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DEPARTMENT OF CHEMICAL ENGINEERING

A MEDIUM THROUGHPUT SCREENING DEVICE WITH ONLINE GROWTH DATA COLLECTION OF MULTIPLE SMALL VOLUME GAS PHASE BIOREACTORS

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ABSTRACT

The national goal for a sustainable production platform for liquid fuels, and the difficulty in achieving economic feasibility on part with fossil fuels, has led to many different options being explored. Large scale corn to ethanol production plants, while a step in the right direction, have run up against an economic wall where the current model is not profitable without government subsidies. Beyond cellulosic biomass to fuel which are currently being deployed, there are next generation efforts to genetically engineer bacteria to produce higher energy fuel molecules like hydrocarbons and butanol from gas feeds like $CO_2/O_2/H_2$ and synthesis gas ($CO_2/CO/H_2$). Since the substrate no longer involves simple addition of sugar to media, gas-consuming organisms present challenges for screening genetically modifications for product formation levels. Additional considerations for scale work are explosion risk (H_2/O_2) or toxicity (carbon monoxide) associated with these gas mixtures. This specific work was motivated by a project to develop liquid hydrocarbon (botryococceene) fuel for the ARPA-E Electrofuels Project. This project has genetically modified *Rhodobacter capsulatus* to produce the triterpene botryococcene—a "drop-in" fuel molecule native to an algae species. The growth modes of this organism includes autotrophic (CO2, H2, O2), therefore, there was a need for a safe method to increase the throughput of the screening process of this potentially explosive gas mixture. Experimental studies demonstrated that this device can obtain online optical density measurements of up to 12 separate 4 mL cultures simultaneously for gas phase fermentations. This device has been used to successfully grow both a chemoautotrophic bacteria as well as a syngas fermenting bacteria (*Clostridium ljungdahlii*). This device can provide k_La values up to 25 hr^{-1} and grow cultures to OD 1.0 while reducing the gas headspace volume of the screening process from 2.4 L in 250 mL shake flasks to only 60 mL. This device should enable faster and more quantitative screening of different organisms and feed gases for biofuels production.

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I would also like to thank Nymul Khan for mentoring me through the research process and teaching me more than I could ever put down on paper. Without his expertise and assistance, this thesis would not have been completed. Nymul also helped obtain data and assisted with the writing of this thesis.

I would like to thank Ryan Johnson for his help in operating the Multiplexor Screening Device. He was an integral part of generating the *C. ljungdahlii* data and troubleshooting the initial growth experiments involving syngas.

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PREFACE

While the work of this thesis was generally in support of gas phase fermentation technology, it included some 'bootstrapping' in related technology, including efforts to fix a mass flow controller (MFC) and initial studies of a scaled down high intensity mass transfer culture device (autotrophinator). When working with the autotrophinator, the device was set up and inoculated alongside Ryan Johnson, a technician for the ARPA-e project. Ryan operated the LabVIEW software. I performed all the data analysis independently. A narrative associated with the overall effort in the lab is articulated in Appendix A, with more specific details in other associated appendices.

The majority of the body of my thesis deals with the specific effort to demonstrate the ability of a multiplexed gas phase growth device which was specifically designed for growth of *R. capsulatus* under autotrophic conditions (CO_2, H_2, O_2). But our intentions were to demonstrate its broader general use for gas phase fermentations, including syngas fermentations in particular as there is interest in utilizing methane as a feedstock that can be converted to syngas (CO, H_2, CO_2) and fermented to biofuels as well. The design of the multiplexor screening device preceded my time in the lab; that work was done entirely by Dr. Wayne Curtis, Nymul Khan, and Ryan Johnson. Although I used the device to grow *R. capsulatus* as part of the training process to take on the more challenging experiment of the syngas fermenting organism, the specific data for the growth of *R. capsulatus* were generated by Nymul Khan and are analyzed in this thesis as part of my contribution to the overall gas-phase fermentation effort.

The work with C. ljungdahlii was conducted together with Ryan Johnson. I performed the initial growth experiments on heterotrophic media independently and designed the weaning experiment with his input based on a review of the literature – noting that this literature is quite

confusing since there are complex media components included in growth media that are referred to as syngas fermentation. The addition of bicarbonate to some cultures was Ryan's suggestion.

Chapter 1

Biofuels and the Future of Energy

Over the past two centuries, fossil fuels have driven the rapid advancement of technology by providing an abundant and cheap source of energy. But more recently, as large swaths of the planet develop, the consumption of fossil fuels has skyrocketed. This increased demand has raised questions about the sustainability of the world energy market. The remaining fossil fuels are proving harder and more expensive to extract which is driving up energy prices, while increases in atmospheric CO_2 have united the scientific community behind the theory of climate change [17].

Unfortunately, the use of fossil fuels is predicted to rise steadily over the next thirty years as seen in **Figure 1-1** [1]. Renewable fuels have seen an increase in production as the global community has begun to recognize the threat of global warming. At the moment, this sector is the fastest growing part of the energy market, growing at 2.5% per year [18]. While renewables will an increase, many of these innovations (like solar and wind power) do not provide portable liquid fuels suitable for use in transportation. Therefore, continued innovation in the field of biofuels is critical to a sustainable and climate friendly global energy market. The

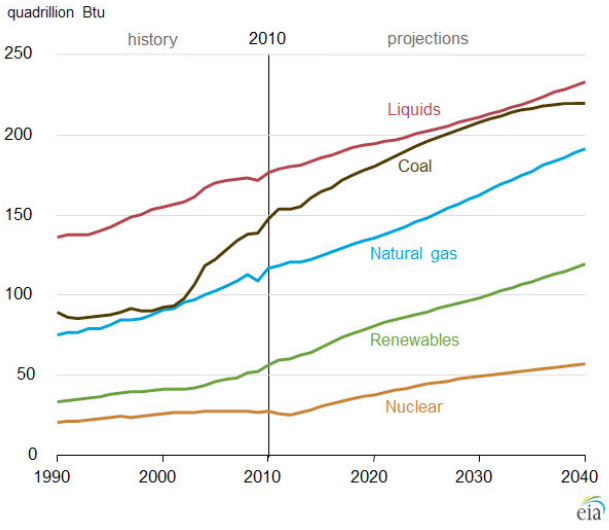

Figure 1-1: World energy consumption by fuel type, 1990-2040

recent push for ethanol is a step in the right direction, but this faces big challenges from a low return on investment and food/fuel competition from corn to ethanol operations [6]. In fact, ethanol has only been able to compete in the U.S. oil market due to extensive government subsidies under the Energy Tax Act of 1978 [2].

One promising route for producing biofuels is by growing them in a bioreactor of genetically modified cells. The Advanced Research Projects Agency – Energy (ARPA-E) Electrofuels project was introduced in 2010 to further the development of advanced liquid fuels from the products of photolysis (O_2 and H_2). This initiative aims to explore new production methods for fuel molecules like ethanol, butanol, biodiesel, and triterpenes.

The ARPA-E Electrofuels Project at Penn State

Under the guidance of Dr. Wayne Curtis, a group at The Pennsylvania State University has worked in collaboration with Dr. Joe Chappell's group at The University of Kentucky to develop a strain of bacteria for use in the production platform depicted in **Figure 1-2** [3]. The goal of this platform is to harness the energy of the sun to split water into oxygen and hydrogen which, when mixed with CO_2 from a power plant, will be fed to *Rhodobacter capsulatus.* This bacterium has unique metabolic capabilities that allow it to grow in three different "trophisms" of growth. It can grow heterotrophically using sugars dissolved in the media as a carbon and energy source, photoheterotrophically using sugars as a carbon source and light as an energy source, and chemoautotrophically using H_2 as an energy source, O_2 as an oxidizing agent, and CO_2 as a carbon source. Additionally, this organism has been successfully genetically engineered to produce the long-chain (C_{34}) triterpene fuel molecule, botryococcene, at high titers while growing in the chemoautotrophic metabolic mode. This work was made possible with the characterization

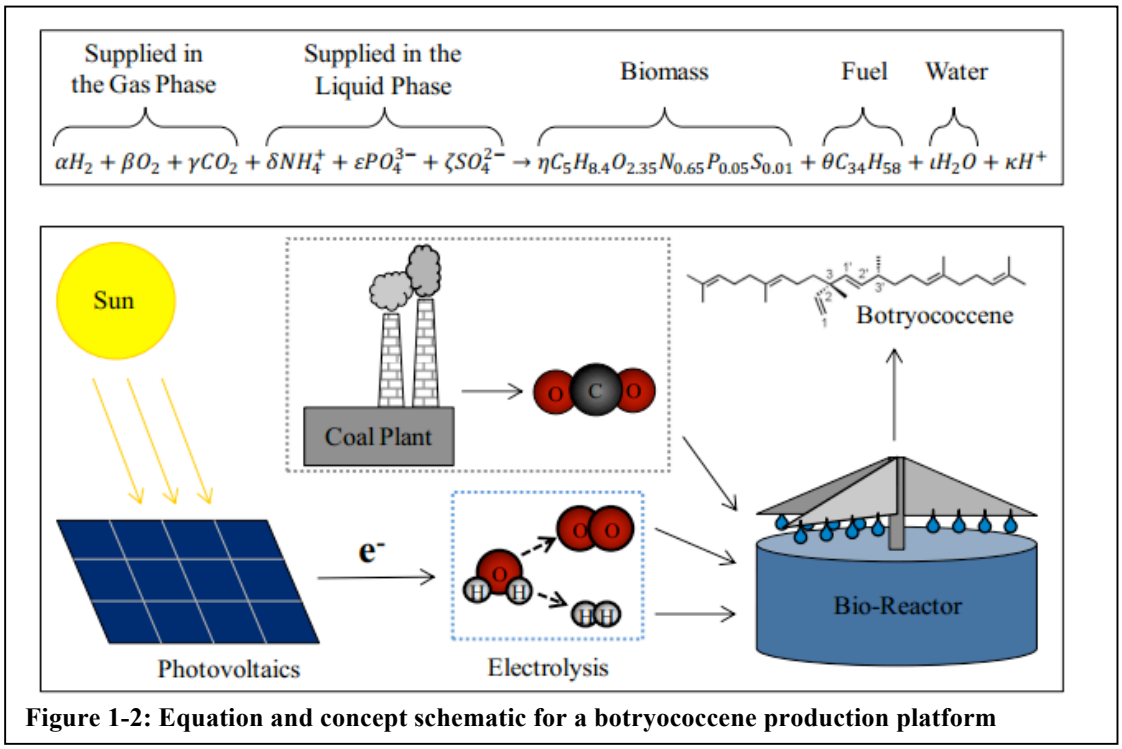

Figure 1-2: Equation and concept schematic for a botryococcene production platform

of the enzymes capable of producing this molecule—squalene synthase and botryococcene synthase—by Dr. Chappell's group at UKy [24] [25].

Botryococcene is a particularly attractive fuel molecule because it is considered a "drop-in" molecule that can be fed directly into current refineries with little to no changes in current refining operations [6] [26]. Botryococcene is also a hydrophobic molecule meaning that it naturally phase separates from water, therefore eliminating the need for expensive separation units like distillation towers. While burning botryococcene will still release carbon dioxide into the atmosphere, the process of producing the fuel molecule will fix carbon that would have otherwise been released, making the overall process carbon neutral.

Synthesis Gas as a Path to Biofuels

The process of fixing carbon dioxide with *R. capsulatus* is one example of using a waste product like CO_2 to create a next generation platform for biofuel production. It has the advantage

of producing an attractive fuel molecule with high energy density. However, the photovoltaic technology that is required to capture solar energy economically on such a large scale is still many years away [6]. Another platform has been explored that aims to produce ethanol (and potentially other fuels through genetic engineering) from a much cheaper feed gas, synthesis gas or syngas, which can be produced by burning anything that contains carbon, oxygen and hydrogen.

Synthesis gas is a mixture of CO, CO_2, and H_2 that is the product of a combustion reaction under limited oxygen conditions. Instead of oxygen fully oxidizing each carbon, the limited supply of oxygen forces some carbon to remain partially oxidized as CO. H_2 is also produced during this process. The energy in syngas can be accessed through directly burning the syngas or by converting it to hydrocarbons via the Fischer-Tropsch process. However, these processes are not cost effective. The syngas can also be consumed by a microorganism, *Clostridium ljungdahlii*, and turned into a liquid biofuel. *C. ljungdahlii* is a Gram-positive,

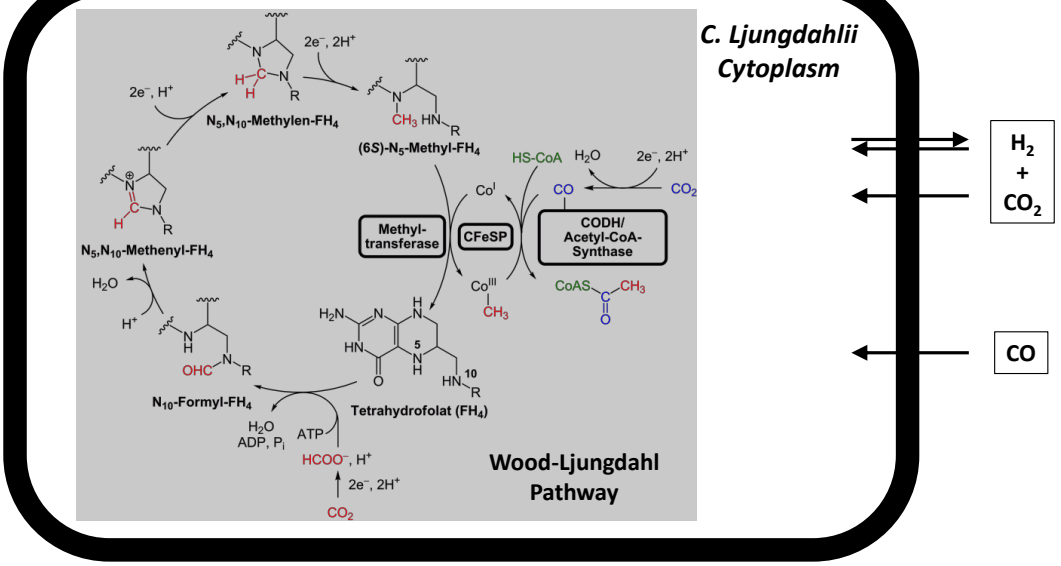

Figure 1-3: Overview of the Wood-Jlundahl pathway in *C. ljungdahlii*. Figure shows the co-transport of CO_2 and H_2 across the cell membrane as well as the uptake of CO. The Wood-Ljungdahl pathway results in Acetyl-CoA for use in general cell metabolism. Image of metabolic pathway credited to Wikimedia Commons.

motile, rod-shaped, and obligate anaerobic bacterium that forms spores [16]. **Figure 1-3** shows the Wood-Ljungdahl pathway in which bacteria can fix CO and CO_2 by obtaining energy from H_2 [5].

C. ljungdahlii naturally produces ethanol and acetate and current efforts are looking at ways to increase the ratio of ethanol to acetate production. Also, production of butanol by *C. ljungdahlii* is being investigated by various laboratories as a logical next step because of higher energy density than ethanol [5] [8]. *C. ljungdahlii* and *R. capsulatus* pose a unique challenge because their production levels must be screened by fermenting under a controlled composition gas phase.

Importance of High Throughput Screening of Genetically Engineered Organisms

The target fuel molecules for these two platforms are produced by some organisms but are not native to the bacteria of interest for a large-scale production platform. The genetic engineering work done at Penn State has focused on *R. capsulatus* and the production of botryococcene. A large number of genetic constructs representing various permutations of the MEP pathway genes and triterpene synthases were created at PSU and UKy, in order to find one that is capable of producing high levels of triterpenes. The dozens of lines created were screened for fuel production levels in triplicate.

Furthermore, a screening of the various genetic backgrounds of *R. capsulatus* was being performed in parallel to the genetic engineering to characterize their growth rates, in case that played a role in their capability for triterpene production. These include *R. capsulatus* SB1003, B10 WT, B10 ΔgtaI, B10 ΔgtaR and B10 ΔgtaRI. SB1003 is a spontaneous mutant of the B10 line, which is one of the earliest lines of *R. capsulatus* to have been isolated. The Δgta mutants are chromosomal knock-outs of gtaI, gtaR, and both genes respectively. The GTA (Gene Transfer

Agent) operon is responsible of packaging bits of DNA and sending them outside the cell and also integrating them into the genome so are responsible for horizontal gene transfers among bacteria [30]. It was hypothesized that the GTA lines would be genetically more stable (due to the absence of the GTAs), an important factor for eventual commercial scale-up of this process.

As a peripheral part of this project, the idea of genetically engineering *C. ljungdahlii* for the possible use of the inexpensive syngas substrate to produce triterpene was being explored. However, the difficulty lies in the fact that *C. ljungdahlii* is an obligate anaerobe and does not readily grow in completely carbon free media (and there are only a small number of literature references that do this) [5] [16]. Much of the reported growth of *C. ljungdahlii* on syngas has been done in media that contains a small amount of yeast extract (1 g/L) which contains a significant level of carbon [5] [11] [15]. As preliminary work with *C. ljungdahlii,* the pathway to carbon free media had to be optimized.

The high volume of screening required for this project called for an innovation to streamline the process and free up time for other research activities. Members of Curtis Lab designed a device that would monitor culture optical density (OD) continuously using an LED and a photodiode sensor. This device provided many benefits over other screening methods and proved to be a pivotal part of the ARPA-E project.

Current Screening Devices

One of the key bottlenecks in genetic research right now is screening variants for the desired metabolic activity. While studies have shown that robots are capable of analyzing up to 100,000 chemical samples a day, no platform exists to produce samples at this volume [19]. Solid media heterotrophic plates can have up to 9,000 sample wells for small cultures, but nothing on this scale exists for fermentations. There have been some significant improvements in mass

transfer of microtiter plates; and it has been shown that a shaken microtiter plate with optimized baffling can achieve k_La values greater than 600 hr^{-1} with culture volumes smaller than 1 mL [20]. But this is not a readily available or cost effective solution for most research budgets.

Two of the most commonly available options for fermentation screening are culture tubes and shake flasks. ProSens, a biotechnology sensor manufacturer, categorized the k_La of both of these devices for a range of shaker RPMs. They found that culture tubes had a k_La range of 0.5-21 hr^{-1} with respective shaking rates of 150-275 RPMs. Shake flasks had a k_La range of 0.7-104 hr^{-1} with respective shaking rates of 80-210 RPM [21]. Culture tubes are not considered airtight so they cannot be used for work with an obligate anaerobe like *C. ljungdahlii.* And neither culture tubes nor shake flasks allow for the collection of online OD measurements. There is a need right now for a cost effective device that can continuously monitor the OD of multiple small fermentation reactors simultaneously.

The objective of this thesis was twofold: (1) to develop and characterize the performance of the multiplexor device using two different species of bacteria growing on different gas phase compositions, (2) to determine the most effective intermediate media composition for transitioning cultures of *C. ljungdahlii* from heterotrophic to chemoautotrophic growth. The multiplexor screening device was designed a built by Nymul Khan, Ryan Johnson, and Dr. Wayne Curtis. The data for the *R. capsulatus* cultures used in this thesis was generated by Nymul Khan. Ryan Johnson assisted in operating the multiplexor screening device for the *C. ljungdahlii* cultures.

Chapter 2

Development of the Multiplexor Screening Device

A device named the "multiplexor screening device" was developed as a way to continuously monitor the OD of multiple small fermenters. This device was born out of earlier work done by Myers, Curtis, and Curtis in 2011 [9]. In that project, they extensively characterized the relationship between dry weight and OD at different wavelengths for different organisms. This was done on both Spectramax and Beckman DU spectrometers with a standard beam path length of 1 cm. Myers, et al. proposed a flow cell device with a smaller path length of 0.1 cm and adjustable intensity LED to increase the applicable range of OD measurements. While this device would require a different LED bulb to produce different wavelength, it could be constructed at a fraction of the cost of commercial spectrometer products. Additionally, many screening projects work with a specific type of bacteria so a standard LED could be used for the duration of the project.

The multiplexor screening device consisted of 12 small volume bioreactors with gas flow connected to all reactors. The small volume was an added safety feature since both O_2/H_2 mixtures and syngas can be flammable. The culture volume in each reactor was only 4 mL with a gas overhead of 5 mL. The flammability risk of this device was greatly reduced when compared to the same number of 250 mL shaker flasks connected in series. Additionally, previous systems required the researcher to physically sample each shaker flask and take offline OD measurements. By connecting this device to a computer with an internet connection, the OD could be monitored from anywhere in the world in real time.

Design and Construction of the Multiplexor Screening Device

The multiplexor device was built using 12 screw cap tubes (VWR Cult 13x100 CS1000, 53283-800) of 9 mL each. The caps of these tubes had 2 small 1/16" holes drilled though them to allow for gas entry and exit needles. The gaps around these holes were sealed with silicone. The gas entry needle dipped down into the 4 mL culture while the gas exit needle was cut short to remain near the top of the reactor. The total number of tubes was divided among multiple sets of two or three, representing biological replicates of the same line and the gas flowed in series through them. The gas flow was split and flowed in parallel among the different sets of replicates (**Figure 2-1**). This was done to avoid cross-contamination between two lines in case some liquid happened to have carried over into the next tube. This sort of parallel splitting of gas is not possible unless the backpressure on each line is nearly the same. However, the backpressure through a chain of two or three liquid-filled tubes cannot possibly be made constant. Therefore, a small orifice (8 μm Bird, #22104) with very high pressure drop was placed before each reactor-

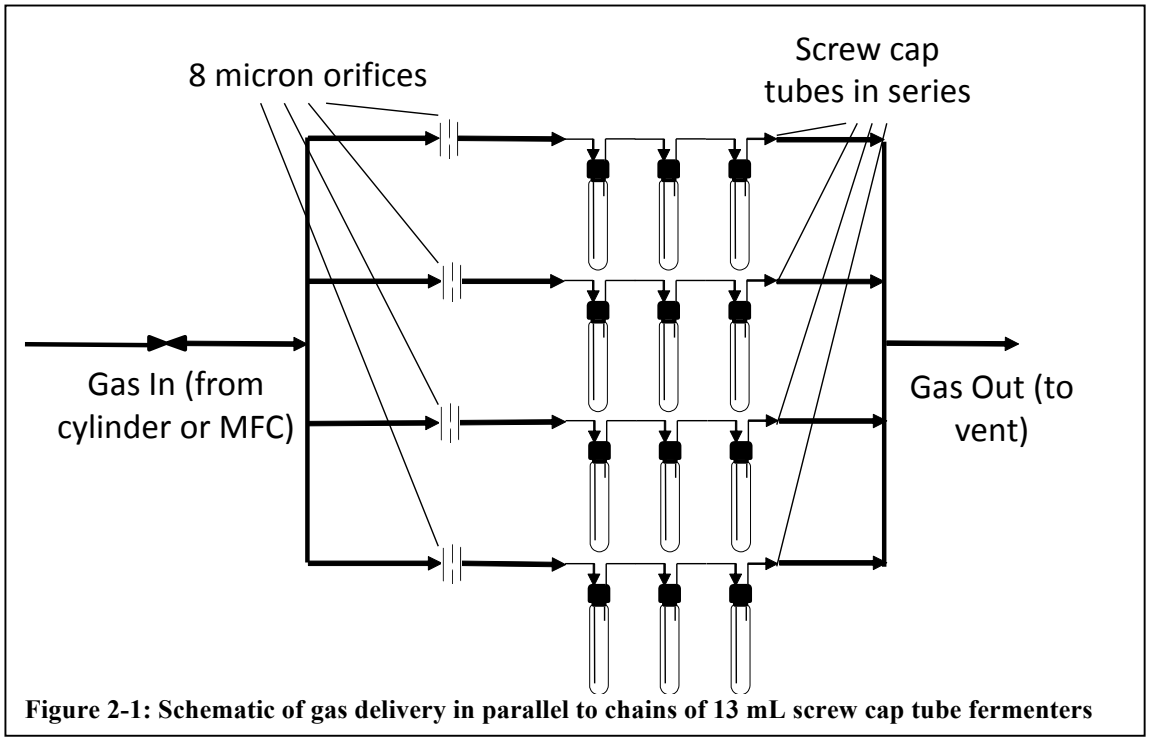

Figure 2-1: Schematic of gas delivery in parallel to chains of 13 mL screw cap tube fermenters

chain so that the fluctuations downstream of this did not affect the backpressure on the line and the gas flow through each set of reactors remained nearly constant.

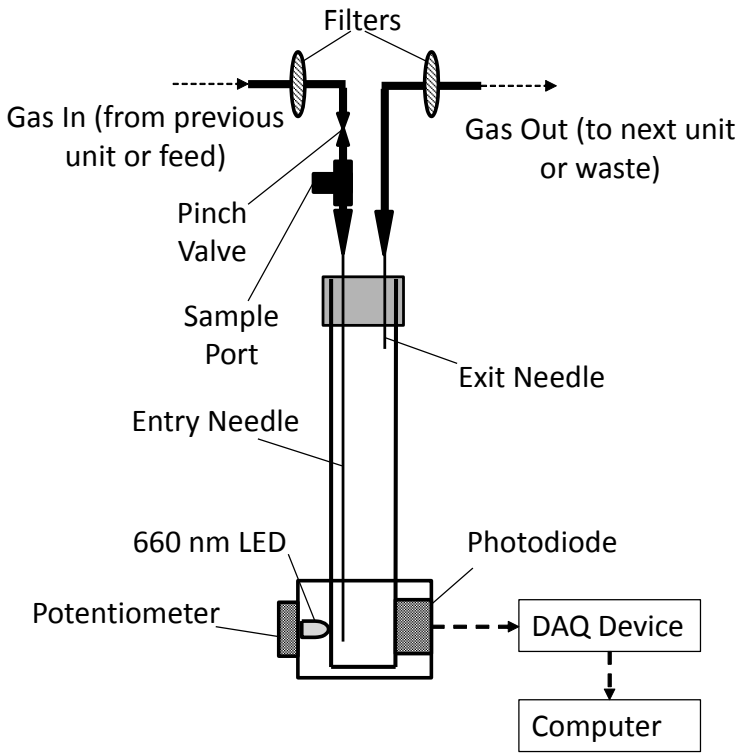

Figure 2-2: Schematic showing the design of an individual reactor on the device. The entry needle was offset to avoid blocking the LED beam path.

Both the entry and exit gas tubes were fitted with filters to prevent small airborne droplets of culture from contaminating adjacent tubes. The entry tube also had a small pinch valve and sample port. A sample could be obtained from a culture by closing its valve and the following culture's valve and pulling liquid up the entry needle to the sample port with a syringe (**Figure 2-2**). The valves prevented the syringe from simply filling with gas or pulling culture from a downstream reactor into the one being sampled.

The base of each tube fit into a small strip of plywood that had a photodiode sensor (Texas Instruments OPT101) on one side and a 660 nm LED (Digi-key #1080-1135-ND) on the other. The wiring diagram for the photodiode can be seen in **Figure 2-3**. This photodiode circuit output a 0-10 V signal to a National Instruments DAQ connected to a LabVIEW program that recorded the output voltage of the photodiode.

Because the voltage reading was only valid within the 0-10 V range, each LED was wired to a manual potentiometer (Trimmer 10kOhm, 0.5W, #TH-3362U-1-103LF) that allowed for the

brightness of the LED to be adjusted. The brightness was adjusted to a low value at inoculation and manually increased via the potentiometer (when the photodiode output fell below a certain voltage) as the cultures grew and become denser. This adjustment was mathematically accounted for during data processing (Eq. 2). The LabVIEW interface allowed for great flexibility with setting the number of samples in each reading, sampling rate, and time between readings. All samples were saved in TDMS file format and opened with a spreadsheet program for data processing. This same wiring and sampling strategy was used for the autotrophinator device briefly described in Appendix D.

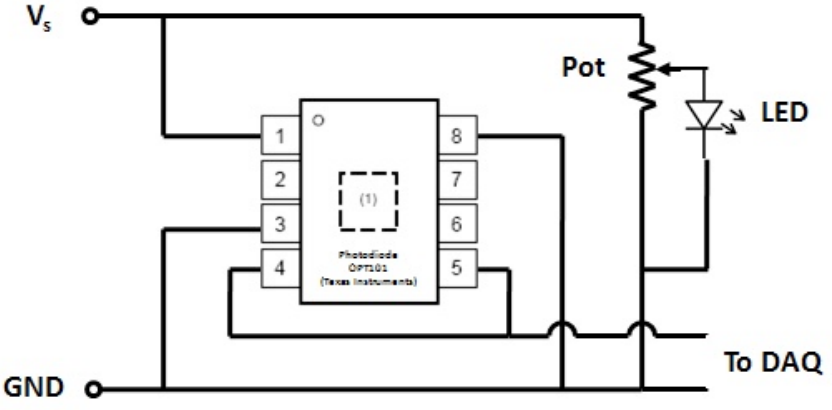

Figure 2-3: Circuit diagram for photodiode sensor in screening device

Since the gas feed tube was submersed in the culture, bubbles from the gas flow would interfere with the LED light path, leading to artificial spikes in OD. To account for this, a 3-way solenoid valve (Gems, #MB345-EB33-L201) was connected to LabVIEW via the DAQ and configured to cut off gas flow to all fermenters and redirect flow to vent at a predetermined time before each reading was taken.

Operation of the Multiplexor Screening Device

The multiplexor screening device can be seen in operation and taking samples of growing lines of *R. capsulatus* in **Figure 2-4**. Operation of the device was similar to the operation of any optical density measurement device. A blank of the media before inoculation was taken at the

start of each run to get a baseline voltage for comparison. Optical density was then calculated by Eq. 1. If the voltage of the LED was increased to account for a dense culture, a new blank was calculated by Eq. 2.

$$OD = LOG(BlankV / SampleV) \qquad \textit{Eq. 1}$$

$$NewBlank = NewSampleV * OldBlank / OldSampleV. \qquad \textit{Eq. 2}$$

This method produced clean and detailed growth curves for two different organisms growing on two different gases. Even with the solenoid valve control, there was still occasional noise in the signal from bubbles leaking out due to back-pressure in the lines. A simple data filter was applied that ignored points that varied by more than a certain percentage from adjacent points. Given the continuous nature of the OD sampling, it was determined that no sample is a given reading should vary by more than 5% from the previous value.

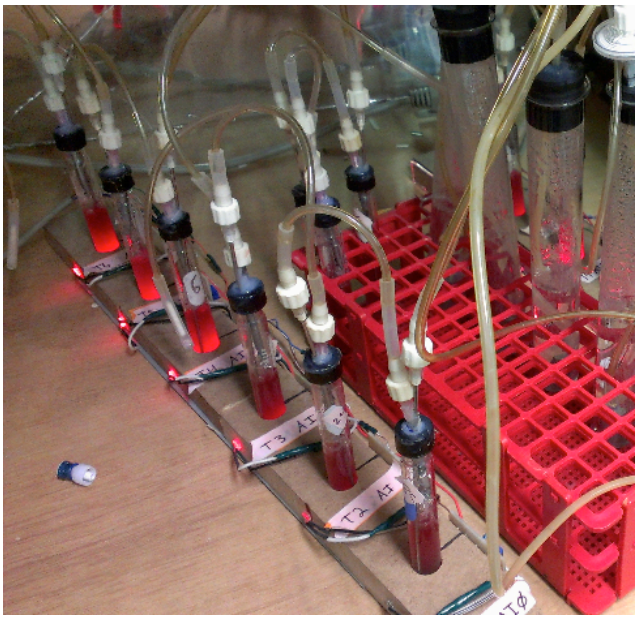

Figure 2-4: Multiplexor screening device in operation growing *R. capsulatus*

$$IF(OR(NewOD>OldOD*1.05,NewOD<OldOD*0.95),NA(),NeOD) \qquad \textit{Eq. 3}$$

An example of the filter that was applied to noisy data using Microsoft Excel can be seen in Eq. 3. Where the first two terms are the conditions of the if/or statement and NA() replaces a noisy data point. An example of an online OD signal before and after filtering can be found in **Figure 2-5.**

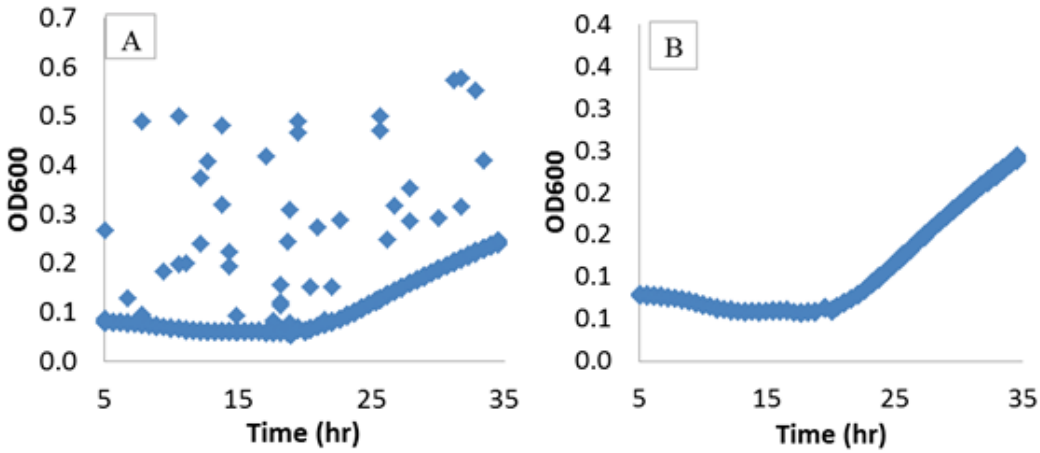

Figure 2-5: Growth curve of *C. ljungdahlii* before and after filtering out bubbles. A – before filtering, B – after filtering.

Limitations of the Multiplexor Screening Device

The multiplexor screening device is a versatile tool with great potential for use, but as with any development prototype, has some limitations. During these experiments, the biggest problem was with reliability of the data collection system. The analog input signal from the photodiode fed into a DAQ connected via USB to a computer where the data was saved. This computer was connected to numerous lab devices, and sometimes the USB ports would send an error to LabVIEW that would cause data collection to stop entirely. While this is not a limitation of the device itself, it was a limitation of the way in which it was set-up during these experiments. If someone was not near the device when the error occurred, a culture could potentially grow for hours without data. The USB had to be manually unplugged and reconnected to fix the error. This

could be addressed by using a dedicated computer for the multiplexor screening device. The DAQ could also be wired to the computer using a connection method other than USB.

Another issue with the device was the complexity of taking samples. The needles delivering gas to and from the reactor were not securely fastened. This was made harder by the proximity between each reactor tube. While the tubes were close together to minimize pressure drop between reactors in series, this small distance increased the difficulty of screwing on the tube caps, making gas connections, and taking samples. If anything puts too much stress on the silicon sealing the needle, then a gas leak could develop with serious consequences. A system was developed to try and prevent a dangerous mixture from entering the lab environment in the event of a leak (Appendix E). The need to close valves to take a sample could be an unnecessary step as well. Check valves placed in-line instead of the pinch valves might serve the same purpose without the need for interaction from the user taking the sample.

Chapter 3

Using the Multiplexor Screening Device to Characterize Growth: Methods

This device was used both as a screening device and as a tool to generate precise growth curves using online data collection. Two separate bacteria were grown using two different gas compositions to illustrate the flexibility of the device. *R. capsulatus* was grown to help determine which host line would be most promising for genetic engineering into a fuel production line. This was important since strong autotrophic growth would be required to create an economical production platform. *C. ljungdahlii* was grown to determine the most effective way to transition a culture from heterotrophic growth onto true carbon free media and synthesis gas. The removal of yeast extract from the media was a step that was not taken in many synthesis gas cultures. This data was also analyzed to characterize the performance of the device in terms of mass transfer values and reproducible growth curves.

Methods: Growth of *Rhodobacter capsulatus*

Cultures of *R. capsulatus* are taken through heterotrophic and photoheterotrophic metabolisms before transitioning to chemoautotrophic. Cultures of *R. capsulatus* were incubated aerobically from cryostock on plates of YCC media at 34°C. YCC media (L^{-1}): 5 g yeast extract, 6 g casamino acids, 5mL YCC Base Concentrate. YCC Base Concentrate (L^{-1}): 11.82 g $Na_2EDTA \cdot 2H_2O$, 0.04 g $CuSO_4 \cdot 5H_2O$, 0.03 g $CoCl_2 \cdot 6H_2O$, 0.02 g $(NH_4)_6Mo_7O_{24} \cdot 4H20$, 19.53 g $MgSO_4$ (anhyd), 0.75 g $FeSO_4 \cdot 7H_2O$, 0.0125 g boric acid, 6.9 g $Ca(NO_3)_2 \cdot 4H_2O$. Final media pH was adjusted to 7 using 5 N NaOH.

Colonies were then taken from plates and inoculated into 13 mL screw cap tubes filled completely with RCVB media to create anaerobic environment with no gas headspace. Cultures were incubated while rotating at 30 RPM under a 60 W halogen lamp that provided photons for photosynthesis and kept the culture at 34°C. RCVB Media (L^{-1}): 4 g D, L malic acid, 1 g $(NH_4)_2SO_4$, 10 mL CA Base Concentrate, 1 mL CA Trace Element, 30 mL CA Phosphates, 1 mL Vitamin Stock (filter sterilized), 1 mL Mg/Ca solution (filter sterilized). Add components in order listed and adjust solution pH to 7.2 before addition of phosphates. CA Base Concentrate (L^{-1}): 2 g Na-EDTA, 1.2 g $FeSO_4 \cdot 7H_2O$. CA Trace Element (L^{-1}): 0.24 g $ZnSO_4 \cdot 7H_2O$, 1.592 g $MnSO_4 \cdot H_2O$, 0.04 g $Cu(NO_3)_2 \cdot 3H_2O$, 0.752 g $Na_2MoO_4.2H_2O$, 2.8 g boric acid. CA Phosphates (L^{-1}): 60 g K_2HPO_4 (dibasic), 40 g KH_2PO_4 (monobasic). CA Vitamin Stock (L^{-1}): 1 g Thiamine-HCl, 0.015 g Biotin. Mg/Ca Solution (L^{-1}): 200 g $MgSO_4 \cdot 7H_2O$, 74.97 g $CaCl_2 \cdot 2H_2O$.

This experiment tested five potential host lines of *R. capsulatus* to determine which strain had the most favorable kinetics under autotrophic growth conditions. The lines tested were: SB1003 WT, B10 WT, B10 GTA-RI, B10 GTA-R, B10 GTA-I. All lines were tested in duplicates A and B.

Cultures were then subcultured to an initial OD of 0.1 in 4 mL tubes of the multiplexor screening device using CA media and incubated at 34°C. CA Media (L^{-1}): 1 g NaCl, 1 g $(NH_4)_2SO_4$, 10 mL CA Base Concentrate, 30 mL CA phosphates, 1 mL CA Trace Element, 1 mL CA Vitamin Stock, 10 mL Mg/Ca Solution. Components were added in order listed and solution pH was adjusted to 7.2 before addition of phosphates. The multiplexor device was then connected to the CO_2, O_2, H_2 (5%, 10%, 85%) gas mixture and continuous OD monitoring began.

Methods: Growth of *Clostridium ljungdahlii*

CGM media, as reported by Hoogendoorn and van Kasteren, was used for these growth experiments since they used this media to take *C. ljungdahlii* to truly carbon-free media [4]. The reducing agent cysteine was added as the last step, and the media was left in an anaerobic chamber for 24 hours to ensure no oxygen remained. Heterotrophic cultures of *C. ljungdahlii* were inoculated from cryostock into 50 mL of CGM media at pH 6.6 in 125 mL serum bottles. Inoculation was done in an aerobic environment, but cultures were immediately moved to an anaerobic chamber to purge any oxygen. The cultures were sealed in the chamber before being moved to an incubator. The atmosphere of the anaerobic chamber was 100% nitrogen at 170 kPa. They were incubated without shaking at 37°C until culture OD was 1.0.

The heterotrophic CGM media has 50 g/L glucose and 5 g/L yeast extract. These two components were varied to determine the most effect method for "weaning" *C. ljungdahlii* off of carbon sources in the media to a carbon-free media. Hoogendoorn and van Kasteren discuss this weaning process but do not give a specific path to follow. To determine the best path forward, the first experiment was done in various media compositions. For the second experiment, all cultures from the first experiment were subcultured into carbon-free media. The carbon free media was prepared as reported by Hoogendoorn and van Kasteren [4]. Both experiments were performed under syngas atmosphere (50% CO, 50% H_2) in the multiplexor screening device. Bicarbonate (2 g/L) was added to some samples to test the effect of free carbon dioxide in the media.

The weaning experiment tested the following conditions in duplicate: A – 0 g/L glucose, 0.5 g/L yeast extract; B – 0 g/L glucose, 5 g/L yeast extract; C – 0.5 g/L glucose, 5 g/L yeast extract, 2 g/L bicarbonate; D – 0 g/L glucose, 5 g/L yeast extract, 2 g/L bicarbonate; E – 0.5 g/L glucose, 5 g/L yeast extract.

The cultures were inoculated into the respective weaning media to initial OD 0.1 from the same heterotrophic culture. This was done in an aerobic environment; but once the tubes were inoculated, they were quickly connected to syngas to purge any oxygen that may have entered the media. It has also been shown that *C. ljungdahlii* is resistant to oxygen concentrations of up to 8% for 12 hours so this brief exposure time did not have any effect on the culture [10]. The screening device was operated for 50 hours to obtain growth curves. Final culture ODs were taken using a Spectramax spectrometer.

All cultures from the weaning experiment were kept in the same tubes and subcultured aerobically into carbon free media at a starting OD of 0.1. The device was again hooked up to syngas to purge any oxygen. This experiment was run for 180 hours, and final OD was taken offline using a Spectramax spectrometer.

Chapter 4

Performance of the Multiplexor Screening Device

Before the experimental results obtained using this device could be trusted, its performance and versatility had to be shown by obtaining accurate growth rates for different species of bacteria growing on different types of gas feed. Additionally, the mass transfer coefficient (k_La) of the device was calculated using the linear growth phase from multiple reactors. For consistency across a screening run, the device had to provide similar k_La values for each reactor connected to the device. Different flow rates were used for each gas composition so the k_La values from *R. capsulatus* were not expected to match the values from *C. ljungdahlii*. However, consistency was expected between reactors in a given experiment.

Device Performance with *Rhodobacter capsulatus*

Using the multiplexor screening device, the growth of *R. capsulatus* on a mixture of CO_2, O_2, and H_2 was readily characterized in both the exponential and linear phases with a high level of precision. **Figure 4-1** shows an example of a growth curve produced by the device. Two different host candidates of *R. capsulatus* produced curves where the phases of interest were easily identified and the kinetic parameters determined from the OD time course.

The growth rate, also known as the doubling time, can be directly extracted from a line of best fit on the exponential phase as seen in Eq. 4, where X(t) is the concentration of cells at any point during exponential growth, X_0 is the concentration before entering exponential growth, μ is the specific growth rate, and t is the time.

$$X(t) = X_0 e^{\mu t} \qquad \textit{Eq. 4}$$

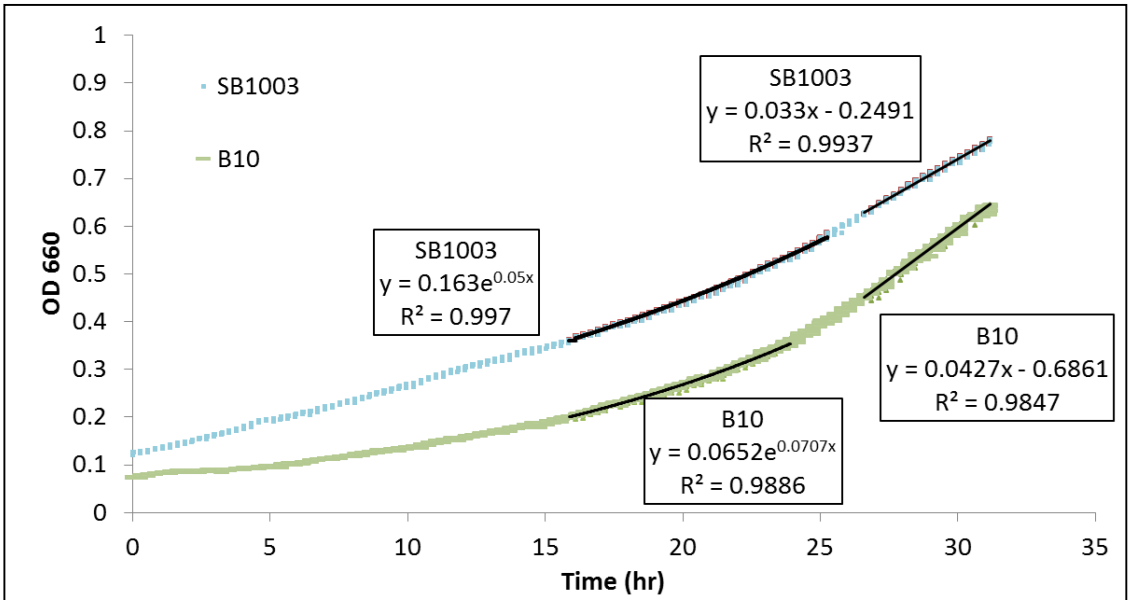

Figure 4-1: Online growth curves of *R. capsulatus* showing clear exponential and linear phases which were used to calculate growth rates and k_La

This exponential phase of growth occurs when all the nutrients in the culture are in excess and the bacteria divide at their characteristic intrinsic rate of growth. They ultimately enter a linear gas mass-transfer limited phase (assuming no liquid phase nutrients are limiting) where their growth rate (dX/dt) equals the rate at which the limiting component of the gas mixture enters the liquid phase. Under the conditions used, it was assumed that the rate limiting component was O_2 [13]. The slope of the linear portion of the growth curve (dOD/dt) is a measure of the rate of change in the mass of cells and can be converted to the latter by a simple dry weight (DW) to OD ratio using Eq. 5. Myers, et al. has a comprehensive compilation of the experimentally measured DW/OD ratios of various organisms studied in Curtis Lab [6]. For Rhodobacter this relationship was found to be to be 0.563 g cell/OD_{660}.

$$dX/dt = dOD/dt * 0.563\ g\ cell/OD_{660} \qquad \textit{Eq. 5}$$

Based on work done by N. Khan, a value of 8.13 g cell/molO_2 was used for the yield of *R. capsulatus* on O_2 [13]. Using Eq. 6 and the yield, the rate of change of cell mass was converted to the rate of consumption of O_2. This value can be combined with Henry's law to determine the mass transfer coefficient. Henry's law (Eq. 7) relates the saturation concentration (C^*_{O2}) of a

Table 4-1: Tabulated kinetic results from screening host strains of *R. capsulatus.* S1-SB1-T1-A never grew beyond OD 0.2, S1-GTA-I-T1-A and B showed some growth but never grew above OD 0.5. The growth rate of B10-GTA-RI1-B was considered an outlier and not analyzed.

R. cap Line	Slope (dOD/dt)	Slope R^2	dO_2/dt	kLa (hr^{-1})	Growth Rate (hr^{-1})	Growth Rate R^2
SB1003 WT-A	**No Growth**	-	-	-	-	-
SB1003 WT-B	0.033	0.99	2.3E-03	17.6	0.050	1.00
B10-A	0.027	0.98	1.9E-03	14.4	0.098	1.00
B10-B	0.043	0.98	3.0E-03	22.7	0.071	0.99
B10-GTA-RI1-A	0.028	0.98	2.0E-03	15.1	0.071	0.96
B10-GTA-RI1-B	0.054	0.98	3.7E-03	28.5	0.369	0.98
B10-GTA-R1-A	0.047	0.99	3.3E-03	25.0	0.073	0.99
B10-GTA-R1-B	0.037	0.98	2.6E-03	19.8	0.060	0.99
B10-GTA-I-A	**No Growth**	-	-	-	-	-
B10-GTA-I-B	**No Growth**	-	-	-	-	-

vapor component in a liquid to the partial pressure (P_{O2}) of that component in the headspace above the liquid through the use of the Henry's law constant (K_H). In this case, the partial pressure of oxygen was 0.1 and K_H for O_2 is equal to 770 mol/L*atm. The k_La of the reactor was calculated using Eq. 8. Since O_2 was known as the rate limiting reactant, it was assumed that the bulk concentration of O_2 in the liquid (C_0) was equal to zero. This is valid if the culture immediately consumes any O_2 that dissolves into the media.

$$dO_2/dt = dX/dt/yield \qquad \textit{Eq. 6}$$

$$C^*_{O2} = P_{O2}/K_H \qquad \textit{Eq. 7}$$

$$dO_2/dt = k_La(C^*-C_0) \qquad \textit{Eq. 8}$$

Using the method described here, the growth rates and k_La values for the *R. capsulatus* host candidates were compiled in **Table 4-1.** The significance of these results is discussed in **Appendix G.** Before the development of this device, all screening was done in non-baffled, glass, 250 mL Erlenmeyer flasks on a shaker at 200 RPM. The gas delivery system was set up very similar to the multiplexor device with shorter series of flasks provided gas in parallel. The 250 mL flask system was capable of delivering k_La values of 120 hr^{-1} and up to 175 hr^{-1} with the addition of metal packing as baffling (Appendix B). The screening device k_La values were about one order of magnitude lower averaging 20 hr^{-1} in the study presented here. These values seem

low compared to a shaker flask, but PreSens has shown that a shaken culture tube can only obtain k_La values of 0.5-21 hr^{-1} making this screening device competitive with current small-scale culture systems [21].

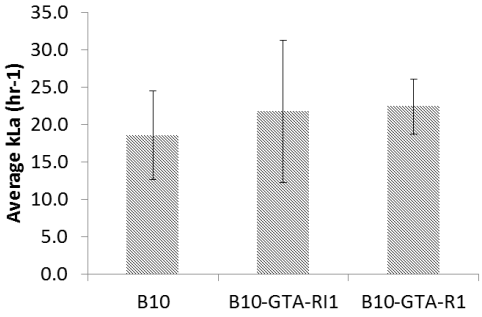

Figure 4-2: Average k_La of reactors in series with standard deviation error.

For this experiment, the gas flow was split in parallel across 5 reactor chains of 2 tubes each. This was to ensure no cross contamination between host lines. This meant that both tubes of a given line should experience the same gas flow rate with slightly different pressure drops. **Figure 4-2** shows that, for lines where both replicates grew, there were slight differences in k_La. But the values were quite comparable considering the range over which k_La can fluctuate for a given device.

While this may seem like a loss of productivity, the screening device offered a few key advantages over the shake flask system: (1) the gas head space volume of a 12 flask system was 2.4 L compared to only 60 mL in the multiplexor screening device which reduced the amount of flammable gases, (2) a shake flask system cannot provide online OD and therefore requires a greater time investment to operate with less precise results, (3) the combination of a shaker and the larger system hold-up volume results in significant utilization of incubator space.

Device Performance with *Clostridium ljungdahlii*

The screening device also provided precise growth curves for *C. ljungdahlii* growing on syngas with various levels of carbon in the media. **Figure 4-3** shows another example of growth curves produced by the device. *C. ljungdahlii* growing on two different media compositions produced curves where the phases of interest were easily identified and analyzed for kinetic data. The biological significance of the curves is discussed in **Chapter 5**. The exponential growth rate was obtained using Eq. 4 as explained above.

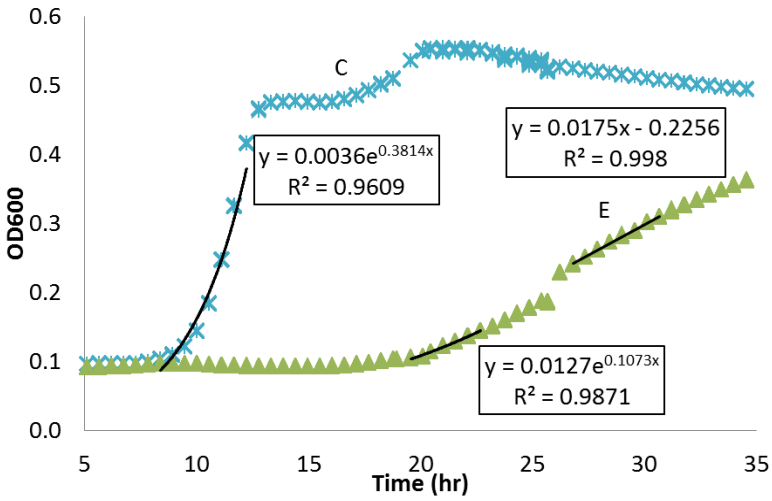

Figure 4-3: Online growth curves of *C. ljungdahlii* showing clear exponential and linear phases which were used to calculate growth rates and k_La.
C - 0.5 g/L glucose, 5 g/L yeast, 2 g/L bicarbonate
E – 0.5 g/L glucose, 5 g/L yeast extract.

The k_La analysis was also quite similar to the calculations done with *R. capsulatus* with only minor changes to account for different constants. The changed constants are tabulated in **Table 4-2.** The mass transfer coefficients in this experiment were much lower than the *R. capsulatus* experiment as seen in **Table 4-3.** The average k_La for the *C. ljungdahlii* cultures was 1.9 hr^{-1}. However, due to concerns with the potential consequences of a CO leak, the flow rate was minimized so that bubbles just came out of the final

Table 4-2: Constants used for calculating kLa in *C. ljungdahlii* cultures

CO Constants	
Yield (gcell/molCO)	8.4
P (CO)	0.5
K_H (mol/L*atm)	1052.63
gcell/OD660	0.472

Table 4-3: Tabulated kinetic results from testing different media compositions for *C. ljundgdahlii* under syngas atmosphere. Curves C and D did not show a strong linear phase of growth so the k_La analysis was not completed.

Condition	Slope	R^2	dCO/dt	kLa	Growth Rate (hr^{-1})	Growth Rate R^2
B	0.013	1.00	7.4E-04	1.6	0.12	0.95
C	-	-	-	-	0.38	0.96
D	-	-	-	-	0.41	0.96
E	0.018	1.00	9.8E-04	2.1	0.11	0.99

multiplexor in each series. This could have contributed to the drop in k_La since fewer bubbles passing through the culture resulted in less surface area for mass transfer. The growth curves of *Clostridium ljungdahlii* obtained by this experiment are hard to interpret. It seems like the cultures exposed to bicarbonate never saw a linear phase, while the cultures without bicarbonate never saw an exponential phase. But even with the strange growth behavior of these cultures, the multiplexor was able to generate growth curves that precisely showed the transition between the different phases of growth.

Chapter 5

Using the Multiplexor Screening Device to Characterize Growth of *Clostridium ljungdahlii*

Weaning *Clostridium ljungdahlii* off of Carbon in the Media

Several conditions were tested for weaning cultures of *C. ljungdahlii* off of the complex carbon source in the media to a truly carbon-free media under syngas atmosphere. Initially, the experiments were conducted with media that contained yeast extract since it seemed to be required for growth (OC+ media). At this time the importance of the CO_2/carbonate was not appreciated, therefore, the inclusion of this yeast extract was presumed necessary to obtain reasonable growth. Given the anticipated role of a CO_2 source, the experiments were also carried out with the addition of bicarbonate (IC+ media). The value of real time OD data from the screening device was immediately apparent from the growth curves of bicarbonate cultures compared to non-bicarbonate cultures. As seen in **Figure 5-1**, cultures B and D have identical media compositions except for the addition of bicarbonate in culture D. The lag phase of culture

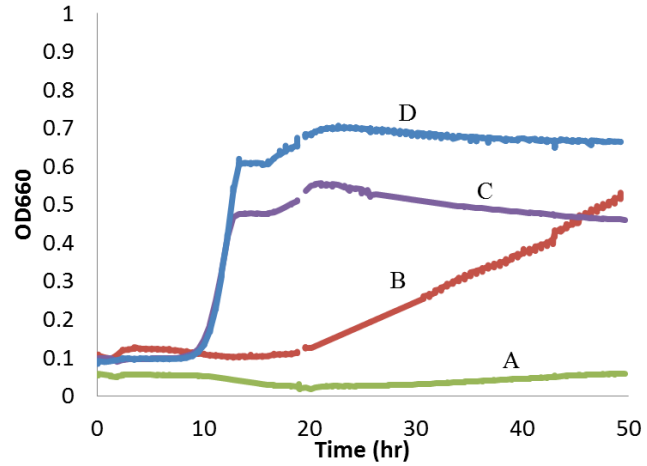

Figure 5-1: Initial attempt to wean *C. ljungdahlii* off of glucose in the media. All cultures were under syngas atmosphere: 50% CO, 50% H_2. CGM media was used with modified levels of glucose and yeast extract. Bicarbonate was added (IC+) to test effect of CO_2 in media.
A – 0 g/L glucose, 0.5 g/L yeast (IC-);
B - 0 g/L glucose, 5 g/L yeast (IC-);
C - 0.5 g/L glucose, 5 g/L yeast, 2 g/L bicarbonate (IC+);
D - 0 g/L glucose, 5 g/L yeast, 2 g/L bicarbonate (IC+).

D is approximately 10 hours while the lag phase for culture B is nearly 20 hours. The addition of bicarbonate (IC+) also induced a pronounced exponential phase where the cultures with bicarbonate grew to 0.5 OD over 2 hours. The CGM media used in this experiment does not have much buffer capacity so the culture grew rapidly until it invariably became inhibited by the pH drop from the acetate byproduct.

One possible explanation for this phenomenon comes from the Wood-Ljungdahl pathway in *C. ljungdahlii*. As seen in **Figure 1-3**, CO_2 co-transports across the cell membrane with H_2. *C. ljungdahlii* can perform the biological analog to the reverse water-gas shift and convert CO_2 to CO by using H_2. The transport of CO_2 across the cell membrane could be faster than the transport of CO. Or the Wood-Ljungdahl pathway could be more efficient when fed from two different pathways by sum means such as the balancing of cellular oxidation and reduction. But the use of CO_2 by the cell would require a higher partial pressure of H_2 since some of the H_2 intended for energy would be consumed by the water-gas shift. The additional consumption of H_2 might alter the process economics of a scaled up platform so an economic analysis should be completed to determine this. The growth curves generated by Kopke et al. seem to support our working hypothesis about the effects of bicarbonate/CO_2 [5]. The syngas provided in their experiments contains CO_2 (strangely without specifying composition) and the curves showed a steep exponential phase much like the one generated in this study with bicarbonate. Additionally, Cotter et al. did not provide any CO_2 in the syngas or bicarbonate in the media and the growth curves from those experiments did not show a steep exponential phase [7]. Those curves were the same linear shape that culture B showed in this study. The final OD of these cultures was consistent with values reported in the literature, which range from 0.5-1.2 OD [5] [7] [4] [9]. The doubling times of the bicarbonate cultures were around 2 hours, which compares well with literature values of 2-5 hours for growth on fructose [29]. However, this result cannot be due to the presence of sugar because the curves with the same organic carbon media composition did not

show this growth pattern. The doubling times for culture B were about 10 hours, which matched closely with reported values of 6-8 hours for growth on CO/H_2 mixtures [29].

In general, we have conducted insufficient systematic work with *C. ljungdahlii* and the composition of the gas phase to draw conclusion about whether the addition of CO_2 increases the growth rate, and our working hypothesis is based largely on the observation of the interconversion of dissolved CO_2 and bicarbonate ion (HCO_3^-):

$$\mathbf{CO_2 + H_2O \leftrightarrow\ H_2CO_3\ \leftrightarrow\ HCO_3^- + H^+}$$

The work does, nonetheless, provide consistency with prior growth observations and show the value of the screening device. The important observation from this initial study is that rapid growth progresses in a media that does not contain glucose. This leads to the obvious question of whether the yeast extract can be removed as well. Unfortunately, this role for CO_2 was not recognized, and the subsequent experiment described below, was carried out with a focus to remove the yeast extract, but not including the bicarbonate (IC+/-) treatment.

Another alternative working hypothesis is that the addition of bicarbonate provided a buffering effect to the cultures. *C. ljungdahlii* is known to change the pH of its environment with the production of ethanol and acetate byproducts. This could be tested in the future by adding a buffer that would not provide CO_2 to the media and analyzing the resulting growth curve. Unfortunately, many of the available buffers are organic which introduces the ambiguity of utilization of that carbon – though arguably unlikely.

The lack of a linear phase in the bicarbonate samples was an unexpected result. *C. ljungdahlii* lowers the pH of its environment as it grows by producing acetate. In many cases it lowers it to a point where it inhibits its own growth [27]. We hypothesize that the bicarbonate samples entered a rapid exponential phase by feeding on the carbon dissolved in the media from the bicarbonate. The cultures entered a stationary phase and then the culture density began to decline.

In a batch reactor, cell density can only decrease when there is cell death, cell aggregation or similar behavior that effect light scattering [9]. This decline was also found in the growth curves generated by Mohammadi et al. [9]. *C. ljungdahlii* is a spore forming bacteria so the drop in density likely occurs when the culture enters stationary phase, senses nutrient shortages, and begins to form spores which are less dense than normal cells [10]. This is also observed in our lab for *Clostridium phytoferementans* cultures which are prolific spore-forming bacteria (Zuroff, personal communication). In this case, the culture had nutrients available in the form of syngas. It seems likely that the pH had dropped too low from the rapid exponential phase of growth of bicarbonate. The culture could not grow in this low pH environment so it began to form spores.

It is worth noting that culture A (0 g/L glucose, 0.5 g/L yeast extract) did not grow significantly during the weaning experiment. This preculture composition simply did not provide enough nutrients in the media for the culture to make the transition from heterotrophic to chemoautotrophic growth. As will be shown below, the benefit of the yeast extract seems to be quite minimal as compared to the requirement for dissolved CO_2. Under low CO_2 conditions, the presence of yeast extract had a strong effect on growth as seen in curves A and B in **Figure 5-1.** Combined with the accelerated growth on CO_2, this might indicate that organic carbon is not required for transitioning to chemoautotrophic growth as long as dissolved CO_2 is available to the culture.

Growing *Clostridium ljungdahlii* in Carbon-Free Media

A final experiment was conducted to further eliminate organic carbon by preculture on various media, and then transfer to a media that had no organic carbon. Utilizing the 'weaning experiment' as inoculum, all cultures were subcultured into carbon free media that contained

neither organic carbon (O-C free) or bicarbonate (I-C free); the absence of CO_2 in the gas phase also further limited the dissolved carbon to carbon monoxide. Unfortunately, the multiplexor screening device experienced an error during this experiment where data recording was stopped for several days. As a result much of the rate data was not obtained, however, the observed growth was slow and linear which is consistent with the bicarbonate free media (IC-) treatments in the prior experiments (**Figure 5-2**). The initial exponential phase seen on cultures C and D was attributed to bicarbonate as carry-over from the preculture media. Offline final OD measurements indicated growth and are presented as demonstration of the ability to grow on media without supplemental carbon beyond carbon monoxide. While bicarbonate without glucose showed the strongest preculture culture growth, culture C (precultured on bicarbonate, yeast extract, and glucose) made the transition to carbon free media with the highest final culture density as seen in **Figure 5-3**. But it is important to note that only one replicate of culture C grew, so the value shown does not have two data points like the other pretreatments.

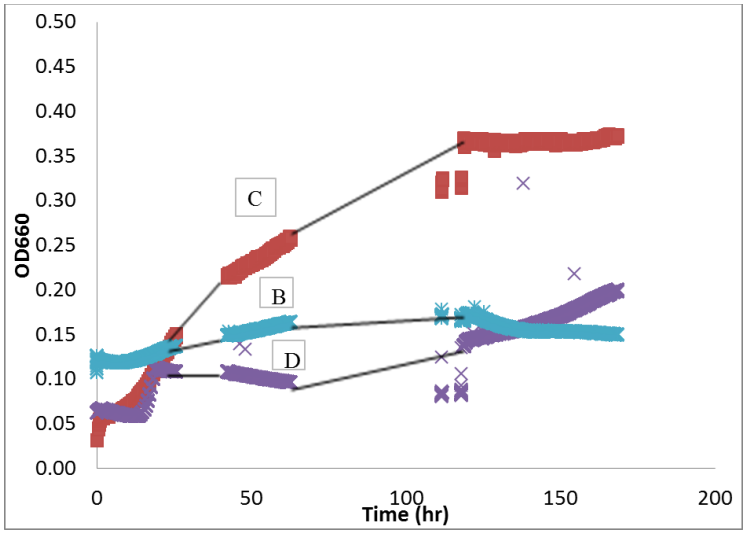

Figure 5-2: Online growth data for *C. ljungdahlii* in IC- and OC- media after growth in preculture on various complex media. Data is incomplete due to error with connection of screening device to computer. Black lines represent missing data. Growth done under syngas atmosphere: 50% CO, 50% H_2. Preculture conditions:
B - 0 g/L glucose, 5 g/L yeast;
C - 0.5 g/L glucose, 5 g/L yeast, 2 g/L bicarbonate;
D - 0 g/L glucose, 5 g/L yeast, 2 g/L bicarbonate.

From the earlier experiment, it seems likely that bicarbonate (IC+) was the key media component for robust growth. However, **Figure 5-3** shows the strong carbon-free final biomass accumulation in cultures C and E, the cultures that were weaned from 0.5 g/L glucose.

Unfortunately, since the rate data was largely lost, and the harvest was at an extended growth time, the data presented in Figure 5-2 does not reflect rates of growth and only final biomass accumulation.

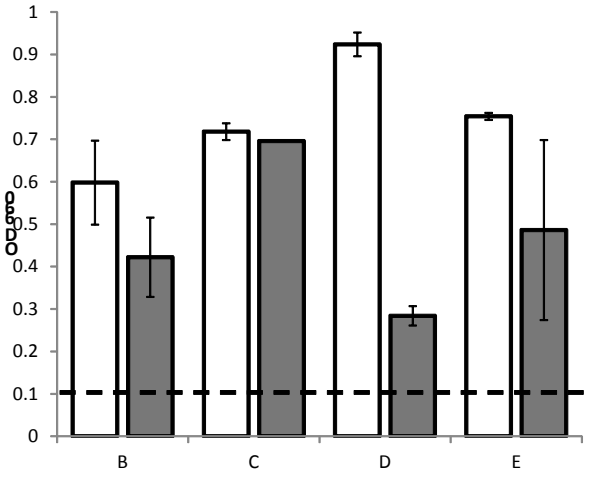

Figure 5-3: Final offline culture ODs for growth on IC-/OC- media (solid bar) after preculture on various complex media (open bar). Dashed line represents initial OD after subculturing. Both experiments were done under syngas atmosphere: 50% CO, 50% H_2. All tests were done in duplicate inoculated at OD=0.1 (horizontal line) and values represent average. One C culture never grew in carbon free for unknown reasons. Standard deviation error presented. Preculture (weaning) conditions:
B - 0 g/L glucose, 5 g/L yeast;
C - 0.5 g/L glucose, 5 g/L yeast, 2 g/L bicarbonate;
D - 0 g/L glucose, 5 g/L yeast, 2 g/L bicarbonate,
E – 0.5 g/L glucose, 5 g/L yeast extract.

These studies suggest that the most efficient way to take *C. ljungdahlii* from heterotrophic to chemoautotrophic growth is with an intermediate step of CGM media with 0.5 g/L glucose, 5 g/L yeast extract, and 2 g/L bicarbonate. The addition of bicarbonate decreases the time required for the intermediate step and the small level of glucose in the intermediate step seems to lead to a healthier culture once in carbon-free media. However, it seems very likely that if bicarbonate had been included in the media, the impact of the preculture media would be trivial by comparison.

Chapter 6

Conclusions and Future Work

The bulk of this thesis focuses on the implementation of a medium throughput growth screening device for gas phase fermentation. A device was designed and built by members of the Curtis Lab that has integrated capability to continuously monitor the optical density of small volume, gas fed fermentations. This device was then operated to characterize the performance of the device when growing two different strains of bacteria—*Rhodobacter capsulatus* and *Clostridium ljungdahlii*—on two different gas compositions. The device is also useful for screening different cell lines and media compositions in small volume batches on defined gas compositions. The work with the syngas fermenting organism, *C. ljungdahlii*, made major progress in unraveling a confusing literature with regard to the media and gas phase compositions; this provides an interesting avenue for future work. This work suggests that the addition of dissolved CO_2 dramatically enhances the metabolism of *C. ljungdahlii* growing under syngas atmosphere. Where we had intended to simply conduct a growth demonstration, the scenario of working with this unusual organism has provided an excellent test bed to demonstrate the versatility of this device. This multiplexed system will be used to explore the effects of bicarbonate on the growth of *C. ljungdahlii.* With the current screening bottleneck in genetic engineering, low cost devices like the one presented here are critical to keep innovation moving forward. The capacity of high throughput screening of heterotrophic cultures has grown exponentially in recent years; and hopefully the capabilities for gas phase screening continue to grow as well.

Future Work

This project generated both unexpected and exciting results for the growth of the syngas fermenting organism *C. ljungdahlii* which is being implemented at demonstration scale by the DOE (INEOS Bio, www.ineos.com) for ethanol production as well as being genetically engineered for advanced biofuels (LanzTech, www.lanzatech.com). However, due to a lack of appreciation for the effects of different variables, there is still more work to be done before the story is completed. The goal of this work was to quantitatively evaluate the performance and diversity of the multiplexor screening device for different types of bacteria on different gas compositions. While growth on organic carbon free media (OC-) media growth was achieved with *C. ljungdahlii*, the device was not operating correctly and online growth curves could not be generated. The process of weaning cultures off of carbon in the media, and onto syngas in carbon free media, will be attempted again so that online growth data of *C. ljungdahlii* in OC- media can be definitively demonstrated. More importantly, the dramatic effects of bicarbonate will be related to the availability of CO_2. Growth on inorganic-supplemented, organic carbon free media (IC+, OC-) will be compared with the growth on IC-, OC- media. To see if it is a buffering effect, a buffer could be used that does not provide a potential source of carbon to *C. ljungdahlii.* The device could also be tested with other autotrophic gas phase fermentations like growing methanogens on CH_4. Noting that a non-synthetic syngas will have a significant level of CO_2 present, the relevance of this demonstration has important implications to the real world application of this organism for syngas fermentation. It also potentially explains the rapid reaction rates claimed for syngas fermentation demonstration projects.

Improvements should also be made to the multiplexor screening device. The reactor tubes should be spaced further apart to allow for easier set-up and sampling. The reliability should be improved by connecting the device to a dedicated computer. If possible, the DAQ should connect

to the computer using a method more reliable than USB. Additionally, adding the capability of online pH monitoring would expand the capabilities of the device. Since *C. ljungdahlii* drastically changes the pH of its environment, pH monitoring would be incredibly useful. A pH probe would need to be found that can fit inside a 13 mm screw cap tube. PreSens has developed a pH microsensor with a 150 µm tip that would be suitable if the sensor is affordable.

If high density growth can be achieved on syngas, this could be another possible route that could be examined in the botryococcene engineering project. The *R. capsulatus* model is hindered by the price of hydrogen, but *C. ljungdahlii* has been shown to grow off industrially produced syngas which can be produced from almost anything [4].

Appendix A

Summary of Undergraduate Research

I began working under Dr. Curtis during the spring semester of my junior year here at Penn State. Earlier in my college career I had explored the idea of going to medical school and had taken several biology classes. While I loved learning about biology, I opted against the medical school path and stuck with engineering. When my adviser was switched to Dr. Curtis in fall of junior year, I was shocked to learn how much biology was going on in the department.

I signed up to help with research under Nymul Khan and I cannot thank him enough for his patience in teaching me the numerous techniques I would go on to use while working in Dr. Curtis' lab. That first semester I did a bunch of odd jobs on the ARPA-E project. I did troubleshooting for several broken mass flow controllers (MFC), which was a great opportunity to learn about how the support devices worked that made the research possible.

I also worked with the autotrophinator to try and determine why cultures would not grow in the device. There was a hypothesis that the exposed magnet on the plunger was leeching ions into the media and killing the cultures. Sealing it with silicon did not work and neither did melting polypropylene over the end. The body of the plunger was made out of Delrin, and melting Delrin over the end finally sealed the magnet and allowed cultures to grow. In addition to those projects, I helped Nymul with whatever was needed, including a complete reorganization of Lab 1A after an EHS safety inspection pointed out some improvements that needed to be made.

At the start of my senior year, I realized I needed to hone in on a project that could be the centerpiece of my thesis. After consulting with Nymul and Dr. Curtis, I decided to help Ryan Johnson with the *Clostridium ljungdahlii* work that is presented in this thesis. It was a challenging project since no one in the Curtis Lab had ever grown these bacteria off of just

syngas. But with the training I had already received, and with the guidance of Dr. Curtis, Nymul, Trevor, and Ryan, I was able to complete the project for my senior thesis. I have enjoyed my time working in Curtis Lab and look forward to hearing of the group's accomplishments in the future.

Appendix B

Characterizing the kLa of Flasks Used for APRA-E Screening

Rationale:

This work was intended to characterize the mass transfer performance of the shaker flasks that were also used for screening of various lines.

Methods:

Four flasks were tested in this experiment: 125 mL Erlenmeyer, 250 mL Erlenmeyer, 250 mL Erlenmeyer with metal column packings, 250 mL Erlenmeyer baffle flask. Cultures were inoculated from a photoheterotrophic wild type *R. capsulatus* to a starting OD660 of 0.1. To avoid the lag phase between photoheterotrophic and autotrophic, no sampling was done on the first round of autotrophic growth. Instead, cultures were grown autotrophically and then subcultured

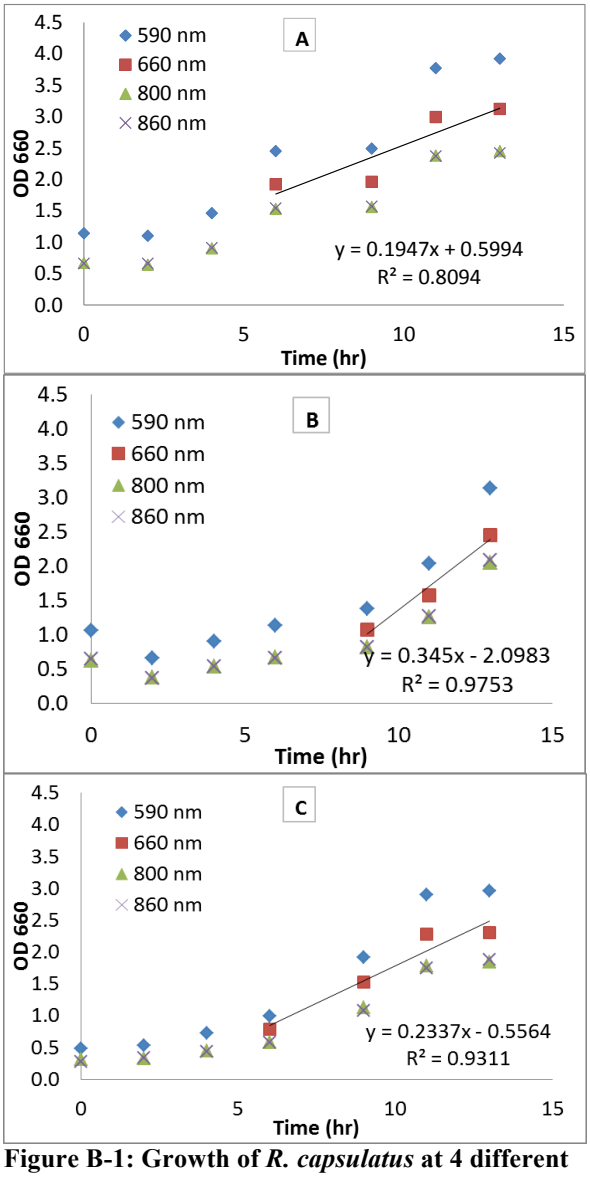

Figure B-1: Growth of *R. capsulatus* at 4 different wavelengths in A.) 125 mL shake flask,B.) 250 mL shake flask, C.) 250 mL shake flask with single layer of 1 cm diameter metal packing. Linear growth was analyzed to determine mass transfer rates.

back into fresh CA media at a starting OD of 0.1. The measurements were taken at least every three hours until a strong linear phase was observed. OD measurements were taken at four wavelengths: 590 nm, 660 nm, 800 nm, 860 nm. This was intended as a teaching point to illustrate how the pigments in *R. capsulatus* can affect an OD signal taken at a different wavelength. Since pigments absorb light, this can made the culture look more dense than it actually it.

Results:

The 250 mL baffle flask never grew during this experiment. These were older polycarbonate flasks that had developed some kind of scale on the inner surface. The other flasks were all glass and grew with excellent results. It was concluded that these polycarbonate baffle flasks are not suitable for growing cultures.

The growth curves for this work can be seen in **Figure A-1** with the tabulated kLa values presented in **Table A-1.** As expected, the highest kLa was the flask with metal column packing spheres because these provided additional surface area for mas transfer to occur. The improvement was only moderate, however. These results show that the flask system has significantly higher mass transfer than the screening device. The screening device could certainly be improved to try and approach the performance of a flask system. But these flask values are consistent with other data for kLa in shake flasks. PreSens, a commercial biotechnology company, has found that shake flasks range from 0.7 hr^{-1} to 107 hr^{-1} with respective shaker frequencies of 80 RPM to 210 RPM.

Table B-1: Tabulated mass transfer data for *R. capsulatus* growing in different shake flask conditions

Flask Type	Slope	R2	dX/dt	dO2/dt	C*	kLa
125 mL	0.195	0.81	0.106	0.013	1.3E-04	100
250 mL	0.234	0.93	0.127	0.016	1.3E-04	120
250 mL packing	0.345	0.98	0.187	0.023	1.3E-04	177

Appendix C

Troubleshooting Broken Mass Flow Controllers

During spring of 2013, there was a need for another set of three Mass Flow Controllers (MFCs) to provide a defined gas mixture to cultures of *R. capsulatus*. There was an attempt to fix two broken MFCs in house. After working through the manual, taking both of them completely apart, and speaking with the engineering department of the manufacturer, it was concluded that this was not a repair within the capabilities of Curtis Lab. The available options for each device are presented below. Nevertheless, it was a great learning experience into how thermal mass flow controllers can precisely regulate the flow to a bioreactor.

Results

Model: Brooks 5850E

Behavior: Output voltage gives a constant reading of 1.4 Volts (Normal Range: 1-5). This occurs no matter what the setpoint is. Flow is minimal.

Diagnosis: Brooks suggested sensor problem. Troubleshooted using the brooks manual. No continuity across HEATER or UPSTREAM T SENSOR. Sensor is broken. Spoke with 'Gary' at Brooks engineering on 4/5/13. He confirmed the broken sensor.

Proposed Solution:

A. New sensor ~$400. Requires recalibration but does not guarantee that sensor is the problem with the device.
B. Brooks Level 3 Service $950. Full service and refurbishment of device with a guarantee
C. New MFC ~$1500

Model: Brooks SLA5850S

Behavior: Output voltage gives a constant reading of 0 Volts (Normal Range: 1-5). This occurs no matter what the setpoint is. NO FLOW

Diagnosis: Brooks could not suggest a problem. Spoke with 'Gary' at Brooks engineering on 4/5/13. No contact with Brooks after identifying the problem. Troubleshooted using Brooks manual. This problem typically indicates either a clogged pathway (sensor or valve) or a broken electronic board. System was purged with high pressure dry nitrogen as recommended in the manual. The valve override pin was then engaged to attempt to force the valve open to better purge the MFC. No flow occurred in any of these cases. Clogging was ruled out. Electronic board is broken.

Proposed Solution:

A. Brooks Level 3 Service $950. Full service and refurbishment of device with a guarantee
B. New MFC ~$1500

Appendix D

Achieving Culture Growth in the 'Autotrophinator'

The goal of this work was getting the autotrophinator growing with *R. capsulatus*. The autotrophinator is a small volume bioreactor designed to deliver high mass transfer rates that are normally not possible on a small scale (kLa > 1000 hr^{-1}) [6]. This device is also connected to an online LED OD sensor much like the one used in the Multiplexor Screening Device. It operates based on a plunger being pulled out and pushed back into a culture by a solenoid coil acting on a magnet in the plunger. There seemed to be some kind of toxicity issue where the plunger's exposed magnet was killing cells. It was proposed by John Myers and Nymul Khan that the magnet was releasing zinc ions into the media and killing the culture.

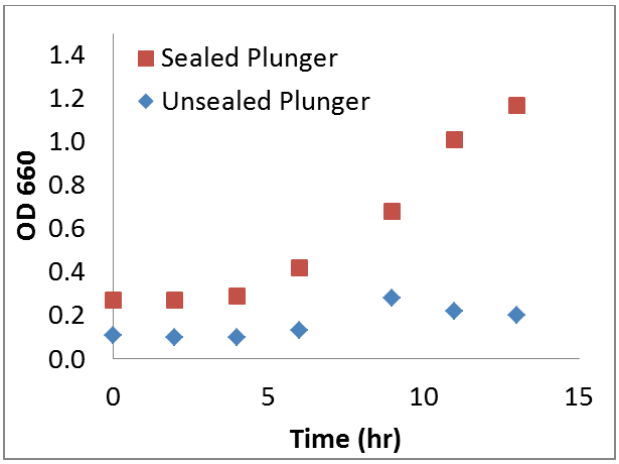

Figure D-1: Growth curves of the unsealed plunger and sealed plunger for the autotrophinator. Plunger was placed in a shaken 250 mL flask. The sealed plunger covered the magnet and allowed for growth of *R. capsulatus*.

The autotrophinator plunger was used as a stir bar in a media bottle to test if the exposed magnet was creating toxicity in the media. The unsealed magnet did indeed result in no growth so the end of the plunger was sealed with silicon. In the next several runs, the silicon fell off the plunger during testing and no growth was observed. Next, the plunger was sealed using melted polypropylene. This type of plastic did not bond to the plunger's Delrin material, and the testing

stage was never reached. Finally, melted Delrin was used to seal the end of the plunger and growth was achieved by placing the plunger in a 250 mL shake flask.

Melting bulk Delrin is a messy way to seal the autotrophinator plunger and a better solution should be found for future plungers. High temperature silicon could be used, or a small cap could be machined from Delrin and melted around the edges to seal. The melted Delrin plunger has since been used in the autotrophinator by Ryan Johnson to perform growth experiments.

Appendix E

Developing a Way to Safely Operate Continuous CO Flow in a lab Environment

When the syngas project began, the first hurdle was developing a safe way to operate a continuous carbon monoxide gas flow in a laboratory environment. The growth rates of *C. ljungdahlii* on syngas are slow and cultures take multiple days to reach final OD. Carbon monoxide is also extremely dangerous to inhale even at low concentrations. Because of this, special considerations were made to ensure that a leak in a multiplexor tube or a faulty gas connection would not result in injury or death.

The first step was to assume a worst case scenario and plan for that event. If the gas were to flow openly out of the device, it needed to be contained and vented safely. Placing the apparatus in a fume hood was considered, but the space in the hood was needed for other research activities. A humidity controlled, atmosphere controlled incubator designed for working with plant culturing was settled on for operating the screening device inside. The unit had the advantage of being designed for airtight operation. It was an older unit, so repairs were made and it was modified to feed in the gas lines.

1. All gas exiting the multiplexor device was sent through a line to vent at the top of the fume hood.
2. A fan was attached to a sample port to blow air from the room into the incubator while another line was open that led to the fume hood. This was to ensure that carbon monoxide did not accumulate inside the incubator and hurt a researcher opening the door.

3. A carbon monoxide alarm was placed directly next to the incubator to alert any bystanders of a gas leak. Similarly, a sign was placed on the door to the lab space instructing anyone passing by how to react to the alarm and who to contact.

This mode of operation was run safely for the duration of this work with syngas. A safety inspection informed the group that redundant carbon monoxide alarms are required for this type of operation. It should be noted that this is a requirement if any future work is done with syngas or carbon monoxide.

Prior to the start of each syngas run, the carbon monoxide detector was actually placed inside the incubator for about an hour to check for leaks. During start-up and sampling the device was moved around the most so those times were determined to be the highest risk for developing a leak.

Appendix F

Overview of Techniques Used

Growing *C. ljungdahlii*

C. ljungdahlii is a motile, rod-shaped bacterium that forms spores. It is capable of growing both heterotrophically and chemoautotrophically. It is also an obligate anaerobe so special care must be taken to avoid exposing it to oxygen. Initial cultures were grown by Nymul Khan. A cryostock was prepared by Reed Taylor and Nymul Khan using filter sterilized 10% glycerol.

For the work presented in this thesis, CGM media, as reported by Hoogendoorn and van Kasteren, was used to grow *C. ljungdahlii* heterotrophically. However, they did not provide a final pH for the media since most of their work was done in a continuous reactor with pH control that tested a range of values. The literature is inconsistent about optimal pH for *C. ljungdahlii.* A low pH (~5) is generally though to inhibit acetate production and boost ethanol production but also inhibits growth. A high pH (~6.5) is thought to promote growth but also increases the levels of acetate produced.

Initial heterotrophic experiments were performed to try and optimize the pH for the *C. ljungdahlii* grown in this project. CGM media was prepared as described with final media pH adjusted to three different values—5.0, 5.8, and 6.6. *C. ljungdahlii* was inoculated into these media types in duplicate. The culture at pH 5.8 was run in triplicate. A small amount of resazurin was added as a dissolved oxygen indicator to test if the dye had any adverse effects on *C. ljungdahlii.*

The only cultures that experienced measureable growth were the ones in pH 6.6 (OD 0.5). The other cultures all had final ODs of less than 0.15. A quick pH measurement was done on the final cultures using pH strips. A picture of the results can be seen in **Figure F-1.** These results seem to indicate that, no matter the stating pH of the culture, the growth of *C. ljungdahlii* adjusts the pH down to between pH 4 and 5 through the production of acetate. This pH seems to be the lower limit for *C. ljungdahlii* growth before spores start to form.

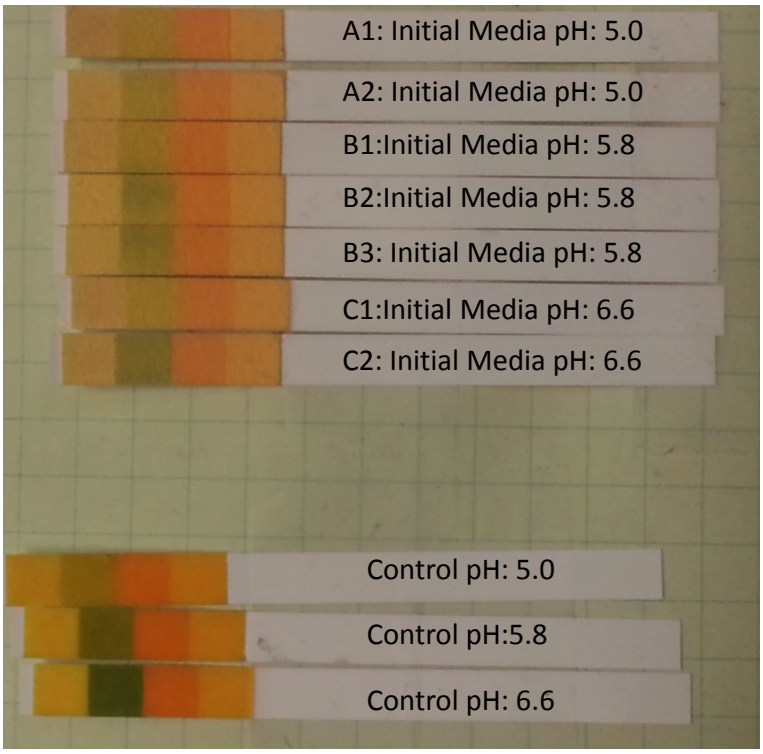

Figure F-1: pH strips showing the final culture OD of *C. ljungdahlii* after heterotrophic growth. Control strips were done in fresh heterotrophic media.

A later test was done with resazurin in a culture with initial pH 6.6. This culture showed no differences from other cultures at the same pH, so resazurin was found be an acceptable oxygen indicator for use with *C. ljungdahlii.*

It is important to note that *C. ljungdahlii* is capable of brief exposures to atmospheric conditions even though it is categorized as an obligate anaerobe. At the suggestion of Trevor Zuroff, inoculations were done outside in a bacterial hood to avoid contamination of chamber. This procedure was validated by work done by Tirado-Acevedo that showed *C. ljungdahlii* was tolerant of up to 8% oxygen for up to 12 hours [10]. All inoculations and subculturing with *C. ljungdahlii* was done aerobically for this project.

Extracting Was Esters

During the Wax Ester project, total lipid extractions were performed on cell cultures to check for the presence of wax esters. These were done on various bacteria where the wax ester gene was either native or had been inserted through genetic engineering.

Cell cultures were spun in a centrifuge at 1000 rcf for 10 minutes and the cells were transferred to glass screw top vials. Methanol and chloroform were added in a 2:1 ratio (1.3 mL MeOH, 0.7 mL CH3Cl). This mixture was vortexed for 30 seconds and then left for 30 minutes to allow cells to lyse. Chloroform and DI water were added in a 1:2 ratio (0.7 mL CH3Cl, 1.3 mL DI H2O). This was then centrifuged at 1000 rcf for 5 minutes to separate phases. The aqueous layer was discarded and the organic layer was dried under a nitrogen stream. The solution used for TLC was 90:7.5:1 of hexane, diethyl either, and acetic acid.

Appendix G

Selecting a Host Strain of *Rhodobacter capsulatus*

The doubling time of *R. capsulatus* was known to be on the order of 6-9 hours from previous experience and literature references [23]. The values obtained from this work were on the order of 15-20 hours which matches more closely with the literature value of 22 hours reported by Tabita [28]. The reported literature values on chemoautotrophic growth cover a large range because *R. capsulatus* can behave differently depending on whether it has adjusted fully to the new metabolic mode. Some lines that have been examined through the APRA-E project have not grown well in an autotrophic culture until they were subcultured into fresh autotrophic media.

The experiments did suggest that the line SB1003 was the quickest to adapt to autotrophic growth. This could prove beneficial for the screening process as it would reduce the down time spent waiting for cultures to move beyond the lag phase. The line SB1003 was eventually chosen as the host line for a variety of reasons. It performed well on later kinetic tests, responded well to plasmid additions, and retained mutations. While this experiment did not conclude the search for a host line, it did prove the capabilities of the multiplexor screening device to generate highly precise growth curves for obtaining kinetic information.

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- Develop and design alpha system that meets cGMP standards for FDA validation
- Purify monoclonal antibodies from commercial pharmaceutical partners

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