Controlling Recombinant Escherichia coli Cultures to the Boundary of Oxidative and Overflow Metabolism (BOOM) for Robust Efficient Growth

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CONTROLLING RECOMBINANT *Escherichia coli* CULTURES TO THE BOUNDARY OF OXIDATIVE AND OVERFLOW METABOLISM (BOOM) FOR ROBUST EFFICIENT GROWTH

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Electrical Engineering

by
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Accepted by:
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Dr. Adam Hoover
Abstract

In the last three decades, biopharmaceuticals such as human growth hormone have been used to treat many diseases from HIV/AIDS to different types of cancer. Controlling the growth of recombinant cells, such as *Escherichia coli* (*E. coli*), is essential for productivity and efficiency of biopharmaceutical manufacturing. The cell growth can be controlled by regulating feed rate as the limiting factor. Hence, a robust and efficient feed controller – that only uses common industrial sensors – is desired.

In this thesis, a controller is presented which can set the appropriate feed rate based on the metabolic state of *E. coli*. A robust metabolic state detector is designed which can detect whether the cells are in oxidative or overflow metabolism. And the controller periodically adjusts the feed rate based on the response of the cells. The controller, named BOOM II, maintains the cells close to the boundary of oxidative and overflow (BOOM) metabolism. Specifically, during a probe interval, the metabolic state detector exponentially increases the feed rate to the bioreactor. Then, by inspecting the real-time estimated oxygen uptake rate (OUR), the metabolic state is evaluated as oxidative if the sensitivity ratio (SR) signal passes a threshold, otherwise the metabolic state is considered to be in overflow.

The performance of the BOOM II controller was validated by several fermentations and benchmarked against a robust and industrially tested controller that uses
an exponential feed controller. Experiments that used the BOOM II controller resulted in higher cell densities, lower waste product levels and utilized less glucose than the parallel fermentation, using the exponential feed controller. The controller also detected metabolic changes to the culture due to growth phase shifts and recombinant protein induction and adjusted the feed rate due to these disturbances. Since the BOOM II controller is based on metabolism fundamental concepts, it has the potential to work on different strains of *E. coli*, other bacteria, yeast and possibly mammalian cells.
Dedication

This thesis is dedicated to my beloved wife. To whom I owe a debt of gratitude for her dignified patience, unceasing encouragements, heartwarming support and above all for her sincere devotion.
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Chapter 1

Introduction

1.1 Biopharmaceutical Manufacturing

The pharmaceutical industry has spent approximately $58.8 billion in 2015 for research and development of new biopharmaceuticals [PhRMA, 2016]. Biopharmaceuticals (or biologics) are drugs produced by modern biotechnology techniques, specifically genetic engineering. In other words, biopharmaceuticals are therapeutic proteins that are produced by “means other than direct extraction from natural (non-engineered) biological sources” [Walsh, 2013]. Traditional pharmaceutical manufacturing uses chemical synthesis to produce small molecule drugs such as aspirin. However, biologics have complex three dimensional structures that are almost impossible to synthesize chemically [edX Inc., 2015].

For many life-threatening diseases such as cancer, hepatitis C and HIV/AIDS, biopharmaceuticals are used for treatment. Hence, the biopharmaceutical manufacturing is becoming more significant recently. Insulin and human growth hormone (hGH) are examples of biologic drugs that are produced by recombinant Escherichia coli (E. coli).
1.1.1 Cells for Manufacturing

The use of cells for manufacturing is not a modern idea. For millennia, people have used yeast, a single-celled microorganism, to brew beer and wine or to leaven bread. However, cells had not been used to produce medications – in large scale – until 1943 when Penicillin was mass produced as the first therapeutic made by a microorganism called *Penicillium chrysogenum* [Aldridge et al., 1999; Zaffiri et al., 2012]. In 1973, the bacterium *E. coli* was the first organism to be genetically modified [Cohen et al., 1973]. Later, the genetically modified *E. coli* was used to produce human insulin in laboratory [Goeddel et al., 1979]. However, it was not until 1982, that human insulin was commercially produced, as the first recombinant protein therapeutic [Quianzon and Cheikh, 2012]. Recombinant insulin has been the major therapy for diabetes type I and II ever since [Leader et al., 2008].

Many other organisms such as *Saccharomyces cerevisiae* (yeast) and Chinese hamster ovary (CHO) cells have also been used as host cells for producing recombinant therapeutics. However, *E. coli* is “the single most common nonmammalian cell type” still used in biotechnology industry [Walsh, 2014]. Additionally, *E. coli* is a model organism for research and development. *E. coli* are extensively studied in molecular biology because these cell have relatively simple genetics, rapid growth and survivability in different growth conditions [Cooper, 2000].

1.1.2 Oxidative and Overflow Metabolism

Metabolism is the set of all biochemical reactions inside a living cell. A metabolic pathway is a chain of these biochemical reactions. Oxidative metabolism (also known as aerobic respiration) is the set of metabolic pathways that oxidizes the substrate (i.e. glucose) and stores its chemical energy in the high-energy phos-
Glucose

Glycolysis

Pyruvate

Acetyl-CoA

Acetyl-P

Acetate

TCA Cycle

PDC

CO2

ATP

NADH

FADH2

GTP

OXPHOS

O2

36 x ATP

CO2

2 x ATP

2 x NADH

6 x NADH

2 x FADH2

2 x GTP

Figure 1.1: The simplified metabolic pathways of *E. coli* for oxidative, overflow and acetate consumption [Voet et al., 2006].

Phosphate bonds in adenosine triphosphate (ATP) molecules. ATP powers almost all energy-requiring processes in the cells from activation energy for chemical reactions to growth and reproduction [Raven et al., 2010]. Oxidative metabolism consists of glycolysis, pyruvate dehydrogenase complex (PDC), tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) pathways (see Figure 1.1).

Overflow metabolism is a set of metabolic reactions that transforms the substrate into waste products even in the presence of sufficient oxygen. For *E. coli*, oversupply of glucose leads to metabolic flux of Acetyl-CoA that consequently causes excretion of acetate as a waste product (see phosphate acetyltransferase (PTA) and acetate kinase (AckA) pathways in Figure 1.1) [Chang et al., 1999].

Carbon atoms of glucose are completely oxidized in oxidative metabolism as glucose turns into carbon dioxide. However, in overflow metabolism the carbon atoms of the glucose are partially oxidized and acetate is the final carbon product instead of carbon dioxide. Therefore, overflow metabolism is less efficient in utilizing glucose
potential energy. Moreover, acetate production has an inhibitory effect on cell growth [Luli and Strohl, 1990]. Hence, for the biopharmaceutical industry – which grows the host cells for biologics – the overflow metabolism is inefficient because it consumes more substrate for the same growth rate. Also, overflow metabolism is undesirable since it has detrimental effects on product quality [Shuler and Kargi, 1992].

The formation of a protein needs transcription of a gene, which is part of a complete process called gene expression. Reagents – such as isopropyl β-D-1-thiogalactopyranoside (IPTG) – can trigger recombinant protein transcription in a process called induction [Shuler and Kargi, 1992].

1.2 Importance of Bioreactor Control

As discussed in Section 1.1, cells are like miniature factories which can transform raw materials such as substrate and oxygen into energy and vital biochemical components such as the proteins. In order to make the cells produce the recombinant proteins (recombinant therapeutics), they need to be cultured under controlled conditions such as incubators or bioreactors. While shaking incubators control temperature and stir speed, bioreactors have sensors and actuators that allow them to control temperature, stir speed, input gas mixture, pH and substrate. For productive biopharmaceutical production, bioreactors are used to grow the host cells to high cell densities.

Bioreactors control the cell growth by controlling the substrate (feed) rate in a process called fed-batch. Fed-batch processes are widely used to grow cells to high cell densities both industrially and in laboratory scale. Fed-batch processes provide feed to the growing cells intermittently or continuously under controlled conditions in order to avoid overflow metabolism [Lim and Shin, 2013]. Pumps are used to provide
the feed solution into the bioreactor.

As living organisms, the behaviors of the host cells are very complex. Intricate sequences of biochemical reactions make cell growth function of many known and unknown parameters. Therefore, an adaptive closed-loop controller is required to compensate the unpredictable metabolic changes of the cells during the growth. The objective of this thesis is to design a feed controller – by using common sensors – for efficient and reliable growth of *E. coli*.

### 1.3 Outline

This thesis is organized in six chapters. Chapter 2 provides a review of *E. coli* growth models. Also real-time adaptive oxygen uptake rate (OUR) estimators are presented, which is necessary for the BOOM controller. Additionally, the previous *E. coli* growth controllers – such as exponential feed controller and the BOOM I controller – are reviewed. Chapter 3 describes the BOOM II feed controller which is the basis of this thesis. Simulations and techniques that are used to tune the empirical parameters of the controller are also presented in this chapter. Chapter 4 describes the *E. coli* strain, bioreactor, equipments and other materials and methods used for the validation experiments.

In chapter 5, the BOOM II controller is validated by experimental results. The BOOM II controller is also benchmarked against the common robust industrial controller for efficiency and robustness. Chapter 6 concludes this thesis with a summary of the BOOM II controller and suggestions for future work including considerations for using the controller to grow recombinant mammalian cells.
Chapter 2

Related Works on \textit{E. coli} Growth Control

2.1 Growth Rate Models

Due to complexities of the cell metabolism, growth models are designed for specific species of microorganisms. Different species do not grow similarly, and models are not available for many species of interest. Yet, models are very useful to test controllers in simulations before the experimental validations. Almost all growth models for microorganisms, such as \textit{E. coli}, use Monod type equations [Monod, 1949]. The two \textit{E. coli} growth models described in this section [Xu, 1999; Rocha, 2003] are flux based models, yet still employ basic Monod type structure for reaction kinetic expressions.
2.1.1 Xu Model

The Xu model [Xu, 1999, Chapter 3.2] describes cell growth in the bioreactor as:

\[
\frac{dX}{dt} = \mu X - DX \tag{2.1}
\]
\[
\frac{dS}{dt} = -qSX + D(S_{\text{feed}} - S) \tag{2.2}
\]
\[
\frac{dA}{dt} = (qA_p - qA_c)X - DA \tag{2.3}
\]
\[
\frac{dV}{dt} = F \tag{2.4}
\]
\[
D = \frac{F}{V} \tag{2.5}
\]

where \( S, X \) and \( A \) are the concentrations of substrate, biomass and acetate in the media, respectively. \( S_{\text{feed}} \) is the substrate concentration of the feed solution. \( F, D \) and \( V \) are feed rate, dilution rate and volume of the liquid media inside the bioreactor.

The specific growth rate \( (\mu) \) is calculated as the summation of growth in oxidative, overflow and acetate consumption:

\[
\mu = (qS_{\text{ox}} - q_m) Y_{X/S,\text{ox}} + qS_{\text{of}} Y_{X/S,\text{of}} + qA_c Y_{X/A} \tag{2.6}
\]

and the oxygen uptake rate (OUR) is given by

\[
\text{OUR} = qO X \tag{2.7}
\]

where \( qO \) is the specific oxygen uptake rate, \( qS \) is the total specific substrate uptake rate \( (qS_{\text{ox}} \) for oxidative and \( qS_{\text{of}} \) for overflow), \( qA_c \) is the specific acetate uptake rate, \( Y_{X/S} \) is the yield coefficient for cells on substrate \( (Y_{X/S,\text{ox}} \) for oxidative and \( Y_{X/S,\text{of}} \) for overflow), \( Y_{X/A} \) is the yield coefficient for cells on acetate and \( q_m \) is the specific
substrate uptake rate for maintenance [Xu et al., 1999]. In summary, Xu proposed that with oversupply of substrate, the specific oxygen uptake rate \( qO \) increases and eventually saturates at maximum specific oxygen uptake rate \( qO_{max} \) and cells go to overflow metabolism afterwards.

### 2.1.2 Rocha Model

The Rocha model [Rocha, 2003, Chapter 5.1.5] extends the Xu model to include that oxygen could be consumed in all three metabolic phases — oxidative (equation 2.8), overflow (equation 2.9) and acetate consumption (equation 2.10).

\[
\begin{align*}
 k_1 S + k_5 O & \xrightarrow{\mu_1} X + k_8 C \\
 k_2 S + k_6 O & \xrightarrow{\mu_2} X + k_9 C + k_3 A \\
 k_4 A + k_7 O & \xrightarrow{\mu_3} X + k_{10} C
\end{align*}
\]  

where \( O \) and \( C \) are concentrations of dissolved oxygen and dissolved carbon dioxide, respectively. \( k_1 \) to \( k_{10} \) are the stoichiometric coefficients. The dynamical system is modeled as:

\[
\begin{align*}
 \frac{dX}{dt} & = (\mu_1 + \mu_2 + \mu_3)X - DX \\
 \frac{dS}{dt} & = (-k_1\mu_1 - k_2\mu_2)X + D(S_{feed} - S) \\
 \frac{dA}{dt} & = (k_3\mu_2 - k_4\mu_3)X - DA
\end{align*}
\]

And the OUR is given by

\[
OUR = (k_5\mu_1 + k_6\mu_2 + k_7\mu_3)X
\]
The growth rule terms $\mu_1$, $\mu_2$ and $\mu_3$ represent three metabolic states — oxidative, overflow and acetate consumption, respectively. In summary, Rocha proposed that with oversupply of the substrate, the specific substrate consumption rate ($q_S$) increases and eventually reaches to the critical substrate consumption rate ($q_{S_{crit}}$) and cells go to overflow metabolism.

2.1.3 Comparison of Xu and Rocha Models

The Xu model differs from the Rocha model in how the inhibitory effect of acetate, especially at high concentrations, is modeled. In the Rocha model, acetate has an inhibitory effect on the specific rate of acetate consumption ($q_{Ac}$), while the Xu model does not include this effect (Equations 2.15 and 2.16).

$$k_4 \mu_3 \overset{Rocha}{=} q_{Ac} \overset{Rocha}{=} q_{Ac,max} \left( \frac{A}{K_A + A} \right) \left( \frac{K_{i,A}}{K_{i,A} + A} \right)$$ (2.15)

$$q_{Ac} \overset{Xu}{=} q_{Ac,max} \left( \frac{A}{K_A + A} \right)$$ (2.16)

where $K_A$ is the acetate constant, $K_{i,A}$ is the inhibition constant of acetate consumption and $q_{Ac,max}$ is the maximum specific acetate uptake rate. In contrast, the Xu model includes the inhibitory effect of acetate on the glucose consumption, while the Rocha model, does not (Equations 2.17 and 2.18).

$$k_1 \mu_1 + k_2 \mu_2 \overset{Rocha}{=} qS \overset{Rocha}{=} q_{S_{max}} \left( \frac{S}{K_S + S} \right)$$ (2.17)

$$qS \overset{Xu}{=} q_{S_{max}} \left( \frac{S}{K_S + S} \right) \left( \frac{K_{i,S}}{K_{i,S} + A} \right)$$ (2.18)

where $K_S$ is the substrate constant and $K_{i,A}$ is the inhibition constant of substrate consumption. Both the Xu and Rocha model use the same Monod equation for-
ulation for the maximum substrate specific consumption rate \( q_{S_{crit}} \) in oxidative metabolism as a function of the maximum specific oxygen uptake rate \( q_{O_{max}} \), as shown in Equations 2.19 and 2.20.

\[
q_{S_{crit}}^{Rocha} = \frac{q_{O_{max}}}{K_{OS}} \left( \frac{K_{i,O}}{K_{i,O} + A} \right) 
\]  
(2.19)

\[
q_{S_{crit}}^{Xu} = \frac{q_{O_{max}}}{Y_{O/S}} \left( \frac{K_{i,O}}{K_{i,O} + A} \right) 
\]  
(2.20)

where \( K_{OS} \) and \( Y_{O/S} \) are both yield coefficient for oxygen consumption on substrate and \( K_{i,O} \) is the inhibition constant of substrate consumption.

The Rocha model has modeled the \( E. coli \) strain JM109(DE3), while the Xu model has modeled \( E. coli \) K-12 strain W3110. The \( E. coli \) K-12 strain MG1655 that was used in the experimental validation of this thesis is very similar to \( E. coli \) K-12 strain W3110 [Pepper, 2015]. Therefore, Xu model is used for simulations and testing the controller before running the cell growth experiments.

2.2 Adaptive Oxygen Uptake Rate (OUR) Estimator

In order to estimate oxygen uptake rate (OUR) in real-time, an adaptive OUR estimator [Wang, 2014; Pepper, 2015; Mayyan, 2017] was employed that uses both off-gas analyzing sensor and dissolved oxygen probe (inside the bioreactor) to estimate unknown parameters during the cell growth and consequently estimate oxygen transfer rate (OTR) and OUR. It is important to distinguish between OUR and OTR. OUR is the rate at which cells consume the oxygen, whereas OTR is the rate at which
Figure 2.1: $b_0$, $b_1$, $b_2$ and $b_3$ are molar concentrations of oxygen in the input gas, surface of the liquid, the headspace and the gas analyzer, respectively. $V_1$ is the liquid volume of the culture and $V_2$ is the headspace volume.

Oxygen transfers from input gas to liquid media. OUR can be calculated as

$$\text{OUR} = \text{OTR} - \dot{C}, \quad (2.21)$$

where $C$ is the dissolved oxygen concentration, $\dot{C}$ is the rate of change of $C$. By using the mass balance of oxygen with the media as the system, OTR can be calculated as

$$\text{OTR} = \frac{M_f \rho_{o_2}}{V_1} (b_0 - b_1), \quad (2.22)$$

where $b_0$ and $b_1$ are molar concentrations of oxygen in the input gas and surface of the liquid media, respectively. $M_f$ is the mass flow rate of input gas, $\rho_{o_2}$ is the oxygen density at working temperature and $V_1$ is the liquid volume of the culture. Also, OTR can be calculated as the rate of dissolution of a gas into a liquid as

$$\text{OTR} = k_L a (C^* - C), \quad (2.23)$$

where $C^*$ is the oxygen saturation in the liquid culture and $k_L a$ is the oxygen volumetric transfer coefficient. $k_L a$ changes with culture time due to many factors in
the bioreactor such as osmolarity of the media. Unfortunately, there is no known universal model for $k_La$ [Klöckner et al., 2013] other than proportional relationship between $k_La$ and the stir speed ($N$)

$$k_La = \alpha_1 N$$  \hspace{1cm} (2.24)

where $\alpha_1$ is an unknown parameter. To get $k_La$ precisely, $\alpha_1$ should be adaptively estimated, during the experiment. To derive the estimator equations, Equations 2.22 and 2.23 are set equal to each other and $b_1$ obtained.

$$b_1 = b_0 - \frac{V_1(C^* - C)N}{M_f\rho_o2}\alpha_1. \hspace{1cm} (2.25)$$

From the mass balance equations for oxygen in the headspace and the first order delay in the gas-analyzer [Wang, 2014], $\dot{b}_2$ and $\dot{b}_3$ can be obtained.

$$\dot{b}_2 = \frac{M_f}{V_2}(b_1 - b_2)$$ \hspace{1cm} (2.26)$$

$$\dot{b}_3 = \frac{1}{\tau_2}(b_2 - b_3)$$ \hspace{1cm} (2.27)

where $b_2$ and $b_3$ are molar concentrations of oxygen in the headspace and gas analyzer, respectively. $V_2$ is the headspace volume and $\tau_2$ is the time delay of the off-gas analyzer. By rearranging Equations 2.26 and 2.27 in the matrix form equation 2.28 is obtained.

$$\begin{bmatrix} \dot{b}_3 \\ \dot{b}_2 \end{bmatrix} = \begin{bmatrix} -\frac{1}{\tau_2} & \frac{1}{\tau_2} \\ 0 & -\frac{M_f}{V_2} \end{bmatrix} \begin{bmatrix} b_3 \\ b_2 \end{bmatrix} + \begin{bmatrix} 0 \\ \frac{M_f}{V_2} \end{bmatrix} b_1 \hspace{1cm} (2.28)$$
And by combining Equations 2.25 and 2.28, Equations 2.29 and 2.30 are obtained as a linear system.

\[
\begin{bmatrix}
\dot{b}_3 \\
\dot{b}_2
\end{bmatrix} = \begin{bmatrix}
-\frac{1}{\tau_2} & \frac{1}{\tau_2} \\
0 & -\frac{M_f}{V_2}
\end{bmatrix} \begin{bmatrix}
b_3 \\
b_2
\end{bmatrix} + \begin{bmatrix}
0 & 0 \\
b_0 & \alpha_1
\end{bmatrix} \begin{bmatrix}
\frac{M_f}{V_2} \\
\frac{V_1(C-C^*)N}{V_2\rho_2}
\end{bmatrix}
\]

(2.29)

\[
y = \begin{bmatrix}
1 & 0
\end{bmatrix} \begin{bmatrix}
b_3 \\
b_2
\end{bmatrix} = b_3
\]

(2.30)

The molar concentration of oxygen in the gas analyzer \((b_3)\) is the only observable output. Based on [Narendra and Annaswamy, 2012], the unknown parameter of the linear system \((\alpha_1)\) can be estimated by transforming the system into the observable canonical form by using

\[
T = \begin{bmatrix}
1 & 0 \\
\frac{M_f}{V_2} & \frac{1}{\tau_2}
\end{bmatrix}^{-1}
\]

(2.31)

The details about the adaptive estimator are explained in [Mayyan, 2017]. Computationally, it takes approximately 8 minutes for the OUR estimator to converge after any disturbance – such as oxygen enrichment of the air.

### 2.3 Previous *E. coli* Growth Controls

There are several open-loop and closed-loop controller to control the growth of *E. coli* by controlling the feed rate. Two common closed-loop control methods are pH-stat and DO-stat. A pH-stat or DO-stat triggers the feed when the culture goes above or below the pH or dissolved oxygen (DO) setpoint. In the case of a DO-stat where the principle is that culture DO will increase when glucose is depleted, thus triggers the feed pump to turn on. DO-stat and pH-stat tend to have slower growth
rate than other control methods. The setpoints and feed rate need to be empirically determined to not cause the culture to go to overflow metabolism.

2.3.1 Exponential Feed Control (μ-set)

Exponential feed controller (also called a growth rate set point controller or simply μ-set) is an open-loop continuous feed controller. It sets the feed rate to a desired specific growth rate (μ_set), which has to be determined empirically. The feed flow rate (F) is calculated by equation 2.32 at any time (t) in the fed-batch [Yamanè and Shimizu, 1984].

\[
F = \frac{\mu_{\text{set}} X_0 V_0}{S_f Y_{X/S}} \exp(\mu_{\text{set}} t)
\] (2.32)

where \(X_0\) and \(V_0\) are the cell density and the culture volume at the beginning of fed-batch, \(\mu_{\text{set}}\) is the desired specific growth rate, \(S_f\) is the glucose concentration in the feed solution and \(Y_{X/S}\) is the yield coefficient of cell on glucose. Typically for \(E. coli\), \(\mu_{\text{set}}\) is less than 0.3 h\(^{-1}\) to avoid overflow metabolism, as this has been shown to be the rate of the TCA cycle at 37°C [Korz et al., 1995]. However, during recombinant protein induction, the maximum growth at the cells can drop below \(\mu = 0.3\) h\(^{-1}\), due to metabolic burden [Bentley et al., 1991] and then manual adjustment of \(\mu_{\text{set}}\) is needed during a fermentation run if the culture is stressed and the cells cannot maintain the growth rate above the initial \(\mu_{\text{set}}\) [Sharma et al., 2007].

2.3.2 BOOM I

This feed controller developed by [Pepper, 2015] is a closed-loop feed controller that uses real-time estimated OUR (see Section 2.2) to detect oxidative and overflow metabolism in order to increase the feed rate. By quantifying the changes of the OUR signal, the BOOM I controller was able to detect the metabolic state of the cells.
2.3.2.1 Ramp

BOOM I periodically (every 15 or 30 minutes) increased the feed rate significantly and in a relatively short time (about 3 minutes) in a process called *ramp*. If the cells were in oxidative metabolism, the cells consumed the excess feed via oxidative pathways and the oxygen uptake rate (OUR) increased accordingly. On the other hand, if the cells were in overflow metabolism, the cells consumed the surplus feed through overflow pathways. Since cells do not consume more oxygen in the overflow pathways (see Section 1.1.2), the relative increase in OUR was smaller.

2.3.2.2 Sensitivity Ratio (SR)

In order to generate a dimensionless signal that could detect changes in OUR signal, sensitivity ratio (SR) is defined by BOOM I as

\[
SR = \frac{\dot{\hat{OUR}}}{\dot{F}} \frac{OUR}{F} \tag{2.33}
\]

where \( \hat{OUR} \) is the estimated oxygen uptake rate and \( F \) is the feed rate. \( \dot{\hat{OUR}} \) and \( \dot{F} \) are time derivatives of \( \hat{OUR} \) and \( F \). \( \dot{\hat{OUR}} \) and \( \dot{F} \) are normalized rate of change in OUR and feed rate, respectively. SR quantifies the normalized rate of change in OUR to normalized rate of change in feed rate in order to detect changes of shape of OUR. A higher SR indicates that the cells are in oxidative metabolism because the rate of change of OUR is high and a lower SR indicates that the cells are in overflow metabolism. BOOM I detected oxidative metabolism when the rising SR signal passes 0.2 threshold and detected the transition from oxidative to overflow at the moment that the diminishing SR crossed the same threshold.
2.3.2.3 Updating Feed Rate

BOOM I kept the feed rate unchanged if the metabolic state was detected as overflow, otherwise it updated the feed rate after each ramp \(F_{\text{new}}\) by using equation 2.34.

\[
F_{\text{new}} = \frac{OUR_{\text{max}}}{OUR_i} F_i
\]

where \(F_i\) and \(OUR_i\) are the feed rate and estimated OUR at the beginning of the ramp and \(OUR_{\text{max}}\) is the maximum estimated OUR during the ramp.

2.3.3 Conclusions

There are controllers that use sensors to measure the concentration of glucose or acetate at real-time and set the feed rate in order to keep the glucose or acetate concentration constant [Kleman et al., 1991; Cheng et al., 2012; Lin et al., 2016]. However, these sensors are expensive and unreliable. As mentioned in [Pepper, 2015], BOOM I is more efficient than DO-stat, pH-stat and exponential feed controller.

Although BOOM I can control the growth better than \(\mu\)-set, it has three shortcomings that needs to be considered — the inappropriate detection of the boundary of oxidative and overflow metabolism, the inability to decrease the feed rate and the improper feed rate profile during the ramp.

The criteria for boundary of oxidative and overflow metabolism needs to be addressed. Specifically, BOOM I detected the metabolic transition from oxidative to overflow later than the actual occurrence. This caused BOOM I to have a feed rate that was higher than the appropriate amount which led to overflow metabolism. Moreover, since BOOM I was not designed to decrease the feed rate in case of overfeeding, the feed rate remained constant even if the BOOM I controller detected that the cells were in overflow. This aggressive monotonic algorithm for feed rate
kept the cells in overflow for a longer time. Although BOOM I did fairly well with temperature decrease of the culture and eventually caught up to meet the feed rate; however, decreasing the feed rate down would have been a better approach. Also, BOOM I increased the feed rate linearly during a ramp. The algorithm for ramp slope was complex and it did not necessarily change the feed rate enough to detect the metabolic state during a ramp.

In order to tackle these three issues, the BOOM II controller is designed as the modified version of the BOOM I controller. Similar to BOOM I, BOOM II only uses the fundamental concepts of metabolism in the cells, so it can robustly adjust the feed rate without relying on specific growth models.
Chapter 3

Research Design: The Controller

3.1 Introduction

The controller described in this chapter uses the fundamental concept of oxidative and overflow metabolism that occurs in all living organisms including \textit{E. coli}. The BOOM II controller detects whether the metabolic state is oxidative or overflow and adjusts the feed rate based on the need of the cells. BOOM II is a closed-loop controller that increases the feed rate in a process called probe in order to detect the metabolic state of the cells. This controller is updated version of BOOM I (see Section 2.3.2) with improved performance. BOOM I pushed the cells slightly into the overflow metabolism, whereas BOOM II keeps the cells close to the boundary of oxidative and overflow metabolism.

3.2 Probe

Probe is a process during which the feed rate is increased to detect the metabolic state of the cells. Length of the probe is variable ranging from 3 to 15
minutes (see Section 3.6 for more details). The probe finishes when the metabolic state detector recognizes overflow metabolism or transition from oxidative to overflow metabolism (see Section 3.4 for more details).

The time interval between two consecutive probes is 8 minutes (Table 3.2) if cells are in oxidative metabolism. The time interval doubles to 16 minutes if the metabolic state is considered to be overflow, so the cells have more time to consume the excess glucose and/or acetate.

During each probe, feed rate increases exponentially

\[ F = (F_i + F_{inc}) \exp(m_F (t - t_0)) \]  

(3.1)

where \( F \) is the feed rate, \( F_i \) is the feed rate at the beginning of the probe, \( F_{inc} \) is the minimum feed increment of the the pump and \( m_F \) is the rate of change of feed during the probe (see Table 3.4). \( t \) is the time and \( t_0 \) is the time at the beginning of the probe. Using an exponentially increasing feed during the probe can reduce the noise in sensitivity ratio calculations. A probe is shown in Figure 3.1 on page 20.

### 3.3 Sensitivity Ratio (SR)

BOOM II employs the same concept of SR as discussed in BOOM I (see chapter 2.3.2.2 for more details). Since the feed is increasing exponentially during the probe

\[ m_F = \frac{\dot{F}}{F} \]  

(3.2)

is constant (see Table 3.4 for its value). By combining Equations 2.33 and 3.2, SR can be redefined as

\[ SR \triangleq \frac{1}{m_F} \frac{O\dot{U}R}{OUR} \]  

(3.3)
Figure 3.1: The probe algorithm in the BOOM II controller (Sample time is 15 seconds).
Using this method reduces the noise in feed rate derivative calculations. However, as shown in Figure 5.1d, the SR signal is still noisy because of noisy estimated OUR (Figure 5.1c) due to improper PID control of stir speed by the DCU (Figures 5.1a and 5.1b) as the media characteristics change during the experiment. Despite the fact that using a low-pass filter on any signal causes undesirable delay, because of too much noise on the estimated OUR signal, using a low-pass filter is inevitable.

\[ H(s) = \frac{1}{\tau s + 1} \]  

where \( \tau \) is the time constant and is approximately 120 seconds. In Figure 5.1 on page 38 the difference between the noisy signals and the filtered ones is illustrated.

### 3.4 Metabolic State Detector

In order to maintain the cells close to the boundary of oxidative and overflow metabolism (BOOM), metabolic state of the cells should be periodically detected. Detection of overflow metabolism is straightforward. If the SR signal does not exceed the threshold (Table 3.4) in a time length known as \( t_{probe} \) (Table 3.2), the metabolic state will be recognized as overflow and the probe finishes. The detection of overflow metabolism is demonstrated in Figure 5.3 on page 41.

If SR crosses the threshold (\( Thresh \)) during the time length of \( t_{probe} \), the metabolic state will be recognized as oxidative, but unlike overflow metabolic state the probe continues. Since the goal of the controller is to grow the cells close to the boundary of oxidative and overflow metabolism, the probe continues to increase the feed rate until the surplus feed changes the metabolic state to overflow.

After detecting oxidative metabolism, the feed rate continues to increase ex-
ponentially. At the same time, SR increases and then reaches to a maximum and eventually starts to fall. The maximum SR represents a turning point at which the metabolic state changes from oxidative to overflow. This turning point is the moment that overflow metabolic pathway is triggered due to overfeeding. The detection of state transition from oxidative to overflow metabolism is shown in Figure 5.2 on page 40.

3.5 Setting Feed Rate

Setting the feed rate is the purpose of the BOOM II controller. In the beginning of the experiment and during the batch phase feed rate is by definition zero. At the start of fed-batch, feed rate is set to be \( F = F_0 \) where \( F_0 \) is shown in Table 3.4. After a delay, BOOM II starts to examine the cells. During each probe, feed rate increases exponentially as explained in Section 3.2. The probe finishes when the metabolic state detector determines a transition from oxidative to overflow metabolism or a complete overflow metabolism (see Section 3.4). These two conditions and the feed update calculations are discussed in this section. After updating the new feed rate, the feed rate remains unchanged until the next probe.

3.5.1 Transition from Oxidative to Overflow Metabolism

If transition from oxidative to overflow metabolism is detected, feed rate \( (F) \) is updated immediately after the probe by using the Equation 3.5, as shown in Figure 3.1 on page 20.

\[
F = \frac{OUR_f}{OUR_i}F_i
\]  
(3.5)
where \(OUR_i\) and \(F_i\) are OUR and feed rate at the beginning of the probe and \(OUR_f\) is OUR at the maximum SR. Increasing feed rate is illustrated in Figure 5.2 on page 40.

### 3.5.2 Completely in Overflow Metabolism

When the cells are in overflow, it means that they have been overfed. In order to bring them back to the boundary of oxidative and overflow metabolism, the feed rate should decrease. Hence the metabolic state detector only uses the estimated OUR and the cells do not consume much oxygen in overflow metabolism, the controller can not assess the excess feed rate amount. An empirical overflow attenuation gain of \(AG\) (see Table 3.4) is used to proportionally decrease the feed rate after overflow detection.

\[
F = AG \times F_i
\] (3.6)

Decreasing the feed rate is illustrated in Figure 5.3 on page 41.

### 3.6 Tuning Controller Parameters

In order to tune the controller parameters, several simulations (see Section 3.8) and controlled growth experiments were performed. Controlled growth experiments are exponential feed rate (\(\mu\)-set) experiments designed to maintain cells in a specific metabolic state. Due to previous experiments and a general experience about the

<table>
<thead>
<tr>
<th>(\mu_{set})</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15 h(^{-1})</td>
<td>Completely in oxidative metabolism</td>
</tr>
<tr>
<td>0.30 h(^{-1})</td>
<td>Boundary of oxidative and overflow metabolism</td>
</tr>
<tr>
<td>0.45 h(^{-1})</td>
<td>Completely in overflow metabolism</td>
</tr>
</tbody>
</table>

Table 3.1: \(\mu_{set}\) values for the controlled growth experiments.
strain of *E. coli*, the $\mu_{set}$ is chosen (as shown in Table 3.1) for three distinct metabolic state categories. During these experiments the probe technique is applied in different durations and intervals and parameters listed in Tables 3.2, 3.3 and 3.4 are defined empirically. Timing parameters such as length of batch, probe and the time interval between two consecutive probes are shown in Table 3.2. Gasmix increments are 5% and disturb the OUR estimator for about 8 minutes. Other parameters for gasmix controller are listed in Table 3.3. $F_0$ is calculated by using Equation 2.32 at time $t = 0$:

$$F_0 = \frac{\mu_{set} X_0 V_0}{S_f Y_X/S}$$

(3.7)

by assuming $\mu \approx 0.25 \text{ h}^{-1}$, $X_0 \approx 2.5 \text{ g(DCW)/L}$, $V_0 \approx 1.45 \text{ L}$, $S_f = 480 \text{ g/L}$ and $Y_X/S \approx 0.5 \text{ g/g}$ at the beginning of fed-batch $F_0$ would be about 3.78 mL/h. Other constant or variable parameters for the BOOM II controller are listed in Table 3.4.

<table>
<thead>
<tr>
<th>Time constant</th>
<th>Description</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{batch}$</td>
<td>Length of batch</td>
<td>9 h</td>
</tr>
<tr>
<td>$t_{delay}$</td>
<td>The delay after batch and before start of probes</td>
<td>20 min</td>
</tr>
<tr>
<td>$t_{probe}$</td>
<td>Average Length of probe</td>
<td>5 min</td>
</tr>
<tr>
<td>interval</td>
<td>Time interval between two consecutive probes</td>
<td>8 min</td>
</tr>
</tbody>
</table>

Table 3.2: The BOOM II time constants.

<table>
<thead>
<tr>
<th>Constant</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>increment</td>
<td>Gasmix increment</td>
<td>5%</td>
</tr>
<tr>
<td>DO$_{SP}$</td>
<td>Dissolved oxygen setpoint</td>
<td>40%</td>
</tr>
<tr>
<td>Blue$_{max}$ O$_2$</td>
<td>Maximum oxygen for gas analyzer</td>
<td>55%</td>
</tr>
<tr>
<td>$N_{max}$</td>
<td>Stir speed threshold for gasmix to increase</td>
<td>1000 rpm</td>
</tr>
<tr>
<td>DO$_{min}$</td>
<td>Dissolved oxygen threshold for gasmix to increase</td>
<td>10%</td>
</tr>
</tbody>
</table>

Table 3.3: Gasmix and stir speed constant parameters.
### Table 3.4: The BOOM II constant and variable parameters.

<table>
<thead>
<tr>
<th>Constant</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$AG$</td>
<td>Overflow attenuation gain</td>
<td>90%</td>
</tr>
<tr>
<td>$F_0$</td>
<td>Initial feed at the start of fed-batch</td>
<td>3.78 mL/h</td>
</tr>
<tr>
<td>$F_{inc}$</td>
<td>Minimum feed increment</td>
<td>1.68 mL/h</td>
</tr>
<tr>
<td>$m_F$</td>
<td>Rate of change of feed during the probe</td>
<td>10 h$^{-1}$</td>
</tr>
<tr>
<td>$Thresh$</td>
<td>Threshold for SR</td>
<td>20%</td>
</tr>
<tr>
<td>$FB$</td>
<td>Signal indicating the fed-batch</td>
<td>0 or 1</td>
</tr>
<tr>
<td>$F_i$</td>
<td>Feed rate at the beginning of the probe</td>
<td>N/A</td>
</tr>
<tr>
<td>$OUR_i$</td>
<td>OUR at the beginning of the probe</td>
<td>N/A</td>
</tr>
<tr>
<td>$OUR_f$</td>
<td>OUR at the maximum SR</td>
<td>N/A</td>
</tr>
<tr>
<td>$SR_m$</td>
<td>maximum SR</td>
<td>N/A</td>
</tr>
</tbody>
</table>

#### 3.7 Feed Controller

The BOOM II feed controller is programmed by using state machines logic in Stateflow inside Simulink software. The simplified version of BOOM II is shown in Figure 3.2, and the complete version is shown in Figure A.1 in Appendix A. All variables and constants are described in Tables 3.2 and 3.4. The program starts with the “batch” sub-state where the feed rate ($F$) is zero. At the start of fed-batch when the FB signal becomes 1, the feed rate is updated to constant value $F_0$. It remains constant until $t_{delay}$ has passed.

Then it goes to the “metabolic state estimator” state to start the probe process. During the probe, the sub-state changes from “unknown” sub-state to “oxidative” sub-state if SR surpasses $Thresh$ in time length of $t_{probe}$; otherwise, it goes to “overflow” sub-state. Feed rate increases exponentially as calculated in Equation 3.1 until the end of the probe. In “oxidative” sub-state, maximum SR is calculated and the status changes to “overflow” sub-state when maximum SR is not updated for one forth of $t_{probe}$. The probe ends at “overflow” sub-state. New feed rate is calculated based on Equation 3.5 if overflow metabolism is achieved with transition from oxida-
tive metabolism, otherwise it is calculated based on Equation 3.6. Feed rate remains unchanged during \textit{interval} until the next probe.

### 3.8 Simulations

Before using the BOOM II controller to control the bioreactor in a real experiment, the controller was tested and tuned in numerous simulations. The simulations in the Simulink use the Xu model (see chapter 2.1.1) to represent the cell growth in the bioreactor. Simulations show many variables that are not measurable during the experiment such as specific oxygen uptake rate ($q_O$) – that saturates at $q_{O_{\text{max}}}$ – and specific growth rate. These variables, that give a better understanding about the cell responses, are used to improve the concept-based controller.

As illustrated in Figure 3.3, the transition from oxidative to overflow happens
at the time that specific oxygen uptake saturates at the maximum specific oxygen uptake rate (Figure 3.3d). It happens at the same time that SR is maximized (Figure 3.3f). After that the acetate builds up which is another sign of overflow (Figure 3.3e). Figure 3.4 compares metabolic state detection of BOOM II with metabolic state based on the Xu model. BOOM II has correctly detected both important situations: overflow (compare Figure 3.4a and 3.4b) and transition from oxidative to overflow (compare Figure 3.4c and 3.4d), in the simulations.
Figure 3.3: Simulation results during the probe time for the BOOM II controller, using Xu model for the cell growth (Sample time is 15 seconds).
(a) Detecting transition from oxidative to overflow metabolism by BOOM II.

(b) Transition from oxidative to overflow metabolism based on Xu model.

(c) Detecting overflow metabolism by BOOM II.

(d) Overflow metabolism based on Xu model.

Figure 3.4: Verification of the BOOM II metabolic state detection with Xu model in simulations (Sample time is 15 seconds).
Chapter 4

Materials and Methods

4.1 *E. coli* Cells

*E. coli* K-12 MG1655 pTVP1GFP was used for all the experiments. *E. coli* MG1655 was obtained from the American Type Culture Collection (ATCC) and the plasmid pTVP1GFP was donated by Dr. A. Villaverde. The plasmid encodes the VP1 capsid of foot-and-mouth disease (FMD) and also encodes a green fluorescent protein (GFP). The plasmid is resistant to ampicillin and can be induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) through *trc* promoter [Baig et al., 2014].

Frozen cell stocks were kept at −80°C freezer. Cells were thawed and then cultured in minimal media, described in [Korz et al., 1995], as overnight cultures (see Appendix C.1) and pre-culture (see Appendix C.2) in an incubator shaker (C-24 classic benchtop incubator shaker, New Brunswick Scientific Company, Inc.) at 37°C and 250 rpm. Pre-cultures were used between the overnight culture and the bioreactor inoculation to ensure that the cell inoculated into the bioreactors were in the exponential phase and approximately at 2 OD (see Section 4.4).
4.2 Bioreactor

The bioreactor (5L autoclavable benchtop glass vessel, B5, B. Braun Biotech International, GmbH) was controlled by a digital control unit (DCU, Biostate B, B. Braun Biotech International, GmbH). The DCU read the sensor data from thermometer (Reference thermometer for B5, B. Braun Biotech International, GmbH), pH meter (EasyFerm Plus HB K8 325, Hamilton Co.) and dissolved oxygen probe (OxyFerm FDA VP 325, Hamilton Co.). In order to maintain temperature, pH and dissolved oxygen at desired setpoints (Table 4.1), the DCU controled a heater, a pump to flow cooling water into the water jacket, a peristaltic pump to add base and a motor to stir the agitator. Depend on the bioreactor vessel, one of the two chillers (Isotemp 3006 and Isotemp 3016S, Fisher Scientific, Inc.) was used to cool the recirculating water.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Setpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>pH</td>
<td>6.95</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>40%</td>
</tr>
</tbody>
</table>

Table 4.1: Setpoints for the PID controllers on the DCU.

A computer was used to send commands to and receive data from the DCU via RS-422 cable every 15 seconds. A Simulink program (Simulink R2015a, The MathWorks, Inc.) on the computer controled the ratio of oxygen to air (gas mixture or simply called “gasmix”) and the feed rate. The feed solution is contained approximately 50% w/v glucose based on [Korz et al., 1995] (see Table B.3 for more details about the feed solution). The details about the software program are discussed in Section 4.5.

Two peristaltic pumps (working at discrete steps of 2%) were used to control
the feed rate more precisely. The pumps use silicone tubings with two different sizes (L/S 13 and L/S 14, Masterflex Tygon Lab tubing, Cole-Parmer Instrument Co.) to change the flow rate from 0 to 426 mL/h with a precision of 1.68 mL/h. An off-gas analyzer (BlueInOne Ferm, BlueSens gas sensor, GmbH) was connected to the computer via USB. It collected and sent the oxygen and carbon dioxide molar concentrations to the computer every 15 seconds. The entire bioreactor setup for the experiments is shown in Figure 4.1.
4.3 Experiments

The objective of the experiments was to compare the BOOM II controller to a commonly employed industrial controller (\(\mu\)-set) for the growth rate and waste product production. A controller that can grow more cells with less acetate – as an undesirable by-product – is the efficient controller. Two paired experiments (Exp 74 and Exp 75) are designed and performed. Each paired experiment uses two bioreactos to monitor performance of BOOM II and \(\mu\)-set — as the benchmark of industrial controller.

The experiments used 1.5 L media with 5 g/L glucose concentration for batch and 2 L substrate with approximately 500 g/L glucose concentration and 0.5 L base with approximately 8% ammonium hydroxide (See Table B.2 for more details about the media and the substrate). The inoculation cell density for the experiments were calculated based on the length of batch and the desired OD at the end of batch (see Appendix C.3). They are all shown in Table 4.2.

After the batch phase, the feed controllers start working in the fed-batch phase. Samples were collected hourly in the fed-batch. Samples were used for real-time OD measurement and off-line acetate measurements (see Section 4.4). Cells were induced by IPTG powder that is dissolved in 5 mL of distilled water to make a 4 mM concentration for the final solution. Time of induction (after start of fed-batch) and weights of IPTG added to the feed and the media are shown for each experiment in Table 4.2. Experiments continued for 24 hours starting from the inoculation. At the end of each experiment, a sample of 50 mL from the final media containing cells was harvested for measuring the ultimate cellular dry weight.
<table>
<thead>
<tr>
<th>Experiment number</th>
<th></th>
<th>74</th>
<th></th>
<th>75</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Induction number</td>
<td></td>
<td>74</td>
<td></td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Induction process</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower induction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Higher induction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controller</td>
<td></td>
<td>BOOM II</td>
<td>µ-set</td>
<td>BOOM II</td>
<td>µ-set</td>
</tr>
<tr>
<td>Inoculation cell density (OD)</td>
<td>0.053</td>
<td>0.053</td>
<td>0.077</td>
<td>0.077</td>
<td></td>
</tr>
<tr>
<td>Batch length</td>
<td>9 h</td>
<td>9 h</td>
<td>9 h</td>
<td>9 h</td>
<td></td>
</tr>
<tr>
<td>Cell density at the beginning of monitoring the controllers (time zero of fed-batch)</td>
<td>4.74 OD</td>
<td>4.64 OD</td>
<td>6.94 OD</td>
<td>6.52 OD</td>
<td></td>
</tr>
<tr>
<td>Induction time (in fed-batch)</td>
<td>7.4 h</td>
<td>7.4 h</td>
<td>7.8 h</td>
<td>7.8 h</td>
<td></td>
</tr>
<tr>
<td>IPTG added to the vessel</td>
<td>1.97 g</td>
<td>1.97 g</td>
<td>2.01 g</td>
<td>1.69 g</td>
<td></td>
</tr>
<tr>
<td>IPTG added to the feed solution</td>
<td>0 g</td>
<td>0 g</td>
<td>1.40 g</td>
<td>1.59 g</td>
<td></td>
</tr>
<tr>
<td>Total IPTG added</td>
<td>1.97 g</td>
<td>1.97 g</td>
<td>3.41 g</td>
<td>3.28 g</td>
<td></td>
</tr>
<tr>
<td>Liquid volume at induction time</td>
<td>2.03 L</td>
<td>~1.84 L</td>
<td>2.07 L</td>
<td>1.74 L</td>
<td></td>
</tr>
<tr>
<td>Final liquid volume</td>
<td>3.31 L</td>
<td>~3.62 L</td>
<td>2.95 L</td>
<td>2.82 L</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2: Inoculation and induction setup

4.4 Measuring Cell and Acetate Concentrations

Determining the cellular dry weight is one of the commonly used methods to measure bacterial cell concentrations in solids-free media [Shuler and Kargi, 1992]. Samples were centrifuged (Z383K benchtop refrigerated centrifuge, Hermle AG), washed and dried in an oven (Isotemp standard lab oven, Fisher Scientific, Inc.) containing desiccants at 80°C for 24 hours. After drying, the dry cell weights (DCW) were measured by using a balance (ME104E, Mettler-Toledo International, Inc.).

Using a spectrophotometer is a fast method to measure bacterial cell density. Spectrophotometer quantifies the opacity of the media containing cells — by measuring the light absorption at the wavelength of 600 nm — in a unit called optical density (OD$_{600}$ or simply OD) [Shuler and Kargi, 1992]. There is a standard curve that relates OD and cell density, which is linear at low OD values. For high OD
values, dilution of the media with distilled water is needed to bring the OD down to the range. The relationship between OD and DCW concentration is given by Equations 4.1 and 4.2 for the spectrophotometer used for the experiments (GENESYS 20, Spectronic Instruments, Inc.).

\[ Y \frac{[g(DCW)/L]}{X[OD]} = k (X [OD]) \text{ (for all } X < 0.5 \text{ OD}) \]  

\[ k \approx \frac{1}{2} \left[ \frac{g(DCW)/L}{OD} \right] \]  

Acetate concentrations were determined by gas chromatography (GC) analysis (Agilent 7890A Gas Chromatograph, Agilent Technologies, Inc.). The GC uses flame ionization detector (FID) with a capillary column (DB-FFAP 123-3223, Agilent Technologies, Inc.).

### 4.5 Bioreactor Control Program

The schematic model for the complete bioreactor control setup is shown in Figure 4.2. Bioreactor, DCU and off-gas analyzer were discussed in Section 4.2. Bioreactor control program was a Simulink program consists of several subparts such as feed controller, OUR estimator and gasmix controller (Figure A.2).

The BOOM II (Figure A.1) and \( \mu \)-set controllers were used as feed controllers. The BOOM II controller has feed rate as the only output and estimated OUR as the only input. The BOOM II controller periodically varied the feed rate and detected the metabolic state of the cells by evaluating the changes in OUR, in order to update the feed rate (see chapter 3 for details). The Simulink program for the bioreactor control is shown in Figure A.3.
Figure 4.2: Schematic model of the bioreactor control setup.
Chapter 5

Results and Discussion

The performance of the BOOM II controller was validated in several experiments and benchmarked against industrial controllers. To make the experimental results of the controllers comparable, only the data for fed-batch cultures are shown in this chapter.

5.1 Metabolic State Detection

As explained in Section 3.3, due to changes in the media characteristics, PID controller of DCU became unstable at some moments during the experiment (see fluctuations of stir speed and consequently dissolved oxygen in Figures 5.1b and 5.1a). However, by using a low-pass filter on estimated OUR (compare Figures 5.1c and 5.1e), the data could be used to detect the metabolic state (compare SR in Figures 5.1d and 5.1f). Data before and after filtration for experiment 75 are compared in Figure 5.1.

Functionality of the metabolic state detector was supported with the experimental results. BOOM II has detected the transition from oxidative to overflow
Figure 5.1: Difference between noisy signals and filtered ones (Exp 75).
metabolism during experiment 74, as illustrated in Figure 5.2. Feed rate has updated according to the amount of change of OUR (see Section 3.5.1) after the end of the probe.

Moreover, BOOM II has detected the overflow metabolism during experiment 74, as illustrated in Figure 5.3. Feed rate has decreased proportionally (see Section 3.5.2) after the end of the probe. Also the interval between the probes has been increased to give time to the cells to consume excess feed and acetate.

5.2 Growth Before Induction

Before induction, BOOM II had a very fast growth in fed-batch. Experimental results for BOOM II, BOOM I (Section 2.3.2) and \(\mu\)-set are compared in Table 5.1. The growth rate was calculated by using least square method (not point-by-point method) for the whole uninduced fed-batch growth profile. The averaged growth rate was much higher for BOOM I and BOOM II than \(\mu\)-set.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>The Controller</th>
<th>Averaged Growth Rate (h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>BOOM II</td>
<td>0.400</td>
</tr>
<tr>
<td>23</td>
<td>BOOM I</td>
<td>0.389</td>
</tr>
<tr>
<td>37</td>
<td>BOOM I</td>
<td>0.368</td>
</tr>
<tr>
<td>74</td>
<td>BOOM II</td>
<td>0.366</td>
</tr>
<tr>
<td>62</td>
<td>(\mu)-set</td>
<td>0.300</td>
</tr>
<tr>
<td>75</td>
<td>(\mu)-set</td>
<td>0.295</td>
</tr>
<tr>
<td>74</td>
<td>(\mu)-set</td>
<td>0.282</td>
</tr>
</tbody>
</table>

Table 5.1: Specific growth rate comparison of uninduced fed-batch cultures in BOOM I, BOOM II and \(\mu\)-set. Higher growth rate is desired.
Figure 5.2: Detecting transition from oxidative to overflow metabolism by BOOM II (Exp 74).
Figure 5.3: Detecting overflow metabolism detection by BOOM II (Exp 74).
5.3 Growth After Induction

After induction, BOOM II continued to grow faster than \( \mu \)-set as shown in Figure 5.4. BOOM II produced much more cells – both per volume and in total – than \( \mu \)-set. Cells secreted recombinant proteins after induction. As demonstrated in [Pepper, 2015], the ratio (yield coefficient) of produced protein per cell is almost constant. Therefore, BOOM II has produced more recombinant therapeutics.

![Growth profile, BOOM II vs \( \mu \)-set. BOOM II has been more successful in increasing the cell density.](image)

Final OD and harvest dry cell weights for induced and uninduced cultures are listed in Table 5.2. The ratio of OD to DCW are relatively consistent in the measurements.

5.4 Acetate Production

A low acetate concentration is one indication of an effective feed control. By measuring the acetate concentration in the samples after the experiment using GC
<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>The Controller</th>
<th>Final OD</th>
<th>Final Dry Cell Weight (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>BOOM II</td>
<td>164.0</td>
<td>75.7</td>
</tr>
<tr>
<td>74</td>
<td>BOOM II</td>
<td>164.8</td>
<td>74.5</td>
</tr>
<tr>
<td>75</td>
<td>BOOM II</td>
<td>153.6</td>
<td>72.2</td>
</tr>
<tr>
<td>74</td>
<td>µ-set</td>
<td>96.8</td>
<td>48.1</td>
</tr>
<tr>
<td>75</td>
<td>µ-set</td>
<td>61.6</td>
<td>31.9</td>
</tr>
</tbody>
</table>

Table 5.2: Cell density at harvest time of uninduced (Exp 70) and induced (Exp 74 and 75) cultures. Higher cell density is desired.

In Figures 5.5 and 5.6. As shown in Figure 5.5, acetate accumulated significantly after the induction for µ-set. However, acetate production was restrained by the BOOM II controller. BOOM II has also produced less acetate per cell especially at cell densities...
Figure 5.5: Acetate concentration, BOOM II vs $\mu$-set. BOOM II has less acetate accumulation at the end of the experiment. Cells are induced at about hour 7.5.

higher than 75 OD (Figure 5.6).

5.5 Efficiency

Figure 5.7 shows estimated biomass per accumulated feed, BOOM II vs $\mu$-set. Where estimated biomass is calculated as

$\text{Estimated total biomass [g]} = (\text{OD}) \times (\text{OD to DCW conversion [g/L]}) \times (\text{volume [L]})$

(5.1)

The BOOM II controller has produced more biomass per feed volume than the benchmark of the industrial controllers. Consequently, BOOM II produced more final products per feedstock for biopharmaceutical industry.

Moreover, the BOOM II controller has produced much less acetate per cell (Figure 5.6) than $\mu$-set, which for biopharmaceutical industry means less waste prod-
ucts per final products.

5.6 Adaptability

BOOM II has adaptively changed the feed rate during the experiments based on the metabolic response of the cells. As illustrated in Figure 5.9, hourly averaged feed rate profiles for BOOM II and $\mu$-set are compared in fed-batch time. BOOM II adjusted the feed rate during the experiment due to metabolic changes such as induction; however, $\mu$-set continued the feeding with predefined growth rate. As demonstrated in Figure 5.9b, BOOM II has detected metabolic changes at least two times during the experiment (induction happened at about hour 8). Comparing the feed profile (Figure 5.9b) with the cell density (Figure 5.8) shows that feed was reduced with the decrease in the growth rate.
Figure 5.6: Acetate concentration at different cell densities, BOOM II vs $\mu$-set. The BOOM II controller has produced less acetate per cell.

Figure 5.7: Estimated biomass vs. accumulated feed, BOOM II vs $\mu$-set. The BOOM II controller has produced more biomass per feed volume.
Figure 5.8: Growth curve in logarithmic scale, BOOM II vs $\mu$-set. BOOM II has been more successful in increasing cell density.
(a) Averaged feed rate for BOOM II and \( \mu \)-set. BOOM II has slowed down the increasing trend of feed rate and has eventually decreased the feed rate.

(b) Averaged feed rate for BOOM II and \( \mu \)-set in logarithmic scale. BOOM II is adaptively adjusting the feed rate based on the metabolic response of the cells.

Figure 5.9: Hourly averaged feed rate profiles for BOOM II and \( \mu \)-set, in fed-batch time (Exp 74).
Chapter 6

Conclusions and Suggestions for Future Research

6.1 Conclusions

The performance of the BOOM II feed controller was better than $\mu$-set – a typical industrial controller – in efficient cell growth with less by-products. The BOOM II metabolic state detector periodically assessed the metabolic state of the cells and assisted the cells to grow fast in oxidative metabolism. Avoiding overflow metabolism is important not only because acetate production inhibits the cell growth, but also because the quality of final products can decline when the host cells are in overflow metabolism.

*E. coli* cells grew fast before and after induction with the BOOM II controller. The controller is efficient for high cell density cultures by using common bioreactor sensors. Stress is one of the important factors that changes the metabolic behaviors of the cells. High cell density culture, i.e., greater than 50 g dry cell weight (DCW) per liter, has more stress which can affect the cell growth.
For more efficiency, cells are grown to a relatively high density and after that they are induced in order to secrete the protein therapeutics as the product. Due to adaptive behavior of the BOOM II controller, it automatically adjusted the feed rate based on the response of the cells. This adaptive change in the feed rate after metabolic changes of the cells kept the cells close to the boundary of oxidative and overflow metabolism which results in abundant drug production and minimum waste production for biopharmaceutical industry.

6.2 Suggestions for Future Research

6.2.1 Using More Signals to Detect the Metabolic States

The metabolic state detector can be considered as a classifier which can periodically detect whether the cells are in oxidative or overflow metabolism. Using more signals other than OUR can improve the precision of the classifier. Controllers such as [Pepper et al., 2014] have also used two or more signals to control the growth.

6.2.1.1 Base Addition Rate (BAR)

Since cells excrete acetate as a by-product when they are in overflow metabolism, acetate production rate (APR) of the cells represent rate of overflow metabolism. By measuring the amount of base addition rate (BAR) and using the Henderson-Hasselbalch equation, APR can be estimated in real-time. There are several complications in using BAR such as considering buffer and base consumption in the culture process. Moreover, acid production happens even when the cells are in oxidative metabolism, so not all acid productions are indicators of overflow.
6.2.1.2 Carbon Dioxide Evolution Rate (CER)

Estimating carbon dioxide evolution rate (CER) is another useful method to increase the number of features for metabolic state classification. There are some challenges in using CER such as the need for dissolved carbon dioxide probe and the complexity of nonlinear parameter estimation of the CER estimator.

6.2.2 Controlling Growth of Recombinant Mammalian Cells

Although *E. coli* and other non-mammalian recombinant host cells such as yeast (*S. cerevisiae*) can produce many useful biopharmaceuticals, they have some considerable drawbacks. When bacteria like *E. coli* produce proteins they put their own hallmark on their products. This hallmark is the extra sugar bonds which are connected to the proteins in a process called glycosylation. These sugar bonds are considered as a hostile antigen by the immune system of the patients who consume the biopharmaceuticals. Moreover, some complicated drugs like monoclonal antibody which are used for diseases such as cancer or Gaucher disease are only produced efficiently by mammalian cells. Therefore, more and more scientists are using mammalian cells such as Chinese Hamster Ovary (CHO) cells as recombinant host cells in order to produce biopharmaceuticals. Since the BOOM technique only uses the fundamental concepts of metabolism that are valid for every single cell – which are oxidative and overflow metabolism – there is a great opportunity to use the BOOM algorithm to control the growth and productivity of more complicated cells such as CHO cells. As mammalian cells are more delicate and therefore more sensitive to culture conditions, controlling the feed rate based on the real-time assessed metabolic state of the cells would have a substantial impact on the efficiency and productivity of the biologic products.
Appendices
Appendix A  Bioreactor Control Model

Figure A.1: Complete version of the BOOM II controller in Stateflow®.

Figure A.2: Gas mixture controller in Stateflow®.
Figure A.3: FermCtrl model: The BOOM II controller, OUR estimator and other controllers in Simulink® program.
Appendix B  \textit{E. coli} Fermentation Checklist

This checklist is based on the 1.5 \textit{L} batch and 2\textit{L} fed-batch fermentation. The minimal media and fed-batch mixtures are from [Korz et al., 1995].

B.1 Preparation of Headplate

\begin{itemize}
  \item Make sure that impellers are in appropriate position.
  \item Make sure headplate O-ring is in place.
  \item Put the headplate in the appropriate orientation
  \item Hand-tighten the headplate screws in a triangular pattern.
  \item Make sure that small feed nozzles are open.
\end{itemize}

B.2 Preparation of Bioreactor Probes

\begin{itemize}
  \item Turn on the DCU and connect temperature and pH probe cables to the DCU
  \item Check if the temperature is about room temperature (about 23$^\circ$C)
  \item Press CALIBRATION until it goes to pH window then hit ”down arrow key” to go to BUFZ
  \item Use wash bottle to rinse the probe then put it in pH 7 (green) buffer
  \item Wait until pH reading stabilizes, then hit ENTER
  \item Wait until the cursor goes to BUFS then rinse the probe
  \item Put it in pH 4 (pink) buffer, wait until it stabilizes and then hit ENTER
\end{itemize}
• Wait until the cursor goes to the next line.

• Disconnect temperature and pH probe cables from DCU

• Cap exposed pH connector, rinse the pH probe and then insert it into the 12 mm port

• Unscrew the cap of the DO probe then rinse the cap

• Add 1.5 mL of electrolyte to the cap then screw the cap

• Screw the DO probe on the headplate slot.

• Since the batch buffer bottle is sterile, pour 150 mL of batch buffer under the hood in a 1L graduated cylinder and use funnel to pour it into the vessel.

• Again, use graduated cylinder and funnel to add 1300 mL of dH₂O to the vessel.

B.3 Preparation of Bioreactor Ports

• To prevent moisture problems, wrap temp cable connection with foil only.

• Check all the tubings of the bioreactor.

• Prepare and check all the boxes in Table B.1.

B.4 Autoclaving

• Make sure that you have the vessel, empty feed bottle, tubings (in a beaker), glucose solution and the stirring bar.

• Open the printer door and position the power switch to ON.
Clamp & Cap  □  Blue & Foil  □  Filter & Mark □

Sparger (Air in)
Exhaust condenser (Air out)
Leftmost port of quad-port □
3 other ports of quad-port □ □
Sample port □ □
2 × LS14, LS13 & Y connection □ □
Empty substrate bottle □ □

Table B.1: Bioreactor ports

- Put the bioreactor in the appropriate orientation on the tray.
- Close the chamber door and hand-tighten the hand wheel.
- Check if that jacket pressure has stabilized at 20 psig then look at the display.
- To Ignore LEAK and DART TESTS, press 2 (for NO) twice.
- Press corresponding number touch pad for LIQUIDS cycle twice.
- Write down date, lab name (Dr. Harcums) and your name on the list.
- Liquid cycle takes about 45 min for the autoclaving and 20 min for cooling down.

B.5 Preparation of Batch Additives and Feed Solution

- Make batch additives and feed solution based on quantities of Table B.2. If the desired feed volume is less than 2 L, use Table B.3 for the feed solution.

B.6 DCU Connections

- Connect DO, pH and temperature probe cables.
Batch additives | Feed solution
---|---
Glucose solution 50% w/v (mL) | 15 mL □ 2 L □
Magnesium sulfate 20% w/v (mL) | 3 mL □ 40 mL □
Trace metals solution 100X (mL) | 15 mL □ 30 mL □
Ferric citrate solution 100X (mL) | 15 mL □ 8 mL □
Ampicillin 1000X (mL) | 1.5 mL □ 2 mL □
Antifoam 1000X (mL) | 1.5 mL □ 3.5 mL □

Table B.2: Volumes of components of batch additives and feed solution for 2 L of fed-batch.

<table>
<thead>
<tr>
<th>Fed-batch volume (mL)</th>
<th>208</th>
<th>260</th>
<th>520</th>
<th>1040</th>
<th>1560</th>
<th>2080</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose solution 50% w/v (mL)</td>
<td>200</td>
<td>250</td>
<td>500</td>
<td>1000</td>
<td>1500</td>
<td>2000</td>
</tr>
<tr>
<td>Magnesium sulfate 20% w/v (mL)</td>
<td>4</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>Trace metals solution 100X (mL)</td>
<td>3</td>
<td>3.75</td>
<td>7.5</td>
<td>15</td>
<td>22.5</td>
<td>30</td>
</tr>
<tr>
<td>Ferric citrate solution 100X (mL)</td>
<td>0.8</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Ampicillin 1000X (mL)</td>
<td>0.2</td>
<td>0.25</td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
</tr>
</tbody>
</table>

Table B.3: Volumes of components of fed-batch solution for different final volumes.

- Check if the temperature is reasonable (less than 100°C).
- Connect chiller to exhaust condenser.
- Place the motor on the agitator shaft.
- Press CONTROL LOOPS until it goes to STIR window, go to SETP line.
- Enter 450 rpm then hit ENTER.
- If MODE is not auto, ALTER it to auto then Hit ENTER.
- Turn on the chiller. Check if the temperature is 12°C and water level is in range.
B.7 Water Connections

- Wait until the fermenter temperature is below 65°C
- Connect water jackets and use Fill Thermostat to fill the water jackets.
- Press CONTROL LOOPS until it goes to TEMP window, go to SETP line
- Enter 36.5°C (purple bioreactor) and 37.9°C (orange one) then hit ENTER.
- If MODE is not auto, ALTER it to auto then Hit ENTER.
- Screw the M12 connector to port A of Blue-In-One to turn it on (see [BlueSens gas sensor, 2017]).

B.8 Calibrating DO Probe

- It takes about 1-2 hours for the DO probe to be polarized.
- Add the batch additives (see appendix B.5) to complete the media.
- Turn N2 cylinder (Black) ON.
- Press CONTROL LOOPS until it goes to STIR window, go to SETP line. Enter 450 rpm then hit ENTER.
- Connect bioreactor air out to bubbler.
- Air circulation order: DCU air out, first sass flow meter, air filter, sparger
- Unclamp the tubings to see the bubbles.
- Press CONTROL LOOPS until it goes GASMX window, go to SETP line. Enter 100%, then hit ENTER
• If MODE is not auto, ALTER it to auto, then hit ENTER.

• Press CALIBRATION until it goes to pO2 window, go to NITR line. Check if it is 0.0%, then hit ENTER.

• Wait until the cursor goes to AIR.

• Press CONTROL LOOPS until it goes to GASMX window, go to SETP line. Enter 0%, then hit ENTER.

• Turn N2 cylinder (Black) off, then open the air valve.

• Exhaust air order: Bioreactor air out, blue-In-One, Second mass flow meter, bubbler

• Press CALIBRATION until it goes to pO2 window, go to AIR line. Check if it is 100.0%, then hit ENTER.

• Wait until the cursor goes to the next line.

B.9 Last Settings

• Use tube size 14 for base and tube sizes 13 and 14 for substrate. Thread all three tubings to the pumps.

• Unclamp the tubes and prime them by using switching pumps to manual.

• Open "Blue-In-One Service Configuration" software and reset the sensor.

• Open BlueVis software, choose purple and 15, then press START.

• Check if $O_2$ is about 20.9 and $CO_2$ is about 0.039. If not, press both buttons on the Blue-In-One for 5 seconds to reset it manually.
• Press CONTROL LOOPS until it goes to
  
  – pH: Check if SETP is 6.95. ALTER the MODE to auto, then hit ENTER.
  
  – SUBS1: Check if SETP is 0. ALTER the MODE to auto, then hit ENTER.
  
  – SUBS2: Check if SETP is 0. ALTER the MODE to auto, then hit ENTER.

• Turn all 3 pump switches to auto.

• Hit remote button on DCU.

B.10 Software Start

• Open MFCS Shell software.

• Click on “Run”, then click on “Operator Services” to open MFCSOPR.

• Open Simulink file (If it is already open, close it and reopen it), then run it.

• On MFCS program, click on “Start batch”. Again, click on “Start batch” and then click OK.
Appendix C  Pre-culture Growth Calculations

Cells needs to be prepared for the experiment in three phases — overnight culture, pre-culture and inoculation. All the calculations are in a Microsoft® Excel file. For each quantity that is mentioned in this appendix, the corresponding Excel cell number (see Figure C.1) is referred in parentheses. In the beginning, estimate all the times for overnight culture (F6), pre-culture (F15) and inoculation (F25).

C.1 Preparation of Overnight Culture

• Remove glycerol stock culture from -80°C freezer and thaw it.

• Pour 50 mL (D7) of minimal media in a sterile 250 mL (D8) Erlenmeyer flask.

• Add 50 µL (D7/1000) of ampicillin and 10 µL (D3) of the cells in the 1.5 mL tube to the media.

• Cap the Erlenmeyer flask with a blue paper by using a rubber band.

• Put it in a shaker incubator to culture at 37°C and 250 rpm

C.2 Splitting the Cells

• Update the time of pre-culture (F15) if needed.

• Remove the flask from the incubator and measure overnight OD and type it in D12.

• Pour 250 mL (D16) of minimal media in a sterile 1 L (D17) Erlenmeyer flask.

• Add 250 µL (D16/1000) of ampicillin and D13 µL of the overnight culture to the media.
Figure C.1: Pre-culture growth calculations (Exp 75).
• Measure the pre-culture OD and type it in D20.

• Cap the Erlenmeyer flask with a blue paper by using a rubber band.

• Put it in the incubator to culture at 37°C and 250 rpm

C.3 Inoculation

• Update the time of inoculation (F25) if needed.

• Remove the flask from the incubator and measure the pre-culture OD and type it in D22.

• Volume of batch media in the fermenter is 1.5 L (D26).

• Use a 10 mL syringe to take sample from the media in the bioreactor. Measure the OD of the media before inoculation and type it in D29.

• The inoculation volume is given in D23. Use pipettes to take D23 mL of the pre-culture.

• If the inoculation volume is more than 60 mL, centrifuge to concentrate the pre-culture (at 8000 RCF for 6 min).

• Inoculate the bioreactor by using a sterile syringe or pipette from the inoculation port.

• Take sample from the inoculated bioreactor. Measure the OD of the media after inoculation and type it in D30.
<table>
<thead>
<tr>
<th>Date</th>
<th>Number</th>
<th>Result</th>
<th>Max OD</th>
<th>Dry cell (g/L)</th>
<th>Controller</th>
<th>µ</th>
<th>Time of probe</th>
<th>Reason of failure</th>
<th>Comments 1</th>
<th>Comments 2</th>
<th>Usefullness</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/21/2016</td>
<td>59</td>
<td>Successful</td>
<td>74</td>
<td>Mu Set</td>
<td>Mu Set</td>
<td>0.18</td>
<td>-</td>
<td>GasMix</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/14/2016</td>
<td>60</td>
<td>Successful</td>
<td>74</td>
<td>Mu Set</td>
<td>Mu Set</td>
<td>0.18</td>
<td>-</td>
<td>Foam Balances</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>3/31/2016</td>
<td>61</td>
<td>Failed</td>
<td>20</td>
<td>BOOM2</td>
<td>BOOM2</td>
<td>0.18</td>
<td>-</td>
<td>#12 tubing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5/16/2016</td>
<td>62</td>
<td>Successful</td>
<td>141</td>
<td>Mu Set</td>
<td>Mu Set</td>
<td>0.3</td>
<td>5 min</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5/23/2016</td>
<td>63</td>
<td>Failed</td>
<td>-</td>
<td>Mu Set + Probe</td>
<td>Mu Set + Probe</td>
<td>0.3</td>
<td>5 min</td>
<td>Clogging</td>
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<tr>
<td>5/26/2016</td>
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<td>Failed</td>
<td>33</td>
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<td>5 min</td>
<td>Clogging</td>
<td>PID-Constant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8/18/2016</td>
<td>65</td>
<td>Failed</td>
<td>4</td>
<td>Mu Set + Probe</td>
<td>Mu Set + Probe</td>
<td>0.3</td>
<td>5 min</td>
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<td>Tom’s prep</td>
<td></td>
<td></td>
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<tr>
<td>8/25/2016</td>
<td>66</td>
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<td>128</td>
<td>45.3</td>
<td>Mu Set + Probe</td>
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<td>5 min</td>
<td>-</td>
<td>BlueSens By-Pass</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9/1/2016</td>
<td>67-A</td>
<td>Failed</td>
<td>5</td>
<td>Mu Set + Probe</td>
<td>Mu Set + Probe</td>
<td>0.3</td>
<td>6 min</td>
<td>Clogging</td>
<td>BlueSens By-Pass</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9/2/2016</td>
<td>67-B</td>
<td>Successful</td>
<td>131</td>
<td>61.5</td>
<td>Mu Set + Probe</td>
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<td>6 min</td>
<td>BlueSens By-Pass</td>
<td>Open Sparger</td>
<td>* Good</td>
<td></td>
</tr>
<tr>
<td>9/8/2016</td>
<td>68</td>
<td>Successful</td>
<td>108</td>
<td>Mu Set + Probe</td>
<td>Mu Set + Probe</td>
<td>0.15</td>
<td>6 min</td>
<td>BlueSens By-Pass</td>
<td>Open Sparger</td>
<td>* Good</td>
<td></td>
</tr>
<tr>
<td>9/15/2016</td>
<td>69-A</td>
<td>Reboot</td>
<td>15</td>
<td>Mu Set + Probe</td>
<td>Mu Set + Probe</td>
<td>0.45</td>
<td>6 min</td>
<td>CCIT imaging</td>
<td>BlueSens By-Pass</td>
<td>Open Sparger</td>
<td>* OK</td>
</tr>
<tr>
<td>9/16/2016</td>
<td>69-B</td>
<td>Successful</td>
<td>136</td>
<td>65.5</td>
<td>Mu Set + Probe</td>
<td>0.45</td>
<td>6 min</td>
<td>BlueSens By-Pass</td>
<td>Open Sparger</td>
<td>* Very Good</td>
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<td>9/22/2016</td>
<td>70</td>
<td>Successful</td>
<td>164</td>
<td>75.7</td>
<td>BOOM2</td>
<td>0.33</td>
<td>8 min</td>
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<td>Open Sparger</td>
<td>* Very Good</td>
<td></td>
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<td>Successful</td>
<td>128</td>
<td>BOOM2</td>
<td>BOOM2</td>
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<td>* Very Good</td>
<td></td>
</tr>
<tr>
<td>10/4/2016</td>
<td>72</td>
<td>Failed</td>
<td>71</td>
<td>BOOM2 + Induced</td>
<td>BOOM2 + Induced</td>
<td>0.34</td>
<td>8 min (Variable)</td>
<td>Hardware</td>
<td>BlueSens By-Pass</td>
<td>Open Sparger</td>
<td>* OK</td>
</tr>
<tr>
<td>10/13/2016</td>
<td>73</td>
<td>Successful</td>
<td>78</td>
<td>BOOM2 + Induced</td>
<td>BOOM2 + Induced</td>
<td>0.27</td>
<td>5 min (Variable)</td>
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<td>Open Sparger</td>
<td>* Good</td>
<td></td>
</tr>
<tr>
<td>10/20/2016</td>
<td>74</td>
<td>Successful</td>
<td>165</td>
<td>BOOM2 + Induced</td>
<td>BOOM2 + Induced</td>
<td>0.36</td>
<td>5-6 min (Variable)</td>
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<td>Open Sparger</td>
<td>* Very Good</td>
<td></td>
</tr>
<tr>
<td>10/27/2016</td>
<td>75</td>
<td>Successful</td>
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<td>BOOM2 + Induced</td>
<td>BOOM2 + Induced</td>
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<td>5 min (Variable)</td>
<td>Probe didn’t stop</td>
<td>BlueSens By-Pass</td>
<td>* OK</td>
<td></td>
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Figure C.2: List of all experiments done for this thesis.
Bibliography


