Optimization of Growth Conditions for Methanol Consumption in *Escherichia coli* Expressing Methylotrophic Genes

by

Michael A Palmer

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Degree in Chemical Engineering with Distinction

Spring 2016

© 2016 Michael A Palmer All Rights Reserved

## **Optimization of Growth Conditions**

## for Methanol Consumption

## in Escherichia coli Expressing

# **Methylotrophic Genes**

by

Michael A Palmer

Approved:

Eleftherios T. Papoutsakis, PhD Professor in charge of thesis on behalf of the Advisory Committee

Approved:

Wilfred Chen, PhD Committee member from the Department of Chemical and Biomolecular Engineering

Approved:

Christopher Kloxin, PhD Committee member from the Board of Senior Thesis Readers

Approved:

Michael Arnold, Ph.D. Directory, University Honors Program

## ACKNOWLEDGMENTS

I would like to thank Dr. Terry Papoutsakis, Dr. Brian Whitaker, Kyle Bennett and the rest of the Papoutsakis lab for their support and assistance over the past year as I was conducting my research. I would also like to thank the ARPA-E program for their generous funding. Finally, I want to thank my friends and family for their emotional support throughout this process.

# TABLE OF CONTENTS

LIST	OF TA	ABLES
LIST	OF FI	GURESvii
ABST	RAC	Гviii
1	INTI	RODUCTION1
	1.1	Goals and Motivation1
	1.2	Overview of Strategy
	1.3	Prior Developments
2	Early	y Strain Development
	•	
3	Co-S	Substrate Determination7
	3.1	Methods
	3.2	Results
	3.3	Analysis
4	Care	Il Saala Chamastat at Variana Vasat Extract Concentrations
4	Sina	ii Scale Chemostat at Various Feast Extract Concentrations
	4.1	Methods
	4.2	Results
	4.3	Analysis
5	Cher	nostat Characterizations at Low Yeast Extract Concentrations
	51	Mathada 10
	5.1	Deculta 20
	5.2 5.3	Analysis 21
	0.0	21
6	Adaj	ptive Evolution
	6.1	Methods
	6.2	Results
	6.3	Analysis
REFE	RENC	CES
	-	

А	Construction of Small-Scale Chemostats	. 28
В	Tabulated Data	. 30

# LIST OF TABLES

Table 1: Average biom	ass yields for the recombinant strain with or without
methanol,	and average rate of methanol loss for the recombinant strain
and the st	rain without the MDH gene (* $p < 0.05$ in unpaired t-test) 13
Table A.1: Optical den	sity over time for the strain containing all methylotrophic
genes in 1	g/L yeast extract with or without 60 mM methanol, as well
as the mer	hanol concentration over time for the culture containing
methanol	
Table A.2: Summary o	f biomass yield (g biomass/g yeast extract) and methanol
loss rate (	g methanol/g biomass*hr) for all large scale batch
experiment	hts performed with yeast extract as the co-substrate, along
with avera	age values, standard deviations, and p values for a un-paired
t-test	32
Table A.3: Optical den	sities and methanol concentrations along with methanol loss
rates for c	ultures at steady-state at various yeast extract concentrations 33
Table A.4: Optical den over time	sity for each reactor in the adaptive evolution experiment

# LIST OF FIGURES

Figure 1: Diagram of methylotrophic metabolic pathway incorporating methanol dehydrogenase and the ribulose monophosphate pathway [Whitaker et al. Current Opinion in Biotechnology (2015)] <sup>8</sup>
Figure 2: Percent survival of cell cultures in various concentrations of methanol with respect to the media only control
Figure 3: Diagram of reactors used for co-substrate determination experiments
Figure 4: Cell density over time for both a reactor containing methanol and yeast extract and a reactor containing only yeast extract, and methanol concentration over time
Figure 5: Diagram of small-scale chemostat used for continuous culture experiments
Figure 6: Steady-state optical densities for yeast extract concentrations of 0.25, 0.50, 0.75, and 1.00 g/L at a dilution rate of 0.2 hr <sup>-1</sup>
Figure 7: Cell concentration over time in continuous culture at varying yeast extract concentrations and a dilution rate of 0.25 hr <sup>-1</sup>
Figure 8: Optical density over time for four chemostat reactors with 0.15 g/L yeast extract and 60 mM methanol at a dilution rate of 0.25 hr <sup>-1</sup>
Figure A.1: Volumetric flowrate vs. pump setting for the pumps used for the small- scale chemostats with 8mm rubber tubing

## ABSTRACT

A variety of experiments were carried out to determine the optimal growth conditions for newly developed strains of *Escherichia coli* expressing methylotrophic genes. Yeast extract was determined to be the ideal co-substrate for methanol incorporation into biomass, providing a statistically significant biomass yield from 0.22 g biomass/ g yeast extract without methanol to 0.28 g biomass/ g yeast extract in the presence of methanol. Experiments were then conducted in continuous culture using varying yeast extract concentrations as a co-substrate with methanol in order to determine optimum growth conditions for methanol consumption. Finally, an adaptive evolution experiment was conducted in an attempt to induce a population of *E. coli* that showed improved growth on methanol over the genetically engineered base-strain.

## Chapter 1

## **INTRODUCTION**

## **1.1** Goals and Motivation

With the global push towards development of alternative fuels there is a plethora of novel techniques being implemented to overcome the challenges presented by attempting to create cheap and renewable sources of energy. Biological avenues of fuel production are rapidly becoming more prevalent, such as the techniques for the production of corn ethanol being used nationwide. It thus follows that, as genetic techniques improve, these are also being incorporated into solving modern energy problems. One such approach makes use of previously established techniques of metabolic engineering to design and create microorganisms capable of production of fuel molecules.

The basic technique of creating organisms capable of non-native metabolism lies in recombinant DNA. Recombinant DNA takes genetic material coding for the desired proteins from organisms that natively contain it, and incorporates these genes into the new organism hosts. These genes allow the organism to produce the proteins necessary for the desired metabolism.

The appeal of using microorganisms for production of fuel lies heavily in the simplicity of implementation once an appropriate organism has been developed. Under the right conditions microorganisms can be very efficient compared with some large-scale chemical processes and they are additionally self-replicating, and are thus able to replace themselves and operate continuously. Furthermore, each microorganism can

operate as a multi-stage process, thus avoiding the necessity for multiple actual stages in a production process.

For our research we used *Escherichia coli* as a platform organism because of its tolerance to genetic alterations. The overall goal of the project is to create a strain of *E. coli* capable of utilizing methanol as a carbon source to produce butanol. The appeal of this type of research lies heavily in the availability of methane and methanol, as the former comprises the bulk of current natural gas stocks while technology is fast advancing to generate the latter. Although much of current natural gas stocks are currently not possible to reach<sup>1</sup>, there is still more than the current market can find use for, thus lowering the price of production and making it a cheap chemical feedstock<sup>2</sup>. Thus, this strain of *E. coli* would be capable of producing energy at a relatively low cost by using methanol as a growth substrate or co-substrate, along with sugar from renewable sources, for the generation of biofuel molecules. This process is made further appealing by the fact that it is a potential natural gas to liquid fuel pathway, which is currently something that is not cheaply available in modern fuel production techniques<sup>3</sup>.

#### **1.2** Overview of Strategy

In order to produce an organism capable of methylotrophy it is necessary to incorporate the metabolic pathway for methanol incorporation. This pathway consists of two major steps: methanol oxidation and formaldehyde assimilation. The methanol oxidation step takes methanol and converts it to formaldehyde while the formaldehyde assimilation step takes the formaldehyde and converts it to fructose-6-phosphate which can then be further processed by the native metabolism of *E. coli*.

Once a strain has been constructed it is necessary to perform reactor characterization to determine efficiency and to optimize the genes by determining which donor organism provides the best genes for the desired product, as well as to determine in what proportion the genes should be expressed in the host organism in order to maximize production. Additionally, optimizing growth conditions, such as substrate concentrations, through reactor characterizations is an important step in determining an overall process that provides the desired results. These types of characterizations were the primary focus of the research that I conducted.

In addition, there are potential benefits to running adaptive evolution experiments in order to possibly drive an organism towards successful growth on a non-native carbon source. The basic principle enlists the strategy of adding selection pressure in a continuous culture system in the hope that a beneficial mutant will arise<sup>4</sup>. This type of experiment is also herein discussed.

#### **1.3 Prior Developments**

Prior research has been conducted to determine the genes coding for enzymes responsible for methanol metabolism in native methylotrophs, in order to identify the optimal genes for applications in synthetic biology. This work is particularly important for projects such as this one, because in order to determine the best metabolic pathway for successful growth on methanol, it is necessary to ensure that the best enzymes are being used.

In 1988, an NAD+-dependent alcohol dehydrogenase, exhibiting particular reactivity with methanol, was isolated from *Geobacillus stearothermophilus*<sup>5</sup>. Similarly, a study in 2013 identified NAD+-dependent methanol dehydrogenase genes

in *Bacillus methanolicus* to determine the full range of effectiveness of the different variations of the enzyme that they code for<sup>6</sup>.

Several nucleic acid sequences were patented for varying degrees of alcohol dehydrogenase activity, and in particular for their ability to dehydrogenate methanol<sup>7</sup>. These results regarding effectiveness of enzymes were used during strain development to create an optimal strain of *E. coli* expressing the desired genes.

## Chapter 2

#### **Early Strain Development**

The two main metabolic steps necessary for an organism to be methylotrophic are the methanol oxidation step and the formaldehyde assimilation step. In our primary strain the methanol oxidation step is accomplished using the methanol dehydrogenase (MDH) enzyme which takes methanol and converts it to formaldehyde. The gene coding for the MDH enzyme in our strain was cloned from *G. stearothermophilus*. The formaldehyde assimilation step is carried out via the ribulose monophosphate pathway (RuMP) which consists of the 3-hexulose-6-phosphate synthase (HPS) and 6phospho-3-hexuloisomerase (PHI) enzymes. The HPS enzyme converts formaldehyde to hexulose-6-phosphate and the PHI enzyme converts the hexulose-6-phosphate to fructose-6-phosphate which is subsequently converted to pyruvate and then to acetyl-CoA by native metabolic pathways present in *E. coli*. Acetyl-CoA can be used by the cell for a variety of functions, including acting as a precursor for a butanol production pathway. The genes that code for the expression of the RuMP pathway enzymes were cloned from *B. methanolicus*.



Figure 1: Diagram of methylotrophic metabolic pathway incorporating methanol dehydrogenase and the ribulose monophosphate pathway [Whitaker et al. Current Opinion in Biotechnology (2015)]<sup>8</sup>

Additionally, both the vector containing the gene for the MDH enzyme and the vector containing the genes for the RuMP pathway, contained genes coding for antibiotic resistance, one for chloramphenicol and one for ampicillin, in order to apply selective pressure and increase the likelihood that the plasmid would be retained. Initially, the strain had the RuMP genes on a high copy vector and the MDH gene on a low copy vector because it was previously determined that the RuMP pathway is the rate determining step in the overall metabolic pathway. This, however, did not prove to provide any benefit in practical use, therefore both sets of genes were cloned on a high-copy vector to increase total amount of protein produced. It is important to note that while other strains were developed using genes from different organisms and with different levels of expression in the *E. coli*, the above strain produced the best results and is therefore what was later used in the kinetic characterizations and co-substrate determination.

## Chapter 3

## **Co-Substrate Determination**

## 3.1 Methods

Kinetic characterization was done in batch experiments utilizing 5 liter, pHcontrolled tank reactors. It was quickly determined from small scale experiments that the strain is not capable of growth on methanol alone, and therefore we examined a variety of co-substrates in order to determine if improved growth on methanol in a cosubstrate in comparison to on the co-substrate alone, could be shown. All experiments were run in M9 minimal media at 37° C and at a pH between 6.8 and 7.2. The tank reactors were agitated at 125 rpm in order to ensure even concentration distributions as well as avoid the buildup of biofilms. We also maintained the dissolved oxygen level between 10% and 20% during the phase of exponential growth, in order to ensure optimal aerobic growth conditions for *E. coli*. The initial methanol concentration during each experiment was also held constant at 60 mM. The value of 60 mM was chosen because a previous experiment comparing cell viability over time in varying concentrations of methanol showed that methanol begins to be toxic to *E. coli* at around 250 mM, and it was desirable to operate significantly below that threshold to ensure that toxicity was not a contributing factor in these experiments.



Figure 2: Percent survival of cell cultures in various concentrations of methanol with respect to the media only control



Figure 3: Diagram of reactors used for co-substrate determination experiments

Throughout the experiments, the cell concentration was monitored using spectroscopy to determine the optical density at a wavelength of 600 nm, which was used as a metric for growth. Substrate concentration, concentration of secondary metabolites, and methanol concentration were monitored over time using high pressure liquid chromatography in order to determine when the co-substrate had been completely consumed as well as to monitor rate of methanol loss.

Two types of control experiments were run alongside these experiments. One type was run using the recombinant strain with complete metabolic pathways and in the same media and environmental conditions, but without methanol, in order to see if the presence of methanol provides growth benefits over the co-substrate alone. The other type was evaporation controls, in which a strain lacking the MDH genes but containing the RuMP genes was run in identical media and conditions to the main experiments in order to examine the background rate of methanol loss due to evaporation and stripping. The intent of this was to provide a baseline rate of methanol loss that could be subtracted from the rate of methanol loss found in the experiments and thus determine if there was any significant incorporation of methanol into the *E. coli*.

The first co-substrate that was assessed was glucose. Glucose was an attractive option at first because of how well *E. coli* grows on it and additionally because it is easily detectable using HPLC and thus its concentration over time could be used to analyze growth kinetics. Glucose is also the standard growth substrate in labs and industry. The starting glucose concentration for all experiments was 4 g/L.

The next co-substrate that was assessed was xylose, also in a concentration of 4 g/L. This was considered as a potentially viable co-substrate due to the fact that the

tac promoter, is not repressed by xylose. Additionally, xylose is one of the most abundant monosaccharides on earth, because it is present in lignocellulosic biomass, making it an attractive industrial option. Thus it was hypothesized that using xylose as a co-substrate would increase the level of non-native protein expression in the cells and increase the ability of the cells to incorporate methanol.

The final co-substrate that was evaluated was yeast extract. We hypothesized that yeast extract would give the *E. coli* enough substrate to begin growth on but not provide resistance to methanol metabolism, as all the yeast extract is providing the cells is amino acids to scavenge. Yeast extract was added to the media in a concentration of 1 g/L.

All co-substrates were analyzed to determine which provided the best conditions for methanol consumption by comparing methanol loss rates as well as cell culture densities over time and thus calculating growth rates and carbon yields. The average yield was calculated using the equation:

(eqn. 1) 
$$Y_{Sx} = \frac{dC_x/dt}{dC_s/dt} = \frac{\Delta C_x}{\Delta C_s}$$

Where  $C_x$  is the concentration of cells, measured in g/L and  $C_s$  is the concentration of the co-substrate also measured in g/L.

Additionally, methanol incorporation rate was calculated using the following equations:

(eqn. 2) 
$$-r_{s} = \frac{dC_{s}}{dt} = \frac{\Delta C_{s}}{\Delta t}$$

(eqn. 3) 
$$q_s = \frac{-r_s}{c_x}$$

#### 3.2 Results

Contrary to initial predictions all the experiments that were run with glucose produced unfavorable results, in which the reactor containing the recombinant strain and methanol showed much lower cell growth than the control reactor containing the strain without the MDH genes. As a result we plated samples of the culture from the failed reactors on different types of agar plates: ones with no antibiotics, chloramphenicol, ampicillin, and with both antibiotics, in order to determine if the decreased cell growth was caused by a loss of a plasmid and thus susceptibility to antibiotics. From these results it was determined that the culture was losing the MDH gene, resulting in death from the antibiotics present in the media.

Similarly, in xylose, the results were not as favorable as initially hypothesized. In this case, although the strain retained both plasmids, there was no significant difference seen between the recombinant strain in methanol and the control for the recombinant strain in the absence of methanol.

The results from the experiments utilizing yeast extract as the co-substrate were clearly far better than those obtained using glucose and xylose, and after the first experiment it could be seen that the recombinant strain grew to a higher cell density in the presence of methanol than it did in the absence of methanol when starting at the same cell concentration. These results were repeated and replicated very well, showing consistant increase in optical density in contrast to the non-methanol control, as well as an increase in methanol loss rate over the evaporation control.



Figure 4: Cell density over time for both a reactor containing methanol and yeast extract and a reactor containing only yeast extract, and methanol concentration over time

The average yield was calculated for the experiment with the recombinant strain in the presence of yeast extract and methanol as well as for the control condition of yeast extract and absence of methanol. Biomass yields were  $0.28 \pm 0.03$  g biomass/g yeast extract and  $0.22 \pm 0.02$  g biomass/g yeast extract respectively. Additionally, these were determined to be statistically significant using an unpaired ttest, with p < 0.05. The average rate of methanol loss was determined in the experiments with the recombinant strain and methanol to be  $0.024 \pm 0.004$  g methanol/g biomass and in the evaporation controls the average rate of methanol loss was  $0.014 \pm .004$  g methanol/g biomass. These results were also determined to be statistically significant with p < 0.05.

Table 1: Average biomass yields for the recombinant strain with or without methanol, and average rate of methanol loss for the recombinant strain and the strain without the MDH gene (\* p < 0.05 in unpaired t-test)

Average biomass yield (g	+MeOH	$(0.28 \pm 0.03)^*$
oronnass, g yeast extract)	-MeOH	$0.22 \pm 0.02$
Methanol Loss (g methanol /g	+MDH	$(0.024 \pm 0.004)^*$
cell dry weight *hr)	-MDH	$0.014 \pm 0.004$

## 3.3 Analysis

The loss of the plasmid containing the MDH gene in the presence of glucose could be explained in a few different ways. The selective loss of the MDH could have been due to degradation of the antibiotic over time. Without the selective pressure, the *E. coli* would shift towards a population without the plasmid, thus avoiding unnecessarily using energy to replicate the DNA. This population would still be susceptible to any remaining antibiotic, thus killing the cells, but the selection pressure would not be enough to retain the plasmid. It is also possible that in the presence of glucose, which is a much more favorable substrate for growth than methanol, the cells shifted towards a population without the methanol incorporation genes due to the large energy difference between glucose metabolism and methanol metabolism. Additionally, glucose represses the tac promoter, which is the promoter used for our strain, thus further decreasing any possible growth benefits from methanol metabolism. After this result it was decided that glucose was not an appropriate co-

substrate for future experiments. Additionally, following these experiments the strain was slightly altered to have a single plasmid construct containing all genes to avoid the loss of vectors in the future.

The results of batch experiments in the presence of xylose indicate that methanol does not provide a significant growth benefit to our strain when using xylose as a co-substrate. Furthermore, the rate of methanol loss was not greater in the experiment than in the evaporation control which further proves that under these conditions methanol is not being incorporated into biomass. The results indicate that while xylose is not an inhibiting the genes required for growth on methanol, as was the case where the MDH vector was being rejected in the presence of glucose, there are no growth benefits associated with xylose as a co-substrate and thus it is also not a viable co-substrate to proceed with in further experiments.

The results of batch experiments in the presence of yeast extract, however, clearly show an improvement in methanol incorporation. The combination of an increased rate of methanol loss over the evaporation control coupled with the data showing a higher carbon yield indicates strongly that methanol is being consumed by the E. coli and being incorporated into biomass. After obtaining these results it was decided that the best way to proceed is to continue to use yeast extract as a co-substrate.

These results are promising because they provide indication that the strain is capable of incorporating methanol into biomass. This also indicates that in the future, genes could be incorporated that would establish a metabolic pathway downstream for the production of a valuable metabolite.

## Chapter 4

## **Small Scale Chemostat at Various Yeast Extract Concentrations**

## 4.1 Methods

After determining the optimal co-substrate for improved growth on methanol, it became desirable to examine how the cells function in a continuous culture. Analyzing relative steady-states in different conditions can provide insight into what is the best environment for methanol incorporation into the cell.

In order to do this, experiments were run at a dilution rate of  $0.2 \text{ hr}^{-1}$  and  $0.4 \text{ hr}^{-1}$ , and at yeast extract concentrations of 0.25 g/L, 0.5 g/L, 0.75 g/L, and 1 g/L. Data were collected after roughly 48 hours following the beginning of the experiment in order to give the system sufficient time to achieve steady-state. The samples were analyzed for optical density at wavelength 600nm as well as for methanol concentration in the steady-state system in comparison to the feed concentration using high pressure liquid chromatography.

It was hypothesized that lowering the yeast extract concentration in the feed media would improve the rate of methanol incorporation into the cells due to increased pressure from the lower quantity of co-substrate.



Figure 5: Diagram of small-scale chemostat used for continuous culture experiments

# 4.2 Results

In these experiments there was a clear difference in the steady-state optical density between the four yeast extract concentrations used, as was expected. The steady-state optical density scaled roughly linearly for these four conditions. This trend was observed in both the  $0.2 \text{ hr}^{-1}$  and the  $0.4 \text{ hr}^{-1}$  dilution rate cases.



Figure 6: Steady-state optical densities for yeast extract concentrations of 0.25, 0.50, 0.75, and 1.00 g/L at a dilution rate of 0.2 hr<sup>-1</sup>

It was, however, observed from the data of methanol concentration over time that it would be nearly impossible to determine any differences in methanol consumption due to the high variability in these systems. The evaporation controls proved that the amount of methanol being stripped from these reactors varies based on which reactor is being examined and also varies over time.

#### 4.3 Analysis

Based on the data for the optical densities at various yeast extract concentrations it can be concluded that the steady-state optical density scales linearly with the yeast extract concentration. This indicates that all of the yeast extract concentrations used for this experiment at both dilution rates were far away from the washout point of our strain but did not provide any evidence that a lower yeast extract concentration in the feed would provide a higher relative yield as was hypothesized. Furthermore, as aforementioned, from these experiments it became clear that it would be impossible to gain any information about relative methanol consumption rates from this type of small-scale chemostat experiment due to the significant fluctuations from the aeration system. This forced the reevaluation of the method for characterization of relative success of a particular strain from rate of methanol consumption to the steady-state optical density versus a control.

## Chapter 5

## **Chemostat Characterizations at Low Yeast Extract Concentrations**

#### 5.1 Methods

The purpose of continuous culture experiments is to examine how different conditions alter steady-state. In our case the new goal was to, over time, decrease the concentration of yeast extract and examine how the steady-state changes at critically low concentrations of yeast extract to evaluate when the cells begin to become strained by the low quantities of co-substrate.

Before this experiment began, it was shown through a  $C^{13}$  flux analysis, that a significant amount of the carbon being incorporated into the cell via our introduced metabolic pathways was being lost as CO<sub>2</sub>. One way that *E. coli* can regenerate ribulose-5-phosphate (Ru5P) (the substrate used in our strain for formaldehyde fixation) is by sending carbon from fructose-6-phosphate through the native oxidative pentose phosphate pathway. This culminates in the formation of Ru5P and the loss of CO<sub>2</sub>. In order to combat this cycle and direct the carbon flux down the glycolysis pathway, two new strains were created, each one with a knock-out of one of the enzymes in the undesired pathway, glucose 6-phosphate-1-dehydrogenase (*zwf*) and phosphoglucose-isomerase (*pgi*) respectively, in addition to still containing the MDH and RuMP genes. These strains were chosen to be used for the adaptive evolution as they should provide improved growth on methanol with minimal carbon loss.

The experiment was run at a dilution rate of 0.25 hr-1 with 60 mM methanol and an initial yeast extract concentration of 0.2 g/L because the previous experiment involving continuous culture showed that these conditions put a strain on the cells, which was illustrated by fairly low optical density at steady-state, but the cells are still fairly far from the washout point, which is a necessary precaution to avoid accidental washout mid-experiment.

After the cells had reached steady-state in 0.2 g/L yeast extract, the yeast extract concentration was lowered to 0.18 g/L. Subsequently, after the steady-state was reached at 0.18 g/L then the concentration of yeast extract was further lowered to 0.15 g/L.

## 5.2 Results

The results from the experiment involving the strains with the knocked out pgi and zwf genes showed the approach to washout in low concentrations of yeast extract. At yeast extract concentrations of 0.2 g/L and 0.18 g/L the cell density remained approximately constant in both strains. However, after dropping the co-substrate concentration to 0.15 g/L yeast extract from 0.18 g/L, the cell density drops by roughly a factor of 2 for both strains.



Figure 7: Cell concentration over time in continuous culture at varying yeast extract concentrations and a dilution rate of  $0.25 \text{ hr}^{-1}$ 

Two of the cultures being used for this experiment became contaminated so only three data points exist for the first steady-state data point after the yeast extract concentration was lowered to 0.15 g/L and only two data points exist for the final sample.

#### 5.3 Analysis

From the results of the experiment in which the yeast extract concentration started at 0.2 g/L and was dropped over time we gained valuable information about the washout point of our strain at a dilution rate of 0.25 hr<sup>-1</sup>. The difference in steady-state optical density between the cultures at 0.2 g/L yeast extract and 0.18 g/L yeast extract was fairly small, but when the yeast extract was lowered to 0.15 g/L the steady-state optical density decreased by roughly 50%, indicating that this is very near the washout point. At this point the maximum growth rate in this substrate-limited condition is

approaching the dilution rate, resulting in lower steady-state concentrations than would be expected based on proportional yield decrease due to less co-substrate.

This data is important moving forward because adaptive evolution experiments are best run at a condition that is very near washout due to the higher selective pressure. By knowing that the washout point is around 0.15 g/L of yeast extract we are able to design an adaptive evolution experiment that will give us the best chance of selecting for a mutant that is better able to utilize methanol as a substrate for growth.

## Chapter 6

## **Adaptive Evolution**

## 6.1 Methods

Following the determination that a yeast extract concentration of 0.15 g/L was fairly close to the washout point at a dilution rate of 0.25  $hr^{-1}$  for our strain, an adaptive evolution experiment was started at that yeast extract concentration and dilution rate with 60 mM methanol. The goal was to determine whether a mutant could be selected for that was better able to utilize methanol as a substrate.

The media was kept at a yeast extract concentration of 0.15 g/L and replaced every 3 days to ensure that the antibiotics were fresh and that the salts in the media did not begin to precipitate.

It was decided that optical density would be used as the metric for measurement of growth success, and that any continuously seen increase from the initial steady-state over time could be indicative of a possible mutant that was better able to utilize methanol as a growth substrate.

## 6.2 Results

Over time the optical density was monitored in a yeast extract concentration of 0.15 g/L and a methanol concentration of 60 mM in order to determine if a strain that was better able to consume methanol as a substrate would emerge. There exists some fluctuation in the data due to the difficulties associated with measurement of such small quantities of yeast extract, however there appears to be a trend in the data after

around 150 hours showing that reactors 1 and 2 achieve a consistently higher steadystate optical density than they did at the start of the experiment.



Figure 8: Optical density over time for four chemostat reactors with 0.15 g/L yeast extract and 60 mM methanol at a dilution rate of 0.25  $hr^{-1}$ 

The other two reactors show roughly the same optical densities for the entirety of the experiment.

The optical densities in reactors 1 and 2 at around 300 hours are around 0.1 which is much higher than the average optical density of 0.077 for the controls done utilizing the same strain but without the MDH enzyme making it incapable of gaining a growth benefit from methanol. Shortly after this boost is observed, however, the optical density decreased to approximately the level at the start of the experiment.

## 6.3 Analysis

From the observed data there appeared to be an increasing trend in the first two reactors towards an increased growth over that at the start of the experiment. This may have been due to fluctuation in media because following about a week of elevated optical densities, the cultures return to approximately the steady-state they were at during the beginning of the experiment.

Adaptive evolution experiments often take months to carry out, depending on the organism being adapted. The variation in length of experiment depends on the doubling time, or generation time, for the individual organism, with those dividing quicker reaching a desired mutation more rapidly. In this case there was not enough time for a successful adaptive evolution experiment to be carried out, and the difficulties inherent in utilizing media with such low concentrations made it difficult to observe changes in steady-state. For future work it may be beneficial to scale up the experiment and therefore reduce the errors and challenges associated with operation on such a small scale.

## REFERENCES

- 1. Blohm A, Peichel J, Smith C, Kougentakis A: **The significance of** regulation and land use patterns on natural gas resource estimates in the Marcellus shale. *Energy Policy* 2012, **50**:358-369.
- 2. Haynes CA, Gonzalez R: **Rethinking biologocal activation of methane and conversion to liquid fuels**. *Nature Chemical Biology* 2014, **10**:331-339.
- 3. Fei Q, Guarnieri MT, Tao L, Laurens LML, Dowe N, Pienkos PT: Bioconversion of natural gas to liquid fuel: Opportunities and challenges. *Biotechnology Advances* 2014, **32**:596-614.
- Lee, Dae-Hee, Bernhard Ø. Palsson. Adaptive Evolution of Escherichia Coli K-12 MG1655 during Growth on a Nonnative Carbon Source, L-1,2-Propanediol. *Applied and Environmental Microbiology* 2010, 76.13 4158-168.
- Sheehan MC, Bailey CJ, Dowds BCA, Mcconnell DJ: A New Alcohol-Dehydrogenase, Reactive Towards Methanol, from Bacillus-Stearothermophilus. *Biochemical Journal* 1988, 252:661-666.
- Krog A, Heggeset TMB, Muller JEN, Kupper CE, Schneider O, Vorholt JA, Ellingsen TE, Brautaset T: Methylotrophic Bacillus methanolicus Encodes Two Chromosomal and One Plasmid Born NAD(+) Dependent Methanol Dehydrogenase Paralogs with Different Catalytic and Biochemical Properties. *Plos One* 2013, 8.
- Brautaset T, HEGGESET TMB, KROG A, Quax WJ, SIBBALD MJJB, VORHOLT J, Müller J, Kiefer P, POTTHOFF E, Wendisch VF: Novel methanol dehydrogenase enzymes from bacillus. World patent publication WO/2013/110797 A1. 2013, Assignees: Sinvent As, Rijksuniversiteit Groningen, Eth Zurich.

 Whitaker, William B., Nicholas R. Sandoval, Robert K. Bennett, Alan G. Fast, and Eleftherios T. Papoutsakis. Synthetic Methylotrophy: Engineering the Production of Biofuels and Chemicals Based on the Biology of Aerobic Methanol Utilization. Current Opinion in Biotechnology 2015 33: 165-75

#### Appendix A

## **Construction of Small-Scale Chemostats**

The main body of the chemostats consists of 16x125mm glass tubes from Chemglass. The opening of the tubes is capped with a rubber stopper which is fixed into place by a plastic screw cap with a 9mm opening in the center.

Air is delivered to the vessel through a 21 gauge needle that reaches to the bottom of the volume, thus ensuring even air distribution to the culture. The air flow also serves as the main source of agitation to the culture. Air is vented from the system using a 21 gauge needle in order to avoid pressure buildup in the vessel. Air is passed through a controller and then through a 4-way splitter, before finally being filtered and entering the reactors.

Fresh media is flowed into the system using a 21 gauge needle that is suspended approximately 1 cm above the height of the liquid. Spent media and culture are removed from the system using an overflow system through a 21 gauge needle positioned at the desired height of the liquid.

The fresh media being flowed into the vessel and the spent media and culture being removed are flowed through Masterflex Puri-flex rubber tubing with an inner diameter of 8mm. The flow is provided by Masterflex L/S peristaltic pumps fitted with multichannel heads. The pumps were calibrated using the tubing to determine curves of volumetric flowrate as a function of the displayed pump setting.



Figure A.1: Volumetric flowrate vs. pump setting for the pumps used for the smallscale chemostats with 8mm rubber tubing

## **Appendix B**

## **Tabulated Data**

The data from an experiment run with 1 g/L yeast extract with or without 60 mM methanol is tabulated below. The majority of the timepoints have entries for two technical replicates for both reactor conditions, indicated as "OD1" and "OD2", with the average optical density for each timepoint listed under "Avg OD". For the reactor containing methanol, the HPLC peak area is tabulated under MeOH peak, and then converted to methanol concentration and shown under "MeOH conc".

	+MDH,+MeOH			+MDH,-MeOH		
Time (h)	OD1	OD2	Avg OD	OD1	OD2	Avg OD
0	0.167	0.154	0.4815	0.151	0.153	0.456
1	0.127	0.116	0.6075	0.114	0.116	0.575
2	0.149	0.141	0.725	0.125	0.131	0.64
3	0.159	0.155	0.785	0.139	0.144	0.7075
4	0.169	0.167	0.84	0.151	0.157	0.77
5	0.124	0.118	0.968	0.105	0.109	0.856
6	0.132	0.121	1.012	0.105	0.11	0.86
7			1.101333333			0.962667
8			1.176			0.994667
13			1.362666667			1.088
22	0.148	0.142	1.45	0.105	0.109	1.07
Time (h)	MeOH Peak	MeOH con	с			
0	42379.8	55.33839				
1	42870.4	55.979				
2	42943.4	56.07432				
3	42809.7	55.89974				
4	42581.7	55.60203				
5	42411.4	55.37965				
6	42239.1	55.15467				
7	41427.2	54.09451				
8	41816.3	54.60259				
13	40357.2	52.69733				
22	37633.3	49.14054				

Table A.1: Optical density over time for the strain containing all methylotrophic genes in 1 g/L yeast extract with or without 60 mM methanol, as well as the methanol concentration over time for the culture containing methanol

The biomass yields and rate of methanol loss were tabulated for all experiments using yeast extract as a co-substrate in order to do statistical analysis. The tabulated data shows the average yield values, the standard deviation, and sample size for the conditions with methanol and for the control, along with the p value generated from an unpaired t-test. The average methanol loss rate, standard deviation, and sample size are also shown for the MDH strain as well as the control lacking the gene, along with the p value generated from an unpaired t-test.

## Table A.2: Summary of biomass yield (g biomass/g yeast extract) and methanol loss rate (g methanol/g biomass\*hr) for all large scale batch experiments performed with yeast extract as the co-substrate, along with average values, standard deviations, and p values for a un-paired t-test

Date	Condition	Yield (g/g)	Q (g/ghr)		Yield (+Me	⊇OH)	Yield(-MeOH)	Q (+MDH)		Q(-MDH)
31-Jul	(+MeOH)	0.26	0.018	avg	0.2725		0.216666667	0.02375		0.0135
31-Jul	(-MeOH)	0.23	-	stdev	0.032016		0.015275252	0.004031		0.003536
17-Aug	(-MeOH) or(-MDH)	0.2	0.016	n	4		3	4		2
17-Aug	(+MeOH)	0.32	0.027							
20-Aug	(-MeOH) or(-MDH)	0.22	0.011		Pvalue	0.0406		Pvalue	0.039	
20-Aug	(+MeOH)	0.25	0.026							
24-Aug	(+MeOH)	0.26	0.024							

The optical densities for steady-state cultures at each concentration of yeast extract and each replicate for the experiment conducted in small-scale chemostat at a dilution rate of 0.2hr<sup>-1</sup>. The average optical density from technical replicates of optical density measurements for each biological replicate is also displayed as "avg OD" with "OD1" and "OD2" being the technical replicates. The HPLC peak area for methanol sampled from the culture is indicated as "MeOH" peak and this value converted into a concentration using a standard curve is indicated as "MeOH conc". The methanol HPLC peak area in the fresh media is indicated as "media peak" while the concentration in the fresh media is indicated as "media conc". The methanol loss rate from the cultures is calculated by multiplying the dilution rate by the difference in concentration in the media and in the culture and is shown under "consumption rate (including evaporation). This value is then averaged between biological replicates and normalized to average optical density and displayed highlighted in yellow.

Table A.3: Optical densities and methanol concentrations along with methano	l loss
rates for cultures at steady-state at various yeast extract concentration	ations

YE conc	OD 1	OD 2	avg OD	MeOH Peak	MeoH conc	media peak	media conc	consumption rate (including evaporation)	Consumpt	tion per OD
.25 (1)	0.194	0.192	0.193	39024.2	50.73146	46933.6	61.01368	2.03073845	10.52196	11.714657
.25 (2)	0.18	0.178	0.179	34620.3	45.00639	43619	56.7047	2.310416225	12.90735	
.5 (1)	0.352	0.358	0.355	32929.3	42.80809	45776.3	59.50919	3.29846725	9.291457	8.736004
.5 (2)	0.352	0.352	0.352	34454	44.7902	45669.4	59.37022	2.87955395	8.180551	
.75 (1)	0.556	0.536	0.546	33425.2	43.45276	45854.2	59.61046	3.19114575	5.844589	5.7974878
.75 (2)	0.572	0.564	0.568	33805.8	43.94754	46527.2	60.48536	3.26621945	5.750386	
1 (1)	0.724	0.724	0.724	33251.6	43.22708	45302.2	58.89286	3.09399155	4.273469	5.3343509
1 (2)	0.956	0.924	0.94	21009.9	27.31287	44423.8	57.75094	6.011518825	6.395233	

The data collected for the adaptive evolution experiment is tabulated as optical density over time for each reactor used in the experiment. Reactors 3 and 4 were terminated early in order to do a control with the strain lacking the MDH gene in order to determine the background biomass yield on yeast extract at the dilution rate being used.

Table A.4: Optical	density for each r	reactor in the ac	Japtive evolutior	n experiment over
time				

Time (hrs)	Reactor 1	Reactor 2	Reactor 3	Reactor 4
0	0.067	0.066	0.058	0.066
24	0.072	0.08	0.046	0.071
96	0.045	0.032	0.028	0.044
120	0.045	0.032	0.028	0.044
129	0.064	0.071	0.035	0.065
144	0.062	0.059	0.038	
192	0.063	0.057	0.04	0.065
216	0.063	0.089	0.073	0.069
225	0.063	0.087	0.072	0.067
264	0.091	0.088	0.052	0.088
288	0.093	0.088	0.052	0.088
312	0.103	0.111	0.058	0.084
336	0.076	0.055		