from planktonic to biofilm growth in response to low N availability. The mechanism was finely-tuned to the external C:N ratio and enabled the reversible sequestration of C in a curdlan-type biofilm matrix. The biofilms also produced high levels of cellulase activities, attached strongly to cellulosic substrates, and degraded the substrate efficiently. This provides a competitive strategy for decomposition and N acquisition despite the predominantly N-limiting conditions of terrestrial environments.

2.3 RESULTS

2.3.1 Effect of N availability on growth and biofilm formation in *Cellulomonas* spp.

We investigated the effect of N availability on the growth of representative species of *Cellulomonas* by culturing the strains in a N-supplemented or a N-limited culture with the cellulose-derived disaccharide, cellobiose, as sole C and energy source. All of the strains tested grew planktonically with N supplementation (Fig. 2.1A) and responded to growth-limiting concentrations of N with a shift from planktonic to biofilm growth (Fig. 2.1B). In contrast to the uniform turbidity of the N-supplemented cultures (Fig. 2.1A inset), the N-limited cultures only supported the development of a white-coloured biofilm, which settled at the bottom of the culture vessel (Fig. 2.1B inset). The biofilms were easily disrupted by vortexing into a suspension of small cell aggregates. This allowed for stable absorbance readings and comparative measurements of biofilm biomass in all N-limited cultures. Interestingly, the response of *Cellulomonas fimi* was not as strong as in the other strains (Fig. 2.1B). However, its growth was poor, with planktonic doubling times in the N-supplemented cultures significantly higher (7 ± 0.5 h) than the average doubling times of the other strains (4 ± 0.6 h). This suggests that the partial response of *C. fimi* to low N availability was due to growth limitation by other nutrients.

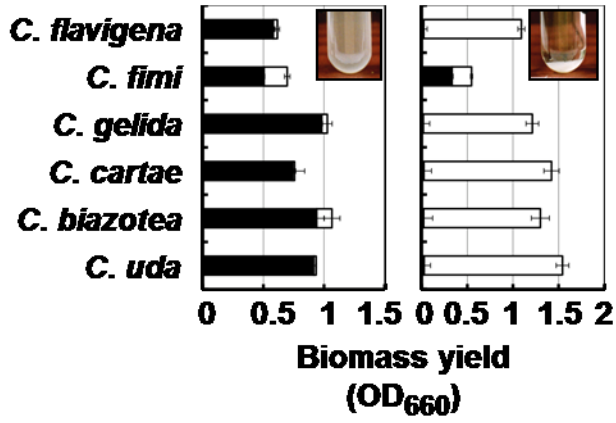

Figure 2.1: Effect of N supplementation (A) or limitation (B) on biomass yields (planktonic, solid columns; biofilm, open columns) of representative species of *Cellulomonas*. Insets in A and B show pictures of *C. uda* planktonic and biofilm cultures, respectively. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

We further characterized this response in *Cellulomonas uda*, which is better known at the physiological level (53), by monitoring its growth and C (cellobiose) use efficiency as a function of N availability (Fig. 2.2A). N-supplementation promoted the exponential growth of a planktonic population at a rate of 0.04 μg total cell protein per h (R^2 = 0.992) and increases in the C:N ratio until N concentrations dropped to ca. 0.1 mM (Fig. 2.2A, left panel). At this point, the cells entered stationary phase and aggregated transiently. In contrast, the N-limited cultures, which had no N supplementation in order to exacerbate the C:N ratio, had no planktonic growth and supported the development of the white-coloured biofilm (Fig. 2.2A, right panel). Increases in biofilm biomass followed a polynomial distribution (R^2 = 0.979), as expected of a process coupled to cell growth, and inversely correlated with decreases in

the C:N ratio (R^2 = 0.973) until all the C and N sources were depleted. Total cell protein also increased linearly and at a rate of ca. 0.02 μg protein per h (R^2 = 0.946) during the linear phase of biomass increase, demonstrating that the biofilm cells were growing and dividing.

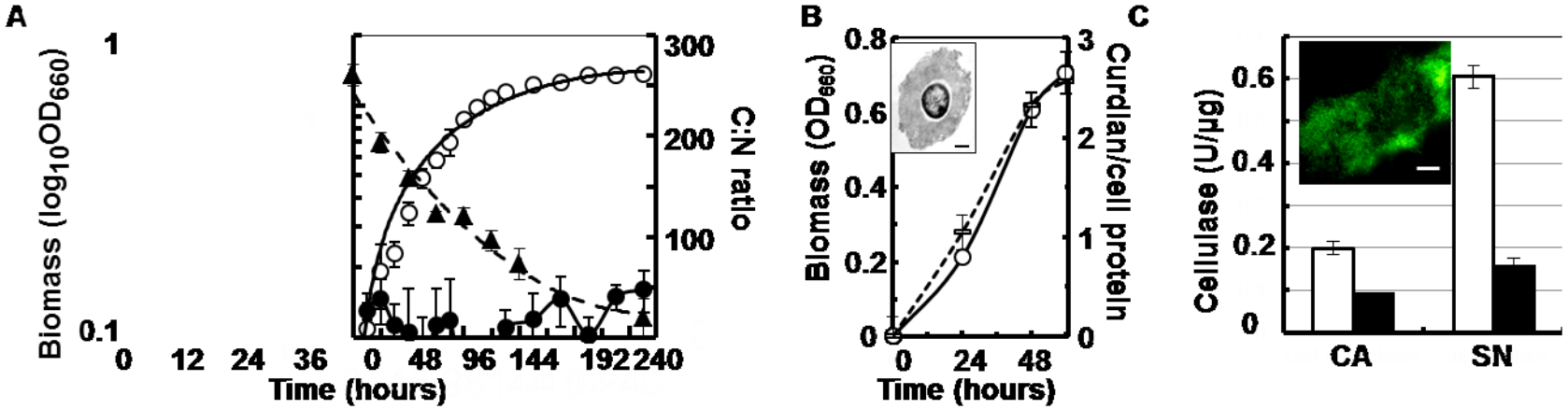

Figure 2.2: (A) Effect of N supplementation (left) or limitation (right) on planktonic (solid circles) and biofilm (open circles) biomass growth (calculated as log_{10} of OD_{660}). C:N (cellobiose: NH_4^+) ratios also are shown (dashed lines with triangles). (B) Curdlan production (dashed line) during the formation of N-limited biofilms (solid line). Inset: TEM thin section showing the ruthenium-stained layer of curdlan around a cell (bar, 0.2 μm). (C) Cell-associated (CA) and supernatant (SN) CMCase specific activities (U per μg of total cell protein) in N-supplemented (black columns) or N-limited (white columns) cultures. Inset: fluorescence micrograph of nitrogen-limited biofilms attached to cellulose fibers in pebbled-milled cellulose. Biofilm cellls were stained with the BacLight™ viability dyes (green, live cells; red, dead cells). Bar, 50 μm.

Biofilm cell growth was not coupled to N_2 fixation, as nitrogenase activity was not detected nor was the biofilm growth dependent on atmospheric N_2 (Fig. 2.3). Rather, the biofilm cells appear to use the low concentrations of N (0.3 ± 0.1 mM NH_4^+ equivalents) available in the medium to support cell growth and to sequester all the excess C in the biofilms.

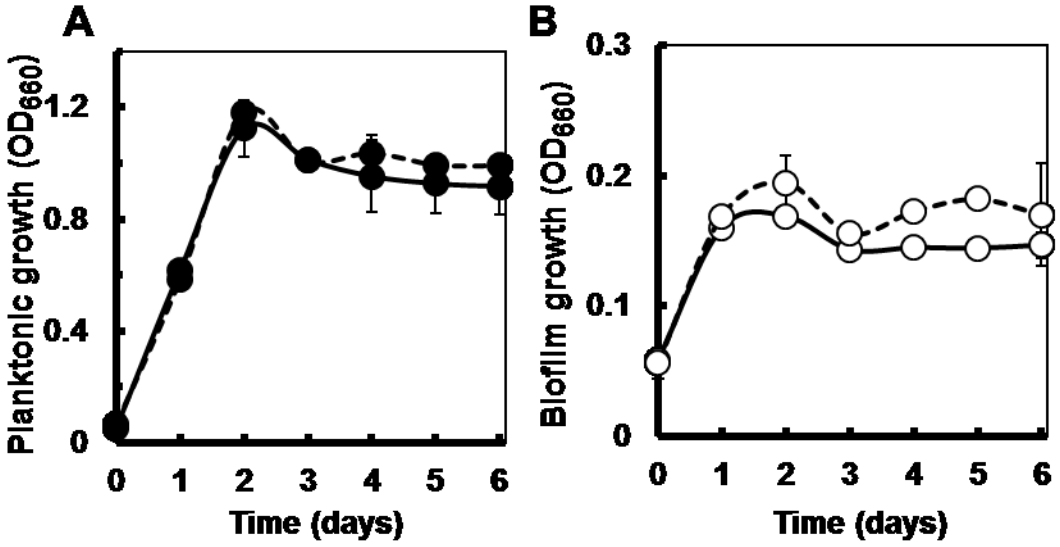

Figure 2.3: *C. uda* growth-independence on atmospheric N_2. Graphs show planktonic (A) and biofilm (B) growth in anaerobic cultures with or without NH_4Cl supplementation, respectively, as a function of the gas atmosphere: N_2 (solid lines) or argon (dashed lines).

2.3.2 C sequestration in a curdlan-type biofilm matrix under N limitation

The rates of C uptake by *C. uda* biofilms (52 moles of C per mol of N) were 1.7-fold higher than in planktonic cultures supplemented with N (31 moles C per mol N). The increased C uptake by the biofilms matched well with the increases in biofilm dry

weight, which were 1.8-fold higher than in the planktonic cells growing in N-supplemented cultures (Table 2.1). By contrast, the dry weight/protein ratio measured in early stationary phase cultures of N-supplemented (planktonic) cultures was ca. 3, which is consistent with the reported accumulation of approximately 3 mg of glycogen per mg of cell protein in stationary-phase cells of *C. uda* (49). High C:N ratios have already been linked to the production of curdlan (β(1-3) glucan) capsules in *Cellulomonas* spp. (51, 58). Thus, we investigated if the biofilms were sequestering excess C as curdlan. We measured linear accumulations of a curdlan-type glucan during biofilm formation at rates (5.8 mg/h; R^2 = 0.976) almost 5-times higher than the rate of cell protein increases (1.3 µg/h; R^2 = 0.977).

Table 2.1: Total cell protein and dry weight content of *C. uda* planktonic cells or biofilms

Culture	Physiology	Dry weight (mg/ml)	Total cell protein (mg/ml)	Ratio (DW/TCP)
N-supplemented (N^+)	Planktonic	0.52	0.18	2.92
N-limited (N^-)	Biofilm	0.95	0.06	16.38
RATIO (N^-/N^+)		1.83	0.33	5.61

The curdlan produced per cell protein matched well with the increases in biofilm biomass measured spectrophotometrically (Fig. 2.2B). These results indicate that biofilm biomass increases resulted from the accumulation of C as a curdlan matrix and, to a lesser extent, from the growth of the biofilm cells. The curdlan accumulated extracellulary as a thick exopolysaccharide (EPS) matrix that stained with the cationic

dye ruthenium red (Fig. 2.2B, inset) and was already visible during the early hours (15 h) of C sequestration (Fig. 2.4). Furthermore, N limitation also resulted in the growth of rugose, rather than smooth, colonies on solidified medium (Fig. 2.4), a phenotypic shift linked to EPS production and biofilm formation (60).

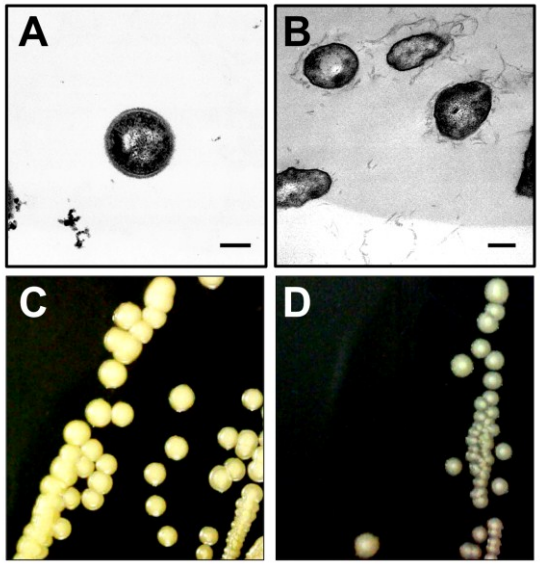

Figure 2.4: (A) TEM micrographs of ruthenium-red stained planktonic (A) and biofilm (B) cells (15 h). (B) Shift from smooth (C) to rugose (D) colonies when N availability limits growth.

2.3.3 Colonization and degradation of cellulosic and chitinous surfaces by N-limited biofilms

Although the N-limited biofilms settled at the bottom of glass or plastic vessels, they did not attach to these materials and were easily detached by gentle agitation. By contrast, the biofilms attached strongly to and grew on cellulose fibers in N-limited cultures supplemented with pebble-milled cellulose as sole C and energy source (Fig. 2.2C

inset). Furthermore, cell-associated and supernatant cellulase (CMCase) specific activities (units, U, per µg of protein) were 2- and 4-fold higher, respectively, in the N-limited biofilm cultures (Fig. 2.2C) as compared to the N-supplemented cultures. The biofilms also attached and degraded other cellulosic substrates such as cellulose-azure (Fig. 2.5A). Furthermore, adhesion was not affected by the roughness of the substrate, as the smooth surface of the dialysis tubing cellulose membranes (regenerated cellulose) was also readily colonized (Fig. 2.5B).

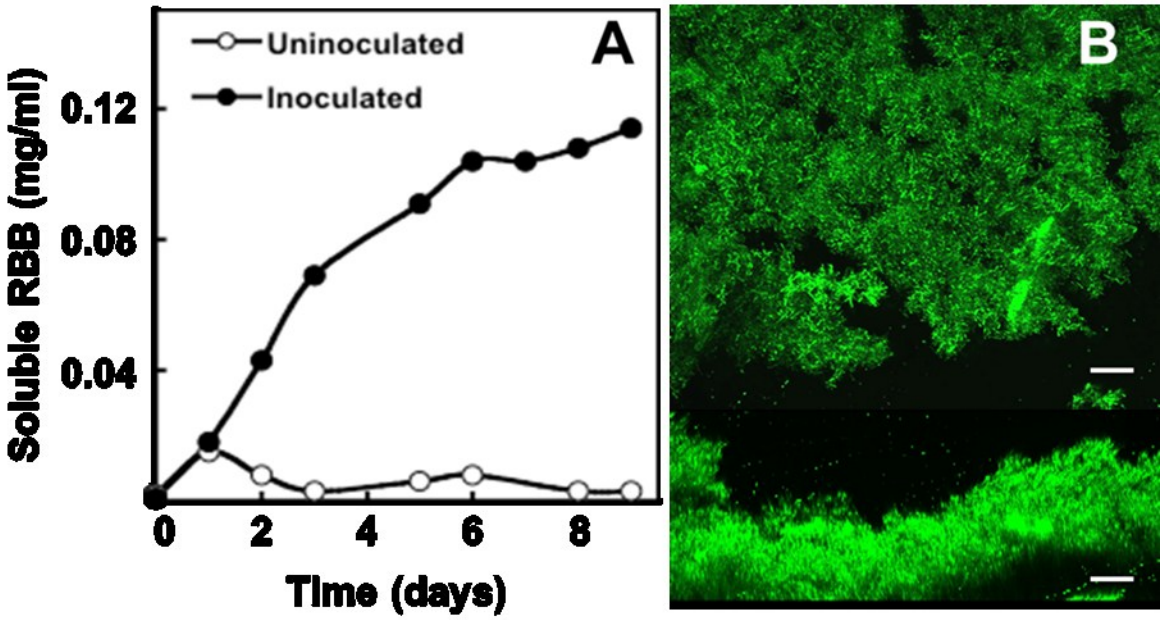

Figure 2.5: (A) Degradation of cellulose-azure and concomitant release of the cellulose-associated dye Remazol Brilliant Blue 5R (RBB) by N-limited biofilms in comparison to uninoculated controls. (B) CLSM micrographs of 72-h old, N-limited biofilms formed on a cellulose membrane (dialysis tubing). The cells were stained in green with the SYTO9 dye. Bar, 20 µm.

The biofilms also colonized and degraded chitinous substrates, which are structurally related to cellulose, and expressed higher levels of chitinase enzymes under N limitation (Fig. 2.6). These results suggest that N limitation induced changes in the adhesive properties of the cells and increases in enzyme production that promoted the colonization and degradation of cellulosic and chitinous substrates.

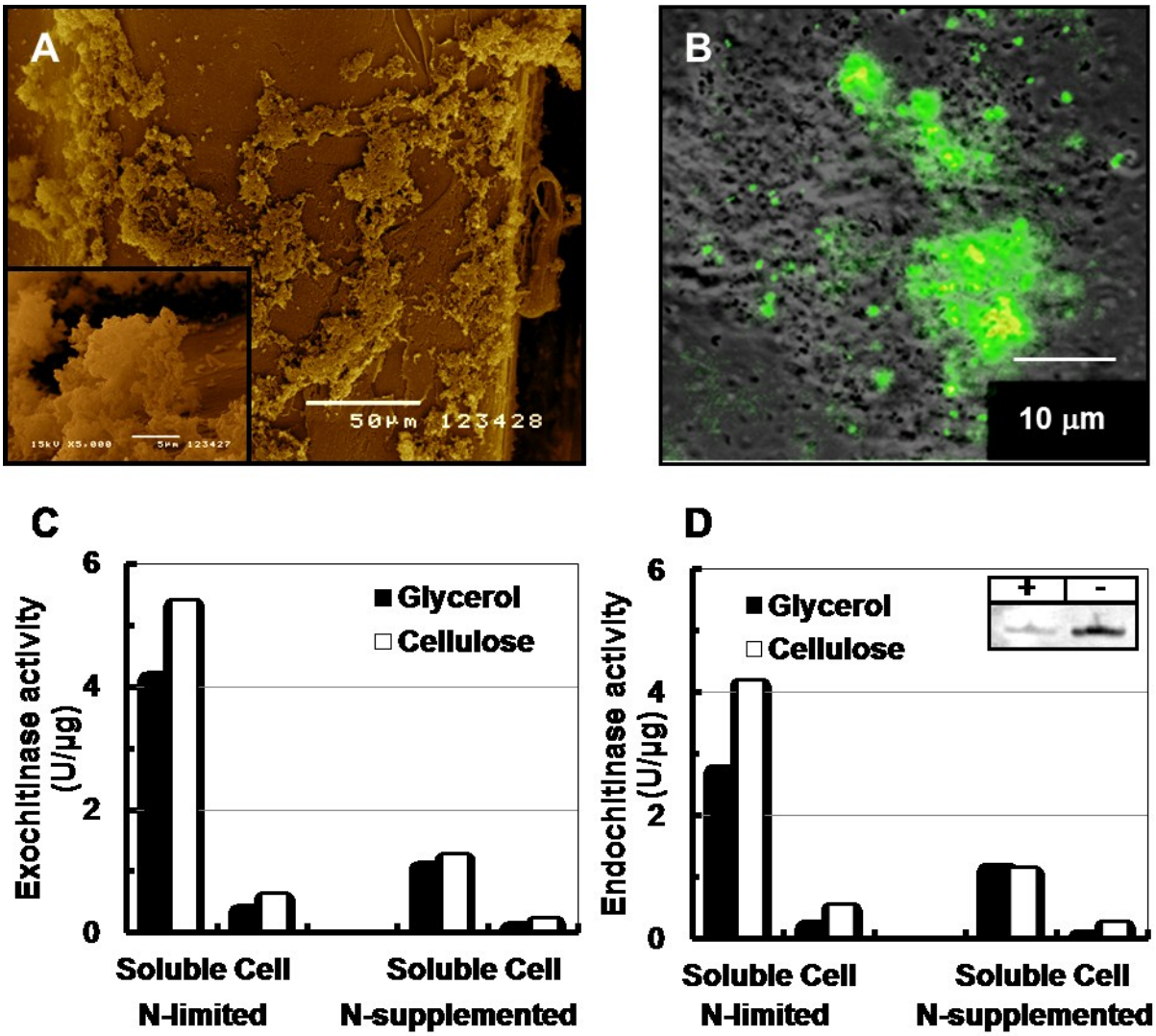

Figure 2.6: (A-B) Attachment of N-limited biofilms of *C. uda* to chitin surfaces (squid pen (A) and colloidal chitin (B)). Biofilms were examined by SEM (A) or by fluorescence

Figure 2.6 (cont'd) microscopy after staining cells with the BacLight viability kit (B). (C-D) Effect of N availability on soluble or cell-associated exochitinase (C) and endochitinase (D) activities in cultures with glycerol or cellulose as sole C and energy source. Inset in (D) shows an immunoblot demonstrating the increased expression of *C. uda* main endochitinase, ChiA, in supernatant fluids from cultures grown in the GS2*-colloidal chitin medium with (+) or without (-) NH_4Cl supplementation.

2.3.4 Biofilm formation and dispersal in response to the C:N ratio

Planktonic and biofilm growth yields inversely correlated with the C:N ratio (Fig. 2.7) independently of the C or N substrates tested (Table 2.2). This is consistent with a physiological response to the C:N ratio rather than to a particular C and/or N substrate.

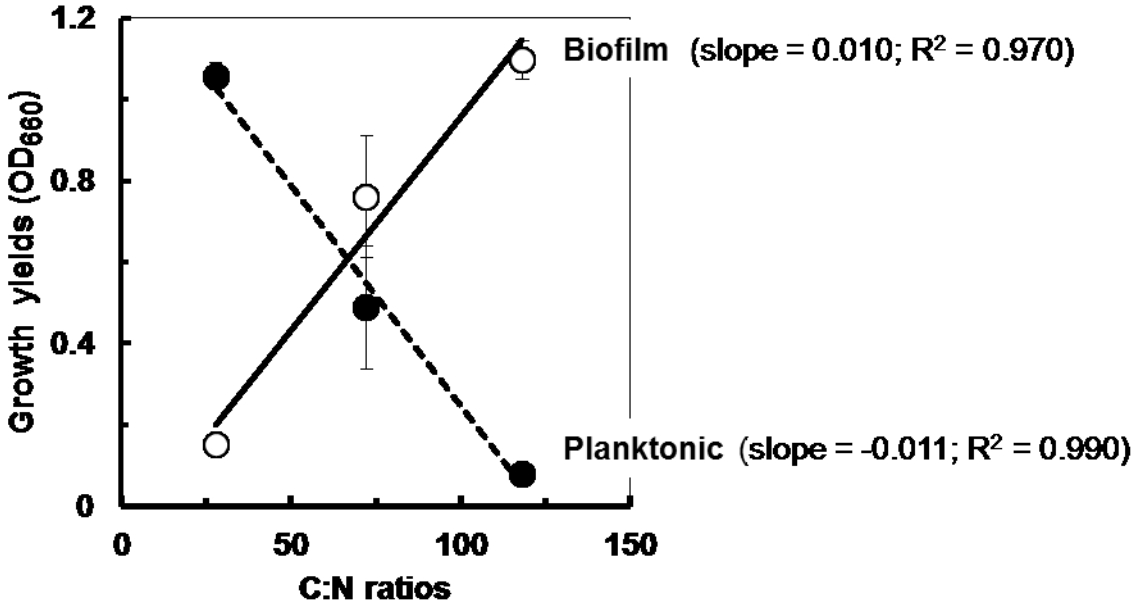

Figure 2.7: Inverse correlation between planktonic and biofilm growth yields as a function of the C:N ratio in *C. uda* cultures grown with 18 mM glucose as a C source and with (0.5 or 2 mM) or without NH_4Cl supplementation.

Table 2.2: *C. uda* planktonic and biofilm growth with labile C and/or N substrates

C source	N source	Growth rates (OD_{660}/h)	Growth yields (OD_{660})	Biofilm growth [a]
Cellobiose	____	NA [b]	NA	+
Cellobiose	NH_4Cl	0.45 ± 0.05	1.12 ± 0.10	-
Cellobiose	Urea	0.43 ± 0.01	1.05 ± 0.03	-
Cellobiose	NAG	0.29 ± 0.03	NA	+/- [c]
Glucose	____	NA	NA	+
Glucose	Urea	0.88 ± 0.02	0.90 ± 0.01	-
Glycerol	____	NA	NA	+
Chitobiose	____	0.50 ± 0.01	1.38 ± 0.04	-
Chitotriose	____	0.35 ± 0.02	1.19 ± 0.01	-

[a] Biofilm growth (+), no biofilm growth (-) (planktonic growth), and mixed cultures composed of cell aggregates and planktonic cells (+/-).

[b] Not applicable (conditions leading to biofilm formation).

[c] NAG, which is assimilated poorly by *C. uda* (45), promoted initial cell aggregation when used as a source of N, consistent with transient N-limiting conditions, but cell aggregates slowly dissolved after three days of incubation.

As a result, the physiological shift from planktonic to biofilm growth, and, therefore, from C utilization to sequestration, was reversible and the biofilm cells dissolved the curdlan matrix and dispersed upon reversal of the C:N ratio by N supplementation (Fig. 2.8).

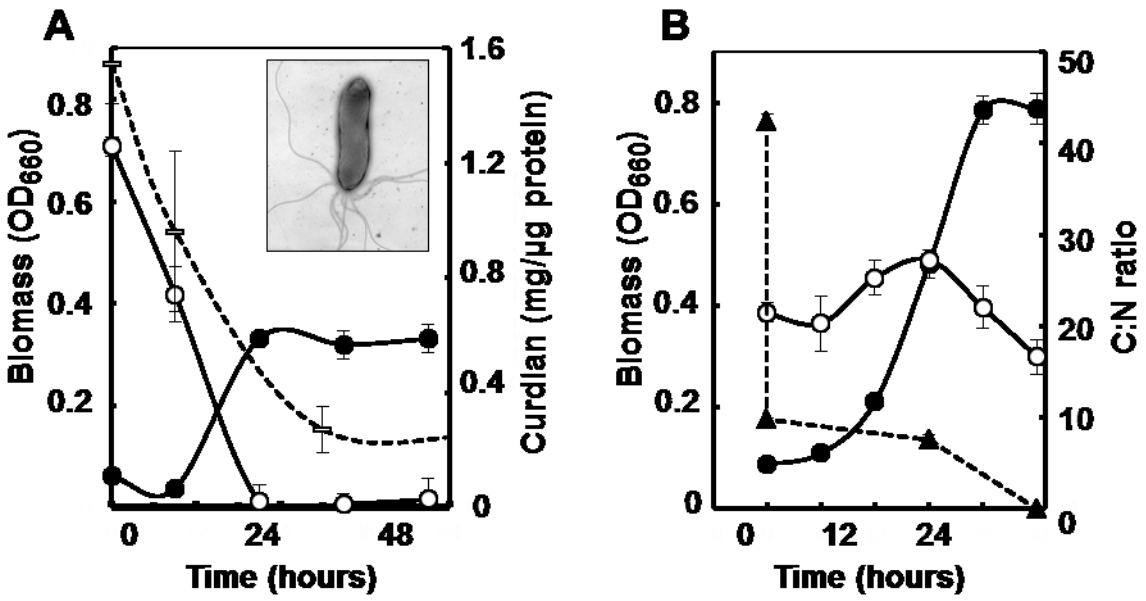

Figure 2.8: *C. uda* biofilm dispersal. (A) Solubilization of the curdlan biofilm matrix (dashed line) coupled to biofilm biomass dispersal (solid line, open circles) and planktonic growth (solid line, solid circles) in the absence of cellobiose in the growth medium. (B) Dissolution of young (28-h) biofilms (solid line, open circles) coupled to planktonic growth (solid line, solid circles) after addition of NH_4Cl to cellobiose-containing N-limited cultures. The C:N ratio (dashed line with triangles) is also shown.

The simultaneous removal of the C source and supplementation with N led to the rapid (less than 24h) solubilization of the curdlan biofilm matrix, which was used as a source of C and energy to support biofilm dispersal and planktonic growth (Fig. 2.8A). Curdlan dissolution was initially observed in localized areas within the biofilm and promoted the release of cell clusters (Fig. 2.9).

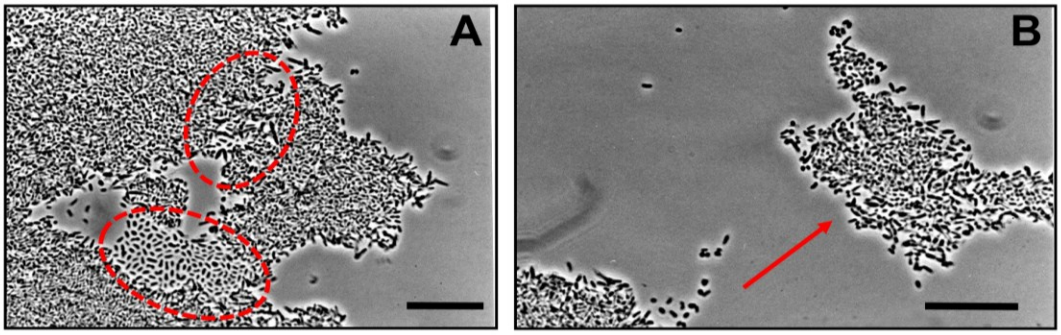

Figure 2.9: Phase contrast micrographs showing islands of curdlan dissolution (red circles in (A)) and dispersal of cell clusters from the main biofilm body (arrow in (B).

Motile cells were also observed during N-induced dispersal and TEM analyses of negatively stained cells from the dispersing cultures revealed some cells with polar multitrichous flagellation (Fig. 2.8A inset). However, motile and/or flagellated cells were not observed in the N-limited biofilms or in the N-supplemented planktonic cultures. This suggests that flagellar motility was a specific response of the cells to N-induced dispersal. Biofilm dissolution responded to the C:N ratio and could be induced at any point in C sequestration and biofilm formation. As shown in Fig. 2.8B, for example, N enrichment of young (28-h old) biofilms growing with cellobiose decreased the C:N ratio and promoted their dissolution and planktonic growth. Furthermore, it was possible to manage N inputs, and, therefore, the C:N ratio, to control the C sequestration capacity of the biofilms (Fig. 2.10).

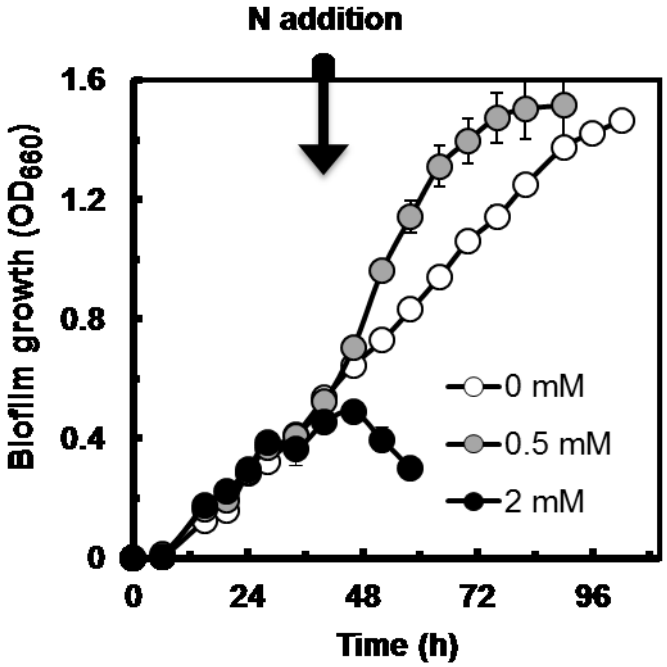

Figure 2.10: Effect of N additions (0.5 and 2 mM additions of NH_4Cl are shown) on the C sequestration capacity and/or dissolution of *C. uda* biofilms. Controls with no N supplementation (0 mM) are also shown.

2.3.5 Degradation of filter paper by N-limited biofilms

The ability of *C. uda* biofilms to degrade cellulose under conditions of N limitation was further investigated using the model cellulosic substrate, filter paper, and in reference to control cultures supplemented with N (provided as NH_4Cl) (Fig. 2.11A). The filter paper substrate supported the growth of *C. uda* (measured as total cell protein) in both the N-supplemented and N-limited cultures, which followed a polynomial distribution (R^2 = 0.939 and 0.967, respectively) as the cells transitioned from exponential to stationary phase (Fig. 2.11A). The degradation of filter paper was also examined to calculate the

yields and rates of decomposition in both cultures (Fig. 2.11A). The degradation of filter paper in the N-supplemented cultures also followed a polynomial distribution (R^2 = 0.921), which inversely correlated with the cell growth. By contrast, decomposition by the N-limited biofilms started after 2 days of incubation, suggesting that the biofilms first colonized and then degraded the cellulosic substrate. After this first phase of colonization, the biofilms degraded the substrate linearly, reaching maximum yields of decomposition in only 4 days. At this point, decomposition stopped and the biofilm cells entered stationary phase. The final yields of decomposition were similar in both cultures (ca. 36% of the filter paper was degraded). However, while the N-limited biofilms degraded the filter paper in 4 days, the N-supplemented cultures needed 6 days of incubation to reach the same yield. Thus, the overall rates of decomposition were higher under N limitation. These contrasting results cannot be explained by differences in cellulase activities, which were similar in both cultures throughout the incubation period (Fig. 2.11B).

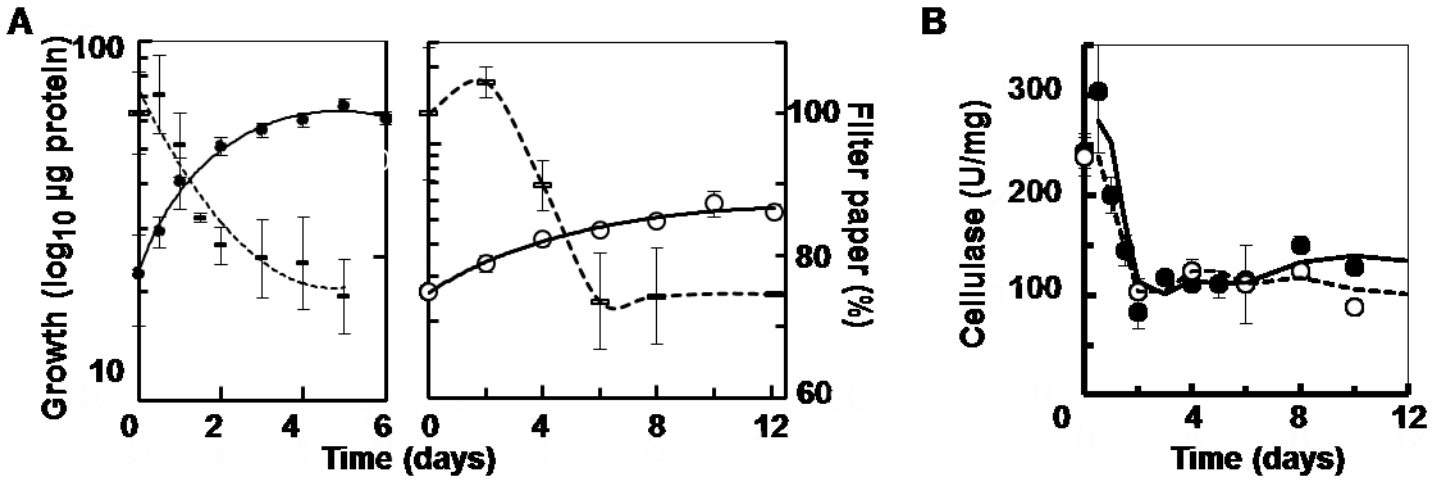

Figure 2.11: Decomposition of filter paper as a function of N availability. (A) Cell growth (log_{10} total cell protein, solid line) and filter paper decomposition (dashed line) in N-supplemented (left) or N-limited (right) cultures. (B) Cellulase (CMCase) specific activities (U per mg of total cell protein) in supernatant fluids from N-supplemented (dashed line) and N-limited (solid line) cultures. The trend lines moving through the average points are shown. All data points in A and B show the average and standard deviations of triplicate samples.

Rather, they reflected a different decomposition strategy as a function of N availability. Planktonic cells from the N-supplemented cultures did not attach to the cellulose fibers and degraded the substrate ‘at a distance’, whereas the N-limited cells attached and formed biofilms on the cellulosic substrate during its enzymatic degradation (Fig. 2.12).

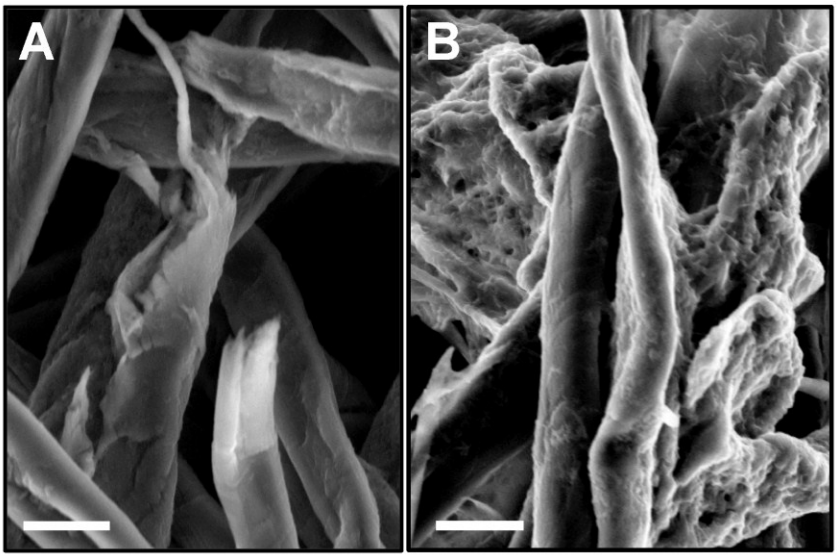

Figure 2.12: ESEM micrographs of the filter paper substrate after 6 days of incubation in the N-supplemented (A) and N-limited (B) cultures (bar, 20 μm). Additional micrographs are shown in Fig. 13.

2.4 DISCUSSION

2.4.1 Biofilm formation and dispersal as adaptive responses of Cellulomonas to C:N disturbances.

Our results show that *Cellulomonas* spp. use biofilm formation and dispersal to adaptively respond to C:N disturbances. As shown in Figures 1 and 2, cells responded to N-limiting conditions and, therefore, to high C:N ratios, by forming biofilms. Biofilm

formation involved the storage of the excess pools of labile C in a curdlan biofilm matrix (Fig. 2.2B), which was dissolved and used as a C source once the C:N ratio was reversed by N supplementation (Fig. 2.8). The formation of a curdlan capsule in *Cellulomonas* spp. was first reported in cultures of *C. flavigena* KU during entry in stationary phase (58) and later studies (24, 51) supported the notion that curdlan production is a general response of *Cellulomonas* spp. to high C:N ratios that leads to cell aggregation. In *C. uda*, curdlan production started rapidly in response to N limitation and was concomitant to increases in biofilm biomass (Fig. 2.2B). This is consistent with curdlan production being the first response to low N availability. In support of this, the degradation of filter paper was only detected after 2 days of incubation in the N-limited cultures (Fig. 2.11A). Furthermore, cell aggregates of *C. flavigena* KU also attached to cellulose surfaces and contained globular protuberances resembling cellulosomes (24). This suggested that the curdlan had a role in cellulose attachment (24). Similarly, N-limited biofilms of *C. uda* bound strongly to cellulose surfaces (Fig. 2.2C) but weakly to abiotic surfaces. However, it is unlikely that the curdlan matrix conferred surface specificity and strong attachment on the biofilms. Curdlan and cellulose surfaces are both anionic in nature, which is expected to result in strong repulsion forces between the biofilms and the surface. Anionic EPS matrices bind divalent cations strongly (12) and can use them to bridge negatively charged functional groups within the EPS (Kim and Jang, 2006). This helps stabilize the EPS matrix but could also allow N-limited biofilms to bind the anionic groups in cellulosic substrates. Yet this attachment is unlikely to be strong and specific, because N-limited biofilms did not attach strongly to other anionic surfaces such as glass and plastic. Furthermore, adhesion was not

influenced by surface roughness, as the biofilms attached strongly to smooth cellulose surfaces such as dyalisis tubing cellulose membranes (Fig. 2.5B). In contrast, the cellulases produced by *Cellulomonas* spp. contain binding domains that function as specific cellulose adhesins (Francisco et al., 1993). Our results show that soluble and cell-associated cellulase production increased in response to low N availability (Fig. 2.2C). Thus, cell-associated and soluble cellulases could provide a mechanism for the specific colonization of cellulosic substrates and for their degradation, respectively.

Curdlan capsules function as short-term C reserve compounds in *C. flavigena* KU during stationary phase (51, 58). Similarly, the curdlan biofilm matrix served as a C source to support biofilm dispersal upon N supplementation (Fig. 2.8A). Biofilm dispersal responded to all the N sources tested (Table 2.1) and could be induced at any point in biofilm formation (Fig. 2.8B). This is consistent with a reversible mechanism in response to the C:N ratio. Curdlan dissolution in response to N availability was not uniform, but rather localized to internal regions of the biofilm (Fig. 2.9). In *P. aeruginosa* PO1, flagella expression increases in response to the sudden availability of C sources to aid cells in dissociating from the biofilm matrix during dispersal (Sauer et al., 2004). Interestingly, we also observed motile cells with polar tufts of flagella (Fig. 2.8A inset) in the localized regions of curdlan dissolution formed during N-induced biofilm dispersal in *C. uda* (Fig. 2.7). This contrasts with earlier reports that *C. uda* is non-motile and non-flagellated (53) and suggests that flagellar motility may be a specific response to N enrichments needed to facilitate cell detachment from the EPS matrix during biofilm dispersal.

2.4.2 N-limited biofilms as an efficient strategy for decomposition

Despite the growth limitation imposed by low N availability, *C. uda* coupled biofilm growth on the cellulosic surface to its degradation (Fig. 2.5 and Fig. 2.11). Furthermore, the N-limited biofilms degraded the same amount of filter paper as planktonic cultures grown with N supplementation and faster (Fig. 2.11A). This argues against classic stoichiometric theory (9) and the accepted notion that all decomposers lower their C-use efficiency to adapt to N limitation (36) unless able to fix N_2 (32). This indicates that N-limited biofilms are an efficient mechanism for decomposition. We detected cell-associated and soluble cellulases in N-limited cultures (Fig. 2.2C), suggesting that the biofilms used both enzymatic strategies during decomposition. Cell-associated cellulases function as specific cellulose adhesins and enable the attachment of the biofilm clusters to the substrate. This concentrates the cells in proximity to the substrate, a mechanism reported to stimulate cellulose hydrolysis, presumably because the cell envelope and/or glycocalyx modifies the physical chemistry of the gap between the substrate and the cell and creates a more favorable microenvironment for the activity of the enzymes (34). The biofilm matrix also concentrates the free enzymes close to the substrate and prevents energy losses from their progressive dilution and nonspecific adsorption (62). The matrix also retains soluble products of cellulose decomposition in the vicinity of the cell (39). This maximizes C-use efficiency and prevents the release of chemoattractants for competing cellulolytic microorganisms (20). As a result, cellulose-attached biofilms can maximize decomposition and C-use efficiency despite the low levels of N available for growth.

2.4.3 Biofilms as a competitive strategy for N acquisition during decomposition.

Natural cellulosic substrates harbor N sources in the form of structural and enzymatic proteins of the plant cell wall (23), which become available during decomposition. The filter paper substrate used in our studies (Fig. 2.11) mimics the natural cellulosic substrates, as it contains NH_4^+ and NH_4^+ salt contaminants within its structure, which are slowly released during its swelling in solution and degradation (29). As a result, cellulose-degrading biofilms provide a strategy for acquiring N in an environment where N sources are scarce. The anionic groups of the curdlan biofilm matrix also bind NH_4^+ cations strongly (12) and increase their concentration in the cell microenvironment. This contrasts with more energy-intensive responses for N acquisition such as chemotaxis and motility (35) or N_2 fixation (32). Hence, N-use efficiency is also maximized in the N-limited biofilms.

By specifically colonizing cellulose substrates, N-limited biofilms also gain access to N sources derived from the activities of N_2-fixing decomposers (32, 35) and of lignin-degrading fungi (16). The ability of N-limited biofilms to attach and degrade chitinous substrates (Fig. 2.6) is significant because chitin is a N-containing carbon substrate structurally related to cellulose and, therefore, also provides a N source for growth. Furthermore, chitin is present in the cell walls of most fungi and the exoskeletons of arthropods. Hence, the colonization and degradation of chitin-containing substrates also confers on the biofilms mycolytic activity and an efficient strategy to prey on the abundant chitin-containing fungal competitors (5) and litter-associated detritivors (14).

2.4.4 Environmental implications of biofilm formation and dispersal in response to C:N disturbances

Our results show that biofilms are a competitive strategy for decomposition and for acquiring N in environments where competition for limited N resources is high. The activity of cellulose degraders controls the trophic interactions that regulate C and N cycling in terrestrial environments and modulates the functional response of the decomposing community to C:N disturbances and feedbacks to the atmosphere (14). The biofilm matrix also enables cell-cell recognition (12) and provides an ideal niche for the coexistence of selected groups of bacteria in close proximity to each other. This favors metabolic interactions such as those that enable microbial consortia to decompose plant litter efficiently and release terrestrial C to the atmosphere (35). In addition, the EPS matrix protects cells from desiccation and rehydration events, such as those resulting from draught and precipitation, and provides extended areas of hydration for solute diffusion and synergistic trophic interactions (39). Inasmuch as N limitation (28) and biofilm formation (40) may occur commonly in terrestrial environments, biofilm formation in response to N limitation may be a general strategy for plant litter decomposition in nature and a significant contributor to C retention in soils. Thus, mechanistic understanding of the activities and C storage capacity of cellulolytic biofilms and their responses to anthropogenic C and N disturbances is important to understand the interplay of C and N cycling in terrestrial environments. The reversible, and potentially manageable, nature of C sequestration by *Cellulomonas* spp. in response to C and N disturbances also provides a potential target for stimulating the C-sequestering capacity of the biofilms *in situ,* a process that could be harnessed to

partially mitigate green house emissions, at least at the regional scale (25, 59).

2.5 EXPERIMENTAL PROCEDURES

2.5.1 Bacterial strains and culture conditions

The following species of *Cellulomonas* were used in these studies: *Cellulomonas flavigena* (ATCC 482), *Cellulomonas fimi* (ATCC 484), *Cellulomonas gelida* (ATCC 488), *Cellulomonas cartae* (ATCC 21681), *Cellulomonas biazotea* (ATCC 486) and *Cellulomonas uda* (ATCC 21399). Unless otherwise indicated, all cultures were grown aerobically in GS2 (Cavedon *et al.*, 1990) or *GS2 (Reguera and Leschine, 2001) medium supplemented with cellobiose (0.2% (w/v)) as the C source (GS2-CB or *GS2-CB medium). Cultures were first grown in GS2-CB medium, a complete medium with 0.2% (w/v) urea and 0.6% (w/v) yeast extract (Cavedon *et al.*, 1990), but without resazurin and cysteine (45). After three transfers in mid-exponential phase, a 10% (v/v) inoculum was transferred to *GS2-CB-NH_4Cl medium, which is the previously described *GS2 medium (Reguera and Leschine, 2001) (a N-limited GS2 medium lacking urea and containing 0.01% (w/v) yeast extract to stimulate growth) supplemented with 0.2% cellobiose as a C source and 2 mM NH_4Cl as a N source. Exponentially growing cells from the *GS2-CB-NH_4Cl cultures were harvested by centrifugation (2,000 x *g*, 15 min) and inoculated to a final OD_{660} of 0.02 into *GS2-CB-NH_4Cl (N-supplemented cultures) or *GS2-CB (N-limited cultures) media. All cultures were prepared in triplicate Nephelo borosilicate flasks (300-ml flasks equipped with a 14-mm side arm, Wheaton) or 18-mm glass test tubes and incubated at $35^{o}C$.

Planktonic growth was monitored spectrophotometrically as the optical density of 660 nm (OD_{660}). Biofilm formation was also monitored spectrophotometrically as increases in biofilm biomass by substracting the OD_{660} of the undisturbed culture (planktonic growth) from the OD_{660} of the culture once the biofilms had been mechanically disrupted with brief (5 seconds) vigorous vortexing.

When indicated, cells were also cultured on solid medium, using the same step-wise culturing protocol and same media with cellobiose but solidified with 1.5% agar. The same culturing protocol and media were also followed to test the effect of other C and N sources on *C. uda* growth in N-supplemented or N-limited liquid cultures. C sources tested other than cellobiose were glucose, glycerol, *N*-Acetyl glucosamine (NAG), chitobiose, or chitotriose (all at 0.2% (w/v) concentrations) or insoluble substrate such as 0.2% (w/v) colloidal chitin (21), 0.2% (w/v) ball-milled cellulose (30) and dialysis tubing cellulose membranes (see *Supplementary Information*). N sources tested other than NH_4Cl were urea or NAG at a final concentration of 0.2% (w/v).

Squid pen (55) or 0.25% cellulose-azure were also tested as C sources, except that CU mineral medium (a defined medium for *C. uda* prepared as *GS2 medium but without yeast extract and supplemented with thiamine-HCl, 10mg/L; biotin, 0.01m/L) was used instead of *GS2 medium.

2.5.2 Nitrogen fixation assays

Nitrogenase activity in *C. uda* cultures was assayed by the acetylene reduction assay (41). Biofilms were grown aerobically or anaerobically to mid-exponential phase in *GS2 medium containing 0.2% cellobiose as sole C and energy source using crimp top

pressure tubes. Reactions were started with the replacement of 10% of the tube headspace with C_2H_2. Formation of acetylene reduction products, C_2H_4 or C_2H_6, was monitored using a gas chromatograph (Varian GC Model 3700) equipped with a flame ionization detector. The gas components were resolved in a stainless steel column packed with POROPACK®N (Supelco Corp.) operated at 50°C and with N_2 (30 ml min^{-1}) as the carrier gas. N_2 fixation was also assayed in growth experiments using anaerobic cultures with cellobiose as C and energy source and a headspace of N_2 as sole N source. Controls with an argon gas atmosphere were also included. Growth was measured spectrophotometrically as absorbance of the cultures at 660 nm, as described above.

2.5.3 Biofilm dispersal

Biofilm dispersal was investigated in two separate assays. In the first assay, we studied the remobilization of curdlan and its use as a source of C to support biofilm dispersal and planktonic growth. For this experiment, 60-h old N-limited biofilms grown in *GS2 medium with cellobiose were harvested by centrifugation (2,000 x *g*, 15 min) and resuspended in fresh *GS2 with 2 mM NH_4Cl and no C supplementation. Biofilm dispersal was also studied by directly adding 2 mM NH_4Cl to N-limited biofilms growing in *GS2 medium with cellobiose for 28 h. The cellobiose and NH_4^+ concentrations before and after NH_4Cl supplementation were measured by HPLC and with the Nessler's reagent, as described below, to monitor the C:N ratios. In both experiments,

planktonic growth and biofilm biomass increases were monitored spectrophotometrically, as described above. All experiments were performed at 35°C.

2.5.4 Carbon, nitrogen, curdlan and total cell protein determination

Cultures were periodically sampled to measure C and N concentrations and total cell protein. Cells and insoluble materials in the samples were separated from supernatant fluids by centrifugation (2,000 x *g*, 15 min). Filtered (0.45-μm) supernatant fluids recovered by centrifugation were used for C and N determinations. C (cellobiose or glucose) concentrations were measured in a High Pressure Liquid Chromatography (HPLC) system equipped with an Aminex HPX-87H ion-exchange column (Bio-rad) and operated at room temperature. The eluent used was 4 mM H_2SO_4. Ammonium equivalent concentrations were determined by measuring the OD_{425} of a 1:2 mix solution of sample and Nessler's reagent (Fluka) using NH_4Cl as standard.

Curdlan in the insoluble material was quantified using a fluorescence dye-binding microassay, as previously described (26). Biofilm cell growth was also measured as total cell protein in the insoluble fraction harvested by centrifugation, resuspended with 2N NaOH and boiled for 1 h to lyse the cells. The lysed samples were cooled at room temperature for 30 minutes and neutralized with an equal volume of 2N HCl prior to measuring its protein content with the Pierce Bicinchoninic Acid (BCA) assay (52) and using bovine serum albumin (BSA) as standard.

2.5.5 Dry weight and total cell protein determination

C. uda cells were grown at 32°C in *GS2 medium with 0.2% cellobiose. A 10% (v/v) inoculum of an exponentially growing culture (24 h) was transferred to fresh medium and further incubated. This culture was used to inoculate 125 ml of fresh medium alone or supplemented with 0.2% (w/v) NH_4Cl. Cultures were prepared in 250-ml side-armed Nephelo flasks to allow for measurements of optical densities of the cultures. The N-supplemented cultures were grown to early-stationary phase (ca. 40 h) and the N-limited biofilms for 72 h before cells were harvested by centrifugation (20 min at 5,000 x g and 4°C) and resuspended in 50 ml of sterile dH_2O. Total cell protein was determined in 300 μl of cell suspensions. Cell suspensions were first boiled in the presence of one volume 1M NaOH and neutralized with one volume of 1M HCl. Cell debris was then sedimented by centrifugation and discarded and the protein content of supernatant fractions was determined as a modification of the method of Bradford (6), using the BioRad protein assay (BioRad Laboratories, Inc.). Bovine serum albumin in 1M NaOH and 1M HCl was used as protein standard. The above-mentioned cell suspension was also used for dry weight calculations. Approximately 55 ml of cell suspension were dispensed in aluminum foil vials, incubated at 100°C overnight, and weighed to calculate the dry weight of the original culture.

2.5.6 Microscopy analyses

Transmission electron microscopy was used to examine thin sections of *C. uda* cells grown in *GS2 medium with 0.2% cellobiose with or without supplementation with

NH_4Cl, as described above. Cells from overnight (15 h) or one-week cultures were fixed and stained with the cationic dye ruthenium red, which stains acidic polysaccharides (57). Thin sections of embedded cells were examined with a JEOL 100S transmission electron microscope operated at 80 kV.

Fluorescence microscopy was used to examine *C. uda* biofilms formed on cellulose fibers (ball-milled cellulose) or colloidal chitin particles after two days of incubation in *GS2 medium at 32°C with gentle agitation. Cellulose- or chitin-associated biofilms were stained with fluorescent dyes using the LIVE/DEAD® BacLight™ Bacterial Viability kit (Invitrogen) and examined using a Zeiss Axioskop. Phase contrast microscopy was used to examine biofilm dispersal using the same microscope.

A Carl Zeiss EVO LS25 variable pressure scanning electron microscope (ESEM) was used to image wet filter paper samples collected from 6-day old cultures grown in *GS-2 with or without supplementation with NH_4Cl, as described below. Six-day old biofilms on squid pen were examined by scanning electron microscopy (SEM) using standard sample preparation procedures and using a JEOL JSM-5400 microscope.

2.5.7 Enzyme and protein assays

Supernatant fluids and resting cell suspensions from exponentially growing cultures were obtained and concentrated as previously described (44, 45). Chitinase activity and chitinase immunodetection are described in detail in the *Supporting Information*. Cellulase activity was measured with the cellulose-azure (19) and carboxymethyl-cellulase (CMCase) (44, 45) assays. In the first assay, exponentially-grown (24 h) cultures of *C. uda* in GS2*-glycerol were inoculated (10% (v/v)) in CU minimal medium

supplemented with 0.25% (w/v) cellulose-azure (Sigma), a cellulose substrate bound to Remazol Brilliant Blue 5R (RBB) dye, which is solubilized during cellulose hydrolysis (19). An uninoculated control culture also was included to detect unspecific dye release from the dyed cellulose. All cultures were incubated at 32°C with gentle agitation and supernatant samples were removed periodically, centrifuged to remove residual cellulose, and the optical density at 595 nm of the soluble dye was measured. The amount of dye release, in mg per ml of culture, was calculated using a standard curve prepared with RBB (Sigma). Cellulase activity was also measured as CMCase activity in supernatant fluids and cell suspensions from cultures grown with 0.2% cellobiose with or without supplementation with 2 mM NH_4Cl, as described elsewhere (44, 45). Supernatant and cell suspensions were also assayed for protein using the Pierce BCA Protein Assay Reagent (52) (Pierce, Rockford, IL), with bovine serum albumin as protein standard.

2.5.8 Filter paper experiments

C. uda was also grown with filter paper as sole C and energy source in *GS2 medium (with or without 2 mM NH_4Cl supplementation), following the step-wise culturing approach described above except that cellobiose was replaced as a C source by a 5-mm diameter disc of filter paper and that microcentrifuge tubes containing 333 μl of *GS2 (N-limited) or *GS2-NH_4Cl (N-supplemented) medium were used for culturing. The filter paper substrate was Whatman 3MM paper discs generated with an office paper hole puncher. Each disc was placed in a microcentrifuge tube before autoclaving for 30 min. The sterile *GS2 or *GS2-NH_4Cl medium was then aseptically dispensed in

the tubes before inoculation. All incubations were performed at 35°C. Several tube replicates of N-supplemented and N-limited cultures were started at once and three replicates from each culture were periodically sacrificed to measure cell growth and filter paper decomposition. The tubes were centrifuged to separate the culture supernatant fluids from the cells and the filter paper disc. The supernatant fluids were used for cellulase enzyme assays, as described above. Cell growth was estimated as total cell protein extracted from the pellets with 2N NaOH and boiling, as described above. This treatment was found not to affect the filter paper analyses. The paper discs were hydrolyzed with 100 µl of 72% sulfuric acid at 30°C for 2 h, then diluted with ddH_2O to 4% sulfuric acid, and the pH raised to 6 with 0.4 g of $CaCO_3$. The amount of reducing sugars in these samples was estimated with the 3,5-dinitrosalicylic acid (DNS) reagent with glucose as standard, as previously described (37). The amount of filter paper degraded was calculated from the amount remaining in the culture in reference to the time 0 samples.

2.5.9 Chitinase activity assays and ChiA immunodetection

Chitinase activity was measured in culture supernatant fluids and resting cell suspensions from N-limited or N-supplemented cultures grown with glycerol or cellobiose as sole C and energy source. Endo- and exochitinase activities were measured using, respectively, methylumbelliferyl-chitotriose or chitobiose, as previously described (44). Emission of fluorescence was detected using a PTI Fluorescence system (Photon Technology International, Inc., NJ) and Felix™ Windows software at a fixed excitation wavelength of 365 nm and a fixed emission wavelength of 460 nm.

The expression of ChiA, the major *C. uda* enzyme component for the solubilization of chitin, was also immunologically detected in supernatant fluids from N-supplemented or N-limited cultures grown with colloidal chitin using mouse polyclonal anti-ChiA antibodies (44). Supernatant proteins were first separated by 10-15% gradient SDS-PAGE, as previously described (44). After electrophoresis, polyacrylamide gels were separated from the GelBond® film support following manufacturer's recommendations (Pharmacia). The gels were equilibrated in transfer buffer (Tris, 50 mM; glycine, 380 mM; SDS, 0.1% (w/v); methanol, 20%; and dH_2O) before they were assembled in the transfer sandwiches, as described elsewhere (15). Proteins in the polyacrylamide gels were blotted onto an Immobilon-NC transfer membrane (Millipore Corporation, Bedford, MA) using a mini Trans-Blot electrophoretic transfer cell (BioRad) operated at 43 V (approximately 181 mAmps) for 35 min. Blocking, antibody binding and immunodetection of proteins on the membranes were performed as described elsewhere (15). Alkaline phosphatase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as secondary antibody. Development was carried out using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) (SIGMA FAST™ BCIP/NBT tablets, Sigma) for approximately 30 min.

2.5.10 Biofilm assays on cellulose membranes

Biofilm formation in response to N limitation was also investigated using 1-inch wide dialysis tubing cellulose membranes (Sigma). Biofilm induction by N limitation followed the same culturing protocol described for other substrates, with the following

modifications. A glass coverslip was inserted inside a 1-inch strip of the membrane to support it and facilitate handling. The membrane-coverslip set-up was placed in a glass Petri dish filled with PBS buffer and autoclaved before transferring it to a well of a 6-well microtiter plate (Corning) containing 5 ml of sterile *GS-2 medium supplemented with 0.2% cellobiose. After 3 days of incubation at 35°C, the membrane was washed in PBS and stained with the fluorescence SYTO9 dye (Invitrogen), following manufacturer's recommendations. The membrane-coverslip was then transferred to a clean well filled with PBS buffer and examined with a Zeiss LSM Pascal CLSM equipped with an Achroplan (40X) immersible objective (excitation at 488, emission at 505-535).

2.6 Acknowledgements

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Chapter 3

Operational and biological approaches for improved consolidated bioprocessing of AFEX-pretreated corn stover to ethanol by *Cellulomonas uda*

3.1 ABSTRACT

Bioethanol is currently the most widely used alternative to petroleum for liquid transportation fuels. Utilization of lignocellulosic biomass to produce ethanol decreases our dependence on foreign oil, improves the economy, and benefits the environment. Lignocellulosic biomass must be pretreated and enzymatically hydrolyzed to enable fermentation of the released sugars to ethanol, which add to the cost of the fuel. Ammonia Fiber Expansion (AFEX) is a low-cost pretreatment method with the potential for high sugar conversion with minimal inhibitory compounds. AFEX-treated corn stover (AFEX-CS) also supports the growth of various cellulolytic microorganisms. In particular, strains of *Cellulomonas* such as *Cellulomonas uda* are especially suited for ethanologenesis from lignocellulose and show promise for the consolidated bioprocessing (CBP) of AFEX-CS into ethanol. Thus, we investigated the operational parameters that promoted fermentative growth of *C. uda* from AFEX-CS. Here we describe the culturing conditions that promote the growth of *C. uda* with up to 5% (w/v) AFEX-CS solids. We also adaptively evolved a strain of *C. uda* with improved fermentative capabilities that increased ethanol productivity from AFEX-CS 12-fold. The results highlight the potential of *C. uda* as a robust CBP organism for the processing of agricultural wastes into ethanol.

3.2 INTRODUCTION

Our current major source of liquid transportation fuel, petroleum, is non-renewable and mostly originates from unstable regions of the world. Biomass, a renewable resource, can be used to produce liquid transportation fuel and provide a domestic supply of

energy. Bioethanol is the most widely used liquid transportation fuel alternative with Brazil, the United States, and Asia contributing the most to its production (7). Ethanol has the potential to reduce our energy carbon footprint as CO_2 emissions could be reduced by as much as 60-90% (7). Corn ethanol, which emits 87-88% less greenhouse gases than gasoline, has a net energy balance of 25% (i.e., the energy output is 25% more than the energy input for production) (19). Lignocellulosic feedstocks have greater potential. Ethanol from switchgrass has, for example, a 500% net energy balance, in addition to improved emission and soil conservation benefits (43). Hence, fuels produced from lignocellulosic substrates have the potential to decrease our reliance on foreign oil, improve our domestic economy by creating new markets, and diminish negative impacts on the environment.

Lignocellulose is composed of lignin, cellulose, hemicellulose and small amounts of protein, pectin, extractives, and ash. Composition and construction of these components varies by plant type. Cellulose and hemicellulose are sugar polymers and could, therefore, be used as substrates for ethanol fermentation. However, the lignin protects the plant cell wall from microbial degradation and blocks the access of microbial enzymes to the carbohydrate polymers (20). The natural recalcitrance of the substrate thus requires pretreatments to increase its digestability (47). The pretreatments break the lignin seal and decrease the crystallinity of cellulose, thus facilitating enzymatic access to the carbohydrate polymers and their hydrolysis (36, 56). Ammonia Fiber Expansion (AFEX) is a physicochemical pretreatment of lignocellulose that utilizes temperature, pressure, and ammonia to solubilize the lignin (9), while decrystallizing the cellulose and increasing the substrate's digestibility (2, 53). The

method allows for the recovery of most of the ammonia catalyst, which reduces costs, and leaves small amounts of residual ammonia that serve as a nutrient for fermentative microbes (1, 45, 57). Furthermore, the substrate does not need to be washed prior to fermentation because inhibitory compounds are only minimally produced (10, 27). After the pretreatment, the exposed cellulose and hemicellulose need to be hydrolyzed into fermentable sugars to produce ethanol. Normally, this step involves costly enzyme cocktails and multi-step methods (16, 31, 44). A cost-effective approach to this process is consolidated bioprocessing (CBP) in which a single organism degrades the substrate and converts both hexose and pentose sugars into ethanol (7, 31). This process is estimated to increase the efficiency of bioethanol production from lignocellulosic substrates and reduce the cost of ethanol production by 50% (30).

In nature, the complex biochemical nature of lignocellulose substrates has selected for organisms that produce multienzyme systems whose catalytic activities act synergistically to efficiently degrade the biomass polymers. Enzymes involved in this process include those cleaving internal (endoglucanases) or terminal (exoglucanases) bonds in cellulose (cellulases), hemicellulose, and pectin (xylanases, arabinofuranidases, mannosidases, etc) (20). Many of these enzymes are organized in domains, often containing catalytic and substrate-binding domains connected through flexible regions or linkers (3). In most anaerobic cellulolytic microorganisms, the hydrolytic enzymes are assembled as protein complexes or cellulosomes using ‘scaffold’ and ‘dockerin’ proteins (3). In addition to evolving complex enzyme system for an efficient and synergistic hydrolysis, native lignocellulolytic organisms have also evolved metabolisms that enable them to ferment the soluble products released during

biomass degradation, thus maximizing energy recovery from the high energy cost associated with the production of hydrolytic enzymes (28). These microorganisms are, therefore, naturally tuned for CBP of lignocellulose substrates. However, fermentative growth is often slow compared to industrial standards and adapted to the environmental rates of biomass processing (14).

Cellulomonas spp. are the only known group of cellulolytic facultative anaerobes (32). They can hydrolyze a wide range of carbohydrate substrates such as cellulose, xylan, starch, and agricultural wastes (51). They can also ferment many biomass-derived sugars such as xylose, arabinose, glucose, mannose, galactose, cellobiose, maltose, and sucrose (50). Ethanol is often a major product of fermentation in these organisms in addition to lactate, acetate, formate, succinate, and CO_2 (50). Some of the strains also have high protein and essential amino acid values and have been used for single cell protein production from agricultural wastes (18, 23, 24). They have also been studied for their hydrolytic enzymes (11, 21, 25, 54). Studies in our lab have shown that *Cellumonas uda* ATCC 21399 is naturally suited for ethanologenesis from AFEX-CS, growing robustly with this substrate and producing ethanol at ca. 50% of the maximum theoretical yield (48). Formate and acetate are also produced during ethanologenesis, along with small amounts of lactate and succinate (48). Although the fermentation byproducts of AFEX-CS metabolism by *C. uda* can cause feedback inhibition (12), they can be removed and used as electron donors by exoelectrogenic bacteria such as *Geobacter sulfurreducens* (49). As a result, AFEX-CS hydrolysis and ethanol fermentation are stimulated during syntrophic growth of *C. uda* with *G. sulfurreducens* in a microbial electrolysis cell (MEC), which also recovers the energy of the waste

fermentation products as hydrogen (48). Energy recoveries from AFEX-CS in the MEC platform were ultimately dependent on the growth robustness of *C. uda*. AFEX-CS hydrolysis efficiencies and ethanol production were, for example, stimulated when the cultures were supplemented with nitrogen sources (48). This is because, as shown in Chapter 2, nitrogen supplementation prevents the accumulation of carbon as a curdlan biofilm matrix (58) and potentially diverts all the carbon towards cell growth, enzyme production, and ethanologenesis. Consistent with this, nitrogen supplementation alone resulted in 2-fold increases in the energy recoveries from the fermentation of AFEX-CS to ethanol (56%) and to ethanol and cathodic hydrogen (73%) (48). These values exceed those reported for ethanol production from acid-treated corn stover using standard fermentative yeast catalysts (29) and highlight the potential of MEC platforms for ethanol production using *C. uda*. Interestingly, ethanol production plateaued before all the nitrogen was removed by *C. uda* (48). Furthermore, despite the low (0.2%, w/v) solid loadings used in this study, only half of the AFEX-CS was degraded. This suggests that nutrients became growth-limiting and, therefore, there is potential for optimizing the CBP of AFEX-CS by *C. uda* by optimizing the culturing conditions.

Here I describe studies to improve the CBP of AFEX-CS to ethanol by *C. uda*. Supplementing the medium with yeast extract, incubating at the temperature optimum, and shaking the cultures during incubation promoted the growth of *C. uda* and ethanologenesis at high (5%) solid loadings of AFEX-CS solids. *C. uda* was also adaptively evolved under ethanol and allyl alcohol pressure to select for a strain with improved fermentation abilities. Improvements in operational parameters alone resulted in 5-fold increases in fermentation yields and 6-fold increases in ethanol yields and

ethanol productivity compared to the previous study (48). Furthermore, ethanol productivity was increased 12-fold in a strain adaptively evolved for robust fermentative growth and ethanologenesis. The results highlight the potential of *C. uda* for the CBP of AFEX-CS and offer valuable insights into the operational and biological constraints that can be targeted for further improvements.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strains and culture conditions

Cellulomonas uda ATTC 21399 was routinely cultured at 35°C in anaerobic GS2 medium (8) with 0.2% cellobiose (GS2-CB). When indicated, cellobiose was replaced with corn stover as sole carbon and energy source, previously processed and pretreated with the AFEX method (27) and ground and sieved as previously described (48) (AFEX-CS). The glucan and xylan contents of the AFEX-CS (glucose and xylose equivalents, respectively, in acid hydrolysates) were estimated to be 36 % (± 8) and 22% (± 4) xylose, respectively. For the experiments with AFEX-CS, a modified GS2 medium (GS3 medium) lacking MOPS was used, as MOPS was found to interfere with HPLC analyses of ethanol. Cells from late exponential-phase GS2-CB cultures were harvested by centrifugation (3220 x g, 10 min, room temperature), resuspended in GS3 medium, and inoculated into GS3-AFEX-CS medium to a final OD_{660} of 0.04. The cultures were incubated at 35°C with gentle shaking (200 rpm) and spectrophotometric readings (OD_{660}) were taken periodically after mixing the cultures by inversion and allowing the solids to settle for 20 min.

3.3.2 Alcohol tolerance of *C. uda*

Late-exponential phase cultures of *C. uda* strains grown anaerobically at 35°C in GS2-CB were inoculated to an initial OD_{660} of 0.02 into GS2-CB medium with varying concentrations of ethanol (between 0 and 5% (v/v)). The cultures were incubated at 35°C and growth was monitored spectrophotometrically as OD_{660} every ca. 6 h.

3.3.3 Adaptive evolution of *C. uda*

An ethanol-tolerant strain of *C. uda* was evolved by continuous subculturing of stationary phase cultures in GS2-CB media supplemented with increasing concentrations of ethanol, starting with concentrations of 1% (v/v) ethanol and increasing them in 0.5% (v/v) increments once the growth rate and *lag* time had stabilized. This approach was successful until reaching 5% (v/v) ethanol. Although the adaptively evolved cultures grew with 5% ethanol, they could not be stably sustained over time, thus signaling the end of the evolution experiment. Two subsets of cultures growing stably and reproducibly at 4% ethanol were also challenged in parallel with allyl alcohol at 10^{-3} M and 10^{-7} M, respectively, in addition to ethanol, in order to select for variants carrying mutations in ethanol dehydrogenase that increase ethanol tolerance and ethanologenic rates (37). Clonal isolates from cultures growing at 4.5% concentrations of ethanol were separated as isolated colonies on solidified (1.5% agar) GS2-CB media. Five colonies were subcultured three times to insure clonal purity and tested for growth and ethanol production in GS3-CB media. The best performing strain was also tested for ethanol tolerance, as described above, to confirm the acquired

phenotypic trait, and for fermentative growth on various carbon sources at 0.2% (w/v) (cellobiose, glucose, xylose, or a combination of glucose and xylose in the ratio [0.12 and 0.08% (w/v) respectively] similar to that calculated for the AFEX-CS.

3.3.4 Batch cultures of *C. uda* on varying concentration of AFEX-CS

Late-exponential phase cultures of *C. uda* grown at 35°C in GS3 medium with cellobiose (0.2%), were inoculated to an OD_{660} of 0.04 in bottles containing anaerobic GS3 medium with varying concentrations of AFEX-CS (0, 1, 3, 5% [w/v]). All cultures were incubated at 35°C with gentle agitation (200 rpm). Aliquots (1 ml) were periodically removed from the culture to monitor growth (OD_{660}) and to analyze the fermentation broth by HPLC, as described below.

3.3.5 Analytical techniques

The xylose content in acid hydrolysates of the AFEX-CS remaining in the cultures after 2 weeks of incubation was used to calculate hydrolysis efficiencies, as previously described (46) and modified (48). The xylose content of the AFEX-CS remaining at the end of the incubation period was also used to estimate the efficiency of the hydrolysis in reference to uninoculated controls. Glucose was not an accurate proxy because of interference from the abundant curdlan exopolysaccharide (β-1,3 glucose) produced by *C. uda* biofilms (58). The glucan and xylan contents of the AFEX-CS (36% glucose and 22% xylose equivalents, respectively) were used to estimate the amount of glucose and xylose solubilized by the CBP strain and, therefore, available for fermentation. Sugars,

alcohols, and organic acids in 1-ml filtered (0.45 μM syringe filters, National Scientific, Rockwood, TN) culture supernatant fluids or AFEX-CS acid hydrolysates were measured by HPLC (Waters, Milford, MA) at 30°C, as previously described (48).

3.4 RESULTS

3.4.1 Hydrolysis and fermentation of varying concentrations of AFEX-CS by *C. uda*

C. uda was cultured in a modified rich medium (GS3) carrying yeast extract to stimulate growth with various concentrations (0.5%, 1%, 3% and 5%) of AFEX-CS solids. The cultures were incubated at 35°C, a temperature previously reported to stimulate fermentative growth (39, 40, 54), with gentle agitation to facilitate mixing and further stimulate growth. The amount of AFEX-CS hydrolyzed increased linearly (R^2 = 0.99) with the solid loadings (Fig 3.1A). As solid loadings increased, hydrolysis efficiencies increased which were approximately 20% at 0.5% solid loadings, 30% at 1% loadings, 40% at 3% loadings, and 60% at 5% loadings. Thus, there was a positive solid effect.

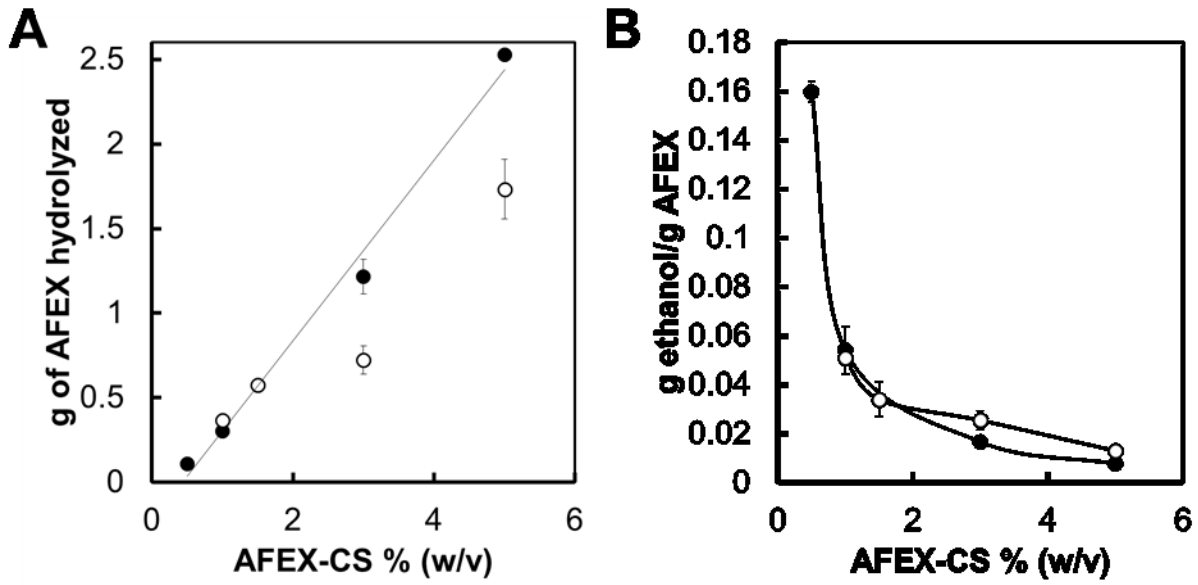

Figure 3.1: Hydrolysis and fermentation efficiencies of *C. uda* (closed symbols) and its variant AA7 (open symbols) on AFEX-CS

(A) Amount of hydrolyzed AFEX-CS (based on xylose equivalents) compared to amount of AFEX-CS provided as substrate (B) Ethanol production normalized to amount of AFEX-CS hydrolyzed

The solid loading had no effect on the fermentation profiles, with formate, acetate, and ethanol being the most abundant products and with only small amounts of succinate and lactate being produced (Table 3.1). Interestingly, titers for fermentation products did not increase proportionally to the amount of AFEX-CS hydrolyzed and available for fermentation. As a result, the conversion efficiency (g of ethanol/g of AFEX hydrolyzed) decreased exponentially (R^2 = 0.92) with the solid loadings (Fig 3.1B). Thus, although substantially more AFEX-CS was hydrolyzed at higher loadings,

fermentation efficiencies remained unchanged suggesting that fermentative growth is naturally slow in this organism and/or fermentative growth is inhibited once some fermentation products reach a threshold.

Table 3.1: *C. uda* fermentation products from AFEX-CS [a]

AFEX-CS [b]	Succinate	Lactate	Formate	Acetate (A)	Ethanol (E)	E/A
0	0.7 (± 0.7)	0.0 (± 0.0)	3.8 (± 1.5)	2.4 (± 1.4)	0.6 (± 1.4)	
0.5	3.8 (± 0.5)	0.4 (± 0.6)	9.8 (± 0.5)	10.2 (± 2.6)	7.5 (± 0.2)	0.74
1	2.8 (± 0.0)	2.1 (± 0.0)	11.1 (± 0.4)	9.7 (± 0.3)	7.1 (± 1.2)	0.73
3	1.9 (± 0.1)	1.2 (± 0.1)	11.4 (± 0.5)	9.0 (± 0.7)	9.0 (± 2.7)	0.99
5	3.0 (± 2.2)	1.3 (± 1.3)	12.8 (± 4.6)	11.6 (± 7.1)	8.6 (± 1.5)	0.74

[a] Shown are averages (and standards errors, in parentheses) of the concentrations of fermentation products (mM) in two replicates.

[b] Concentration of AFEX-CS (w/v)

3.4.2 Adaptive evolution of *C. uda* for improved fermentation

As the ethanol titers did not increase proportionally to the amount of AFEX-CS hydrolyzed as the solid loading increased, we investigated the ethanol tolerance of *C. uda*. Growth rates decreased rapidly in the presence of even low concentrations of ethanol (Fig 3.2A). The growth rates of cultures with 1% and 2% (v/v) ethanol were, for example, 75% and 13% of those grown without ethanol. Higher concentrations inhibited growth altogether (Fig 3.2A). Although *C. uda* tolerance to ethanol is naturally low, the ethanol titers measured from AFEX-CS fermentation (Table 3.1) are within the ranges (less than 0.5% (v/v)) that are tolerated by this bacterium. Hence, it is unlikely that ethanol accumulation inhibited fermentation from AFEX-CS.

As acetate and formate accumulation can also feedback inhibit fermentation and enzyme production (12), we designed an experiment to adaptively evolve *C. uda* cultures for improved fermentative growth. We initiated the experiment by serially transferring cellobiose-grown cultures in the presence of increasing concentrations of ethanol to concomitantly evolve ethanol tolerance. Once the cultures were adaptively evolved to grow at 4% (v/v) of ethanol, additional pressure with allyl alcohol was introduced to improve fermentative rates (37). We evolved separate cultures (AA3 and AA7) with both ethanol and 10^{-3} (AA3) or 10^{-7} M allyl alcohol, respectively, in parallel to cultures with only ethanol. This resulted in three lines of adaptively evolved cultures: ethanol, ethanol + AA3, and ethanol + AA7. All the three culture lines evolved tolerance to up to 5% (v/v) ethanol over the course of 18 months. The growth rates of clonal isolates from the three evolved lines were similar in GS3-CB media (Fig. 3.2B) but ethanol yields were higher in the clones derived from cultures selected under allyl

alcohol pressure (Fig. 3.2C). The strain with the best growth rate and ethanol titer was from the culture with 10^{-7} M allyl alcohol. It was designated strain AA7 and was chosen for further studies.

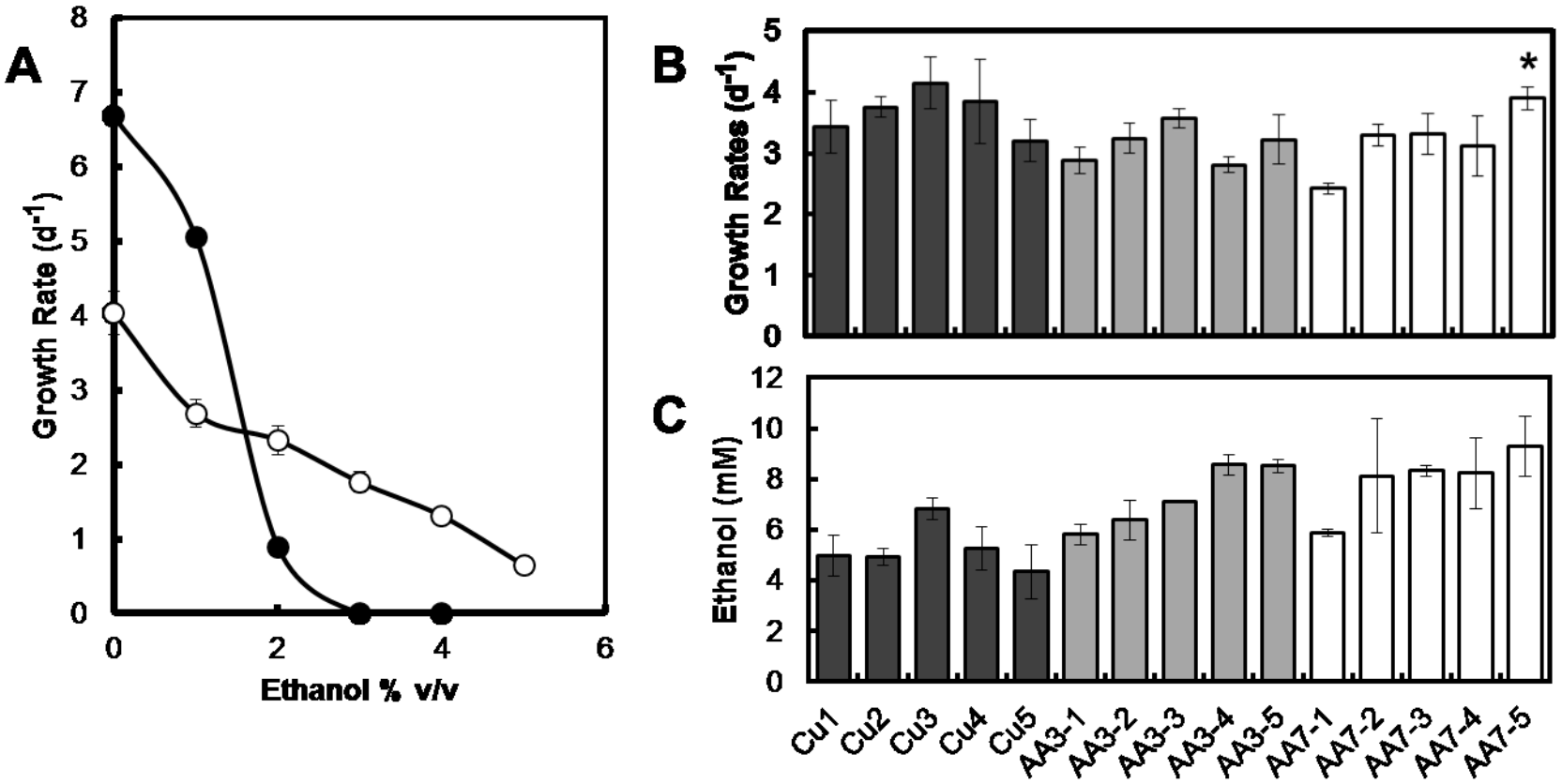

Figure 3.2 Ethanol Tolerance of *C. uda* and adaptively evolved strains

(A) Growth rates of the parental and the adaptively evolved AA7 strains of *C. uda* (closed and open circles, respectively) in triplicate at increasing concentrations of ethanol. (B-C) Growth rates (B) and ethanol yields (C) of clonal isolates of strains of *C. uda* adaptively evolved with ethanol alone (dark grey; Cu strains) or together with high (light grey; AA3 strains) or low concentrations of allyl alcohol (white; AA7 strains).

As shown in Fig. 3.2A, strain AA7 grew at concentrations of ethanol as high as 5% (v/v) in GS3-CB medium. The fermentative growth of *C. uda* AA7 was also investigated with different sugars and in reference to the parental strain. Cultures were grown in GS3-CB to late exponential phase before being transferred to GS3 with cellobiose, glucose, xylose, or a combination of glucose and xylose in a ratio mimicking that calculated for the glucose:xylose content in AFEX-CS. Growth with cellobiose resumed rapidly without an apparent *lag* phase in the parental and AA7 strains and growth rates were similar in both strains (Fig. 3.3A). Growth with glucose and xylose or a combination of both did not start immediately and the cultures had *lag* phases of variable lengths (Fig. 3.3B-D), as they transitioned from growing with cellobiose to other fermentable sugars. However, the *lag* phases were significantly reduced in the adapted strain AA7 compared to the parental strain. Thus, strain AA7 was able to rapidly switch its metabolism to growth with the new sugar, lagging for only 18 h with glucose (Fig. 3.3B) and about 24 h with xylose (Fig. 3.3C) or with both glucose and xylose (Fig. 3.3D). By contrast, the parental strain lagged for 80 h with glucose (Fig 3.3B) and about 55 h with xylose and the combination of glucose and xylose (Fig 3.3C and D, respectively).

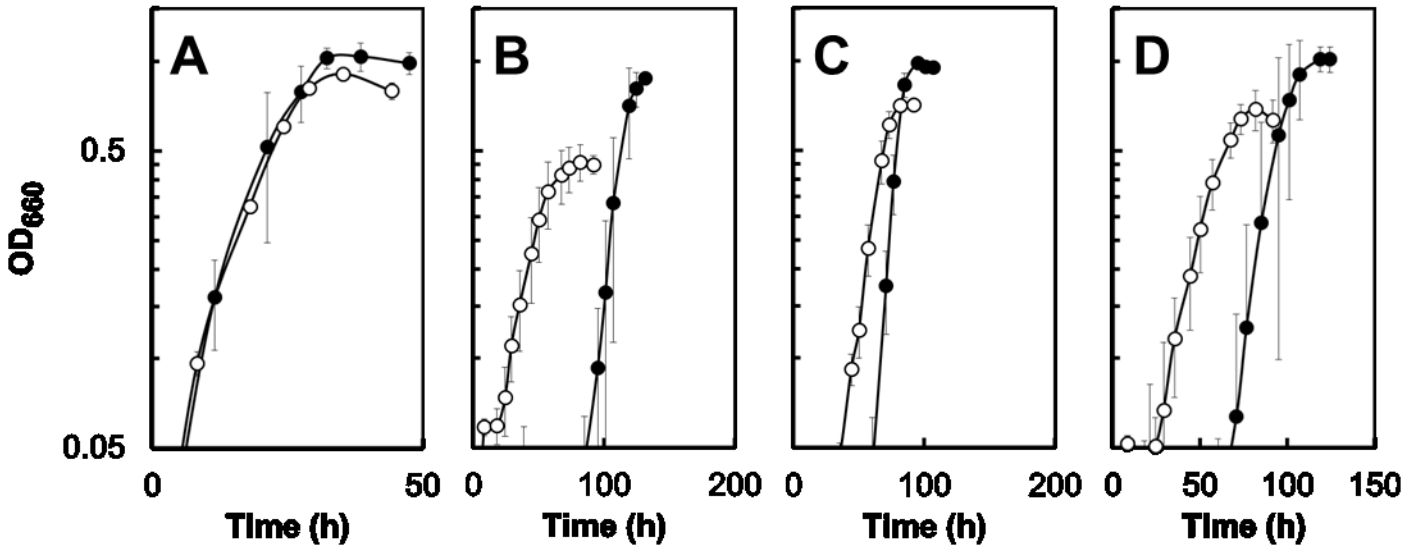

Figure 3.3 Fermentative growth of the parental (closed circles) and AA7 (open circles) strains of *C. uda* with cellobiose (A), glucose (B), xylose (C), and glucose and xylose (D)

3.4.3 Fermentation of AFEX-CS by the adapted AA7 strain of *C. uda*

Strain AA7 also grew in medium with AFEX-CS at solid loadings as high as 5%. At low solid loadings, hydrolysis efficiency was comparative to the parental strain, however, the percentage of AFEX-CS that was hydrolyzed at the different solid loadings was similar and averaged 33 (±6) %. The resulting hydrolysis efficiencies thus decreased at high solid loadings compared to the parental strain. Ethanol titers were also similar in the cultures regardless of the solid loading (Table 3.2) and comparable to those measured in the parental strain (Table 3.1). As a result, the ethanol conversion efficiencies (g of ethanol/g of AFEX), though decreasing exponentially with increased solid loadings (R^2 = 0.96), showed small improvements at higher solid loadings compared to the parental strain (Fig 3.1B).

Table 3.2: *C. uda* AA7 fermentation products of AFEX-CS [a]

AFEX-CS [b]	Succinate	Lactate	Formate	Acetate (A)	Ethanol (E)	E/A
0	1.5 (± 0.2)	0.7 (± 0.0)	4.8 (± 0.3)	7.7 (± 0.2)	4.4 (± 0.4)	
1	1.9 (± 0.1)	0.8 (± 0.1)	8.7 (± 0.0)	11.5 (± 0.8)	8.1 (± 0.2)	0.61
1.5	1.8 (± 0.1)	0.8 (± 0.2)	10.6 (± 0.0)	12.9 (± 0.4)	7.4 (± 2.1)	0.57
3	1.9 (± 0.3)	1.1 (± 0.1)	12.5 (± 0.0)	12.2 (± 0.2)	8.0 (± 0.3)	0.54
5	2.2 (± 0.6)	1.3 (± 0.5)	14.1 (± 0.2)	12.7 (± 2.5)	9.8 (± 0.6)	0.69

[a] Shown are averages of concentrations of fermentation products (mM) and, in parentheses, standard deviations of two replicates.

[b] Concentration of AFEX-CS (w/v)

Although the yields of fermentation products of strain AA7 (Table 3.2) were similar to the parental strain (Table 3.1), the adapted strain reached those yields far more rapidly (Fig 3.4). On average, it took the parental strain 4-6 days to complete the fermentation whereas *C. uda* AA7 reached the same yields in half that time (Fig. 3.4). Thus, the selective pressure with ethanol and allyl alcohol produced a strain with improved fermentative abilities that was able to rapidly adapt its metabolism to ferment the available sugars from AFEX-CS.

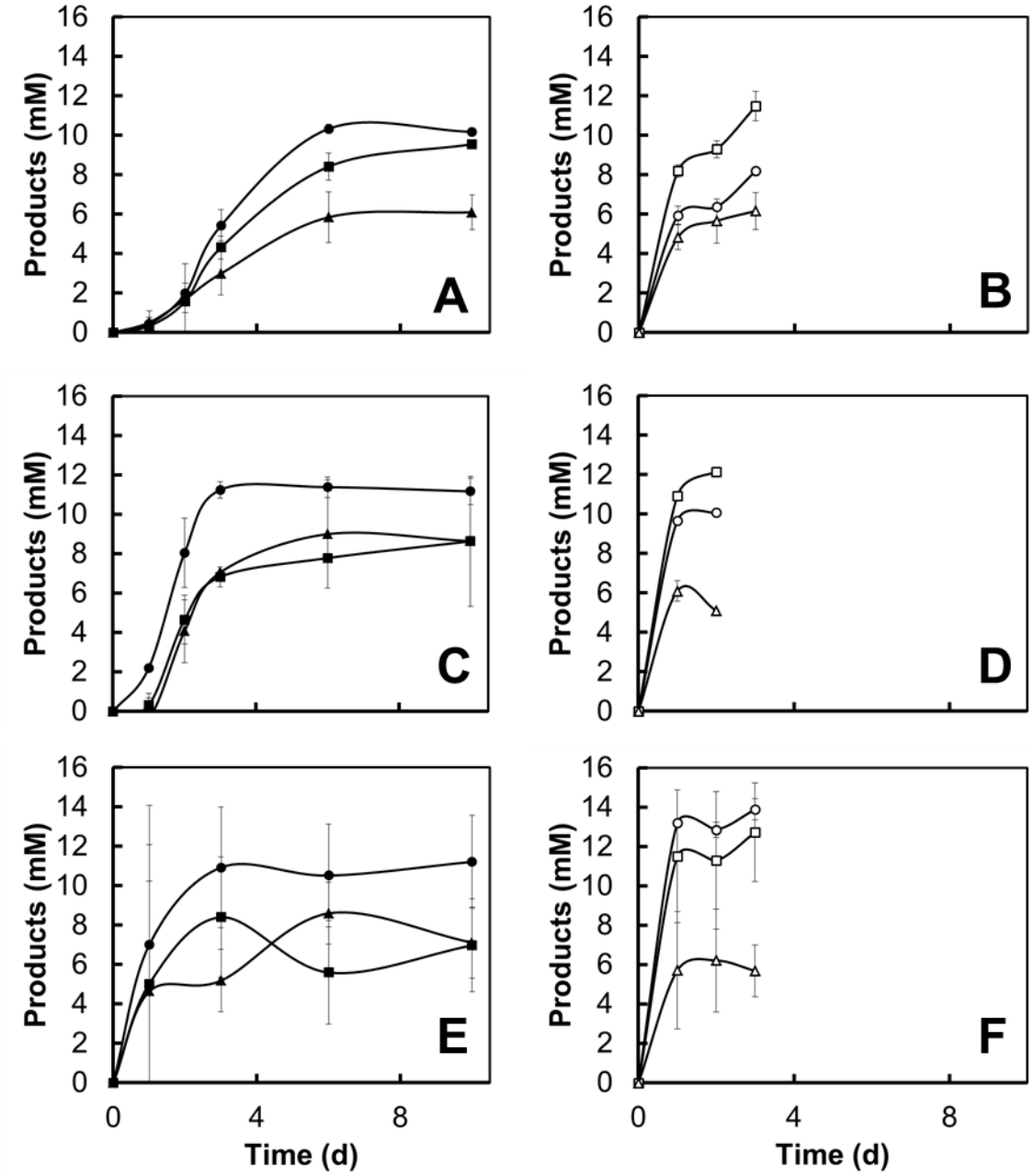

Figure 3.4 Fermentation products of 1% (A-B), 3% (C-D) and 5% (E-F) AFEX-CS by the parental or adapted AA7 strains of *C. uda* (closed and open symbols, respectively). Shown are formate (circles), acetate (squares), and ethanol (triangles).

The fermentation yields and rates of ethanologenesis of the parental and AA7 strains on 5% (w/v) AFEX were also compared to the previously reported study (48) that used minimal medium and 0.2% (w/v) AFEX-CS and incubations at 30°C (Fig 3.5). By optimizing operational parameters such as temperature and agitation and providing yeast extract to satisfy any growth requirements, *C. uda* was able to grow and ferment higher (5%) solid loadings. This resulted in 5-fold increases in the yields of fermentation products, with ethanol titers increasing 6-fold. Ethanol production per day was also 6-fold higher in the parental strain when grown under the optimized culturing conditions at 5% solid loadings. Furthermore, strain AA7 produced ethanol 2-fold faster than the parental strain under the same optimized conditions or 12-fold faster compared to minimal conditions.

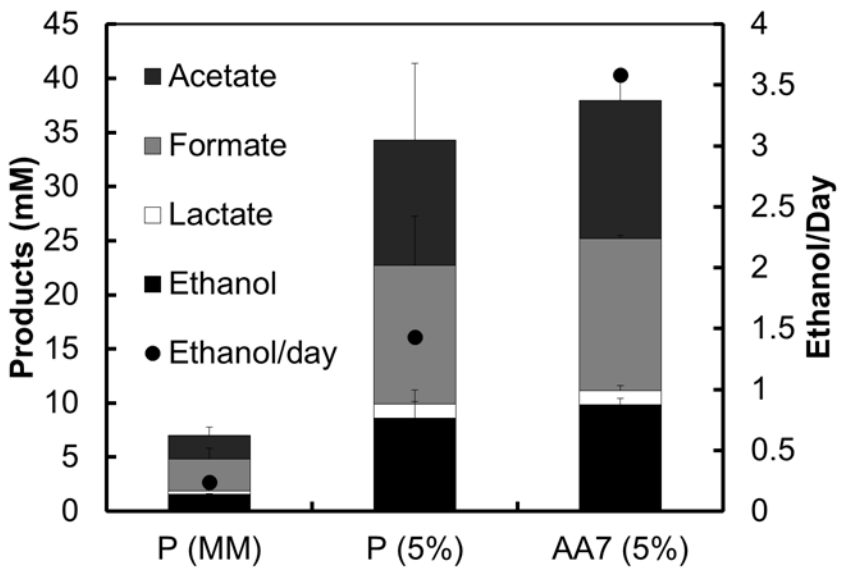

Figure 3.5 Comparison of fermentation products (columns) and ethanol productivity (line) produced by the parental (P) and adapted (AA7) strains of *C. uda* under the optimized conditions reported in this study (5%) in reference to previously reported (48) conditions (MM)

3.5 DISCUSSION

The results show that the CBP of AFEX-CS by *C. uda* can be improved significantly by optimizing the culture conditions and improving the fermentative rates of the bacterium via adaptive evolution. At increased solid loadings (0.5-5% w/v), *C. uda* efficiently degraded AFEX-CS for conversion to ethanol, but hydrolysis was never complete even in the best performing cultures. Plant cell walls are recalcitrant by nature, and studies suggest that the crystallinity of the cellulosic substrate can limit hydrolysis efficiencies (15, 41). However *C. uda* is able to degrade amorphous and crystalline regions of cellulose simultaneously (11). Yet the hydrolysis of even pure forms of cellulose by *C.*

uda is often incomplete (11). This has been linked to the accumulation of cellobiose, the final product of cellulose hydrolysis by *C. uda*, which acts as a feedback inhibitor of enzyme production (11). Additionally, compounds released during pretreatment (vanillin, syringic acid, syringyaldehyde) or fermentation (formate) can also inhibit the activity of xylanases (6, 20, 38). Alternatively, the enzyme system of *C. uda* may not be able to hydrolyze specific components of the AFEX-CS substrate. Hence, studies of the composition of the unhydrolyzed substrate and the enzyme system produced by *C. uda* for the hydrolysis of AFEX-CS could provide valuable information to genetically engineer strains with improved enzyme systems.

Several factors could explain the poor ethanologenesis efficiency observed at increased solid loadings (Fig. 3.1B). AFEX degradation products have been shown to affect xylose metabolism (27). Similarly, feedback inhibition by fermentation products could decrease fermentation efficiency. Acetate has been shown to be a non-competitive inhibitor of cellobiose metabolism in *C. uda*, presumably because it interferes with cellobiose uptake (12), As acetate levels increase during fermentation, cellobiose accumulates in the fermentation broth and feedback-inhibits cellobiose metabolism and cellulase synthesis (4, 12). Formate is also a major product of the fermentation of AFEX-CS and can inhibit xylanases activity (6, 20, 38). As sugars accumulate in the fermentation broth, they can also directly repress the activity of the hydrolytic enzymes and prevent further enzymatic hydrolysis. Glucose, for example, is a well-known inhibitor of *Cellulomonas* enzymes, and its accumulation results in decreases in β-glucosidase activity and the activities of other cellulases, whereas cellobiose can reduce endoglucanase activity (17, 52, 54, 55).

Ethanol is also a strong feedback inhibitor in *C. uda*, as evidenced by the reduced tolerance to ethanol of the parental strain (Fig. 3.2A). This feedback inhibition is a result of a lack of native pressure for high product tolerance as fermentation products diffuse rapidly and are utilized by neighboring organisms in nature (33, 34). Adaptive evolution was used to develop ethanol tolerance and improve fermentation efficiencies. Allyl alcohol pressure was also introduced to select for improved ethanol producers as alcohol dehydrogenase converts the allyl alcohol into a toxic compound, acrolein, which kills the cell (37). Thus, the presence of allyl alcohol selects for variants that preferentially use acetaldehyde, rather than allyl alcohol, as a substrate for alcohol dehydrogenase, thereby producing ethanol instead of acrolein (37). While improved ethanologenesis was not observed in the adapted strain, *C. uda* AA7 (Fig. 3.1B), the approach selected for variants with improved fermentation as seen by rate of fermentation (Fig 3.3) and ethanol productivity (ethanol/day) (Fig. 3.5). The adapted strain adjusted to changes in sugar availability faster than the parental strain. This is likely to reflect a decrease in *lag* phase, evolved to initiate fermentation and minimize the metabolism of allyl alcohol and the accumulation of the toxic acrolein. Many proteins involved in the transport and metabolism of sugars are not constitutively expressed and substrate utilization and cell growth cannot be initiated until the proteins are expressed, thus resulting in *lag* phases (5, 13, 26). For instance, cellobiose phosphorylase is synthesized de novo in response to cellobiose availability (42). Our experiments used inocula grown in cellobiose (Fig. 3.4). Thus, when transferred to fresh media with cellobiose, the cells resumed exponential growth immediately and no *lag* phase was observed. Cellobiose is a non-competitive inhibitor of glucose uptake in *C. fimi* (22). As

a result, cells grown on cellobiose transport glucose at one half the rate of glucose-grown cells. Hence, strain AA7 has likely evolved cellular mechanisms to deregulate and improve the transport and/or metabolism of sugars. Some of the proteins that are involved in the transport of sugars and that are produced de novo may, for example, be constitutively expressed in the adapted strain AA7. This strategy enables a faster response to changes in sugar availability but it is energetically costly to the cell as it devotes protein components and pathways to a metabolism that may not be utilized at all times. The decreased growth yields observed in the strain AA7 with all the sugars tested (Fig. 3.3) is consistent with this notion. We also observed faster fermentation of AFEX-CS in *C. uda* AA7 compared to the parental strain (Fig. 3.4) but reduced hydrolysis efficiencies (Fig. 3.1), suggesting a diversion of energy resources towards sugar fermentation at the expense of hydrolysis.

As ethanologenesis rates are an essential aspect to the productivity of the biofuel industry, strain AA7 shows promise as a robust CBP organism. Together with small changes in the operational parameters and culturing conditions, we have observed notable improvements in the CBP of AFEX-CS by *C. uda* over previous studies (Fig 3.5). Feedback inhibition of *C. uda* fermentation by acetate and formate, and its indirect effect in the inhibition of enzyme production and activity, remain the bottleneck of the process. We are currently sequencing the genome of the parental and adapted AA7 strains of *C. uda*, which will provide critical information about carbohydrate metabolism and regulation. This, together with the promising results of a genetic system for the closely related *C. flavigena* (Appendix A), suggest that genetic engineering could be used to develop strains with improved traits for the CBP of AFEX-CS. Feedback

inhibition can also be alleviated, and the hydrolysis and fermentation of AFEX-CS can be stimulated, during syntrophic growth of *C. uda* with the exoelectrogen *G. sulfurreducens* in a MEC (48). This approach provides an attractive platform for the CBP of agricultural wastes with high energy recoveries.

3.6 ACKNOWLEDGMENTS

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Chapter 4

Fermentation of glycerol into ethanol and simultaneous hydrogen production in microbial electrolysis cells

The material presented in this chapter was generated through equal collaboration with Allison M. Speers. All experiments were a joint effort except for adaptive evolution experiments with *Clostridium cellobioparum,* which were performed by J.M.Y., and adaptive evolutions experiments with *Geobacter sulfurreducens,* which were performed by A.M.S.

4.1 ABSTRACT

Biodiesel is a promising alternative to petroleum-based transportation fuels as it is chemically analogous to petrodiesel and can be used in compression engines and distributed using the existing infrastructure. However, its production results in large amounts of glycerol-containing wastewater, the treatment of which is an economic liability for the industry. The 'in situ' generation of enhanced-value co-products from crude glycerin has been identified as a promising step to simultaneously reduce the cost of biodiesel production and generate additional revenue for the industry through product diversification. Consequently, we developed a microbial consortium for the conversion of glycerol into ethanol and H_2 in a type of bioelectrochemical system (BES) called a microbial electrolysis cell (MEC). The ethanol can be recycled as a feedstock for the transesterification reaction, whereas the fermentative and cathodic hydrogen can be used to partially offset the energy needs of the biodiesel plant. The platform consists of a glycerol-fermenting bacterium, *Clostridium cellobioparum*, and the exoelectrogen *Geobacter sulfurreducens*. *C. cellobioparum* ferments glycerol into ethanol and other fermentative byproducts including lactate, acetate, formate, and H_2. *G. sulfurreducens* converts the fermentative byproducts into hydrogen in the MEC, thereby reducing feedback inhibition and improving glycerol fermentation. Both organisms were adaptively evolved for tolerance to industrially relevant glycerol concentrations (10% w/v). Co-cultivation of the evolved strains stimulated microbial growth, glycerol consumption, ethanologenesis and the conversion of fermentative byproducts into cathodic H_2 in the MEC with 10% (w/v) glycerol. Additionally, a metabolic shift in

fermentation from ethanol to 1,3-propanediol was observed compared to batch monocultures of *C. cellobioparum*. Optimization of the platform by increasing the buffering capacity of the media and removal of fermentative H_2 and CO_2 resulted in glycerol consumption near 50 g/L, making this an attractive platform for the pretreatment of glycerol-containing wastewater.

4.2 INTRODUCTION

Global energy use and environmental concerns have led to the development of renewable energy sources, in particular those that can replace liquid transportation fuels. The transportation sector accounts for 60% of oil consumption globally each year and is estimated to increase 1.8% per year. This would result in depletion of petroleum reserves in the next century (1). Biodiesel is an attractive alternative fuel as it is derived from renewable resources and can be used in the existing infrastructure either alone or blended with petrodiesel fuel (35).

As an alternative to petrodiesel, biodiesel has several beneficial characteristics including a higher lubricity which reduces long-term engine wear (11, 27), as well as lower emissions of sulfates, carbon monoxide, particulates, hydrocarbons, and aromatic compounds (13, 48). Biodiesel has a lower toxicity, higher biodegradability (13, 15, 48), and a higher flash point enabling it to be classified as a non-hazardous fuel (51). As a promising biofuel, biodiesel has a high cetane rating in the range of 45-70 (51) which reflects a lower ignition point following fuel injection (1) and a higher energy content compared to a competing fuel alternative, ethanol (13). Additionally, burning biodiesel contributes only small amounts of net atmospheric CO_2 due to the closed loop of the

CO_2 cycle (35). However, there are some challenges to biodiesel usage, including poor cold fuel properties (pour point and cloud point) that limit usability especially in winter months (51), a heat of combustion that is 93.4% lower than conventional diesel, higher emissions of nitrogen oxides (NO_x), and faster fuel degradation by both oxidation and microbial contamination (51). If biodiesel blends above 20% are used, engine modifications are required to facilitate fuel injections due to the 10-15 times higher viscosity of the biodiesel (1, 15), as well to prevent corrosion (51).

Biodiesel is produced from triacylglycerides, an energy rich form of reduced carbon that is very abundant and renewable (i.e. produced using energy from the sun). Triacylglycerol feedstocks include a wide variety of edible and non-edible plant oils, animal fats, and microbe-derived oils (1). As feedstock cost represents at least 75% of biodiesel production costs, inexpensive and productive sources will be essential for the economic sustainability of the biodiesel industry (33). Microalgae have potential to become the next generation feedstock for biodiesel production, due to their efficient conversion of solar energy to biomass (57) and projected oil yields per land area (58,700-136,900 L ha^{-1} $year^{-1}$), which are higher than for any other feedstock (1). They can grow with low nutrient input and in saline or harsh conditions, and they do not compete with food crops for land (57). Currently, economic challenges limit the potential to scale up microalgae production, as the energy inputs to assure robust growth rates and oil yields need to be reduced (40). If these challenges are overcome, microalgal oils are likely to be the feedstock with the greatest potential to supply the global biodiesel supply chain (1).

Triacylglycerides have three fatty acid chains (18-16 carbons in length) esterified to glycerol. They are too viscous to use directly as a fuel, and therefore need to be converted into the less viscous fatty acid esters via transesterification (Fig 4.1). The transesterification process occurs with an alcohol, usually methanol or ethanol, and a catalyst to produce fatty acid methyl/ethyl esters (FAMEs and FAEEs) (13). Using ethanol as the alcohol feedstock for transesterification has many benefits. FAEEs eliminate dependence on non-renewable energy as methanol is produced from natural gas derived from fossil fuels while ethanol is made from renewable sources (corn, sugarcane, lignocellulose) (42). Other benefits of FAEE include higher cetane number and energy content (as FAEEs have one more carbon per molecule than FAMEs), lower density, pour point, and cloud point (improving handling and transportation efficiencies), and lower NO_x emissions (42). Furthermore, evidence also shows that FAEEs may store better than FAMEs as they are more resistant to oxidation, light, temperatures, and metals (42).

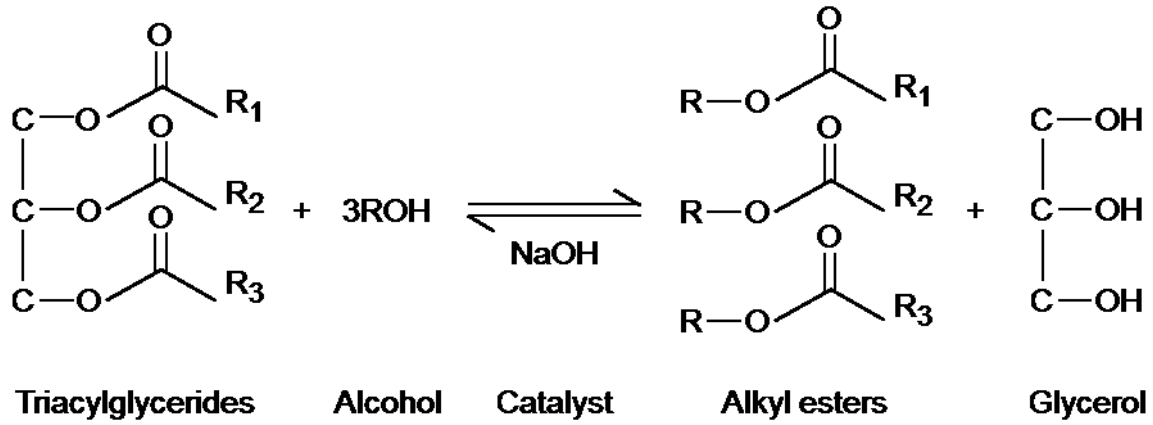

Figure 4.1: Transesterification reaction of triacylglycerides and alcohol to produce either fatty acid methyl esters (R, CH_3) or fatty acid ethyl esters (R, CH_3CH_2). Adapted from (5).

In addition to biodiesel (fatty acid esters), the transesterification process also yields 10 lb of glycerol for every 100 lb of biodiesel generated (61). To meet international standards of fuel quality the glycerol must be thoroughly removed from the biodiesel (1). The density difference between glycerol and biodiesel allows for their separation after centrifugation. The separation from glycerol is improved and almost complete when FAMEs rather than FAEEs are used (5). Water at pH 4.5 is often added to the crude biodiesel to improve the phase separation and remove additional contaminants such as residual catalyst and alcohol, unreacted mono-, di-, and triglycerides, and soaps (20). The biodiesel undergoes one more round of centrifugation to separate the water and is then vacuum dried until the final moisture content is below

0.050% (v/v) (20). Glycerol waste streams contain on average 10% glycerol and 5% alcohol, and require treatment prior to disposal. Soaps are converted to free fatty acids by hydrochloric acid treatment, which are then removed by centrifugation and disposed of as sewage. The waste stream is then neutralized and distilled to recover residual alcohol for reuse in the transesterification reaction (20). The resultant crude glycerol is partially purified to 80% (w/v) by distillation and sold to commercial refineries for further treatment. However, as biodiesel production has increased, the glycerol byproduct has become an environmental and economic liability (14, 26). Between 2000 and 2010, the global production of biodiesel increased 22-fold (56), dropping the price of glycerol from US $0.20-0.25 per pound in 2004 to US $0.06-$0.011 per pound in 2012 (36, 61). This has mostly impacted small and medium scale producers as profit margins decrease with the added cost of waste disposal and loss of byproduct revenue (20, 61).

While glycerol has value in the food, cosmetic and pharmaceutical industries (38), the combination of the glut caused by the biodiesel industry and the high cost of processing and purification of crude glycerol from biodiesel waste streams (20, 61) creates the need for alternative uses. Recent research has therefore focused on finding uses for crude glycerol, which will both remove glycerol from the waste stream and provide added value to the biodiesel refineries. The reduced nature of glycerol compared to other sugars also makes an attractive substrate for microbial conversion into value added products such as ethanol or 1,3-propanediol (Fig. 4.2; (45, 61)). Several organisms have been identified that can ferment glycerol including several species of *Klebsiella, Bacillus, Clostridium, Lactobacillus, Enterobacter, Propionibacteria* and *Citrobacter* (2, 3, 12, 21, 23, 34, 37, 39, 44, 54). However, many of

these bacteria are also classified as opportunistic pathogens, which greatly limits their practical applications. *E. aerogenes* (44) and *B. subtilis* (37) have been shown to couple the degradation of low concentrations of glycerol (1% and 0.1%, respectively) with current production in microbial electrolysis cells (MECs), however coulombic efficiencies were low. In the case of *E. aerogenes,* current production from crude glycerol was enabled by the addition of a mediator, thionine. However, this approach increases the cost of MEC operation and makes large scale flow-through system difficult to attain. In another MEC platform, *Shewanella oneidensis* was genetically engineered to convert glycerol to ethanol by knocking out the native phosphate acetyltransferase gene and heterologously expressing four *E. coli* genes for glycerol consumption and two ethanol production genes from *Zymomonas mobilis* (17). The fermentation proceeded with stoichiometric conversion of glycerol to ethanol and acetate in the presence of a poised electrode, which was required to consume the excess reducing power of the fermentation. However, low (ca. 0.5% w/v) concentrations of pure glycerol were used in this study and antibiotic supplementation was required to maintain the expression vectors (17). Similarly, an adaptively-evolved strain of *G. sulfurreducens* carrying a single point mutation in a transcriptional regulator was able to convert glycerol into electricity in a microbial fuel cell (MFC), yet concentrations of glycerol processed by the exoelectrogen were even lower (0.05% w/v) (29). BECs driven by naturally-established microbial communities have been reported, to partially recover energy from glycerol-containing wastewaters. However, power densities were still low and the efficiency of glycerol removal was never assessed (9). All glycerol-fermenting species are able to consume the excess reducing equivalents by reducing glycerol to 1,3-propanediol (Fig.

4.2), which is a valuable precursor to a new formulation of polyester (polypropylene terephthalate) as well as biodegradable plastics (19, 45). *Clostridium acetobutylicum* for example, while unable to ferment glycerol naturally, was engineered to produce 1,3-propanediol through the introduction of genes from *Clostridium butyricum*, the resulting strain was able to consume ca. 6% (w/v) glycerol and produce primarily 1,3-propanediol (molar yield of 0.64), yet lactate, acetate, formate, butyrate and H_2 were produced as waste products (19).

The waste products that are made during glycerol fermentations for ethanol and propanediol (Fig. 4.2) can act as feedback inhibitors and/or acidify the fermentation broth, thus inhibiting cell growth. As a result, extensive genetic engineering is often required to reroute the native metabolisms. For example, the efficient conversion of glycerol into ethanol by *E. coli* required nine gene knockout mutations to reduce the cell's central metabolism from over 15,000 possible pathways to a total of 28 glycerol-utilizing pathways and adaptive evolution was still needed to divert the metabolic flux towards ethanol production (54). Some of the waste products are coproduced to maintain the cell's redox balance and therefore cannot be eliminated by genetic engineering and must be otherwise removed to prevent inhibition of cell growth and fermentation. H_2, for example, is produced by *Clostridium cellobioparum* concomitantly to ethanol during fermentation (Fig. 4.2) and is a potent feedback inhibitor of growth (8). However, feedback inhibition can be alleviated and growth and fermentation stimulated by cocultivating *C. cellobioparum* with the methanogen *Methanobacterium ruminantium*, which removes the H_2 and converts it into methane (8).

In this chapter, a coculture approach is described in which *C. cellobioparum*, a robust glycerol-fermenting bacterium, is cocultured with the exoelectrogen *G. sulfurreducens* in a microbial electrolysis cell (MEC). *G. sulfurreducens* converted the waste fermentation byproducts into electricity, which was electrochemically converted into cathodic H_2. This allowed for the energy in the waste products to be recovered as ethanol feedstock and H_2 and also stimulated glycerol consumption and fermentation by *C. cellobioparum.*

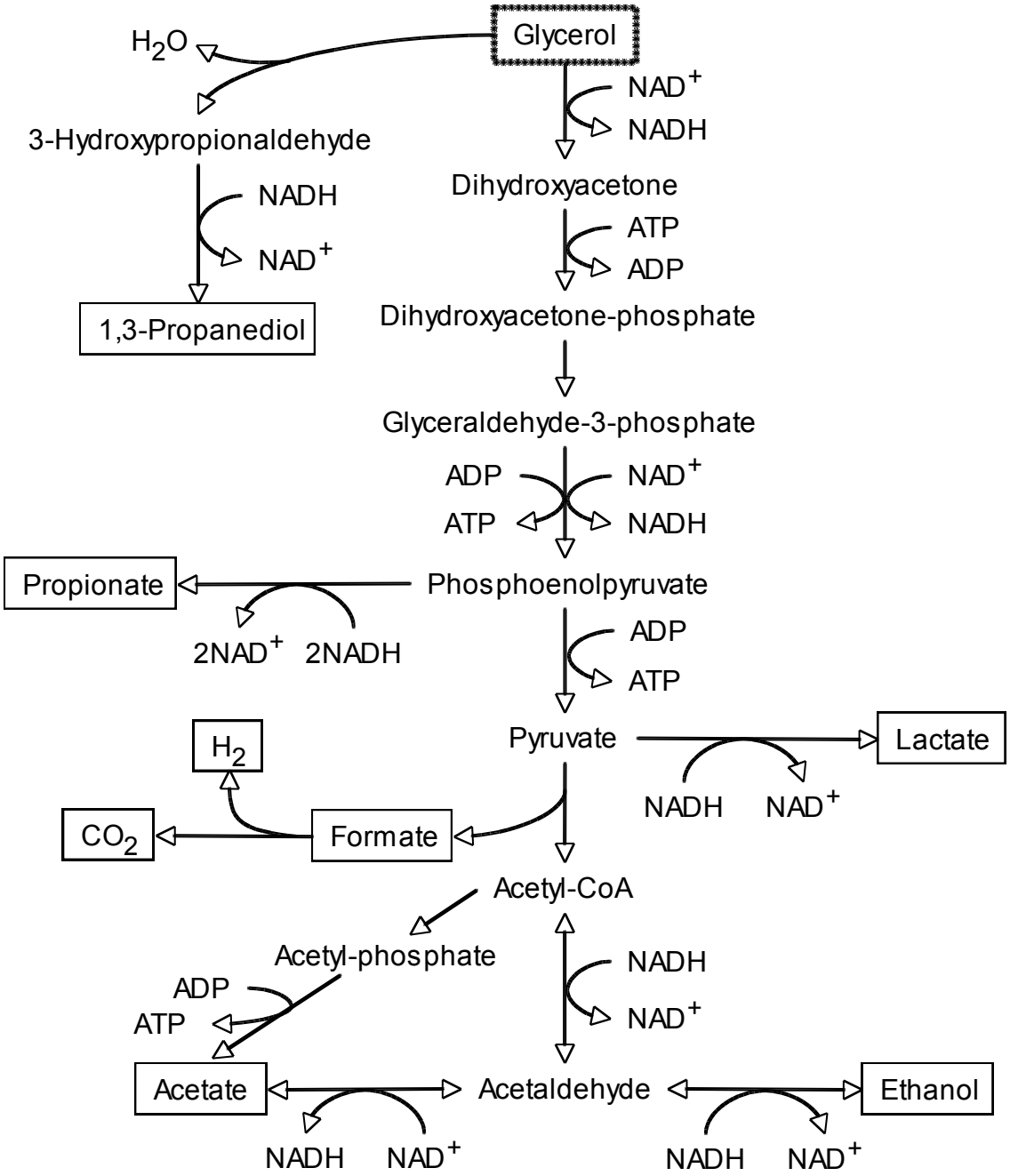

Figure 4.2: The fermentative metabolism of glycerol into ethanol and 1,3-propanediol and the associated fermentative byproducts. Adapted from (2, 31, 43, 61).

4.3 MATERIALS AND METHODS

4.3.1 Bacterial strains and culture conditions

Geobacter sulfurreducens PCA was routinely cultured at 30°C in anaerobic minimal medium (DB medium; (50)) with 20 mM acetate and 40 mM fumarate (DB-AF). Ethanologenic, cellulolytic strains from our culture collection (Table 4.1) were routinely grown at 35°C in anaerobic GS2 medium (6) supplemented with 0.2% cellobiose (GS2-CB) or glycerol (GS2-glycerol). When indicated, 3-(N-morpholino)propanesulfonic acid (MOPS) was omitted from the media (GS3 medium).

4.3.2 Screening of fermentative strains for glycerol consumption

Ethanologenic strains from our culture collection (Table 4.1) were screened for their ability to produce ethanol from glycerol at 35°C in anaerobic GS2 medium supplemented with 0.25% (w/v) glycerol. Cultures were grown anaerobically in GS2-CB before inoculating to an initial optical density at 660 nm (OD_{660}) of 0.04 into triplicate tubes with 10 ml GS2-glycerol medium. Growth was monitored spectrophotometrically (OD_{600}) every 12 h.

4.3.3 Batch cultures of *C. cellobioparum* and *G. sulfurreducens*

Late-exponential phase cultures of *G. sulfurreducens* and *C. cellobioparum* grown anaerobically at 30°C in DB-AF and GS2-CB media, respectively, were inoculated to an initial OD_{660} of 0.02 in the same (coculture) or separate (monoculture) tubes containing 10 ml GS2 medium supplemented with 0.25% (w/v) glycerol and 40 mM fumarate.

Control monocultures of *G. sulfurreducens* and *C. cellobioparum* were also prepared in GS2 medium without glycerol to account for any growth from the yeast extract present in the medium or from nutrients carried over in the inoculum. Controls of *G. sulfurreducens* in DB-AF with and without 0.25% (w/v) glycerol were also included. All cultures were incubated at 30°C and growth (OD_{660}) was monitored every 6 h.

4.3.4 Alcohol tolerance of *C. cellobioparum* and *G. sulfurreducens*

Late-exponential phase cultures of wild-type *C. cellobioparum* and *G. sulfurreducens* grown anaerobically at 30°C in GS3-CB and DB-AF media, respectively, were inoculated to an initial OD_{660} of 0.02 in the same (coculture) or separate (monoculture) tubes with 10 ml GS3 medium containing 40 mM fumarate in the presence of glycerol (concentrations ranging from 0 and 10% (w/v)) or ethanol (concentrations between 0 and 5% (v/v)). The cultures were incubated at 30°C and growth was monitored every 12 h.

4.3.5 Adaptive evolution of *C. cellobioparum*

A glycerol-tolerant strain of *C. cellobioparum* was evolved through serial transfers of stationary phase cultures in GS3 medium supplemented with increasing concentrations of glycerol, starting at 6.3% (w/v). The glycerol concentration was increased to 8.8% (w/v) glycerol and finally 10% (w/v) once the growth rate and *lag* time of the cultures had stabilized. After approximately 16 months, a culture was adapted that grew with 10% (w/v) glycerol and clonal representatives were isolated on solidified (1.4% agar) GS2-CB using roll tubes (22). Individual colonies were subcultured three times to insure

the purity of the clone. Five clones were tested for growth, glycerol consumption and fermentation product yields in medium with 10% (w/v) glycerol. The best performing strain was designated $CcelA_{10G}$ and its glycerol tolerance was assessed as described above.

4.3.6 Adaptive evolution of *G. sulfurreducens*

An ethanol-tolerant strain of *G. sulfurreducens* was evolved by serially transferring stationary phase cultures in DB-AF medium supplemented with increasing concentrations of ethanol (between 1 and 5% v/v). The strain was routinely transferred in the same concentration of ethanol at least seven times or until growth rates improved and stabilized, before increasing the ethanol concentration by 0.5% increments. Cultures adapted for growth at each ethanol concentration were routinely preserved at -80°C in anaerobic vials containing 10% dimethyl sulfoxide (DMSO). Once a culture was adapted that grew at 5% (v/v) ethanol after several transfers, clonal individuals were recovered as isolated colonies on solidified NBAF media (10) in an anaerobic glove bag (Coy Laboratory Products, Inc.). Ten colonies were subcultured three times to ensure purity and the one with the most robust growth in DB-AF medium with 5% (v/v) ethanol was designated $GsulA_{5E}$ and was selected for further study. $GsulA_{5E}$ was tested for tolerance to increasing concentrations of glycerol and ethanol, as described above.

4.3.7 MECs

Dual-chambered, H-type MECs were set up as described previously (50) and incubated at 30°C. They were autoclaved before the addition of 90 ml DB medium to the anode

and cathode chambers. The medium in the anode chamber was supplemented with 1 mM acetate. The reference electrode (3 M Ag/AgCl, Bioanalytical Systems Inc.) was sterilized in 70% ethanol for 1 min and rinsed with sterile water before being added to the anode chamber. Before inoculation, the anode electrode was poised at 0.24 V with a VSP potentiostat (BioLogic) and the chambers were sparged with filter-sterilized N_2:CO_2 (80:20) gas. Once the current was stabilized, the anode chamber was inoculated with 10 ml of a cell suspension of the wild-type (GsuI) or ethanol-tolerant ($GsuIA_{5E}$) strain of *G. sulfurreducens* in DB medium. The cell suspension was prepared by harvesting cells from a 40% (v/v) volume of an early stationary-phase culture grown at 30°C in DB-AF by centrifugation (6,000 x *g,* 6 min, 25°C) under anaerobic conditions and washing them once before resuspending them in 10 ml DB medium.

Once all the acetate was consumed in the anode chamber (i.e., when the current declined to baseline level), the medium was replaced with GS3 medium containing 10% (w/v) glycerol, and the chamber was inoculated with *C. cellobioparum* (the wild-type CceI or the adapted $CcelA_{10G}$ strain). The CceI or $CcelA_{10G}$ cells were harvested anaerobically by centrifugation (6,000 x *g*, 6 min, 25°C) from a 40% (v/v) inoculum of late exponential-phase culture grown at 30°C in GS3-glycerol, washed once, and resuspended in 10 ml GS3-glycerol medium. The anode chamber was sparged briefly with N_2 following inoculation to ensure anaerobiosis, but no additional sparging was used to prevent the evaporation of fermentative ethanol. By contrast, the cathode chamber was sparged continuously with N_2:CO_2 (80:20) to prevent the crossover of H_2 into the anode chamber. The percent of cathodic H_2 recovered in the MEC system was

determined by discontinuing the sparging of the cathode chamber, sampling the headspace and analyzing the gas composition by gas chromatography (GC), as described below. Alternatively, other types of media were used in place of GS3-glycerol to test their effect on MEC performance. Where indicated, GS2-glycerol or GS3-glycerol supplemented with 200 mM phosphate buffer were used as media in the MECs to improve the buffering capacity of the medium in the anode chamber. Alternatively, anode chambers with GS2-glycerol medium were continuously sparged with N_2 to remove the fermentative H_2 and CO_2 and investigate the potential effect of feedback inhibition or medium acidification, respectively, on MEC performance.

Controls with $GsulA_{5E}$ monocultures to test the glycerol tolerance in the MECs were performed as described above by first growing $GsulA_{5E}$ anode biofilms in DB media containing 1 mM acetate. After current production declined, the medium was switched to fresh DB medium containing 1 mM acetate with and without 10% (w/v) glycerol. The anode supernatant was analyzed by high pressure liquid chromotrography (HPLC) as described below and the efficiency of acetate conversion into current (coulombic efficiency, CE) was calculated as the coulombs recovered divided by the total coulombs in the substrate (eq. 1).

$$CE = \frac{\int_0^t I\, dt}{8F\,\Delta A} \qquad (1)$$

The integral of the current (*I*) over the duration of the experiment (*t*) is given in coulombs (A*s). The number 8 represents the number of moles of electrons in 1 mol of acetate, *F*

is Faraday's constant, and ΔA is the decrease of acetate (moles) over the duration of the experiment.

Controls with CcelA$_{10G}$ monocultures were also grown in the MECs. CcelA$_{10G}$ cells were harvested as described above and inoculated into MECs containing 90 ml GS3 medium and 10% glycerol. Where indicated, the anode electrode was poised at 0.24 V vs. Ag/AgCl and current production was monitored.

4.3.8 Energy recovery

Energy recovery η (%) for the MECs was calculated by dividing the energy outputs by energy inputs (59), as described in the following equation:

$$\eta = \frac{W_E + W_{HA} + W_{HC}}{(W_G)m_G + (W_A)m_A + W_P} \qquad (2)$$

The energy outputs in eq. 2 included the amount of energy recovered as ethanol (W_E), which was calculated as the heat of combustion of the ethanol produced (upper heating value 23.4 MJ/L; (47)), and the energy recovered as H_2 at the cathode (W_{HC} in eq 2) plus the energy recovered as fermentative H_2 in the anode (W_{HA}), which were determined using the heat of combustion of H_2 (upper heating value 285.83 kJ/mol; (7)). The recovery of cathodic H_2 from the system was calculated as the number of moles of H_2 measured in the headspace of the cathode chamber at the end of the experiment

divided by the maximum theoretical coulombic H_2 recovery (r_{CE}), which was obtained from the amount of current (I) produced in the MEC as follows:

$$r_{CE} = \frac{\int_0^t I\, dt}{2F} \qquad (3)$$

Where F is Faraday's constant and 2 represents the number of moles of electrons per mol of H_2 (7).

The energy inputs in eq. 2 included the energy input from glycerol, which was determined by the heat of combustion of glycerol (W_G; 17,961 J/g; (53)) multiplied by the mass of glycerol consumed over the duration of the experiment (m_G), and the energy input from acetate which was determined by the heat of combustion of the acetate (W_A; 870.28 kJ/mol; (7)) multiplied by the moles of acetate (m_A) consumed over the duration of the experiment. The electricity input from the potentiostat to maintain the cell voltage (W_P in eq. 2) over the duration of the experiment (t) was calculated as:

$$W_P = \int_{t=0}^{t} I\, E\, dt \qquad (4)$$

Where I is the measured current and E is the cell voltage (28). The applied potential of the cathode was measured with respect to a reference electrode (3 M Ag/AgCl, Bioanalytical Systems Inc.) inserted in the cathode chamber. The cell voltage was calculated as the difference between the measured cathodic potential and the applied potential at the anode electrode.

The energy recovery for the MFCs was calculated similarly (eq. 5), by taking into account the energy outputs from ethanol and fermentative H_2 as described above, as well as the power produced from the fuel cell (W_F) which is a product of the voltage and current over the duration of the experiment as seen in eq. 4. The energy inputs were from the amount of glycerol and acetate consumed over the duration of the experiment, as described above.

$$\eta = \frac{W_E + W_{HA} + W_F}{(W_G)m_G + (W_A)m_A} \quad (5)$$

The energy recovery from the *C. cellobioparum* fermentation was determined as seen in eq. 6 and took into account only the energy inputs from the glycerol consumed and the fermentative ethanol and H_2 produced.

$$\eta = \frac{W_E + W_{HA}}{(W_G)m_G} \quad (6)$$

4.3.9 Confocal Laser Scanning Microscopy (CLSM)

Anode biofilms were examined by CLSM at the end of the MEC experiments as previously described (50), except that *G. sulfurreducens* (Gram negative) and *C. cellobioparum* (Gram positive) cells were differentially stained in green and red, respectively, with the BacLight Gram Stain Kit (Invitrogen) following the manufacturer's recommendations. The electrodes were imaged with an Olympus FluoView FV1000 inverted microscope (Olympus; Center Valley, PA) equipped with a PLAPON 120X oil immersion objective (Olympus; numerical aperture [NA], 1.42**).** The excitation

wavelength was 488 nm for both dyes. The emission spectra were detected with a BA505-525 band pass filter (SYTO 9, green) and a BA650IF long pass filter (hexidium iodide, red). Image stacks were collected every 0.4 μm and image projections were generated using the FV10-ASW 3.0 software (Olympus).

4.3.10 Analytical techniques

At the end of each experiment when the cultures had reached stationary phase, the composition of the fermentation broth and the headspace atmosphere were analyzed by HPLC and GC, respectively. Alcohols and organic acids in culture supernatant fluids were measured by HPLC (Waters, Milford, MA) at 30°C, as previously described (32) except that samples were filtered with 0.45 μm syringe filters (National Scientific, Rockwood, TN) prior to analysis. GC analyses were performed in a Varian CP-4900 Micro Gas Chromatograph (Agilent, Santa Clara, CA). Where indicated the pH was also measured with an Orion Aplus pH meter (Thermo Electron, Beverly, MA).

4.4 RESULTS

4.4.1 Identification of a glycerol-fermenting ethanologenic strain

Seven ethanologenic strains from our laboratory culture collection grew in rich media containing 0.25% (w/v) glycerol at 35°C. Only *C. cellobioparum* coupled growth to the fermentation of glycerol to ethanol (Table 4.1), growing to an OD_{660} of 0.37 ± 0.01 (OD_{660}), consuming 28.9 (± 0.7) mM glycerol (84.5 (± 2.0)% of the glycerol provided), and producing 31.3 (± 1.2 mM) ethanol as the main product of fermentation followed by acetate (19.7 ± 0.8 mM), lactate (10.8 ± 0.5 mM) and H_2 (7.7 ± 0.2 mM). The amount of glycerol fermented corresponds well with the amount of ethanol produced (0.29:0.31 mmol). The maximum theoretical molar conversion of glycerol to ethanol is 1:1; therefore the high (more than 100%) fermentation yields from glycerol suggest that substrates available in the rich medium and/or carried over from the inoculum were also used for fermentation. In fact, the GS2 medium without glycerol supported the growth of *C. cellobioparum* to yields of 0.21 ± 0.01 (OD_{660}) and production of ethanol (4.2 ± 0.9 mM), acetate (3.9 ± 1.7 mM), formate (1.9 ± 1.5 mM), lactate (1.1 ± 0.5 mM) and H_2 (2.7 ± 0.3 mM). Although glycerol was not efficiently consumed by the other strains tested, they still grew in the GS2 medium (Table 4.1).

Table 4.1: Screening of fermentative strains for glycerol consumption.[a]

Strain (designation)	Glycerol consumed (mM)	Ethanol produced (mM)	Growth rate (d^{-1}) [b]
Cellulomonas uda (ATCC 21399)	0.9 (± 1.5)	6.0 (± 1.1)	1.5 (± 0.1)
Cellulomonas biazotea (ATCC 486)	1.8 (± 1.5)	4.8 (± 0.5)	1.0 (± 0.1)
Cellulosimicrobium cartae (ATCC 21681)	0.3 (± 0.3)	5.1 (± 0.3)	1.8 (± 0.1)
Cellulomonas gelida (ATCC 488)	0.4 (± 0.5)	2.7 (± 0.8)	1.7 (± 0.1)
Clostridium cellobioparum (ATCC 15832)	28.9 (± 0.7)	31.2 (± 2.7)	1.5 (± 0.1)
Cellulosilyticum lentocellum (ATCC 27405)	0.6 (± 0.9)	0.8 (± 0.4)	14.7 (± 1.4)
Clostridium papyrosolvens (NCIMB 11394)	0.7 (± 0.7)	2.1 (± 1.1)	5.3 (± 0.2)

[a] Shown are averages and, in parentheses, standard deviations of three replicates.

[b] Determined by optical density at 660 nm of planktonic growth.

4.4.2 Syntrophic growth of *G. sulfurreducens* and *C. cellobioparum* in batch cultures with fumarate as the electron acceptor

The syntrophic growth of *C. cellobioparum* and *G. sulfurreducens* was investigated in batch cultures grown at 30°C in GS2-glycerol supplemented with fumarate to serve as the terminal electron acceptor for *G. sulfurreducens* (Fig. 4.3A). Monocultures of *C. cellobioparum* in medium with and without glycerol, and monocultures of *G. sulfurreducens* in medium with glycerol were included as controls (Fig. 4.3A). A small amount of growth (OD_{660}, 0.22) was also seen in *C. cellobioparum* monocultures without glycerol (Fig. 4.3A), suggesting that growth was being sustained by the yeast extract or carryover from the inoculum. Consistent with this, small amounts of fermentation products were also detected (Fig. 4.3B; ethanol, 1.1 ± 0.6 mM; lactate, 0.5 ± 0.01 mM; acetate, 3.6 ± 0.3 mM; formate, 1.2 ± 0.2 mM; H_2, 3.0 ± 0.6 mM; CO_2, 1.1 ± 0.2 mM). The basal GS2 medium with glycerol and fumarate was unable to support high growth yields for *G. sulfurreducens* (OD_{660}, 0.15) and glycerol was not consumed. Furthermore, fermentation products were not detected in the culture broth. *C. cellobioparum* monocultures were able to couple glycerol fermentation to growth and reached higher yields (OD_{660}, 0.47) (Fig 4.3A). The *C. cellobioparum* monoculture consumed 22.7 ± 0.6 mM glycerol (82.8 (± 2.3)% of the glycerol provided) and produced ethanol (30.3 ± 1.2 mM), lactate (15.6 ± 0.9 mM), acetate (10.2 ± 0.8 mM), formate (2.4 ± 2.3 mM), H_2 (7.4 ± 0.4 mM) and CO_2 (3.1 ± 0.3 mM) (Fig 4.3B). In the coculture, 23.5 ± 0.4 mM glycerol was consumed (85.8 (±1.5)% of the glycerol provided). Coculture supernatants contained ethanol (26.6 ± 2.6 mM) and lactate (14.5 ± 1.1 mM), while no

acetate or formate were detected (Fig. 4.3B). H_2 concentrations in the headspace decreased (1.4 ± 0.2 mM) and CO_2 production increased (4.3 ± 0.2 mM) in the coculture compared to the *C. cellobioparum* monoculture. The observed consumption of the preferred electron donors for *G. sulfurreducens* (acetate, formate, and H_2) coupled with the stimulated growth yields (OD_{660}, 1.2; Fig. 4.3A), correlates well with the increases in CO_2 production observed in the cocultures (Fig. 4.3B). Furthermore, we also measured 1.3-fold increases in growth rates in the coculture (2.3 ± 0.1 d^{-1}) compared with the *C. cellobioparum* monoculture (1.7 ± 0.1 d^{-1}), consistent with the stimulation of growth when the two strains are growing syntrophically. The growth of *G. sulfurreducens* was unaffected by the addition of 0.25% (w/v) glycerol as growth rates were similar in GS2 with acetate and fumarate in the presence (2.4 ± 0.1 d^{-1}) and absence (2.2 ± 0.1 d^{-1}) of glycerol (data not shown).

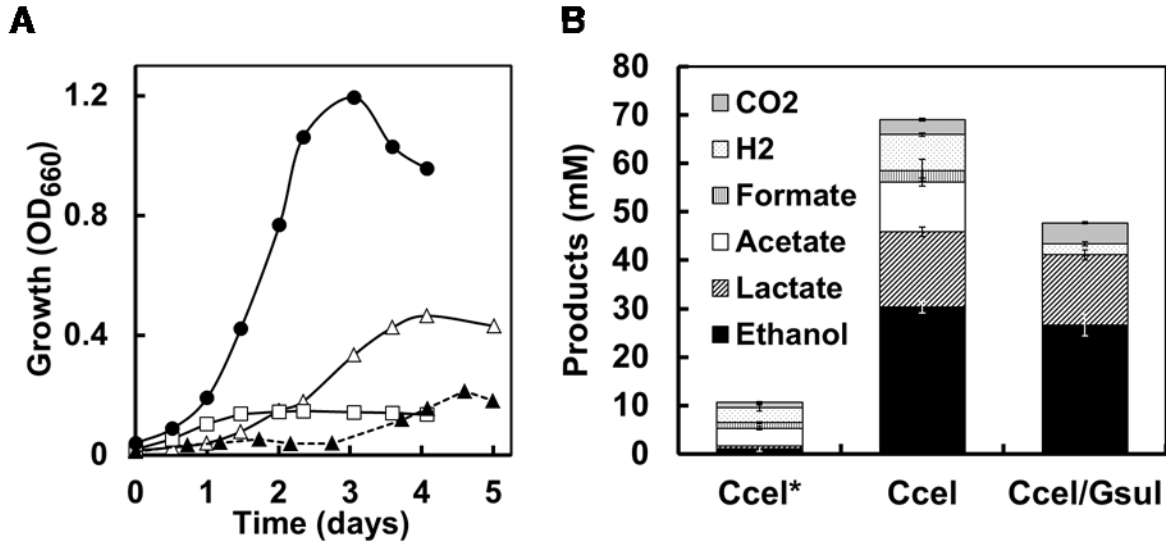

Figure 4.3: Syntrophic growth of *G. sulfurreducens* and *C. cellobioparum* in batch cultures with glycerol and fumarate at 30°C. (A) Growth of the coculture (solid circles), and *C. cellobioparum* monocultures with glycerol (open triangles), *C. cellobioparum* monocultures without glycerol (solid triangles) and *G. sulfurreducens* monocultures (open squares). Growth was monitored as optical density at 660 nm (OD_{660}). (B) Glycerol fermentation products at the end of the experiment in *C. cellobioparum* monocultures with (Ccel) and without (Ccel*) glycerol and in the coculture (Ccel/Gsul).

4.4.3 Tolerance of wild-type strains to glycerol

The glycerol tolerance of wild-type *C. cellobioparum* was investigated in batch cultures incubated at 30°C with glycerol concentrations ranging from 0-10% (w/v) (Fig 4.4A). The growth rate increased 1.4-fold as glycerol concentrations increased from 0.25% to 1%, as more substrate was available for growth. Growth rates were ca. 79% of the maximum for cultures grown with glycerol concentrations between 3 and 7% and no growth was observed at 10% glycerol concentrations. The tolerance of the wild-type strain of *G. sulfurreducens* to increasing concentrations of glycerol was also investigated (Fig 4.4A). *G. sulfurreducens* tolerated up to 3% (w/v) glycerol concentrations with growth rates 81% of the maximum. At 5% glycerol concentrations the growth rates were 47% of the maximum and no growth was observed at 7% and 10% glycerol. The two strains were also cocultured with increasing concentrations of glycerol to investigate the glycerol tolerance of the coculture (Fig 4.4A). The highest growth rates were observed at glycerol concentrations of up to 3% and then declined to 69% of the maximum growth rate in cultures with 5% glycerol. No growth was observed at 7 and 10% glycerol. These results suggest that the *G. sulfurreducens* strain is more sensitive to glycerol than *C. cellobioparum,* and that the growth of *G. sulfurreducens* was the bottleneck that drove the growth of the coculture.

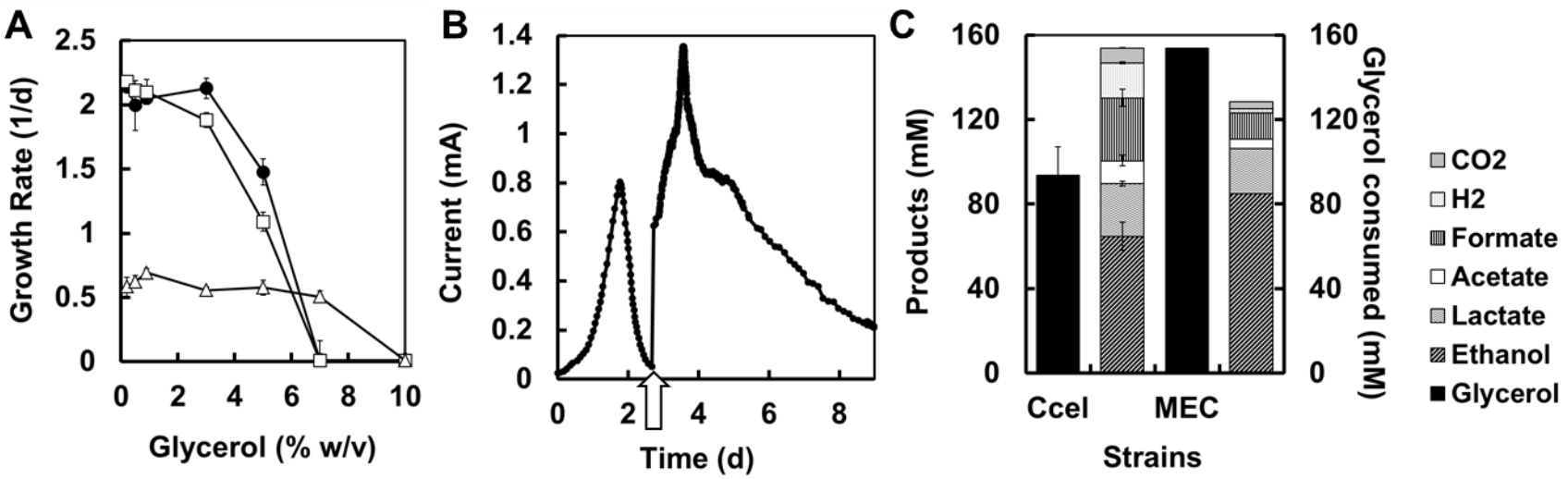

Figure 4.4: (A) Tolerance of wild-type *C. cellobioparum* (open triangles), *G. sulfurreducens* (open squares) and the coculture (closed circles) to increasing concentrations of glycerol. Error bars show standard deviations from three replicate cultures. (B) Current production of a sequentially inoculated MEC. *G. sulfurreducens* was inoculated first and supplemented with 1 mM acetate, the anode media was then exchanged (arrow) for GS3 media supplemented with 3.8% (w/v) glycerol and inoculated with *C. cellobioparum.* (C) Glycerol consumption and fermentation products from the MEC shown in panel B compared with a *C. cellobioparum* monoculture (Ccel) in 90 ml GS3-3.8% glycerol medium. Error bars show standard error of two replicates.

4.4.4 Fermentation of glycerol to ethanol in a MEC

We investigated the ability of the coculture of *G. sulfurreducens* and *C. cellobioparum* to couple the fermentation of glycerol with current production in a MEC. A sequential inoculation strategy was followed in which *G. sulfurreducens* was first inoculated in the MEC and supplemented with 1 mM acetate to provide optimal conditions for biofilm formation on the anode electrode (Fig. 4.4B). The current increased exponentially at a rate of 3.1 d^{-1} and peaked at 0.8 mA after 42 h, before declining once the acetate was consumed. As the current reached baseline levels below 0.1 mA, the anode medium was replaced with GS3 containing 3.8% (w/v) glycerol, a concentration that supported robust growth of the coculture (Fig. 4.4A), and the anode medium was inoculated with *C. cellobioparum* (Fig. 4.4B). The current resumed immediately and peaked at 1.34 mA before declining to below 0.2 mA over a period of ca. 5 d and yielding a total of 3.4 mmol of electrons. As a monoculture control, we also grew *C. cellobioparum* into duplicate serum bottles containing 90 ml GS3-glycerol media, following the same procedure used to inoculate the anode chamber of the MEC. HPLC analysis of the culture supernatant fluids at the end of the experiment showed a nearly stoichiometric conversion of glycerol (94 $\pm$ 14 mM) into ethanol (65 $\pm$ 7 mM), lactate (25 $\pm$ 1 mM) and acetate (11 $\pm$ 2 mM), with the concomitant production of formate (30 $\pm$ 4 mM), H_2 (17 $\pm$ 1 mM) and CO_2 (7 $\pm$ 0.2 mM) in the *C. cellobioparum* monoculture (Fig. 4.4C). Glycerol fermentation was stimulated 1.6-fold in the MEC driven by the coculture, with approximately 154 mM of glycerol being consumed. Ethanol production was also stimulated in the MEC (1.3-fold) and ethanol yields were 85 mM. The concentrations of

acetate (5 mM), formate (12 mM), and H_2 (2 mM) were significantly lower (45, 40 and 12%, respectively) than in the monoculture, consistent with the removal by *G. sulfurreducens* of much of the waste fermentation products that could be used as electron donors. The concentration of lactate was only slightly lower (21 mM or 84%) than in the monocultures (Fig. 4.4C), in agreement with previous studies (50) that reported that lactate is not a good electron donor for *G. sulfurreducens.* The concentration of CO_2 (3.5 mM) was half of that measured in the monoculture. This was unexpected because both *C. cellobioparum* and *G. sulfurreducens* produce CO_2 during their metabolism and more CO_2 production is expected with increased glycerol fermentation. This reduction may however reflect limitations derived from the use of MECs, which are not tight seal systems and can leak some of the headspace gases during operation.

4.4.5 Adaptive evolution of *C. cellobioparum*

Adaptive evolution was used to increase the tolerance of *C. cellobioparum* to industrially-relevant glycerol loadings (10% w/v, Fig. 4.5). We had previously observed robust growth of the wild-type strain of *C. cellobioparum* up to 3% glycerol and slower growth at up to 7% concentrations (Fig. 4.4A). Consequently, adaptive evolution was initiated at glycerol loadings of 6.3%, where growth rates were diminished but growth was sustainable over many serial transfers. The strain was continually subcultured from stationary phase cells in order to take advantage of the higher mutation rates induced by the expression of the error-prone, stationary phase DNA polymerase IV, which lacks

5'-3' proofreading ability. The increased rates of mutation in the stationary phase cultures allowed us to select for strains with "growth advantage in stationary phase" (GASP) mutations (16). The strain was transferred in medium containing 6.3% glycerol for ca. 2 months. During this period of time, the cultures reached stationary phase faster (from 16 to 7 days) (Fig. 4.5A), as a result of decreases in the duration of the *lag* phase from 120 to 24 h (Fig. 4.5B), and increases in growth rates from 0.5 to 0.8 d^{-1} (Fig. 4.5C). Furthermore, growth yields also increased 2-fold (from 0.40 to 0.81 OD_{660} units) during the evolution experiment (Fig.4.5D).

The culture that was adapted to faster growth with 6.3% concentrations of glycerol was then transferred to cultures with 8.8% glycerol concentrations. After ca. 1 month the cultures recovered the growth rates (0.9 d^{-1}), reduced *lag* phases (24 h) and robustness (7 days from inoculation to stationary phase) of the 6.3% cultures while maintaining growth yields of 0.64 OD_{660} units (Fig 4.5). The glycerol concentration was then increased to the industry target of 10%. Although the initial growth rates were lower (0.7 d^{-1}), they improved (1.3 d^{-1}) and were maintained stably after serial transfers for approximately 13 months (Fig. 4.5). At the end of the adaptive evolution experiment, the growth time to stationary phase was reduced to 4 days and the *lag* time to 12 h, whereas the growth yields increased to 0.93 (OD_{660} units).

Following the adaptive evolution of *C. cellobioparum* for enhanced growth with glycerol, five single colonies were isolated in roll-tubes (22) and subcultured 3 times to ensure the purity of the strains. Each clonal culture was tested for growth, glycerol

consumption, and fermentation product yields on 10% (w/v) glycerol-containing media. The best performing strain grew at a rate of 1.1 d^{-1}, consumed 3.5% of the glycerol provided (or the equivalent of 37.9 ± 1.8 mM) and produced 39.5 (± 0.9) mM ethanol, 21.2 (± 1.8) mM formate, 5.5 (± 0.3) mM H_2 and 5.9 (± 0.4) mM CO_2. Interestingly, unlike the wild-type strain (Fig. 4.3B), no lactate or acetate was produced. Thus, the adapted strain had become optimized for stoichiometric ethanol production with the concomitant formation of formate, H_2 and CO_2. The strain was designated $CcelA_{10G}$ and chosen for further studies.

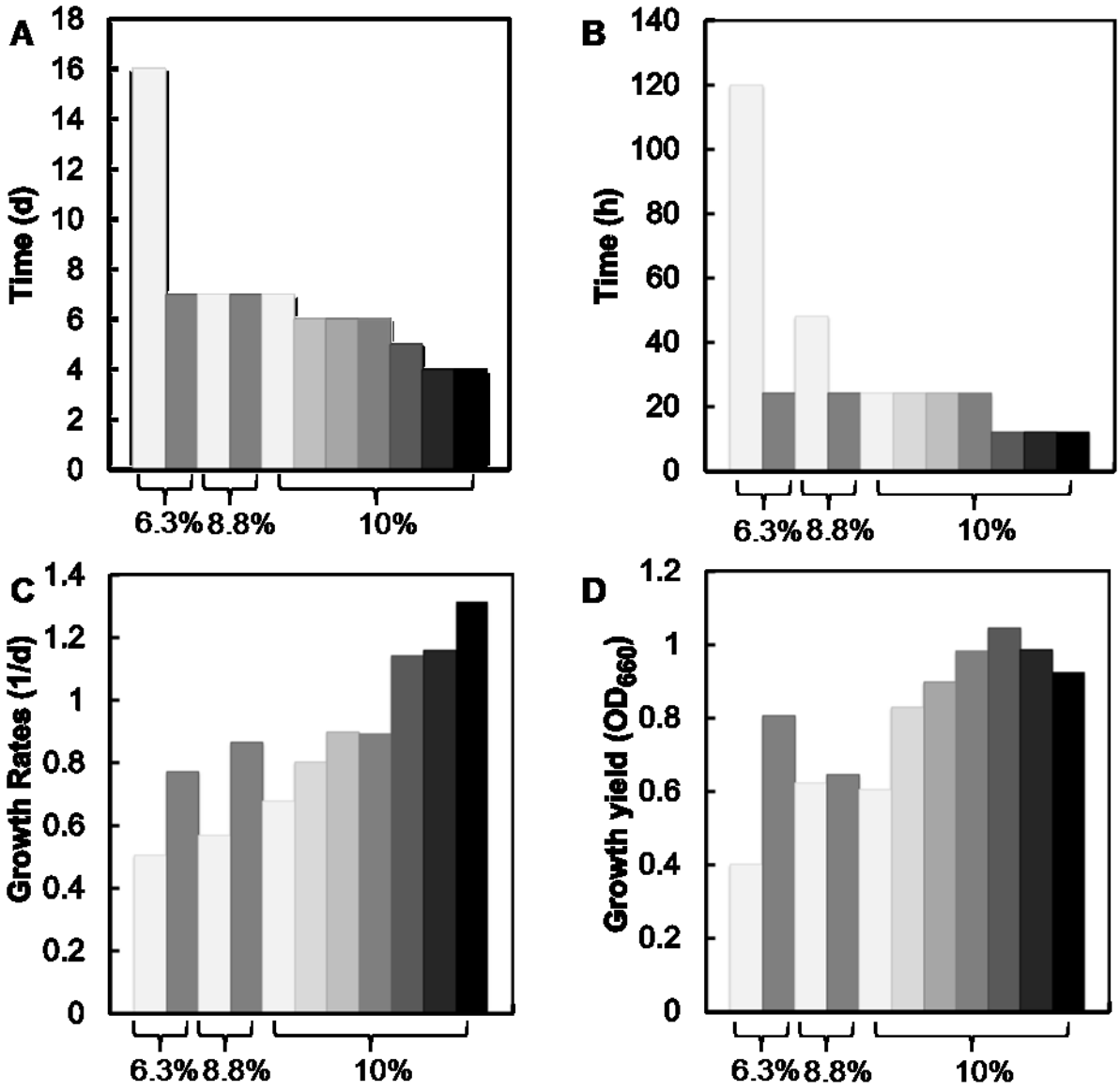

Figure 4.5: Adaptive evolution of glycerol tolerance in *C. cellobioparum*. Points shown are representative transfers at approximately 2-month intervals of the adaptive evolution at 6.3% (w/v) glycerol, 8.8% (w/v) glycerol and 10% (w/v) glycerol. (A) Time the cultures took to grow to stationary phase; (B) duration of the *lag* phase; growth rate (C) and growth yield (D) determined from OD_{660} of planktonic growth.

4.4.6 Adaptive evolution of *G. sulfurreducens*

Adaptive evolution was also used to improve the tolerance of *G. sulfurreducens* to high concentrations of alcohol. As *G. sulfurreducens* would be exposed to high ethanol concentrations during coculture with *C. cellobioparum*, and because an ethanol-tolerant *G. sulfurreducens* strain would also be a valuable component of the consolidated bioprocessing platform described in Chapter 3, ethanol tolerance was the first goal of the adaptive evolution. The tolerance of the wild-type strain to ethanol was investigated with increasing concentrations of ethanol from 0 to 5% (v/v) (Fig. 4.6A). Growth rates decreased dramatically to 37% of the maximum as ethanol concentrations increased from 0 to 1%, and continued to decline steadily until reaching the inhibitory concentration of 5%, where no growth was observed. The adaptive evolution experiment was therefore started at 1% ethanol loading. The strain was continually transferred from cells in stationary phase cultures to take advantage of GASP mutations as described above for *C. cellobioparum.* Cultures were serially transferred a minimum of 7 times at any given concentration of ethanol before transferring it to cultures 0.5% higher concentrations of ethanol. After approximately 10 months, the strain was able to stably sustain growth in media containing 5% ethanol. At this point, the cultures were plated in solidified medium in an anaerobic glove bag to isolate individual colonies. Ten colonies were subcultured three times to ensure that they came from a monoclonal population. The ten strains were then grown in the presence and absence of ethanol to identify the strain with the most robust growth. Interestingly, two growth phenotypes were observed for the clonal variants: three of the strains grew planktonically whereas the remaining seven strains grew as microcolonies attached to the walls of the glass

tubes (Fig 4.6B). The strain with the highest growth rate in the presence (0.6 d^{-1}) and absence (4.7 d^{-1}) of ethanol ($GsulA_{5E}$) was among those that grew planktonically and was chosen for further studies. Ethanol concentrations in the cultures of $GsulA_{5E}$ grown with acetate and fumarate did not decrease during the course of the experiment (data not shown), indicating that ethanol was not being used as a source of carbon or as an electron donor by $GsulA_{5E}$.

$GsulA_{5E}$ was tested for ethanol tolerance at varying concentrations (0-6%) as described above for the wild-type strain (Fig. 4.6A). Interestingly, the growth rate of the adapted strain in the absence of ethanol was 1.3-fold higher compared to the wild-type strain (Fig. 4.6A), although the growth yields were lower (0.62 ± 0.01, OD_{660}) than the wild-type strain (0.74 ± 0.02, OD_{660}). Although the growth rates at 2 and 3% ethanol concentrations were 63 and 45%, respectively, of the maximum, they were 4-fold higher than the wild-type strain rates at the same ethanol concentrations (Fig. 4.6A). The growth yields of the $GsulA_{5E}$ at 2% ethanol (0.47 ± 0.02, OD_{660}) were similar to those of the wild-type (0.48 ± 0.02, OD_{660}), but higher (0.46 ± 0.01, OD_{660}) than the wild-type strain (0.33 ± 0.02, OD_{660}) at 3% concentrations. And while the wild-type strain growth was inhibited at 5% concentrations of ethanol, the adapted strain grew with both 5% (0.38 ± 0.02, OD_{660}) and even 6% ethanol, although with reduced yields (0.19 ± 0.04, OD_{660}).

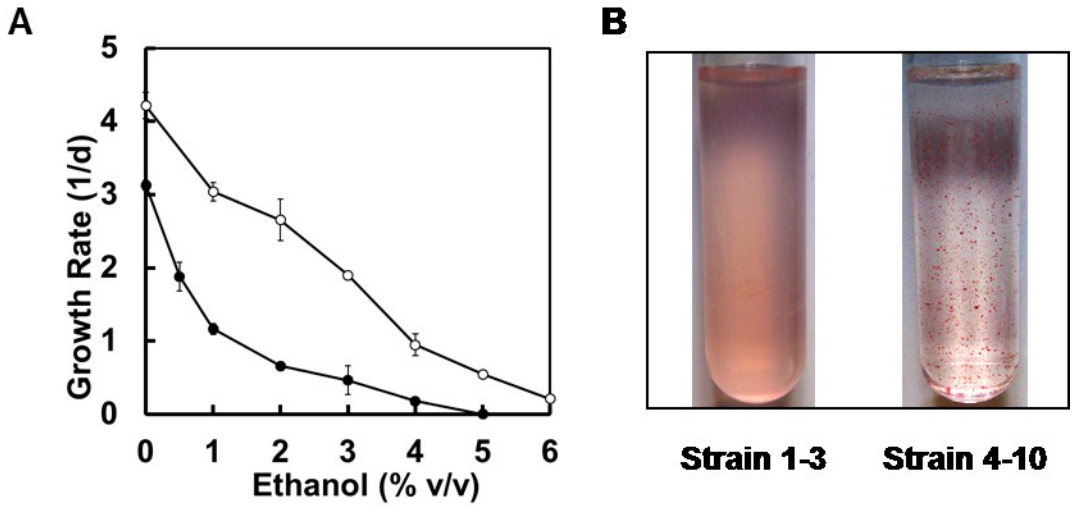

Figure 4.6: (A) Tolerance of wild-type *G. sulfurreducens* (solid symbols) and the ethanol-adapted strain ($GsulA_{5E}$, open symbols) to increasing concentrations of ethanol. (B) Growth phenotypes obtained from the adaptive evolution of *G. sulfurreducens* for ethanol tolerance. Three strains grew planktonically (left image) while seven grew as microcolonies (right image).

4.4.7 Glycerol tolerance of $GsulA_{5E}$ and $CcelA_{10G}$

It was hypothesized that the ethanol-adapted strain $GsulA_{5E}$ would display a nonspecific tolerance to other alcohols, including glycerol. The tolerance was tested as described previously for the wild-type strain at glycerol concentrations between 0 and 10% (w/v) (Fig. 4.4A). The growth rate for $GsulA_{5E}$ was similar to the wild-type at glycerol concentrations between 0 and 5%. At higher concentrations of glycerol, decreases in growth rate up to 61% of the maximum as glycerol concentrations were observed. At glycerol concentrations greater than 7%, the wild-type strain was unable to grow whereas $GsulA_{5E}$ was able to sustain growth rates greater than 39% of the maximum (Fig. 4.7A). $CcelA_{10G}$ was also tested for glycerol tolerance. The growth rate of the adapted strain increased 2-fold as the glycerol concentration increased from 0.6% to 2.5%, and was constant at approximately 1.0 d^{-1} between glycerol concentrations of 2.5% and 10% (Fig. 4.7A). This represents a significant improvement compared to the wild-type strain, which was unable to sustain growth at 10% glycerol loadings (Fig. 4.4A).

The adapted strains $GsulA_{5E}$ and $CcelA_{10G}$ were grown together at varying concentrations of glycerol to investigate the tolerance of the coculture to glycerol (Fig. 4.7A). The growth rates of both the wild-type coculture (Fig. 4.4A) and the adapted coculture increased 1.6-fold as concentrations of glycerol increased from approximately 0.5 to 3%. At 5% and above, the adapted coculture out-performed the wild-type coculture with growth rates that only decreased to 76% of the maximum at 10%

glycerol. Interestingly, the growth rate of the adapted coculture at 10% glycerol (1.5 ± 0.1 d^{-1}, Fig. 4.7A) was higher than that of either the $CcelA_{10G}$ or $GsulA_{5E}$ monocultures (1.1 ± 0.1 d^{-1} and 0.9 ± 0.1 d^{-1}, respectively), suggesting that the growth was stimulated by the synergistic action of the two strains. Consistent with this, glycerol consumption (Fig. 4.7B) and ethanol production (Fig. 4.7C) were both stimulated approximately 1.4-fold in the coculture compared with $CcelA_{10G}$ monocultures when starting glycerol concentrations were high (i.e. greater than 5% w/v).

In addition to experiments in batch culture, the effect of 10% glycerol loadings on the $GsulA_{5E}$ anode biofilms in MECs was investigated (Fig. 4.8). The anode biofilms were first grown with medium containing 1 mM acetate. When the acetate was depleted and the current declined to less than 0.1 mA, the medium in the anode was replaced with fresh medium containing 1 mM acetate with or without 10% glycerol. The current immediately resumed in all the fuel cells regardless of the presence of glycerol. While the MECs without glycerol reached a higher current (1.46 ± 0.01 mA) than those with glycerol (1.12 ± 0.02 mA), the coulombic efficiency for acetate conversion to current was similar in the presence (92 ± 1%) or absence (88 ± 2%) of glycerol. No glycerol was consumed over the duration of the experiment. These results indicate that the electronic efficiency of $GsulA_{5E}$ anode biofilms is not inhibited by 10% glycerol loadings.

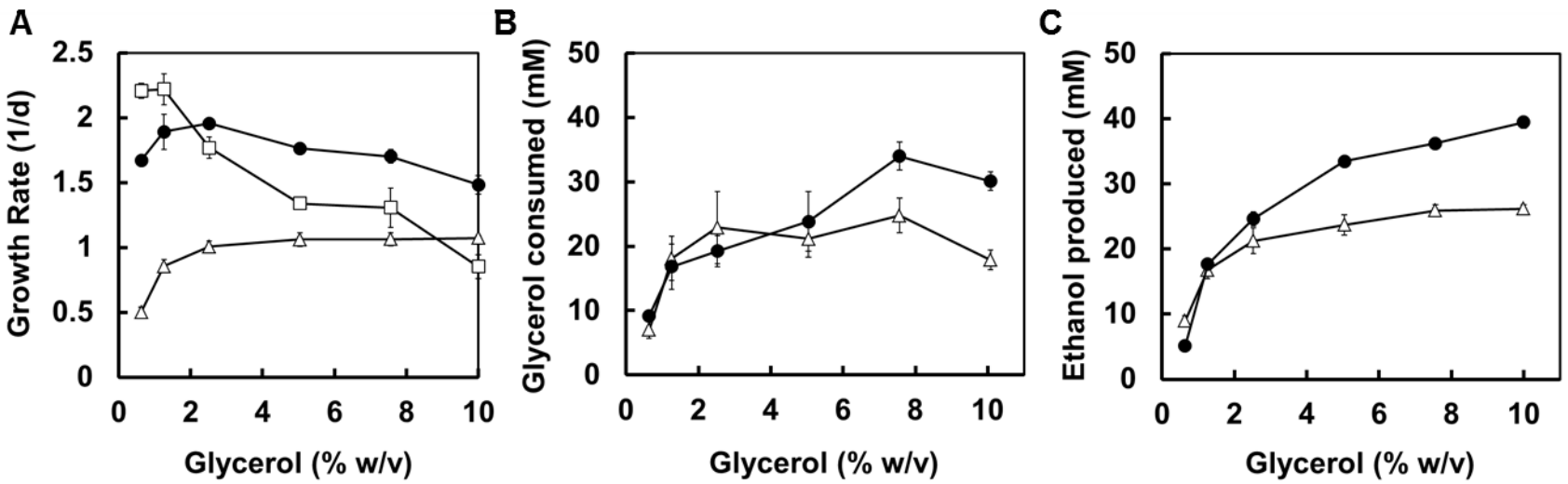

Figure 4.7: (A) Growth rates of CcelA$_{10G}$ (open triangles), GsulA$_{5E}$ (open squares) and the coculture of the adapted strains (solid circles) when grown in increasing concentrations of glycerol. (B-C) The amount of glycerol consumed (B) and ethanol produced (C) in the CcelA$_{10G}$ monocultures (open triangles) and cocultures (solid circles) shown in (A). Error bars show standard deviations from three replicate cultures.

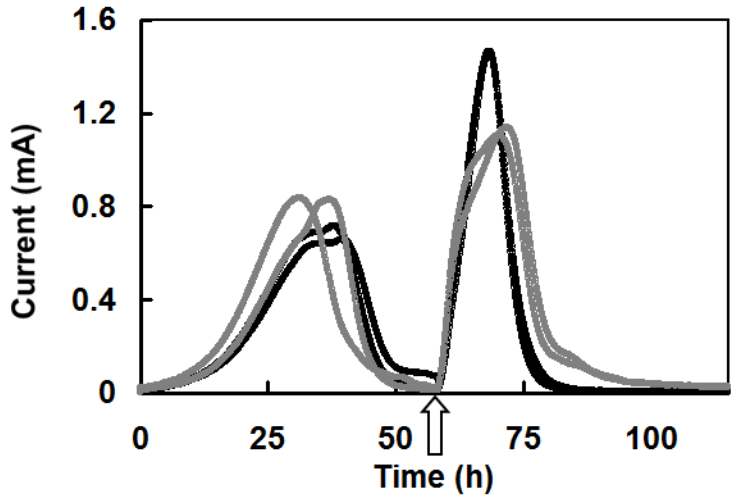

Figure 4.8: Tolerance of $GsulA_{5E}$ in anode biofilms to glycerol in a MEC. $GsulA_{5E}$ was initially grown with 1 mM acetate. When the acetate was consumed and the current declined, the media was replaced (arrow) with media containing 1 mM acetate (black lines, two replicate experiments shown) or 1 mM acetate and 10% (w/v) glycerol (gray lines, two replicate experiments shown).

4.4.8 MECs with 10% (w/v) glycerol loading

The adapted strains, $GsulA_{5E}$ and $CcelA_{10G}$, were shown to be tolerant to industrially relevant glycerol concentrations (10% w/v) and to syntrophically couple glycerol fermentation by $CcelA_{10G}$ with fumarate reduction by $GsulA_{5E}$ in batch cultures (Fig. 4.7A). We next investigated the ability of the coculture to couple the fermentation of 10% glycerol with current production in a MEC. As described previously, a sequential inoculation strategy was followed in which $GsulA_{5E}$ was first inoculated into the MEC

and supplemented with 1 mM acetate to provide optimal conditions for biofilm formation (Fig. 4.9A). The current increased exponentially at a rate of 6.9 (± 1.0) d^{-1}, peaked at 1.1 (± 0.2) mA after approximately 30 h, and then declined once the acetate was consumed. When the current was below 0.1 mA, the anode medium was replaced with GS3 containing 10% (w/v) glycerol, and the MEC anode was inoculated with $CcelA_{10G}$ (Fig. 4.9A). Following inoculation with $CcelA_{10G}$, the current immediately resumed and peaked at 1.4 (± 0.2) mA before declining to below 0.2 mA over a period of ca. 3 days, producing 2.0 (± 0.2) mmol e^- (Fig. 4.9B). For comparison, $CcelA_{10G}$ was grown as a monoculture in the MECs, however no current production was observed (not shown). At the end of the experiment, the anode electrode was removed from the MEC, stained with fluorescent dyes that differentially stain Gram positive (red) and Gram negative (green) cells, and imaged using confocal microscopy (Fig. 4.9C). Both red ($CcelA_{10G}$) and green ($GsulA_{5E}$) cells were visible on the anode electrode with the $GsulA_{5E}$ positioned closer to the electrode and $CcelA_{10G}$ forming a layer on top. While the anode culture broth was visibly turbid, a result of $CcelA_{10G}$ growth, the close proximity of the cells on the anode electrode may be facilitating interspecies metabolite transfer.

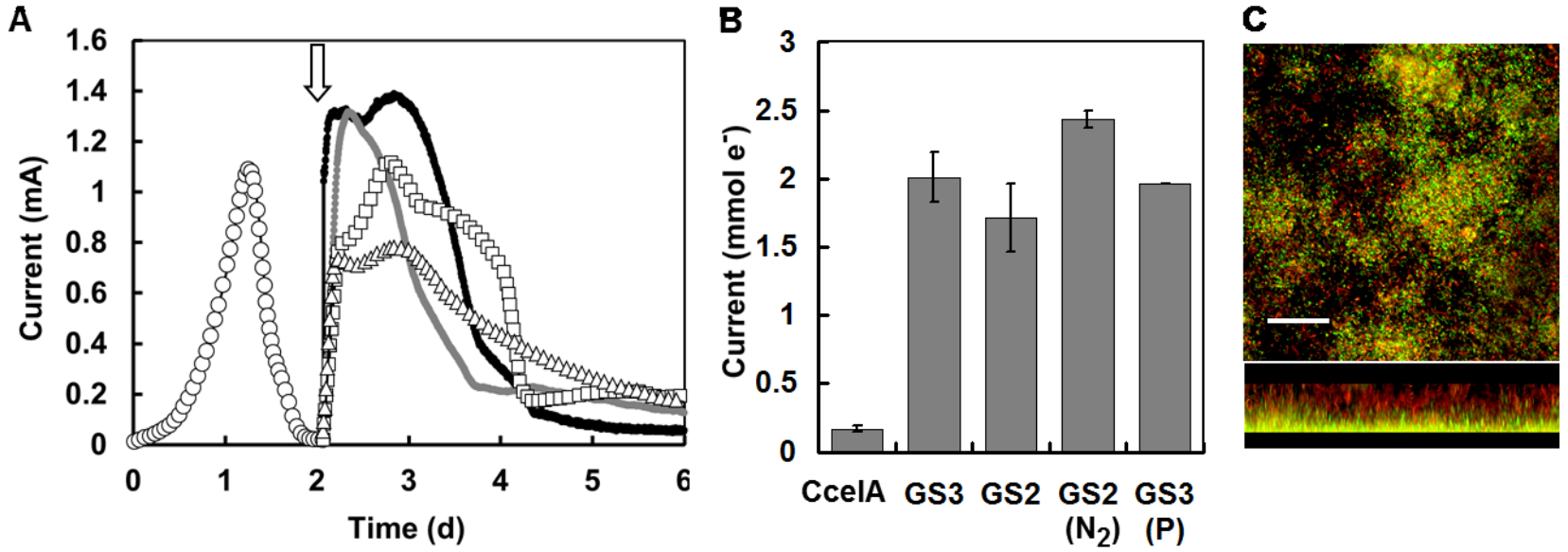

Figure 4.9: Current production of sequentially inoculated MECs. (A) $GsulA_{5E}$ was inoculated first and supplemented with 1 mM acetate (open circles), the medium was then exchanged (arrow) for GS3 (black line), GS2 (grey line), GS3 with 200 mM phosphate buffer (open triangles), or GS2 medium that was then sparged with N_2 over the duration of the experiment (open squares). All MECs were supplemented with 10% (w/v) glycerol and inoculated with $CcelA_{10G}$. (B) Current production following the inoculation of $CcelA_{10G}$ into the MEC and the addition of 10% (w/v) glycerol. $CcelA_{10G}$

Figure 4.9 (cont'd) monocultures, Ccel; MECs sparged continuously with N_2; MECs with 200 mM phosphate buffer, P. (C) The anode biofilm shown in panel A (black line) was stained with the BacLight Gram Stain Kit (green, Gram negative, *G. sulfurreducens*; red, Gram positive, *C. cellobioparum*) and imaged with CLSM. Top view and corresponding projection in the *x* axis (bottom) is shown; scale bar, 10 μm.

At the end of the MEC experiment when the current had declined below 0.1 mA, the composition of the anode supernatant was analyzed by HPLC and compared to $CcelA_{10G}$ negative controls grown in GS2-10% glycerol medium in the MECs in the presence and absence of a poised anode electrode (Fig. 4.10B). Although less than 0.2 mmol e^- were produced from $CcelA_{10G}$ grown with a poised electrode (Fig 4.9B), growth in the presence of the poised anode increased glycerol consumption 1.9-fold from 72.3 (± 18.8) mM to 140.5 (± 53.8) mM (Fig. 4.10A). Ethanol production also increased by 2.2-fold from 46.2 (± 10.1) mM to 101.8 (± 43.9) mM. This improved performance is accompanied with an increase in pH from 5.3 (± 0.4) to 5.7 (± 0.4) (Fig. 4.10A), although the cause of the pH increase is uncertain. Both $CcelA_{10G}$ cultures grown in the MECs produced acetate and lactate which was unexpected as previous experiments with $CcelA_{10G}$ grown in batch culture in 10 ml pressure tubes following the adapted evolution produced only ethanol, formate, H_2 and CO_2 produced.

Glycerol consumption was similar in the MEC with the CcelA$_{10G}$ monoculture with a poised electrode and the MEC pregrown with GsulA$_{5E}$ (151.6 ± 10.4 mM; Fig. 4.10A) both in GS3. Interestingly, ethanol production decreased in the coculture (29.0 ± 2.8 mM) due to some of the carbon being shunted to the formation of 1,3-propanediol (22.7 ± 4.4 mM) and propionate (7.7 ± 1.0 mM), which were not produced in any of the CcelA$_{10G}$ monocultures (Fig. 4.10B). The production of ethanol, acetate and lactate began immediately after inoculation of CcelA$_{10G}$ into the anode chamber with GsulA$_{5E}$, while the production of propionate and 1,3-propanediol were first detected in the culture broth approximately 5 days after inoculation and at the same time as the ethanol production was reaching a plateau. This suggests that there was metabolic switch that diverted fluxes away from the production of ethanol and toward 1,3-propanediol and propionate (Fig. 4.2). The energy recoveries for the CcelA$_{10G}$ monocultures were high (poised anode, 62.4 ± 2.3%; unpoised anode, 57.6 ± 3.2%) because approximately 68% of the glycerol was converted to ethanol. The energy recoveries in the MEC, calculated assuming cathodic H_2 recovery efficiencies of 72% as described previously (49), were lower (16 ± 2%) because only ca. 19% of the glycerol consumed was recovered as ethanol. The energy recovered from the fermentation alone made up 15% (3.44 ± 0.22 kJ) of the energy recovered while cathodic H_2 production from the MEC contributed only 1% (0.27 ± 0.03 kJ).

Current production ceased in the MEC even though acetate, lactate, formate and H_2 remained in the anode chamber at the end of the experiment (Fig. 4.10B), indicating

that something was limiting the performance of the *G. sulfurreducens* anode biofilm. The pH of the MEC anode broth at the end of the experiment was 5.1 (± 0.1), which was substantially lower than that of the $CcelA_{10G}$ monoculture (pH, 5.7 ± 0.4). Because the thermodynamics of energy conversions at the anode are sensitive to pH changes (28, 37), we hypothesized that this had caused the drop in current production despite the abundance of electron donors available for *G. sulfurreducens*. Consequently, we repeated the MEC experiments and used MOPS-buffered GS2 with 10% glycerol to replace the medium in the anode chamber at the time of $CcelA_{10G}$ inoculation (Fig. 4.9). As before, the current increased immediately upon inoculation of the $CcelA_{10G}$, peaking at 1.3 (± 0.1) mA before decreasing over a period of ca. 3 d to <0.2 mA. The pH of the anode broth at the end of the experiment (5.1 ± 0.2) was similar to that of the GS3 medium despite the additional buffering. This was possibly due to the increased accumulation of CO_2 in the anode chamber (26.0 ± 3.4 mM) compared with the GS3 MECs (8.0 ± 0.4 mM), which, when dissolved into the medium, results in the production of carbonic acid. Likely as a consequence of the low pH, the predicted increase in current production was not observed (Fig. 4.9B). There was, however, a 1.3-fold increase in the amount of glycerol consumed (193.5 ± 7.1 mM), and a 2.2-fold increase in total fermentation products detected at the end of the experiment (Fig. 4.10). The percent of the glycerol consumed that was recovered as ethanol increased to ca. 30%; the energy recoveries also increased accordingly to 27% (± 2). As seen previously, the energy recoveries were primarily due to the fermentation (26%) with only a small contribution from the MEC (1%).

Because we suspected that CO_2 accumulation in the anode headspace resulted in the acidification of the medium, we repeated the MEC experiment with GS2 as described above, and following the inoculation of $CcelA_{10G}$, the anode and cathode media were sparged continuously with N_2 gas (Fig. 4.7). As before, the current immediately resumed upon inoculation of $CcelA_{10G}$, reaching ca. 0.8 mA, however it took an additional day for the current to reach a maximum of 1.1 (± 0.1) mA. This difference is likely due to the fact that hydrogen was removed from the anode vessel by sparging, and that it took some time for the $CcelA_{10G}$ to start fermenting glycerol into products that could be used as electron donors by $GsulA_{5E}$. The current declined to ca. 0.2 mA over a period of 3.7 days and was maintained at ca. 0.2 mA for approximately 6 days. It was this sustained low level of current production that resulted in higher electron recoveries (2.4 ± 0.1 mmol e^-) (Fig. 4.9B) than were observed in either of the other MEC experiments. Removing the H_2 by sparging the anode did not decrease the current production, which indicates that *G. sulfurreducens* was preferentially using the organic acids as electron donors. However, lactate (5.8 ± 0.2 mM) and acetate (28.2 ± 5.9 mM) still remained in the anode supernatant at the end of the experiment and were not converted into current. As expected, removing CO_2 resulted in increased pH (6.0 ± 0.2, Fig. 4.10A) compared with MOPS-buffered medium alone (5.1 ± 0.2). The glycerol consumed by $CcelA_{10G}$ increased 2.4-fold compared with the unsparged GS2 MEC, and ca. 43% of the glycerol that was provided was removed (Fig. 4.10A). This increase

is likely due to the removal of H_2 as a feedback inhibitor of the fermentation (8). Correspondingly, 1.7-fold more fermentation products were detected at the end of the experiment, however this number does not include the H_2 and CO_2 that were produced, which were removed by sparging. Interestingly, formate was not detected at the end of the experiment. As H_2 and CO_2 are in equilibrium with formate through the action of formate dehydrogenase, their removal probably drove the reaction in the direction of H_2 and CO_2 production. Some formate may also have been removed by *G. sulfurreducens.* Approximately 40% of the glycerol consumed was recovered as ethanol (Fig. 4.10) or the equivalent of 187.8 (± 48.5) mM, although it is worth noting that this may be an underestimate of ethanol production because some ethanol may have evaporated during the sparging. Energy recoveries also increased compared with the GS2 medium without sparging to 34% (± 8); 33.6% was a result of the fermentation while 0.4% was from cathodic H_2 production in the MEC.

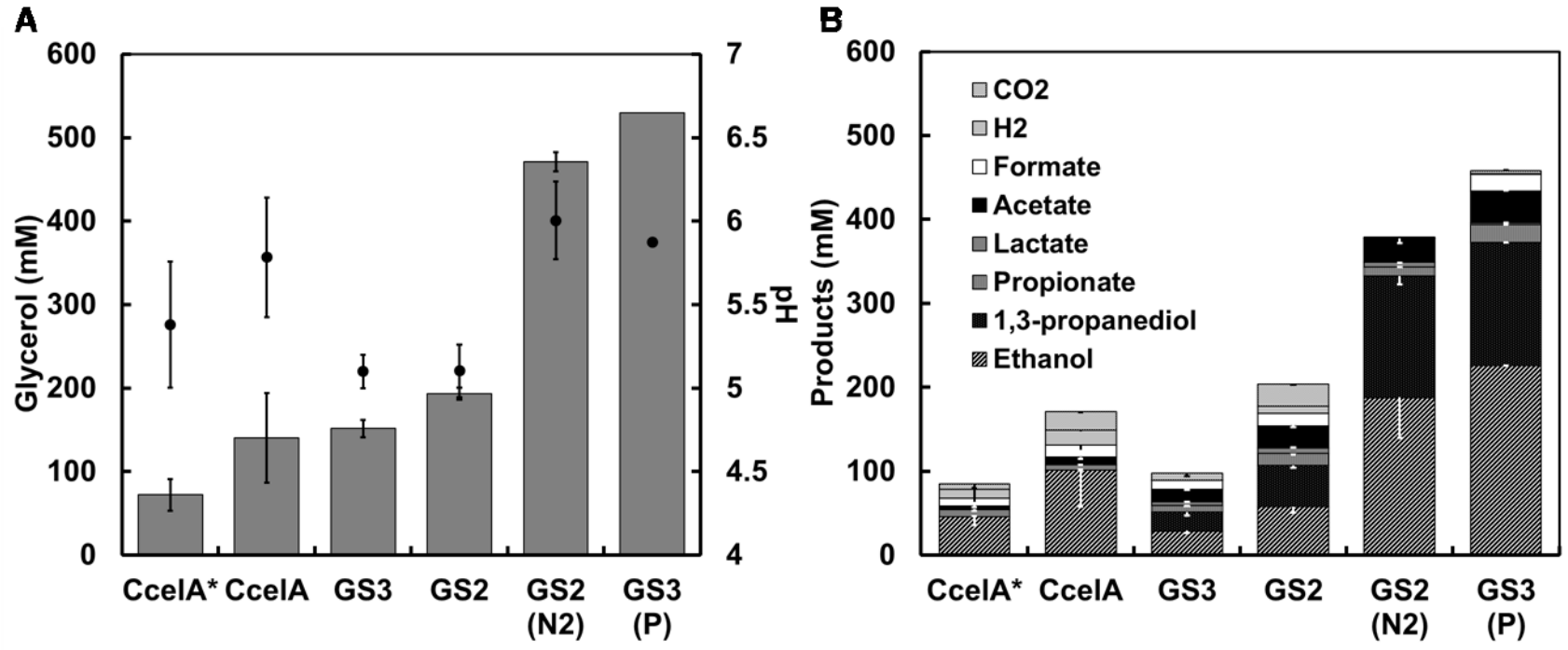

Figure 4.10: Glycerol consumed (bars in panel A), fermentation products produced (B) and final pH (line in panel A) measured at the end of the experiment in each of the MECs shown in Fig. 4.9A (N_2, MECs sparged continuously; P, MECs with 200 mM phosphate buffer). Monocultures of $CcelA_{10G}$ grown in GS3 medium with 10% glycerol in MECs with (CcelA) and without (CcelA*) a poised anode electrode are shown for comparison.

MOPS buffer is a useful laboratory chemical that, because of its price, does not lend itself well to widespread industrial applications. We therefore tested the effectiveness of 200 mM phosphate buffered GS3 medium in the MECs (Fig. 4.9A). As seen previously, the current immediately resumed following the replacement of the medium and inoculation of $CcelA_{10G}$, however the maximum current production (ca. 0.77 mA) was only 60% of that seen in the MECs with standard GS3 medium (Fig 4.9A), suggesting that the phosphate buffer was detrimental to the *G. sulfurreducens* anode biofilm. Consistent with this, large amounts of electron donors (lactate, 2.2 mM; acetate, 37.8 mM; formate, 19.8 mM) remained in the supernatant after the current production stopped. Despite this difference, the total current production (ca. 2 mmol e^-) was similar to that of GS3 medium without the phosphate buffer (Fig. 4.9B). The pH of the anode medium at the end of the experiment (5.9) was similar to that of the MEC that was run with continuous sparging (Fig. 4.10A), demonstrating that the phosphate buffer was effective at counteracting the fermentative CO_2 production. Glycerol consumption was stimulated 3.5-fold compared to standard GS3 medium, and approximately 50% of the glycerol that was provided in the medium was consumed (Fig. 4.10A). 43% of the glycerol consumed was converted to ethanol and correspondingly the energy recovery increased to ca. 36%. Consistent with the poor conversion efficiency of the electron donors into current, only 0.2% of the energy recovery was due to the MEC.

4.5 DISCUSSION

In this chapter, we identify *C. cellobioparum* as a robust glycerol fermenting, ethanologen. Consumption of glycerol (29 ± 1 mM) matched ethanol production (31 ± 1 mM) corresponding to a 1:1 fermentation yield showing great potential as an industrial strain. *C. cellobioparum* is a cellulolytic strain within Cluster III of the *Clostridiaceae* family (60) later reclassified as *Ruminococcaceae* (30). To our knowledge, no other strain in this clade is able to ferment glycerol. *C. papyrosolvens*, another member of this cluster, was tested in this study and no glycerol fermentation was observed.

C. cellobioparum demonstrated robust growth at low concentrations of glycerol. Growth rates increased 1.4-fold as glycerol concentrations were increased from 0.25 to 1% (w/v) due to the additional substrate provided (Fig. 4.4A). However, as glycerol concentration increased (3%-7% (w/v)), growth was negatively affected (Fig. 4.4A), likely due to the increases in viscosity and osmotic pressure of the medium (58). The cell membrane is semi-permeable to glycerol, and at high concentrations, glycerol can also enter the cell and affect enzyme activity (58). No growth was observed at 10% (w/v) glycerol (Fig. 4.4A). As crude glycerol wastewater is generally at least 10% glycerol (w/v), *C. cellobioparum* was adaptively evolved on increasing concentrations of glycerol (Fig. 4.5). Over the course of 16 months, *C. cellobioparum* developed robust growth at 10% (w/v) glycerol. Overtime, growth rates and yields increased (Fig. 4.5C-D), while lag phase and duration of fermentation (time to stationary) decreased, resulting in a glycerol-tolerant strain ($CcelA_{10G}$) able to ferment industrially relevant glycerol loadings.

G. sulfurreducens was more sensitive than *C. cellobioparum* to glycerol, with growth rates decreasing dramatically at glycerol concentrations above 3.8% (w/v). Adaptive evolution of alcohol tolerance in *G. sulfurreducens* resulted in a strain tolerant to 5% (v/v) ethanol and 10% (w/v) glycerol ($GsulA_{5E}$), which could be grown in coculture with *C. cellobioparum* at high glycerol loadings and at high rates of ethanologenesis (Fig. 4.6A and 4.7A). In general, adaptations that confer solvent tolerance are complex, requiring multiple genetic changes, which affect general cellular stress response mechanisms as well as changes in membrane fatty acid, phospholipids and protein composition (25, 46, 62).

In addition to ethanol, wild-type *C. cellobioparum* also produced lactate, acetate, formate, hydrogen, and CO_2 via fermentation of glycerol (Fig. 4.3B). Interestingly, the adapted strain, $CcelA_{10G}$, only produced ethanol, formate, hydrogen, and CO_2 from glycerol fermentation. Lactate and acetate were no longer observed in improved fermentation efficiency of glycerol to ethanol with the concomitant production of formate hydrogen, and CO_2. At higher glycerol concentrations (3.8% (w/v) for WT and 10% (w/v) for $CcelA_{10G}$), both strains only metabolized a fraction of the glycerol provided. Fermentation products can act as feedback inhibitors and acidify the medium preventing further glycerol metabolism. Hydrogen, in particular, has been shown to inhibit growth of *C. cellobioparum* (8). The ability of WT *C. cellobioparum* and WT *G. sulfurreducens* to grow syntrophically was demonstrated on low concentrations of glycerol (0.25% (w/v)) using fumarate as the terminal electron acceptor (Fig. 4.3A). Acetate, formate, and most of the hydrogen produced by *C. cellobioparum* were consumed by *G. sulfurreducens* in

the coculture, leaving behind ethanol and lactate. Following adaptive evolution of the two strains, syntrophic growth was investigated at high glycerol loadings. Growth rates of the adapted consortia exceeded the growth rates of either species alone (Fig. 4.7A). Additionally, glycerol consumption and ethanol production increased in the coculture compared with the $CcelA_{10G}$ monoculture when the starting glycerol concentration was above 5% (w/v) (Fig. 4.7B-C). This suggests that the consumption of fermentation products by $GsulA_{5E}$ decreased feedback inhibition and stimulated the fermentation of $CcelA_{10G}$.

The ability of *G. sulfurreducens* to consume fermentation products in batch coculture was limited by the amount of fumarate provided as the terminal electron acceptor. However, *G. sulfurreducens* can consume fermentative byproducts and convert them to electrical current in MECs where an electrode acts as an unlimited electron acceptor. Additionally, the energy lost in the fermentative byproducts (i.e., non-ethanol products) can be recovered at the cathode as H_2 fuel, thereby contributing to the overall energy recovery of the system. Syntrophic growth of WT *C. cellobioparum* and WT *G. sulfurreducens* with glycerol (3.8% (w/v) was demonstrated in a sequentially inoculated MEC, and resulted in current production (and therefore cathodic H_2 production) by *G. sulfurreducens* (Fig. 4.4B). Glycerol consumption and ethanol production were improved within the MEC compared to *C. cellobioparum* in batch culture (Fig. 4.4C).

The adapted strains were also tested in the MECs. Controls of CcelA$_{10G}$ were grown alone in the MEC with and without a poised anode electrode to test for current production by the fermentative strain. Some species of *Clostridia* such as *C. propionicum* have been shown to produce current with the help of mediators (63). Under the conditions used in our system, CcelA$_{10G}$ does not appear capable of current production (Fig. 4.9B). However, an increase in glycerol fermentation compared to the batch culture was observed when a positive potential of 0.24 V was applied at the anode. Additionally, the unpoised electrode resulted in a further increase in glycerol consumption and ethanol production (Fig. 4.10) although energy recoveries were similar (62.4 and 57.9%, respectively). Despite this improvement, only ca. 20% of the glycerol was consumed by CcelA$_{10G}$ (Fig. 4.10A).

Co-culturing the adapted strains in the MECs in GS3 medium resulted in increased current production due to the presence of GsulA$_{5E}$ (Fig. 4.9B), however, the energy recovery was lower (16%) compared to the CcelA$_{10G}$ monoculture, this is a result of redirection of fermentation products away from ethanol to 1,3-propanediol and propionate. Energy recovery in this system was due primarily to ethanol production, and only 19% of the glycerol consumed went towards ethanol, compared to 68% in the monocultures. Furthermore, the pH at the end of the MEC run was low (5.1), which we believe contributed to both the decline in current production by GsulA$_{5E}$ and potentially the diversion of products away from organic acids by CcelA$_{10G}$. The nature of this metabolic shift is not yet understood and would benefit from further investigation

through the use of enzyme activity assays and the quantification of intracellular $NADH/NAD^+$ pools as described previously (19).

Stabilization of culture pH was attempted using the addition of MOPS buffer (GS2), GS2 with sparging to eliminate CO_2 acidification of the media, and adding a high concentration of phosphate buffer to GS3 (P). GS3 medium (with MOPS) was unsuccessful at improving the pH (final pH 5.1). GS2 and continuous sparging and GS3 with phosphate buffer resulted in improved pHs of 6.0 and 5.9 respectively. While the presence of MOPS alone did not stabilize the pH of the medium, glycerol fermentation was stimulated 1.3-fold, and fermentation products increased 2.2-fold compared to the GS3 medium without MOPS. The conditions with improved pH (sparging and phosphate buffer, respectively) showed noted improvements in glycerol consumption (2.4- and 3.5-fold increases) and fermentation products (1.7- and 4.7-fold increases) respectively compared to their base medium (GS2 and GS3 respectively). The pH also influenced the metabolic shift. The lower pH cultures result in less ethanol produced as a percentage of glycerol consumed compared to the more pH controlled cultures. The ethanol conversion for the original MEC culture was 19%, the addition of MOPS improved it to 27%, the addition of sparging increased it to 40%, and the best fermentative scenario of phosphate buffering resulted in 50% conversion of glycerol consumed to ethanol. This corresponded to increases in energy recoveries as well (16, 27, 34, and 36% respectively).

Interestingly, $GsulA_{5E}$ current production was not greatly affected by the buffer optimization (Fig 4.9B). The increases in pH did seem to increase the duration of

current production (Fig 4.9A), but the total current produced did not increase. Several factors may be limiting current production by *G. sulfurreducens*. While pH stabilization was improved, the best scenarios still had a pH around 6. which is not optimal (28). The high concentration of glycerol is unlikely to have inhibited the current production of $GsulA_{5E}$, as control MECs with $GsulA_{5E}$ alone using acetate as the electron donor showed that the presence of 10% (w/v) glycerol did not decrease the coulombic efficiency (Fig. 4.8).

Genome sequencing of both of the adapted strains is underway to ascertain the genetic basis for our improved glycerol tolerance. Additionally, the sequence of $CcelA_{10G}$ and its parental strain could lead to metabolic engineering of *C. cellobioparum*. Currently, this strain does not have a genetic system, but closely related species *Clostridium thermocellum* (55) and *Clostridium cellulolyticum* do (4, 24, 52). The shift in metabolism creates an interesting potential of customized fermentation via metabolic engineering.

The primary fermentation product of *C. cellobioparum*, ethanol, is one of the main types of alternative fuel (41), but it can also be used as a feedstock for the transesterification process, thereby reducing production costs for biodiesel (5). The other fermentative products, propionate and 1,3-propanediol, which were produced in the MECs during coculture with *G. sulfurreducens* are marketable as well. Propionate is a chemical used in antifungal agents, herbicides, solvents and perfumes (31), and 1,3-propanediol is used as a precursor to renewable plastics (19, 45). 1,3-propanediol has great potential to improve the economics of the biodiesel industry and its production has been widely studied (2, 18, 31, 34). By determining the mechanism for the observed metabolic shift,

and/or by directly manipulating metabolism, we could potentially direct the fermentation of glycerol to the product that will return the most value to our wastewater treatment platform: ethanol as a feedstock to reduce production costs, ethanol as a fuel to recover energy, or propionate and 1,3-propanediol as commercial products to add value to the wastestream.

Genetic engineering of the *C. cellobioparum* strain could be used to target the metabolic pathway for either ethanol production or 1,3-propanediol production by knocking out the genes required for competing pathways. For example, eliminating the pathways to lactate, acetate, propionate and 1,3-propanediol production could result in increases in ethanol yields (46, 55), up to the maximum theoretical yields of 1 mol of ethanol per mol of glycerol. Alternatively, the highest theoretical yield for 1,3-propanediol (0.72 mol per mol glycerol) is achieved when acetate is coproduced for ATP synthesis (31). This could be achieved by eliminating the pathways for ethanol, lactate, formate, and propionate production. In either scenario of metabolic engineering, fermentative waste products are produced (formate and H_2 during ethanol fermentation, or acetate during 1,3-propanediol fermentation) which could be used by *G. sulfurreducens* for electricity production in BESs.

Energy recovery from fermentative and cathodic H_2 was also investigated, however, H_2 yields were low and therefore did not contribute much to our system (ca. 1%). Many of the electron donors available for *G. sulfurreducens* remained in the supernatant at the end of the experiments, indicating that something was limiting *G. sulfurreducens* current production. It is possible that some of the fermentative products

(e.g., propanediol or propionate) were inhibiting *G. sulfurreducens* metabolism and should be further investigated. Further improvements in the MEC system will be needed to improve energy recoveries from this part of the platform. Alterations in MEC design should be implemented and scaling up will be essential. Improvements will include removing expensive components such as the proton exchange membrane, as well as design features such as decreasing the distance between the anode and cathode electrodes, and increasing the ratio of anode surface area to reactor volume (28). In addition, better control of the pH conditions could prolong current production.

Currently, we use refined glycerol in our coculture MEC platform, but future directions for the research will include testing crude glycerol wastewater from the biodiesel industry. Glycerine wastewater has high salinity and contains contaminants that may inhibit the growth of *C. cellobioparum* and *G. sulfurreducens*. It will be interesting to see if the cellular improvements gained from glycerol tolerance (most likely membrane changes) will confer tolerance to more osmotic stress and harsh conditions of the wastestream.

Developing a use for the crude glycerol produced as a major byproduct of the biodiesel industry without the need to purify it has the potential to improve the economic viability of the industry. Conversion of crude glycerol into ethanol would have the added benefit of supplying the biodiesel refinery with up to 1/3rd of the ethanol feedstock required for the transesterification reaction, thereby reducing costs of the chemical inputs (presently 12% of the cost of biodiesel production) (1), and making the production of biodiesel increasingly independent of fossil fuel inputs. In this study, we have demonstrated interspecies metabolite transfer between *C. cellobioparum* and *G.*

sulfurreducens when grown with industrially relevant glycerol loadings, which resulted in the production of value-added products (ethanol, propionate, 1,3-propanediol, and H_2). Additionally, we have demonstrated notable improvements in our system that shows great potential for this platform as a method for wastewater treatment for the biodiesel industry.

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Chapter 5

Conclusions and Future Directions

5.1 CONCLUSIONS

Consolidated bioprocessing (CBP) microorganisms are key drivers of plant matter decomposition in terrestrial environments and show great promise for the industrial bioprocessing of agricultural wastes and plant-derived substrates into biofuels such as ethanol. One of the CPB microorganisms that I studied, *Cellulomonas uda*, serves as a great laboratory model for ecological studies that link carbon cycling to climate and anthropogenic nitrogen disturbances. Further understanding the mechanisms that enable this microorganism to finely tune its physiology to carbon and nitrogen inputs will provide valuable insights for the development of more sensitive climate models and remediation strategies for carbon mitigation.

The microorganisms and processes that enable the decomposition of plant matter in nature can also be harnessed in the bioenergy industry. We harnessed the cellulolytic and fermentative capabilities of *C. uda* to develop a novel platform for bioethanol and biohydrogen from agricultural wastes via consortia growth with *Geobacter sulfurreducens*. Using the knowledge gained on nitrogen responses of *C. uda*, our lab was able to improve the energy recoveries in this system with nitrogen supplementation (Allison M. Speers, personal communication). Despite high energy recoveries, limitations remained. Using operational and biological approaches, I was able to improve rates and yields of ethanol production of *C. uda* from AFEX-pretreated corn stover.

In a similar platform, we harnessed the ability of *C. cellobioparum* and *G. sulfurreducens* cocultures to process glycerol to ethanol and biohydrogen for the treatment of glycerin wastewaters derived from the biodiesel industry. Improvements in the operational parameters of the platform led to the consumption of up to 50 g/L of

glycerol and the production of the value-added products of ethanol, 1,3-propanediol, and biohydrogen. My research shows that understanding native capabilities of microorganisms is the first step to utilizing them in industry.

5.2 FUTURE DIRECTIONS

5.2.1 *Cellulomonas uda*

Carbon sequestration and substrate attachment in nutrient-limited conditions give *Cellulomonas* spp. an adaptive advantage. Specific colonization of cellulose substrates gives *C. uda* better access to both carbon and nitrogen derived from plant cell walls (Chapter 2). Understanding these mechanisms will provide potential targets for improved cellulose degradation in industry, which is necessary for high levels of ethanol production. Allison M. Speers observed low energy recovery under nitrogen limiting conditions in a platform using a coculture of *C. uda* and *G. sulfurreducens* to convert agricultural waste at low concentrations of Ammonia Fiber Expansion pretreated corn stover (0.2% (w/v) AFFEX-CS) into ethanol and hydrogen in a microbial electrolysis cell (MEC). This was attributed to carbon being used for storage (curdlan) instead of fermentative conversion to ethanol. Nitrogen supplementation resulted in improved energy recoveries (10).

Using this knowledge, I improved ethanol yields and rates of *C. uda* on AFEX-CS by adjusting the operational parameters, increasing the solid loading (up to 5% (w/v)), and adapting *C. uda* for fermentative productivity. Our system is still limited in terms of complete substrate hydrolysis and fermentative efficiency. We are in the process of developing a genetic system for this strain (Appendix A). We are currently sequencing

the genome of both the parental and adapted strains of *C. uda*. In addition, I have performed preliminary tests to reproduce the genetic methods used to transform *Cellulomonas flavigena* (8), that hopefully will be applicable to *C. uda* as well. These two strains are closely related (11), so I anticipate similar results in this strain. Currently, we have a method to make electrocompetent cells as well as an electroporation protocol that have both proved successful. Potential targets for strain improvement include enzymes and metabolic pathways. If the limitation in hydrolysis seen is due to a missing enzyme that would complete the AFEX-CS degradation, a simple gene insertion could improve the strain. Alternatively, if feedback inhibition is the limitation, overexpression of the enzymes already produced could increase hydrolysis. Both methods have been attempted for recombinant cellulolytic strategies (7). There are several strategies used to promote the production of ethanol by metabolic engineering. Enzymes can be added to redirect metabolism and diminish pyruvate accumulation (3), and knocking out genes responsible for organic acid formation (such as acetate and lactate) would decrease production of undesirable products and increase the production of ethanol (9).

Additionally, *G. sulfurreducens* in coculture with *C. uda* in MECs could result in improvements in *C. uda* fermentation, as we hypothesized feedback inhibition as a cause of limitations in fermentation yields of *C. uda* on AFEX-CS (Chapter 3). Consortia growth with *G. sulfurreducens* would reduce the feedback inhibition as acetate and formate are converted to hydrogen at the cathode. This has the potential to improve hydrolysis and fermentative yields as formate reduces xylanase activity (5) and acetate disrupts cellobiose utilization (2). Adjustments in MEC parameters will be necessary as

our current system does not work well with high solid loadings. A flow-through system in which *C. uda* and AFEX-CS are separate from the anode chamber of the MEC, for example, could help avoid the mechanical stress of AFEX-CS on the anode biofilm (personal communication with Allison M. Speers).

5.2.2 *Clostridium cellobioparum*

We have identified a robust glycerol-fermenting ethanologen, *C. cellobioparum*, capable of consortia growth with *G. sulfurreducens*. Adapted strains and improvements in operating parameters have led to the production of ethanol, 1,3-propanediol, and biohydrogen from glycerol in a MEC. However, remaining limitations in the system include the incomplete consumption of glycerol and the low current production by *G. sulfurreducens,* which resulted in biohydrogen production having a minimal contribution to energy efficiencies.

To enhance biohydrogen production in the MEC by *G. sulfurreducens*, improvements in the MEC system will be necessary. Such improvements may include removing components such as the proton exchange membrane, which contributes to the high internal resistance of the MEC, as well as design features such as decreasing the distance between the anode and cathode electrodes, and increasing the ratio of anode surface area to reactor volume (6).

We are currently sequencing the genomes of our parental and adapted *C. cellobioparum* strains. While no genetic system is available for this organism, we believe the genetic systems in closely related organisms could be applicable to our

strain. Two other members of the *Clostridiaceae*/*Rumminococcaceae* clade including *C. thermocellum* and *C. cellulolyticum* have established genetic protocols (1, 4, 12).

We observed interesting metabolic changes in the MEC cocultures compared to MEC monocultures and batch cultures. In our best performing MEC, *C. cellobioparum* consumed ca. 50% of the glycerol available and produced ethanol and 1,3-propanediol. About 43% of the glycerol consumed was converted to ethanol. While both ethanol and 1,3-propanediol are value-added products, it would be beneficial to direct fermentation in one direction to allow for higher titers of the desired product. This creates an interesting potential for customizable fermentation via metabolic engineering. To produce 1,3-propanediol and acetate, the pathways for ethanol, lactate, formate, and propionate could be eliminated. Alternatively, to only produce ethanol and formate, elimination of lactate, acetate, propionate, and 1,3-propanediol could be accomplished through genetic engineering.

Lastly, to apply this system to wastewater treatment for the biodiesel industry, we will need to test crude glycerol wastewater as a growth substrate. Refined glycerol used in this study does not have high salinity or contaminants, such as those present in biodiesel wastewater, which could inhibit the growth of *C. cellobioparum* and *G. sulfurreducens*. It is possible that the adaptations that conferred glycerol tolerance to both *C. cellobioparum* and *G. sulfurreducens* (most likely membrane changes) will serve to increase tolerance to the greater osmotic stress and harsh conditions of the waste stream. Given the successful adaptive evolution strategies presented in this dissertation it is likely that continued adaptive evolution would be possible for these conditions.

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APPENDIX

Appendix

Investigations towards the development of a Genetic System in *Cellulomonas flavigena*

A.1 BACKGROUND

As the bioenergy field expands, the need for improved microbial catalysts becomes increasingly important. *Cellulomonas* spp. are promising candidates for the consolidated bioprocessing of lignocellulose substrates, but further strain improvement is necessary. Genetic engineering is a targeted approach for strain improvement that could greatly enhance the metabolic attributes of *Cellulomonas* spp. for applications in bioenergy. However, no genetic systems have been reported for any *Cellulomonas* strains. The genome of the type strain *Cellulomonas flavigena* has recently been sequenced by the Joint Genome institute (1). Reports are also available that indicate that this strain could be transformed by electroporation (6). Hence, I began investigations towards the development of a genetic system in this organism.

A.2 MATERIAL AND METHODS

A.2.1 Bacterial strains and culture conditions

Cellulomonas uda (ATCC 21399) and *Cellulomonas flavigena* (ATCC 482) were routinely cultured aerobically in GS2 (3) medium supplemented with cellobiose (0.2% (w/v)) as the C source (GS2-CB). When indicated, 1.5% (w/v) agar was added. Cultures were incubated at 35°C.

A.2.2 Antibiotic screening and minimum inhibitory concentration (MIC)

C. uda and *C. flavigena* were tested for sensitivity to an array of antibiotics including kanamycin, chloramphenicol, gentamicin, streptomycin, ampicillin, novobiocin, erythromycin, tetracycline, and vancomycin. All antibiotics were tested using the paper

disc method (4) except for vancomycin, which was tested using a gradient strip (Etest®, Biomerieux). Paper discs of Whatman 3MM filter paper were saturated with varying concentrations of antibiotic (10, 50, 100, and 250 µg/ml) and pressed into an agar plate streaked for confluent lawn growth. Zones of growth inhibition around the discs were measured to determine the minimum inhibitory concentration (MIC) for each antibiotic.

A.2.3 DNA Manipulations and Plasmid Construction

Genomic DNA (gDNA) was routinely isolated from cells harvested by centrifugation (16873 x *g* for 5 min at 25°C) from 4.5 ml of late exponential phase (ca. 18h) cultures using the Masterpure DNA purification Kit (Epicentre), following the manufacturer's instructions. The gDNA was resuspended in sterile water and its concentration was determined using a Nanodrop (Eppendorf). Primers for PCR amplification of DNA fragments of *C. flavigena* were designed using sequence data from the Joint Genome Institute. PCR amplification was performed using Herculase II Fusion DNA Polymerase (Agilent Technologies) according to the manufacturer's instructions. Plasmid DNA was purified using Qiagen midi-plasmid purification kits and resuspended in sterile water.

Plasmid pJYP11 was constructed using the In-Fusion HD Cloning Kit® (Clontech) that carried an insertion of a ~1.7 kb recombinant PCR construct into the MCS of the pCR2.1®TOPO® vector (Invitrogen). The insert contained the PCR-amplified upstream (~0.3 kb) and downstream (~0.4 kb) regions of the *C. flavigena* gene Cfla3152, which encodes a 1,3-β-glucanase, flanking an erythromycin-resistance cassette. The upstream region of Cfla3152 was amplified from *C. flavigena* gDNA using primers

Cfla3152 F1 (CGACACAGCTCGCGTACC) and Cfla3152 R1 (GTGGTCGTCGTGCTCGTCCATATGGCTACCCGACACCTCGAT), whereas primers Cfla3152 F2 (ATCGAGGTGTCGGGTAGCCCATATGGAGGAGCACGACGACCAC) and Cfla3152 R2 (CTCTACGTGCAGGGCGAGTA) were used to PCR-amplify the downstream region. The upstream and downstream regions were sewn together using primers Cfla3152 F1 and Cfla3152 R2 and introduced into the MCS of the plasmid pCR2.1®TOPO® using *Escherichia coli* Stellar™ competent cells (Clontech). The plasmid was then purified and linearized with FastDigest® NdeI. An erythromycin resistance cassette was amplified from plasmid pORI19 (5) using primers Inf Erm1 (TCGTGCTCCATATGAGCACAGTTCATTATCAACCAAA) and Inf Erm2 (TGTCGGGTAGCCATATGATGCAGTTTATGCATCCCTTA). The linearized vector and the erythromycin resistance cassette were then "infused," using the In-Fusion HD Cloning Kit® and Stellar™ competent cells (Clontech). This resulted in the final plasmid pJYP11 containing the erythromycin resistance cassette within the upstream and downstream region of the 1,3-β-glucanase gene of *C. flavigena* (Cfla3152).

A.2.4 Preparation of electroccompetent cells and electrotransformation

Cells were harvested by centrifugation (3220 x *g* 8 min at 4°C), washed with ice-cold sucrose electroporation buffer (SPB: 272mM sucrose, 7mM sodium phosphate buffer [pH 7.4], 1mM $MgCl_2$) and resuspended in 1/20 of the original volume of SCP as previously described (2, 6). The cell suspension was either used fresh or mixed 1:1 with 50% glycerol (v/v) and stored at -80C.

Electrotransformations were performed following previously published protocols (2, 6) using an Eppendorf 2510 Electroporator using ~1 µg/µl of DNA. Electrocompetent *C. flavigena* cells were either used fresh or thawed on ice. The cells were pulsed with 2500V before resuspension in pre-warmed (35°C) GS2 and incubated overnight at 35°C in liquid medium (GS2-CB) before plating on agar-solidified GS2-CB supplemented with 50 µg/ml erythromycin.

A.3 RESULTS

A.3.1 Antibiotic resistance profile of *C. uda* and *C. flavigena*

No growth inhibition was observed for either *C. uda* or *C. flavigena* in the presence of kanamycin, chloramphenicol, gentamicin, streptomycin, novobiocin, and tetracycline (Table B.1-2). Thus, these strains exhibit resistance to these antibiotics up to concentrations of 250 µg/ml. By contrast, both strains were sensitive to ampicillin at concentrations of 50 µg/mL and higher and to erythromycin at concentrations of 10 µg/mL and higher (Tables B.1-2). The zones of inhibition observed for *C. flavigena* in the presence of increasing concentrations of erythromycin are shown in Fig. B.1. Vancomycin was also tested for both strains using Etest® strips, and inhibition was observed at concentrations as low as 0.125 µg/mL (Fig. B.2).

Table A.1: Antibiotic resistance profile of *C. uda*

Antibiotic	10 (µg/ml)	50 (µg/ml)	100 (µg/ml)	250 (µg/ml)
Kanamycin	-	-	-	-
Chloramphenicol	-	-	-	-
Gentamicin	-	-	-	-
Streptomycin	-	-	-	-
Ampicillin	-	2 mm	5 mm	***a***
Novobiocin	-	-	-	-
Erythromycin	2 mm	5 mm	8 mm	11 mm
Tetracycline	-	-	-	-

a Zone of inhibition too large to measure

Table A.2: Antibiotic resistance profile of *C. flavigena*

Antibiotic	10 (µg/ml)	50 (µg/ml)	100 (µg/ml)	250 (µg/ml)
Kanamycin	-	-	-	-
Chloramphenicol	-	-	-	-
Gentamicin	-	-	-	-
Streptomycin	-	-	-	-
Ampicillin	-	1 mm	4 mm	***a***
Novobiocin	-	-	-	-
Erythromycin	2 mm	5 mm	14 mm	20 mm
Tetracycline	-	-	-	-

a Zone of inhibition too large to measure

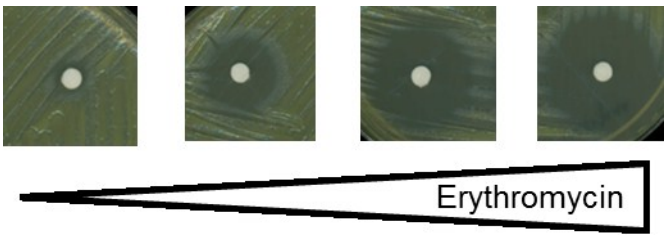

Figure A.1: Sensitivity of *C. flavigena* to erythromycin at increasing concentrations (10, 50, 100, and 250 µg/ml).

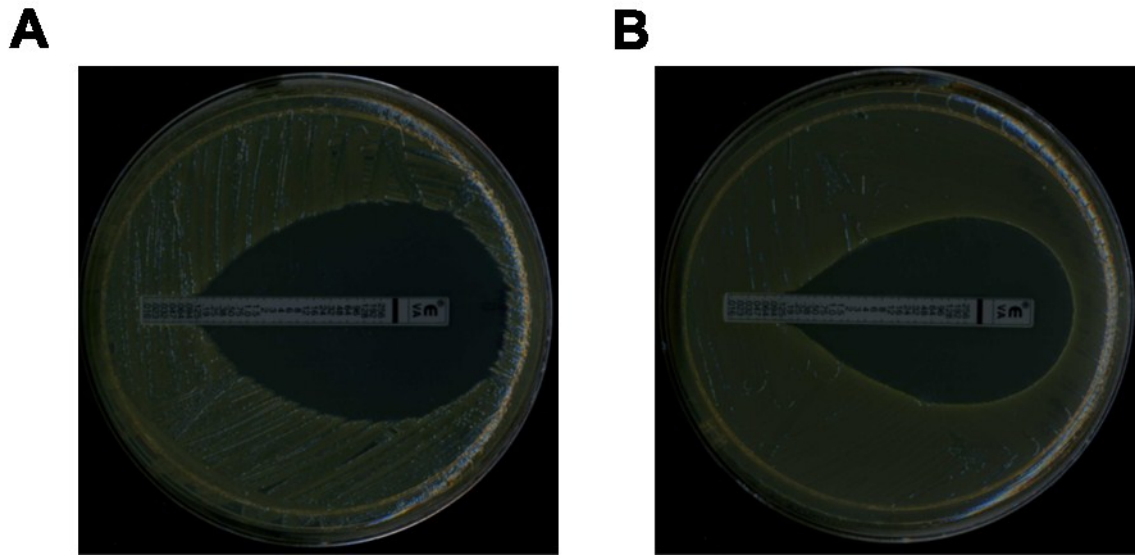

Figure A.2: Sensitivity of *C. uda* (A) and *C. flavigena* (B) to vancomycin

A.3.2 Transformation of *C. flavigena*

Based on the MICs determined during the antibiotic sensitivity screening, erythromycin was chosen as marker for genetic manipulations. An erythromycin-resistance cassette was amplified from plasmid pORI19 (5) and used to construct a recombinant PCR fragment for targeted deletion of gene Cfla3152 from *C. flavigena*. Gene Cfla3152

encodes a 1,3-β-glucanase, which break down the curdlan (1,3-β-glucan polymer) exopolysaccharide. The recombinant PCR construct contained the erythromycin-resistance cassette flanked by the upstream and downstream regions of the Cfla3152 gene and was inserted in the MCS of the pCR2.1®TOPO® vector. The resulting pJYP11 plasmid was transformed into electrocompetent cells of *C. flavigena*. DNA-free controls were also included. Whereas control cells did not produce any erythromycin-resistant colonies, the cells transformed with plasmid pJYP11 did, averaging ~400 CFUs per µg of DNA. Approximately 10 erythromycin-resistant colonies were randomly picked and cultured in liquid medium (GS2-CB). The clones were designated *C. flavigena* P11. gDNA was isolated from all the clones and the recombination of the recombinant insert of plasmid pJYP11 was evaluated by PCR amplification using primers Cfla3152 F1 and Cfla3152 R2. The WT gene with up and downstream regions results in a fragment ~2.8 kb while the expected recombined fragment containing the erythromycin resistance cassette in place of the Cfla3152 gene would be ~1.7 kb. The PCR-amplifications did not yield a product of the expected size (ca. 1.7 kb) in the P11 cultures, but, rather, a 2.8 kb fragment as in the control samples with WT gDNA, consistent with the amplification of the uninterrupted chromosomal Cfla3152 gene (Fig B.3). This suggests that the plasmid replicated in *C. flavigena.*

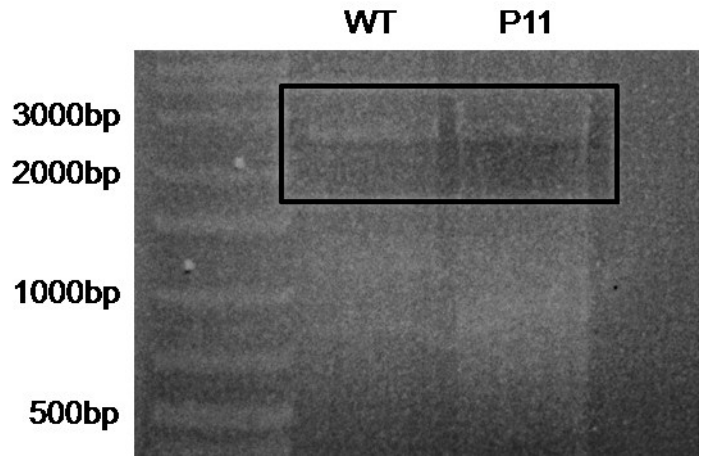

Figure A.3: Amplification of gene Cfla3152 in *C. flavigena* WT and P11

A.4 DISCUSSION

The combination of an antibiotic resistance profile and an effective transformation method shows promise for a tractable genetic system in *Cellulomonas* strains. *C. flavigena* was used in this study as a transformation system had been previously described, and the genetic sequence is now available. In Chapter 2 and 3, I demonstrated the promise of this clade of organisms and in particular *C. uda* as a candidate for bioenergy industrial applications. Although improvements have been made in our strain via adaptive evolution, limitations in ethanol yield will need to be overcome for use in industry. This work could lead to improved fermentation strains either by directed mutagenesis for metabolic engineering or a transposon mutagenesis screening for improved strains.

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