ABSTRACT

CHOE, KISURB. Development of Glycerol Feedback Control, Cell Density Measurement Device, and Supervisory Control and Data Acquisition (SCADA) Software for Automation of High Cell Density *Pichia pastoris* Fermentations in a 30L Bioreactor. (Under the direction of Dr. Michael C. Flickinger.)

Timely and accurate bioprocess control is necessary for process reproducibility and efficiency, and an inexpensive and flexible control system is especially needed for automating pilot scale fed-batch experiments. A SCADA software and hardware system were developed for controlling glycerol feeding to a 30L *Pichia pastoris* fed-batch culture performing at-line biomass monitoring by exact mass dilution without *in situ* probes. The glycerol fed-batch control system uses an at-line analyzer to maintain glycerol level within ±10 percent of a set point throughout *Pichia pastoris* exponential growth phase to achieve cell densities of 140 OD$_{600}$. Using the SCADA system, a prototype at-line electromechanical device combining automated sample collection, precision dilution, and cell density measurement was developed correlating automated OD$_{600}$ measurement to manual off line OD$_{600}$ measurement of 30L *P. pastoris* fermentations. Based on this correlation, the device was able to predict OD$_{600}$ results at OD 42 to OD 72 with less than 14 percent deviation during the exponential growth phase of a *Pichia pastoris* fed-batch fermentation. The control system can be utilized as part of an at-line sensing and control strategy for optimizing protein production using high cell density *Pichia pastoris* fed-batch fermentation.
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by
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DEDICATION

I dedicate this thesis to my family. Dad, mom, and Insurb I love you all.
BIOGRAPHY

After graduating with BA Biological Sciences from Rice University in May 2010, Kisurb Choe worked as a life science research assistant at Stanford University. There he discovered a passion for yeast technology and programming and decided to pursue graduate studies. While continuing thesis research, he studied both upstream and downstream biomanufacturing within his Professional Science Master’s degree program. During his graduate studies Kisurb was also involved in metabolic engineering research funded by DARPA (Defense Advanced Research Projects Agency) gaining experiences in synthetic biology at Duke University Department of Biomedical Engineering. He also performed fermentation procedures and built software for the BioAg Alliance as a summer intern at Novozymes (Salem, VA). He was a part-time bioinformatics programmer for NCSU Center for Integrative Fungal Research implementing and maintaining data analysis programs in a publically available web server. He is currently an active member of the International Society for Pharmaceutical Engineering and Korean-American Scientists and Engineers Association.
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CHAPTER 1: Introduction

1.1. *Pichia pastoris* as an Expression Host for Heterologous Protein Manufacture

*Pichia pastoris*, a highly productive methylotrophic yeast, has been one of the primary platforms for industrial production of recombinant protein (Ahmad *et al.*, 2014). There are several important advantages of *Pichia pastoris* as an expression host which include: 1) high protein expression levels, 2) rapid growth speed, 3) simple culture conditions, 4) high levels of secreted heterologous recombinant proteins, 5) no endotoxin, 6) extensive post-translational modification of proteins, and 7) simple induction method of protein expression by addition of methanol (Broadway, 2012). The genome sequences of the expression strains CBS7435 and GS115 became available around 2010 so the genome mining for strain engineering such as searching for proteases and foldases became very feasible. Also, the recent classification as Food and Drug Administration (FDA) GRAS (generally recognized as safe) for recombinant protein production makes *Pichia pastoris* very attractive as an industrial expression host for recombinant proteins (Ahmad *et al.*, 2014).

1.2. History of *Pichia pastoris*

Koichi Ogata discovered the ability of *Pichia pastoris* to utilize methanol as the single source of carbon and energy (Cereghino *et al.*, 2000). During the 1970s, Phillips Petroleum Company developed a continuous culture process and media for growing *P. pastoris* cheaply on methanol to produce animal feed products. However, the oil crisis increased the cost of methanol dramatically, therefore the process lost economic competitiveness. In the 1980s, Phillips Petroleum contracted with the Salk Institute Biotechnology/Industrial Associates Inc. (SIBIA, La Jolla, CA) to create *Pichia pastoris* as a recombinant protein
production system. Phillips sold the *Pichia pastoris* expression system to RCT and later licensed the technology to Invitrogen (Cregg, 2017).

### 1.3. The Global Market Demand for Biologics

The global demand for biologics is expanding. The biopharmaceutical market reached $162 billion in 2014 and is projected to grow up to $278 billion by 2020. In order to meet the growing need, it is important to develop efficient and reliable biomanufacturing processes (Persistence Market Research, 2015).

### 1.4. Phenotypes of *Pichia pastoris* Methanol Utilization

The hyperactive promoter of *Pichia pastoris* Alcohol Oxidase I (PAOX1) gene can be used to tightly control production of recombinant proteins. Alcohol oxidase is the enzyme catalyzing the first step in methanol assimilation pathway which is conversion of methanol to formaldehyde. This enables using methanol as the sole carbon source. There are two alcohol oxidases in *Pichia pastoris* which are alcohol oxidase I (AOX1) and alcohol oxidase 2 (AOX2). The AOX1 promoter is around 10 times more active than AOX2 promoter. There are three different *Pichia pastoris* types differing in methanol utilization phenotypes. The first type is the wild type having methanol utilization plus phenotype (Mut+) which means both AOX1 and AOX2 are active. The second type contains only AOX2 gene (MutS) and the last type has no alcohol oxidase gene meaning both AOX1 and AOX2 genes are deleted (Mut-) (Looser *et al*., 2015).
1.5. *Pichia pastoris* Transformation

Typically genomic integration of a gene or genes of interest for recombinant expression in *P. pastoris* is performed by homologous recombination. The expression cassette containing the gene of interest and an antibiotics resistance marker can be flanked by sequences identical to a region of the target AOX gene in the *Pichia pastoris* genome. Restriction enzymes cutting the sites of the target sequences contained in the plasmid can generate two regions on the linearized DNA that can bind to the target site of the genomic DNA for homologous recombination, resulting in stable transformation.

![Diagram of genomic recombination of *Pichia pastoris* expression cassette](image)

**Figure 1**: Genomic recombination of *Pichia pastoris* expression cassette (Invitrogen Co. 2010).

Transformation of *P. pastoris* starts from preparing electrocompetent cells which are washed cells collected from the log-growth phase in a liquid cell culture. A gene insert linearized by restriction enzyme is inserted into the cell via electroporation which is transformation through a brief electrical shock. Lastly, the population of transformed cells are then grown on an agar plate containing antibiotics selecting for the cell containing the insert in the genome.
1.6. Defined Media for Recombinant Protein Expression in *Pichia pastoris*

Chemically defined media is often used for recombinant proteins production in *Pichia pastoris* due to reduced variability in bioprocess yield. Common components of defined medium for *Pichia pastoris* include glycerol as carbon source and ammonium hydroxide or ammonium sulfate as nitrogen source. Glycerol is the preferred carbon source because the glucose assimilation pathway is fermentative resulting in ethanol production, which is known for repressing AOX promoter activity. Defined amounts of basal salts can be provided through highly optimized salt recipes like BFM21 and FM22 developed at BTEC (Sigmon, 2013). Vitamins are known for increasing the production yield of recombinant proteins from *Pichia pastoris*, thus critical vitamins like biotin are typically included in the growth medium. Overall, the optimized media for *Pichia pastoris* consist of economically attractive components making *Pichia pastoris* an attractive expression system (Marquard et al., 2016).

1.7. Bioreactor Fermentation of *Pichia pastoris*

Protein production yield and biomass achieved in a bioreactor is typically much higher than achievable by shake flask experiments because process parameters like oxygen level, temperature, and pH can be precisely controlled for the optimal growth and production of proteins. Especially, *Pichia pastoris* achieves cell densities above 100 OD\(_{600}\) units or higher requiring high agitation and aeration rates for maintaining pO\(_2\) of 30 % of dissolved oxygen tension (DOT) required for high cell viability (Sigmon, 2013). Also temperature is a critical element for maintaining cell growth and protein production. For example, *Pichia pastoris* achieves an optimal growth rate at 30ºC but too high a temperature results in protein misfolding and aggregation which reduces recombinant protein yield. Lastly, maintaining
pH in the range of 3 to 7 is reported to enable cell growth and protein production (Sigmon, 2013).

1.8. Fed-batch Fermentation of *Pichia pastoris*

A fed-batch fermentation process is the preferred method of cell culture for industrial production of recombinant protein. This is because fed-batch fermentation achieves high cell biomass and protein yield while enabling precise control of cell culture conditions necessary for regulatory requirements needed by the industry. There are three phases of fed-batch culture of *Pichia pastoris*: the glycerol batch and fed-batch phases, and the methanol induction phase (Looser et al., 2015). During the glycerol batch and fed-batch phases, the biomass of recombinant *Pichia pastoris* is maximized for greater protein production. Glycerol is used for the biomass generation because cells grow rapidly on glycerol compared to methanol. The glycerol concentration of the batch phase typically starts with 40 g/l. During the glycerol fed-batch phase, glycerol media is continuously supplied to the culture to maintain glycerol concentration and the growth rate of *Pichia pastoris*. After achieving high cell densities of 100~200 OD, the methanol induction phase starts where precisely controlled addition of methanol is performed to maintain the methanol level needed for cell survival and continued recombinant protein production. Monitoring and maintaining methanol at a constant level in the fermentation media is critical because there is a delicate range of methanol concentration that achieves maximal pAOX1 induction while minimizing methanol toxicity for cells. For example, it is recommended to maintain methanol level to around 0.3 % v/v continuously for maximal performance (Invitrogen Co. 2002).
1.9. Substrate Feeding Strategy

The *Pichia pastoris* fermentation requires PAT control of two substrates: glycerol level during glycerol fed batch phase and methanol level during the methanol induction phase. Fluctuations in these substrates affect the quality and quantity of the model protein produced in this study, recombinant Human Serum Albumin (rHSA) (Sigmon, 2013). For example, low methanol concentration results in lack of fully induced AOX promoter activity and high concentration can be toxic to the cells. There are two methods for substrate concentration control. The first is feedforward control which is determination of substrate feed by measuring the causative agent of disturbances on the substrate concentration. This requires process understanding and mathematical models predicting effects of various disturbances on the substrate concentration. The second is feedback control which is determination of substrate feed by measuring its actual change on the substrate concentration. Since feedback
control achieves better robustness and simplicity in control, it was chosen to be the control scheme for glycerol and methanol level control in this study.

1.10. Process Analytical Technology (PAT)

1.10.1. Background

Conventionally, biopharmaceuticals have been manufactured through rigidly set procedures and laboratory testing of collected samples from the process. All manufacturing process validation and product quality control was performed through laboratory testing which is time consuming and costly. Also it takes around 10~15 years and $800 million to > $1 billion to develop a new drug or biologic. Due to tight FDA regulatory requirements and costly drug development process, biopharmaceutical companies have been reluctant to develop and apply innovative manufacturing approaches. Overall, development and investment in the manufacturing sector of pharmaceutical industry stalled while the burden on regulatory authorities increased due to the amount of quality documentation generated. (Lynch et al., 2016)

1.10.2. FDA Regulatory Framework and Process Analytical Technology

To encourage adaptation of the latest manufacturing technology and science for improving efficiencies in manufacturing process and regulatory compliance, the FDA started a new initiative called “Pharmaceutical CGMPs for the 21st Century: A Risk-Based Approach.” This defined new desired states of pharmaceutical manufacturing processes: 1) Product quality is assured by implementation of effective manufacturing processes based on sound process understanding. 2) Continual real-time quality measurements. In the official document published by the initiative, one of the chief enabling technologies for the above
goals is PAT. This document defines PAT as “a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality” (FDA. 2004). PAT consists of 1) Process analyzers, 2) Process control tools, 3) Multivariate tools for process design, data acquisition and analysis (FDA. 2004). There is a considerable need for development of these tools for the next regulatory landscape.

1.10.3. Types of Process Monitoring and PAT

The physical distance and temporal difference between the process and location of the measurement classify process measurements into three types: On-line, at-line, off-line. On-line devices are integrated with the vessel either internally (in-situ) or externally (ex-situ) and perform real-time measurements. At-line devices are physically separated from the vessel but located near to the reactor and have a temporal gap between measurement and the process. Off-line measurement has indefinite physical and temporal gap between the process and monitoring. Since PAT requires timely measurement of the process, PAT includes on-line and at-line monitoring.
1.10.4. \textit{Pichia pastoris} Fermentation Process Variables

Process variables involved with \textit{Pichia pastoris} fermentation can be grouped by properties: physical, chemical, and biological. Physical properties include pressure, aeration, agitation, culture viscosity, and temperature. Chemical properties include pH, dissolved gas type and concentration, substrate type and composition, product titer, and media composition. Biological properties include biomass, cell size and morphology. This thesis project develops PAT for two of the most important process variables that directly impact product quality and quantity: sensing and maintaining glycerol level during glycerol fed-batch phase, sensing and maintaining methanol level for the methanol induction phase, and at-line determination of biomass level at high OD600 by exact mass dilution.

1.10.5. Supervisory Control and Data Acquisition (SCADA) for PAT

SCADA is the central software package that enables real-time bioprocess data acquisition, data analysis, process control, and data logging needed for PAT. It communicates with at-
line and on-line sensors for process monitoring and with bioprocess controllers. Accumulating process knowledge needed for establishing the process design space or for establishing the impact of process variables on the criticality of one or more product quality attributes (Quality by Design, QbD) is achieved by utilizing database systems like Structured Query Language (SQL). Finally SCADA includes a proper user interface that presents summarized data to the bioprocess operators who can direct the course of the bioprocess through the same simple interface (Inductive Automation, 2017). The benefits of massive data acquisition and processing are reaped by multivariate analysis and mathematical modeling (Bhatnagar, 2014). SCADA communication with diverse and disparate devices is handled by an industry standard software interface protocol called OLE for Process Control (OPC) (OPC Foundation, 2017).

The SCADA system used for this project was BioPAT® MFCS/win which is an industry standard system developed by Sartorius for supervisory bioprocess control and data acquisition. It is OPC-enabled control software equipped with a user interface and mathematical analysis module. It can control physical and chemical parameters like agitation, aeration, temperature, pH, and dissolved oxygen within BIOSTAT® D-DCU3 vessel as shown in Figure 4.
1.11. Objectives

*Pichia pastoris* is one of the standard heterologous protein expression systems for the biopharmaceutical industry. Pilot-scale bioprocesses often lack inexpensive flexible system for controlling glycerol level, and cell density which are critical for high product yield and quality. This thesis shows development of 1) feedback control system for glycerol level, 2) an automated cell density measurement device for at-line biomass monitoring at high optical densities (OD600 >50), 3) and a control software for directing the systems. Then the performance of these systems in actual *Pichia pastoris* cell culture runs is discussed.

For reproducibility of high product yield and quality during cell culture process development, precise automatic control of nutrients and biomass is needed. Glycerol and
methanol consumption rate changes rapidly and dynamically during the fermentation course, and such a control system should be able to adjust accordingly. Moreover, very precise maintenance of glycerol and methanol level is needed to achieve maximal product yield while avoiding cell death coming from substrate effects.

The objectives of this thesis to achieve precise automated control of glycerol and biomass include:

- Development of a system and algorithm for controlling nutrient addition
- Creation of a SCADA software for controlling nutrient level and biomass with a proper user interface for the operator to decide process parameters
- Creation of an electromechanical device automating at-line cell density measurement using exact mass dilution
- Development of Standard Operation Procedures for running the control systems
CHAPTER 2: Development of an At-Line Exact Mass Dilution High Cell Density Measurement Device for Controlling a Fed-Batch Culture of *Pichia pastoris*

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A version of this chapter will be submitted to:

*Fermentation*
2.1. Abstract

Timely and accurate bioprocess control is necessary for process reproducibility and efficiency, and an inexpensive and flexible control system is especially needed for automating pilot scale fed-batch experiments. We have developed an inexpensive software and hardware system for controlling glycerol feeding to a 30L *Pichia pastoris* fed-batch culture that includes at-line glycerol fed rate and at-line biomass monitoring without *in situ* probes. The glycerol fed-batch control system uses an at-line analyzer to maintain glycerol level within ±10 percent of a set point throughout *Pichia pastoris* exponential growth phase to achieve cell densities of 140 OD$_{600}$. A prototype at-line electromechanical device combining automated sample collection, precision exact mass dilution, and cell density measurement was developed for up to 80 OD$_{600}$ range using a flow through spectrophotometer. A calibration curve correlating automated OD$_{600}$ measurement to manual off line OD$_{600}$ measurement performed on 30L *P. pastoris* fermentations was made. Based on this correlation, the device was able to determine OD$_{600}$ results in the range of OD 42 to OD 72 with less than 14 percent deviation during the exponential growth phase of a *Pichia pastoris* fed-batch process. Our system can be utilized as part of an at-line sensing and control strategy for optimizing protein production using high cell density *Pichia pastoris* fed-batch fermentation.

2.2. Introduction

*Pichia pastoris* has been used as an expression host for producing recombinant protein therapeutics and industrial enzymes. *Pichia pastoris* is a highly productive methylotrophic yeast with a strong methanol inducible alcohol oxidase promoter (AOX1), has the ability to produce gram amounts of recombinant protein per liter, and achieves very high cell densities
(OD600 > 50) (Ahmad et al., 2014). Some of these advantages are that it can be engineered to express heterologous proteins with human-like glycosylation and perform efficient protein secretion. Carbon sources for cell growth such as glycerol and methanol repress and induce the AOX1 promoter, respectively (Looser et al., 2015). High cell density cultures of Pichia pastoris for production of recombinant proteins typically consists of an extended fed-batch cell growth phase on growth substrates like glucose and glycerol followed by methanol adaptation and induction phase (Looser et al., 2015).

When engineered yeast strains are constructed and are ready to be evaluated for industrial protein production, process control, automation, and optimization techniques are applied to improve the fermentation performance in the following aspects: (1) increase recombinant host cell concentrations in the fermentor to high density by extending the growth phase; (2) effectively induce heterologous protein yield and maximize accumulation; (3) decrease operation costs by relieving the workloads of heat-exchanger/coolant demand and oxygen supply (Gao et al., 2013). Robust on-line process sensing and control can aid in achieving the above performance aspects. This is achieved by online or at-line measuring and tightly controlling key parameters such as cell density, substrate concentration and protein induction. Analytical instrumentation tools exist for on-line control of a range of values pertaining to substrate concentration and protein induction, but cell density is one factor for which there are restrictions when it comes to robust control, mainly because there are few cell density measuring systems that provide on-line or at-line OD600 measurements greater than 50. For this reason, this project includes design, implementation and optimization of a novel at-line inexpensive exact mass dilution and flow through spectrophotometer cell density measurement device used for monitoring and control of glycerol feeding of high cell density P. pastoris fermentations. The development of this system is supported by design and implementation of an at-line glycerol concentration and control system during an
extended glycerol fed-batch phase for generating high levels of *Pichia pastoris* biomass in a 30L defined media culture.

2.3. Materials and Methods

2.3.1. Fermentation of *Pichia pastoris*

Fed-batch fermentation of *Pichia pastoris* GS115 His+ Mut⁴, a strain with a slow methanol utilization phenotype, was performed on 20L of modified BFM21 media with 87ml PTM4 solution within an agitated 30L bioreactor. The BFM21 media contained 3.5ml/L 85% H₃PO₄, 0.12g/L CaSO₄, 2.4g/L K₂SO₄, 1.95g/L MgSO₄·7H₂O, KOH 0.655g/L, 2.94g/L sodium citrate·2H₂O, 6.00g/L (NH₄)₂SO₄, 1.54g/L NaOH, 40ml/L 1M acetate buffer, and glycerol 40.0g/L (Sigmon, 2013). The PMT4 trace metals solution contained 1ml/L H₂SO₄, 2g/L CuSO₄·5H₂O, 0.08g/L NaI, 0.02g/L H₃BO₃, 0.5g/L CaSO₄·2H₂O, 0.5g/L CoCl₂, 7g/L ZnCl₂, 22g/L FeSO₄·7H₂O, and 0.2g/L biotin. BFM21 media was sterilized at 121°C for 30 minutes, and PTM4 was filter sterilized. The fed batch media contained 110g/L (NH₄)₂SO₄, 800g/L glycerol, and 87ml PTM solution/L. For inoculum generation, *Pichia pastoris* colonies collected from a YPD agar plate were inoculated into 100ml YPD media which consisted of 20g/L bacteriological peptone, 10g/L yeast extract, and 20g/L glucose. After incubation (< 4 hours), the inoculum was aseptically injected into the bioreactor by using a sterile syringe through the bioreactor inoculation septum port located in the head plate, completed in 3 minutes to minimize cell death by lack of oxygen during the transfer
Figure 5: Bioreactor system for testing of glycerol feedback control and automated cell density measurement device. (1,2) Glycerol fed-batch supply, (3) Seg-Flow® 4800 on-line sampling system, (4) YSI MBS 7100 biochemistry analyzer, (5,6) base supply (20% NH₄OH), (7,8) cell density module, (9) Pendotech SPEC-600L flow through spectrophotometer, (10) waste container, (11,12,13,14) BCpreFerm inlet and exhaust gas sensors (BlueSens™, Herten, Germany), (15) dissolved methanol probe (Cueva, 2012), (16) dry air supply, (17) Figaro TGS-822 methanol sensor, and (18) Micro850 programmable logic controller (Rockwell Automation, Milwaukee, WI).

The bioreactor system used to test the control system is shown in Figure 5. The *P. pastoris* strain was cultured in a 30L Biostat® C stirred bioreactor (Sartorius Stedim Biotech GmbH, Göttingen, Germany) at 30 °C, pH 5 adjusted and controlled by 20% NH₄OH and 85% H₃PO₄, with dO₂ set point at 30% of air saturation, and pressure at 400 mbar.
2.3.2. In situ Dissolved Methanol Probe

The *in situ* sterilizable hydrocarbon gas sensor used for measuring dissolved methanol during the yeast culture is shown in Figure 6 (Sigmon, 2013).

![Figure 6: In situ Dissolved Methanol Probe (Sigmon, 2013)](image)

The methanol probe consists of a 650mm x 14 mm stainless steel rod which includes two 1.59 mm outer diameter stainless steel hollow tubes serving as dry air inlet and outlet for an air duct passing through the steel rod. Two meters of SILASTIC® Laboratory Tubing 508-003 (Dow Corning, Midland, Michigan, United States) wraps around the outer surface of the steel rod and connects the air inlet and outlet stainless steel tubes generating an ~95 cm² surface area for dissolved methanol diffusion from the culture medium into the lumen gas.
The inner diameter and the outer diameter of the silicone tube was 2.41 mm and 1.57 mm respectively. The methanol probe is installed in the BIOSTAT C-DCU bioreactor through a threaded port in the headplate and directly contacts the yeast culture. Methanol dissolved in the broth diffuses through the silicon tube wall into the gas flowing through the silicon tubing. The dry gas sweeps the diffused methanol into a hydrocarbon sensor located outside of the bioreactor for methanol measurement. An inexpensive tin dioxide (SnO\textsubscript{2}) semiconductor (Taguchi Gas Sensor (TGS) model 822, Figaro Inc., Japan) was for detecting methanol. It has low conductivity in clean air but has high conductivity when exposed to hydrocarbon gases. A linear methanol calibration was established over a range of dissolved methanol concentrations from 8.1mM to 161.1mM in sterile media under identical temperature and agitation conditions used for the yeast fermentation (Sigmon, 2013). The increased voltage resulting from higher conductivity is measured by LabVIEW system for quantifying the methanol level (Cueva, 2012). This probe was also used for accurate determination of methanol stripping rate during the yeast culture as a function of aeration rate and lumen gas flow rate (Sigmon, 2013). The methanol level data is used by a methanol feedback control system consisting of a Micro850 programmable logic controller (Rockwell Automation, Milwaukee, WI), a 550-0213 peristaltic pump head (Autotrol, Crystal Lake, IL), and a control program written in Python\textsuperscript{©} (Rossum, 1995).

2.3.3. Glycerol Feedback Control During Glycerol Fed-Batch Phase

A Seg-Flow\textsuperscript{®} 4800 on-line sampling system (Flownamics\textsuperscript{®}, Madison, WI) was used to collect cell-free culture media from the 30L bioreactor and deliver samples to the MBS 7100 Biochemistry Analyzer (YSI Inc., Yellow Springs, OH) for at-line glycerol concentration measurement. The in-situ FISP\textsuperscript{®} sampling port (Flownamics\textsuperscript{®}, Madison, WI) filtered out \textit{P. pastoris} cells during sample collection for glycerol determinations. For maintaining dissolved glycerol concentration during rapid yeast growth, a lab-developed control
software activated a Rockwell 850 PLC to switch on a peristaltic pump (520 du, Watson Marlow, Wilmington, MA) for a duration calculated by a glycerol addition algorithm. Throughout this study, the glycerol set point during fed-batch exponential growth was maintained at 30g/L.

The mass of glycerol addition in each feedback loop \( (A_{\text{current}}) \) for maintaining glycerol concentration in the bioreactor was calculated by two variables: \textit{Pichia pastoris} glycerol consumption rate per a single feedback cycle \( (R_{glycerol}) \) and glycerol mass adjustment to cancel out glycerol level offset \( (A_{\text{adjustment}}) \).

\[
A_{\text{current}} = R_{glycerol} + A_{\text{adjustment}} \tag{1}
\]

\( R_{glycerol} \) is the change in glycerol mass during each feedback cycle:

\[
R_{glycerol} = (C_{\text{previous}} \times M_{\text{broth}} + A_{\text{previous}}) - C_{\text{current}} \times M_{\text{broth}} \tag{2}
\]

where \( C_{\text{current}} \) is current glycerol concentration measured, \( M_{\text{broth}} \) is the mass of the bioreactor as measured by the Biostat C control system, and \( A_{\text{previous}} \) means the mass of glycerol added during the previous glycerol feedback loop. Lastly, \( C_{\text{previous}} \) is the glycerol concentration reported in the previous feedback loop. The amount of glycerol adjustment \( (A_{\text{adjustment}}) \) for canceling out the offset from setpoint \( (SP) \) is:

\[
A_{\text{adjustment}} = (SP - C_{\text{current}}) \times M_{\text{broth}} \tag{3}
\]

Therefore, the amount of glycerol addition to be performed during the current feedback loop \( (A_{\text{current}}) \) was determined by Equation 4 which is the sum of Equation 2 and Equation 3:
\[ A_{\text{current}} = (SP - 2C_{\text{current}} + C_{\text{previous}}) \times M_{\text{broth}} + A_{\text{previous}} \]  

(4)

2.3.4. Control Software System

A *Pichia pastoris* fermentation control program was developed in Python© (Rossum, 1995) for device control, bioprocess execution, data logging, data visualization, and set point management. pySerial©, which is a Python© module for RS232 serial communication, was used to control a programmable logic controller Micro850, Arduino Uno (Arduino Foundation, Italy), electronic balances including AV3102 (Ohaus Scales, Parsippany, New Jersey), EB60EDE-L (Sartorius, Göttingen, Germany) and a flow-through spectrophotometer SPEC-600L (Pendotech, Princeton, NJ). For 30L Biostat C bioreactor control, the control software utilized Autohotkey™ (Autohotkey Foundation LLC, Indiana) which automates software interaction to control the MFCS 3.0 (Sartorius, Göttingen, Germany) which is the control software for the bioreactor. This enables dynamic changing of the pH, dissolved oxygen percent, and the temperature set point during cell culture and real time process data acquisition for glycerol feedback control. Acquisition of bioreactor glycerol concentration data from the YSI Biochemistry Analyzer was performed by utilizing functionalities of Splinter© and BeautifulSoup© which are Python© modules that automate web browser actions. These modules programmatically activate and read from the internal web server of the Seg-Flow® 4800 sampling system which controls and reports the glycerol level determined by the YSI Biochemistry Analyzer.

A Django© (Django Software Foundation, Lawrence, Kansas) local server host was used as a user interface based on HTML© (World Wide Web Consortium, Cambridge, Massachusetts) and Javascript™ (Oracle, Redwood City, CA) which enabled remote bioprocess control. This platform accepts user inputs for dissolved glycerol levels, target
biomass, and hardware communication configurations. Lastly, real

time visualization of bioprocess data is performed by combining

dcharacters, and hardware communication configurations. Lastly, real time visualization of bioprocess data is performed by combining Matplotlib (The Matplotlib development team) and JQuery (jQuery Foundation) which plot time series graphs and periodically renders the plot on a web browser, respectively. The user interface of the Python control software designed for this system is shown in Figure 7.
Figure 7: User interface designed for controlling the Python® control software.
2.3.5. Cell Density Module

**Figure 8**: Electromechanical system for automated at-line optical density measurement at high cell density using exact mass dilution: (1) BIOSTAT C bioreactor, (2) PK-0802-NC-3 solenoid valve, (3) Rainin RP-1 peristaltic pump, (4) sample bottle, (5) Ohaus AV3102 scale, (6) Watson Marlow 330du peristaltic pump (7) Pendotech 600nm UV-Vis-NIR spectrophotometer, (8) waste container, (9) 0.1M sodium acetate container, (10) 70% ethanol container, (11) filter, (12) 20 psi purge air supply, (13) Arduino UNO, (14) PC, (15) waste container.
The configuration of the prototype electromechanical device developed to achieve automated at-line measurement of cell density at high cell density by exact mass dilution is shown in Figure 8. To control each pump and valve, Arduino Uno was used to convert serial data sent from the PC into analog signals controlling the pumps and valves of the device. Cell culture sample dilution by mass, mixing, and cleaning were all done on a 250mL Corning® PP centrifuge conical tube (Corning Inc., Corning, NY, USA) mounted on an Ohaus platform scale.

Each cycle of OD measurement started with the sample duct from the 30L bioreactor being purged by pressured filtered air. Then approximately 0.5 gram of cell culture sample was delivered to a sample bottle, after which the scale measured and reported the mass of the sample added to the bottle with a resolution of 0.01 gram. 0.1 M sodium acetate solution was then pumped to the sample bottle to generate an approximately 250 fold dilution of the sample based on mass. Sample dilution by weight (exact mass) was done to accommodate for the limited linear OD$_{600}$ measurement range of the flow through microspectrophotometer (OD$_{600}$ range 0.00 to 1.00). The mass of the sample ($M_{\text{sample}}$) and the mass of the diluent ($M_{\text{diluent}}$) was used to precisely calculate the dilution factor ($DF$) using Equation 5:

$$DF = \frac{(M_{\text{sample}} + M_{\text{diluent}})}{M_{\text{sample}}}$$  \hspace{1cm} (5)

For one minute, the diluted sample was pumped through a recirculation loop which was both a route to the flow-through spectrophotometer and a sample mixing mechanism by creating liquid turbulence. After recording the spectrophotometer signal through serial communication, the diluted sample was pumped to drain and the vessel was washed by diluent, followed by sample duct purging by pressured filtered gas. Finally the automated
OD$_{600}$ value ($OD_{600\text{auto}}$) was calculated by multiplying the dilution factor ($DF$) by the spectrophotometer reading ($A_{600\text{nm,auto}}$) following equation within the Python$^\circ$ code:

$$OD_{600\text{auto}} = DF \times A_{600\text{nm,auto}}$$ (6)

This cycle of bioreactor sample collection, dilution by mass, turbidity measurement, draining, and washing was repeated three times for triplicate OD$_{600}$ measurements. Completion of the triplicate measurements required ~30 minutes. Software codes driving all of the above electromechanical steps of the device are provided in the supplemental materials.

2.3.6. Evaluating Cell Density Module

After establishing the glycerol feedback control, a *Pichia pastoris* fed-batch culture was performed during which both off-line and automated at-line OD measurements were measured in triplicate. The off line measurements were diluted by volume. The at-line measurements were diluted by mass. The average value of triplicate offline and automated at-line exact mass diluted OD$_{600}$ measurements were used to form a scatter plot relating automated at-line OD$_{600}$ to off-line OD$_{600}$ values. A correlation equation relating offline OD$_{600}$ measurement to automated OD$_{600}$ measurement was derived from this plot. This equation was used to predict offline OD$_{600}$ measurements during the subsequent and independent *Pichia pastoris* fermentation by using the exact mass dilution cell density module. Then the offline OD$_{600}$ measurement and predicted OD$_{600}$ values were compared for validation.
2.4. Results

2.4.1. Glycerol Feedback Control:

For 20.5 hours, the entire duration of glycerol feedback control during glycerol fed-batch phase of *Pichia pastoris* fermentation, the average value of the glycerol concentration was 30.6 g/L with standard deviation value of 0.61 g/L, with a measured minimum value of 29.7 g/L, and a maximum value of 32.2 g/L (Figure 9). During the glycerol control, the biomass measured in OD$_{600}$ increased from 9 to 133, and 6.2 Kg of glycerol fed-batch media was added by the system. This control system was used for maintaining glycerol level at 30g/L in subsequent fermentation runs.

![Figure 9: Performance of glycerol concentration feedback control for *Pichia pastoris* biomass generation during the fed-batch phase. Triangles (▲) represent glycerol](image)

Figure 9: Performance of glycerol concentration feedback control for *Pichia pastoris* biomass generation during the fed-batch phase. Triangles (▲) represent glycerol
concentration (g/L), circles (●) represents the cumulative grams of the fed-batch media added, and rectangles (■) represent cell density measured off-line at OD. Glycerol feedback control started at time 21.8 hours and ended at hour 42.8.

2.4.2. Automated OD vs Offline OD Calibration Curve

As shown in Figure 10, the automated exact mass at-line OD measurements by the cell density module displayed the following polynomial correlation with off-line OD data measurements diluted by volume (Equation 7) in another fed-batch cell culture where \( OD_{600}^{\text{auto}} \) stands for automated OD measurement and \( OD_{600}^{\text{manual}} \) means manual offline OD measurement.

\[
OD_{600}^{\text{auto}} = 36.855 \cdot \sqrt{OD_{600}^{\text{manual}}} - 57.651 \quad \text{with } R^2 = 0.991 \tag{7}
\]

**Figure 10:** (a) Calibration of off-line optical density (OD) and the automated at-line cell density module (OD) in *Pichia pastoris* fermentation. (b) Linearized calibration graph correlating OD and \( \sqrt{OD_{600}^{\text{manual}}} \) which includes the linear fit line for equation 7.

\( OD_{600}^{\text{auto}} \) displayed a non-linear correlation with offline OD above 80 OD (data not shown).
2.4.3. Cell Density Module Performance

The comparison between the off-line OD measurement predicted by the cell density device using equation 7 and the exact mass dilutions at-line OD measurements is shown in Figure 11. Because of the differences between the two dilution methods as culture density increases to high cell concentrations, these two different methods were expected to not agree precisely. When the acceptable range of predicted OD\textsubscript{600} was defined as no more than 15% difference from the measured value, the working range of the cell density module is 72 \geq OD\textsubscript{600} \geq 42. The greatest difference between the predicted and measured OD occurred at OD\textsubscript{600} = 29.4 where the predicted value was 33% less than the measured value.

![Figure 11: Comparison of automated at-line and offline measurement of OD\textsubscript{600}.](image)

Figure 11: Comparison of automated at-line and offline measurement of OD\textsubscript{600}.
2.5. Discussion

2.5.1. Glycerol Feedback Control during Glycerol Fed-Batch Phase

Glycerol consumption rate changes rapidly during the exponential growth phase of *Pichia pastoris*. Fluctuation of glycerol concentration due to lack of adequate glycerol feedback control during the biomass exponential growth can impact cell growth rate, oxygen consumption rate, cell physiology, and the methanol induction scheme, resulting in inconsistent protein product yield and inefficiency in upstream bioprocess development. Our glycerol feedback control system was confirmed to be capable of maintaining glycerol concentration with less than 5 percent deviation from a set point of 30g/L throughout the glycerol fed-batch extended exponential phase. Also, the standard deviation of the glycerol level during the control system run (0.61g/L) was less than 2% of the average glycerol concentration value of 30.6g/L, which also shows a high stability of the system. To the knowledge of the authors, there is a lack of publications specifically reporting on alternative glycerol feedback control systems for pilot-scale fed-batch culture of *Pichia pastoris*. Therefore, this at-line analyzer could be a good model for developing glycerol concentration control system for *Pichia pastoris* fermentations independent of bioreactor scale using a commercially available autosampler, a micro flow through spectrophotometer, and a conventional at-line biochemistry analyzer.

2.5.2 Characterization of Cell Density Module

As expected, the calibration of the exact mass dilution cell density module showed lack of linearity between off-line volumetric dilution and automated at-line OD\textsubscript{600} measurements. The lack of linearity between the manual and automatic OD\textsubscript{600} measurement may also be from cell aggregates forming at high OD\textsubscript{600}, an imperfect sample tube purging method, and
insufficient cleaning cycles for the flow-through spectrophotometer cell between measurements. However, the exact mass cell density module combined with the empirical formula derived from Equation 7 proved sufficiently accurate for OD$_{600}$ monitoring of *Pichia pastoris* cell density from OD$_{600}$ 42 to OD$_{600}$ 72. The likely causes of the nonlinearity between OD$_{600}^{\text{auto}}$ and OD$_{600}^{\text{manual}}$ above OD$_{600}$ 80 are due to volumetric dilution errors, the changing culture density at high OD, cell aggregation (visible in the cell density transfer tubing from the bioreactor at high OD) or probable fouling of the spectrophotometer. *Pichia pastoris* can flocculate during cell culture forming clumps, which was observed during our cell culture runs. At cell density of above 80 OD$_{600}$, highly flocculent cells may resist homogenization by liquid turbulence, which would have created measurement errors by the spectrophotometer. Also, the rinsing of the spectrophotometer with 0.1M sodium acetate solution between measurement runs at very high cell density may not have been sufficient to prevent fouling of the optical sensor. Additional spectrophotometer sanitation steps with 0.1M NaOH and including deflocculant in the 0.1M sodium acetate diluent may greatly expand the optimal OD$_{600}$ measurement range.

At-line measurement of OD$_{600}$ in the range of OD$_{600}$ 42 to 72 during exponential growth phase enables more precisely timed glycerol fed-batch supply to the bioreactor for the purpose of controlling the level of biomass entering the glycerol-limited methanol induction phase. Because the initial biomass concentration upon methanol induction is a significant factor affecting recombinant expression yield, the ability of the cell density module to precisely stop glycerol feeding can be an important aid for product yield improvement (Davies *et al.*, 2010). There are several *in situ* on-line optical sensor systems for measuring high optical density of microbial fermentation, some of which reliably perform up to ~300 OD$_{600}$ (Mettler-Toledo International Inc., 2017; Applikon Biotechnology, 2017; Aquasant Messtechnik AG, 2017; Optek-Danulat Inc., 2017; Wedgewood Analytical, 2017; Cerex Inc., 2017). However, there is no optical sensor that is specifically developed and tested for
Pichia pastoris fed-batch fermentation by the companies, which may be part of the reason for the differences between the cell density module and the alternative devices in the maximal range in OD measurement. Also, compared to other systems, the measurements of the cell density using the apparatus described here will be unaffected by bioreactor conditions such as agitation rates and bubble sizes at the cell concentration of OD\textsubscript{600} 42 to 72. Overall, the device is shown to be a good model system for creating an at-line flow injection analysis device for measuring high cell density utilizing conventional miniature flow through spectrophotometers.

2.6. Conclusion and Outlook

Our study shows a simple exact mass dilution flow through spectrophotometric cell density module that can be used for at-line monitoring of pilot scale cell culture of Pichia pastoris. The polynomial correlation between offline OD\textsubscript{600} and the automated OD\textsubscript{600} device enabled OD\textsubscript{600} determination from OD\textsubscript{600} 42 to OD\textsubscript{600} 72. In addition, supporting systems for Pichia pastoris culture such as control software and glycerol feedback control were confirmed to be suitable for maintaining stable glycerol concentration throughout the glycerol fed-batch phase up to a final OD\textsubscript{600} of 140. Hardware configurations providing greater range of accurate at-line OD\textsubscript{600} determination by mass-based dilution will be investigated further by including spectrophotometer sanitization steps and adding deflocculant in the 0.1M sodium acetate diluent.
2.7. References


CHAPTER 3: Future Work

3.1. Glycerol Level Fed-Batch Control System

The current glycerol feedback control system for the bioreactor has been tested on one set point. Future research will need to be focused on testing and tuning the system at different glycerol set points needed for *Pichia pastoris* cell culture process development. Also, acute or gradual change of glycerol level during glycerol fed-batch phase may be needed for cases like initiating transition into the methanol induction phase. A more accurate measures of changes of glycerol level to better control dissolved glycerol levels will be implemented by developing glycerol feeding algorithms utilizing proportional integral control. Gradual change in glycerol level can be achieved by incremental change of the glycerol set point.

3.2. Automated Cell Density Measurement

Our automated cell density measurement device using exact mass dilution is shown to perform reasonably well at a limited range of OD$_{600}$ 42. It was not expected that this measurement would be linearly related to off-line measurements of OD600 measured by volumetric dilution since a 200-fold dilution is required for the linear region of the spectrophotometers and different spectrophotometers were used for the off-line and at-line measurements. The probable cause of this behavior is due to cell aggregation or probable fouling of the spectrophotometer at high cell density. Future research will focus on increasing measurement range by either adding deflocculant in the diluent to resolve the cell flocculation problem. Also, additional sanitization steps like rinsing the entire liquid circuit of the device by 0.1M NaOH and 70% ethanol can be added to reduce possible spectrophotometer fouling. Based on the additional improvement, a calibration will be
made again by correlating manual OD$_{600}$ to automated OD$_{600}$ measurement, and cell density module performance will be measured again by predicted OD$_{600}$ value and measured OD$_{600}$.

3.3. Testing of the Methanol Control System

For methanol induction of AOX1 promoter and simultaneously avoiding methanol cytotoxicity, methanol concentration should be maintained precisely during the methanol induction phase of the *Pichia pastoris* fermentation. For methanol level monitoring, a dissolved methanol probe was built inexpensively by utilizing a semiconductor sensor for hydrocarbon (model TGS-822, Figaro, Osaka, Japan) combined with an *in situ* steel rod containing methanol permeable silicone tubing was made by previous researcher (Cueva, 2012). In this setup, dissolved methanol diffuses through the permeable tubing wall and air flow through the tube carries methanol gas to the hydrocarbon sensor. Tubing wall thickness, length, gas flow rate, and bioreactor agitation rate were optimized by a previous BIOM researcher (Sigmon, 2014).

Through this thesis project, a feedback control system for maintaining methanol level in the methanol induction phase of *Pichia pastoris* fermentation was developed based on the methanol monitoring system (Figure 12).

![Figure 12: Summary of the methanol feedback control system.](image)
In this system, a LabVIEW system (National Instruments, Austin, TX) first saves the voltage measurement of the dissolved methanol probe into a text file. Then the control program for methanol induction phase within the SCADA software reads the newest data appended to the text file and compares them to the set point submitted by the user through the web page. If the methanol level measurement drops below the set point, the control program sends RS232 serial command to Micro850 PLC which switches on a relay (model 13366, Weidmüller, Richmond, VA) for activation and deactivation of a methanol pump (model 550-0213, Autotrol, Crystal Lake, IL). After methanol pumping, the control program waits for 130 seconds which is the response time of the methanol probe, and checks the change in methanol voltage level. To adjust for rapid change in methanol consumption rate during cell culture, the control program dynamically adjusts the duration of methanol pumping by measuring voltage change resulting from methanol addition. Also, the program records the amount of methanol added by recording the serial data sent from a platform scale (model EB60EDE-L, Sartorius, Göttingen, Germany). Finally, the methanol control program turns off the Seg-Flow / YSI system 3 hours after the initiation of the methanol induction phase to confirm glycerol is removed completely from the fermentation during the methanol growth phase.

When the methanol feed media is developed and optimized for the methanol induction phase of 30L *Pichia pastoris* fermentation in bioreactor, the methanol control system should be tested for maintaining the methanol level in actual fermentation runs. The methanol pumping rate and frequency may need to be adjusted for optimizing the robustness of the control system. Two parallel peristaltic pumps may be needed to accommodate for changing methanol consumption rate during the methanol induction phase. The air flow rate of the methanol probe can be impacted by the air purging step of the cell density sensing device,
impacting the methanol measurement results. A programmatic solution can be implemented by having methanol feedback loop to pause until the gas purging steps complete.

3.4. Measurement of Recombinant Protein Expression

The model organism used for developing the control system was *Pichia pastoris* strain GS115 His+ Mut^S that secretes human serum albumin (HSA) (Invitrogen Co., 2010). For quantifying the HSA concentration in cell culture broth, an Enzyme-Linked Immunosorbent Assay (ELISA) procedure was implemented and tested during the thesis project (Genway, 2017). The next researchers will use this method to measure HSA level in Pichia pastoris cell process development experiments based on the control system.
CHAPTER 4: References


APPENDICES
Appendix A: Assembly of Automatic Cell Density Sensing Module
1. PURPOSE:

1.1. This procedure provides directions on how to create automatic Cell Density Sensing (CDS) device.

2. SCOPE:

2.1. This procedure applies to all Operations staff, faculty, students and other BTEC staff.

3. RESPONSIBILITIES:

3.1. All students or BTEC staff attempting to create CDS device are responsible for following the established procedures.

4. SAFETY:

4.1. Follow all general lab safety procedures
4.2. Follow all safety procedures listed in SOPs related to a material, tools or equipment in question.

5. MATERIALS:

5.1. 20 psi air supply
5.2. SILASTIC® laboratory tubing (Dow Corning part # 508-007 )
5.3. C-Flex® tubing (Cole-Parmer part # 06422-05)
5.4. Rubber tubing with luer locks (Flownamics part # A-T-10 1.6mm ID)
5.5. Low pressure fitting kit (Bio-Rad part # 731-8220)
5.6. T-connector for C-Flex® tube
5.7. Stepped tubing connector up to 5mm (Fisher scientific model # S50700C)
5.8. Two peristaltic pumps (Watson-Marlow model # 323Du)
5.9. One peristaltic pump (Rainin part model # Dynamax RP-1)
<table>
<thead>
<tr>
<th>Item</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.10. 7 electric valves (Takasago Electric Inc part # PK-0802-NC-3)</td>
</tr>
<tr>
<td>5.11. In-line .2 micron hydrophobic PTFE filter (Millipore model # 50 mm Millex)</td>
</tr>
<tr>
<td>5.12. Extra small zip ties pack (e.g. McMaster-Carr part # 7130K101)</td>
</tr>
<tr>
<td>5.13. Regular size zip ties pack (e.g. McMaster-Carr part # 7130K53)</td>
</tr>
<tr>
<td>5.14. Small electronic scale (Ohaus model # AV3102)</td>
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<tr>
<td>5.15. Small cardboard box for the scale</td>
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<td>5.16. Sampling port for BIOSTAT-C bioreactor</td>
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<tr>
<td>5.17. General-purpose laboratory labeling tape (e.g. VWR part # 89098-068)</td>
</tr>
<tr>
<td>5.18. Laboratory stand (Zoro part # G8554183)</td>
</tr>
<tr>
<td>5.19. Two test tube clamp for laboratory stand (Science lab supplies part # 3380-15)</td>
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<tr>
<td>5.20. 250ml conical centrifuge tube (Corning part # 430776)</td>
</tr>
<tr>
<td>5.21. 250ml conical centrifuge tube holder (Fisher scientific # 75008144)</td>
</tr>
<tr>
<td>5.22. 15ml conical tubes (Falcon part # 3521969c91)</td>
</tr>
<tr>
<td>5.23. Super glue (e.g. Henkel model Loctite 430)</td>
</tr>
<tr>
<td>5.24. 2ml plastic serological pipette pack (Corning Inc model # 357507)</td>
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<tr>
<td>5.25. Two 2 liter plastic carboy with volume scale</td>
</tr>
<tr>
<td>5.26. 1.7kg of sodium acetate</td>
</tr>
<tr>
<td>5.27. Large magnetic stirrer and stirring rod for the 20L carboy</td>
</tr>
<tr>
<td>5.28. 2L media storage bottle (Corning model PYREX™ 13952L)</td>
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<tr>
<td>5.29. Tube connector for the 2L media storage bottle</td>
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<tr>
<td>5.30. Bleach (e.g. Clorox bleach)</td>
</tr>
<tr>
<td>5.31. Small air hydrophobic filter 1.25&quot; (BD Biosciences model # 343538T)</td>
</tr>
<tr>
<td>5.32. Two open jaw screw compression clamps (Hatch part # 63301)</td>
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<tr>
<td>5.33. Scissor</td>
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<tr>
<td>5.34. One plastic container</td>
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<tr>
<td>5.35. Arduino Uno (Arduino)</td>
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<tr>
<td>5.36. Large breadboard (Sparkfun part # PRT-12614)</td>
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<tr>
<td>5.37. 50 of breadboard pin header</td>
</tr>
<tr>
<td>5.38. 14 of 330Ω resistors</td>
</tr>
<tr>
<td>5.39. 14 of diode 1N4148</td>
</tr>
<tr>
<td>5.40. 14 of NPN transistor</td>
</tr>
<tr>
<td>5.41. 14 of 5A relay (Sparkfun part # COM-00100 model # JZC-11F)</td>
</tr>
<tr>
<td>5.42. 200 of male-to-male breadboard jumper wires</td>
</tr>
<tr>
<td>5.43. 2 of 9-pin male to female serial cable</td>
</tr>
<tr>
<td>5.44. AC to DC power adapter 24 volt 5 ampere laptop charger</td>
</tr>
<tr>
<td>5.45. 2 of electrical extension with 8 outlets that are horizontally oriented</td>
</tr>
</tbody>
</table>
5.46. 2 of USB to Serial Adapter (Startech part # ICUSB2321F)
5.47. USB Hub (D-Link part # DUB-H7)
5.48. USB cable A to B at least 5 feet
5.49. 2 of USB cable A to A at least 5 feet
5.50. 2 of DB25 male-to-female serial RS232 extension cable
5.51. 2 of DB25 male breakout board
5.52. Wire stripper
5.53. 8 vinyl coated wires, each containing two smaller wires with > 1 watt capacity
5.54. Vinyl electrical tape
5.55. Mini screwdriver set
5.56. Regular screwdriver set
5.57. Iron solder set
5.58. Electrical multimeter
5.59. 3-store stainless laboratory cart (e.g. Nasco model # Z09515U)
5.60. 2 of 14" X 14" X 10" plastic containers with lids
5.61. 5 inch by 5 inch a plastic plate
5.62. Turbidity Flow Cell & Measurement Unit (Pendotech)
  5.62.1. Measurement unit with transmitter operating at 600nm
  5.62.2. Low hold-up absorbance flow cell
  5.62.3. Fiber optics cable
  5.62.4. PressureMAT™ Sensor Monitor (Pendotech)
  5.62.5. 12 Volt 2 ampere power adapter
  5.62.6. 24 Volt 2 ampere power adapter

6. Setting Up the Laboratory Cart

6.1. Set the laboratory car on a flat open space
6.2. Set the 14" X 14" X 10" plastic container on the top of the cart
6.3. Set the second 14" X 14" X 10" plastic container on the second level of the cart
6.4. Put two extension outlets and the USB hub in the container on the second floor
6.5. All of the electronic devices discussed below will connect to the extension outlets after which the plastic container lids will cover the upper opening of the boxes. For this, the plastic covers should be truncated to accommodate the wires.

7. Building Arduino Control System
7.1. Connect power lines of the breadboards to 5V Arduino power supply and to the 24 V adapter. Do not power on Arduino and the adapter.

7.2. In the breadboards, build seven Arduino-controlled relay circuits for the solenoid valves. Breadboard wiring and circuit diagram are shown below. Refer to 7.4 for Arduino pin to valve table for the correct linking.
7.3. In the breadboards, build 4 Arduino-controlled relay circuits for peristaltic pumps. Breadboard wiring and circuit diagram are shown below. Refer to 7.4 for Arduino pin to pump table for the correct linking. Point A and point B should connect to the corresponding pins of the breakout boards connected to the Watson-Marlow peristaltic pumps. The pin information will be discussed later.
7.4. Connect Arduino pins to corresponding relay circuits

<table>
<thead>
<tr>
<th>Hardware controlled by relay circuit</th>
<th>Arduino pin #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent Pump (Watson-Marlow 323du #1)</td>
<td>2</td>
</tr>
</tbody>
</table>
Drain pump (Watson-Marlow 323du #2) | 3
Valve #1 (Ethanol valve) | 4
Sample pump (Rainin RP-1) | 5
Drain pump direction (Watson-Marlow 323du #2) | 6
Valve #2 (Sample port valve) | 7
Valve #3 (Recirculation valve) | 8
Valve #4 (Drain valve) | 9
Sample pump direction (Rainin RP-1) | 10
Valve #5 (Gas purging valve) | 11
Valve #5 (Sample input valve) | 12
Valve #6 (Sample drain valve) | 13

7.5. Connect Arduino to the USB hub
7.6. Connect USB hub to the PC through A-to-B USB cable
7.7. Open the Arduino Software (IDE) and copy and paste Arduino code ‘sketch_jul03a’ to the text field of the software
7.8. Click Tools>Serial Port>COM# corresponding to Arduino. If not clear, unplug and plug back in Arduino to see the appearance of the COM number.
7.9. Click the button for compilation and verification
7.10. Click the upload button
7.11. Put the Arduino + breadboard into the plastic container 14” X 14” X 10” and set them on the top floor of the laboratory cart

8. Setting up peristaltic pump control

8.1. Setting up Watson-Marlow 323du control
8.1.1. Set the both pumps on the top floor of the laboratory cart
8.1.2. Connect to the Watson-Marlow 323du pumps to the extension outlet
8.1.3. Connect the DB25 serial cable to the DB25 female port on the pump
8.1.4. Connect the DB25 male breakout board to the DB25 serial cable
8.1.5. Locate the relay circuits connected to pin #2 or pin #3 of Arduino
8.1.6. Connect pin A in step 7.3 to the 0V ground line of the breadboard
8.1.7. Connect pin B to pin 14 of the breakout board of the corresponding pump
8.1.8. Connect pin 4 of the breakout board to 5V line of the breadboard
8.1.9. For the relay circuit controlling drain pump direction, refer to page 9 of the 323Du pump manual

8.1.10. Press ANA button on the front of the peristaltic pump

8.2. Setting up Rainin RP-1 control

8.2.1. Set the pump on the top floor of the laboratory cart
8.2.2. Connect the RP-1 pump to the extension outlet
8.2.3. Connect pin A and pin B of the relay circuit for sample pump direction control to pin # 5 and pin #6 of the analog control port of the pump
8.2.4. Connect pin A and pin B of the sample pump switch relay circuit to pin # 5 and pin #6 of the analog control port of the pump

8.2.5. By using the control panel of RP-1 at the front side, increase the pump speed to max

9. Setting up solenoid valve circuits
9.1. On the 5 inch by 5 inch a plastic plate, use soldering iron to create three evenly spaced holes (approximately 3 inches) and one bigger hole
9.2. Mount three valves on the plate using valve bolts through the smaller holes
9.3. Use the bigger hole to hang the setup on the sample port
9.4. Set four valves on the top floor of the laboratory cart
9.5. Strip a vinyl wire to expose two smaller wires and connect each wire to the wires of the electrical valves. Repeat this to all valves
9.6. Use electrical tape to wrap the wire-to-wire contact
9.7. Solder a single pin header to the end of the wires connected to the valves
9.8. Connect the wires to the relay circuits made in step #2, remembering the relay-Arduino pin correspondence

10. Setting up connectivity of the scale

10.1. Set the Ohaus scale on the top floor of the laboratory cart
10.2. Plug in the 9-pin male to female serial cable to the scale
10.3. Connect the USB to Serial adapter to the serial cable
10.4. Connect the A to A USB cable to the adapter
10.5. Connect the USB cable to the USB hub

11. Setting up the spectrophotometer

11.1. While caring not to fold the fiber optics cable, connect the flow cell to the fiber optics cable
11.2. Attach the flowcell to a piece of cardboard to enable vertical liquid flow
11.3. Plug in the fiber optics cable to the absorbance transmitter unit
11.4. Connect the transmitter unit to the PressureMAT™ sensor monitor
11.5. Put all of the spectrophotometer setup on the second floor of the cart
11.6. Connect in the 9-pin male to female serial cable to the sensor monitor
11.7. Connect the USB to Serial adapter to the serial cable
11.8. Connect the A to A USB cable to the adapter
11.9. Connect the USB cable to the USB hub

12. Setting up sample collection system

12.1. Setting up sampling port circuit

12.1.1. Install one 4 inch of C-Flex tubing through each of valve #2, #1, and #5.
12.1.2. Install in-line Millipore model # 50 mm Millex to the 15 Psi gas supply tube
12.1.3. Connect 5 inch of C-Flex Clear Tubing (1/8"ID x 1/4"OD) to the other end of the filter
12.1.4. Using piece 731-8223 and 731-8225 of the low pressure fitting kit, connect C-Flex clear tubing to SILASTIC® laboratory tubing (Dow Corning part # 508-007 )
12.1.5. Insert the SILASTIC® laboratory tubing through the C-Flex clear tubing installed in the valve #5.
12.1.6. Connect another SILASTIC® laboratory tubing to the 70% ethanol supply and have it pass through the C-Flex tubing installed in valve # 1.

12.1.7. Connect a SILASTIC® laboratory tubing to the bioreactor sampling port and have it pass through the C-Flex tubing installed in valve # 2. The length of this tubing should be as short as possible.

12.1.8. Wrap a C-Flex tube around the connecting point between the SILASTIC® laboratory tubing and bioreactor sampling port and fasten the C-Flex wrapper with a mini zip tie.

12.1.9. Using the piece 732-8302 of low pressure fitting kit, connect the SILASTIC® laboratory tubing for gas supply, ethanol supply, and sampling port into a single circuit as shown in the figure below.

12.2. Setting up RP-1 peristaltic sampling pump

12.2.1. Connect the SILASTIC® laboratory tubing from the sampling circuit to the piece 732-8300 of low pressure fitting kit.
<table>
<thead>
<tr>
<th><strong>BTEC, NC STATE UNIVERSITY</strong></th>
<th><strong>DOCUMENT TYPE</strong></th>
<th><strong>SOP</strong></th>
<th><strong>Document Number:</strong></th>
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<td><strong>Title:</strong> Assembly of Automatic Cell Density Sensing Module</td>
<td><strong>Revision:</strong></td>
<td>1</td>
<td><strong>Effective Date:</strong></td>
<td>5/31/17</td>
</tr>
</tbody>
</table>

12.2.2. Connect the piece 732-8300 to Flownamics PharMed rubber tubing. For joining, use the superglue.

12.2.3. Wrap a side-truncated 0.5 inch piece of the C-Flex tube around connection region between Flownamics PharMed rubber tubing and the SILASTIC® laboratory tubing.

12.2.4. Mount a mini zip tie at the C-Flex tube piece. The precise location of the C-Flex tube piece for mounting the zip tie should be the region where the SILASTIC® laboratory tubing touches the 732-8300 connector.

12.2.5. Using the zip tie, very strongly tighten the C-Flex tubing with the zip tie. This setup will function as an anchor preventing the rubber tubing from moving with the direction of the peristaltic rotor.

12.2.6. Connect the rubber tube back into 2 feet of SILASTIC® laboratory tubing through the piece 732-8300 of low pressure fitting kit and superglue.

12.2.7. Cut 5 inch piece of C-Flex tubing and another 1 inch piece of C-Flex tubing. Wrap the side-truncated 1 inch tube around one end of the 5 inch piece. Use superglue and mini zip tie to firmly attach them.

12.2.8. Install the 5 inch piece of C-Flex tubing onto RP-1 peristaltic pump as shown in the figure below and check if the rollers in the peristaltic pump are forced rolling. This reduces friction applied on the PharMed tube increasing tube life span.

12.2.9. Install the rubber tube on the RP-1 peristaltic pump. Make sure the zip tie and C-Flex tube piece goes behind the black clump, as shown in the figure below.
12.2.10. Set the direction of the pump rotation to be clockwise.

12.3. Setting up the final end of the sample delivery circuit

12.3.1. Connect the SILASTIC® laboratory tubing from RP-1 pump to the T-connector (piece 732-8302 of low pressure fitting kit).

12.3.2. Connect two separate 10 inch SILASTIC® laboratory tubing to the T-connector.

12.3.3. Install 5 inch of C-Flex tube pieces through valve #5 and valve #6.

12.3.4. Have the SILASTIC® laboratory tubing to pass through the C-Flex tube on the vales in configuration as shown below. The distance between the T-connector and the valves should be as short as possible. Also, the tube
path leading from RP-1 to the dilution bottle should be short and set horizontally.

12.3.5. Prepare the waste bottle and 100ml of bleach solution and connect the SILASTIC® laboratory tubing from valve 5 to the bottle.

13. Setting up the sample dilution and drain system

13.1. Dilution bottle setup

13.1.1. Set an open-headed cardboard box around the Ohaus scale
13.1.2. Set a 250ml conical centrifuge tube holder on the scale
13.1.3. Using a soldering iron, cut the top of the 250ml conical centrifuge bottle
13.1.4. Set the open-headed centrifuge bottle inside the tube holder
13.1.5. Set a laboratory stand right next to the scale
13.1.6. Set two clamps on the laboratory stand
13.1.7. Make three shortened 2ml plastic serological pipettes, relative size of which is shown in figure A. Tube 1 is for injecting liquids from the recirculation circuit, tube 2 is for fresh diluent delivery, and tube 3 is for draining liquid from the bottle for liquid recirculation or sample disposal.
13.1.8. Tightly hold the pipet tips together by using laboratory labeling tape, maintaining the height differences relative to the bottom.
13.1.9. Put the connected pipet pieces into the two clamps and tighten the clamps.

13.1.10. Connect 2 feet C-Flex tube to tip 1, connect 2 feet C-Flex tube to tip 2, and connect 3 feet C-Flex tube to tip 3, as shown in figure B.

13.1.11. By adjusting the height of the clamps from the bottom, adjust the distance from the bottom tip of pipet 3 to the bottom of the bottle to be ¼ inch. After this, strongly tighten the grip of the clamp on the lab stand. Care should be taken to see if tip #3 touches the wall of the dilution bottle, impacting the reading of the scale.

13.1.12. Tare the scale

---

13.2. Preparing sample diluent

13.2.1. Set a 20L carboy on a large magnetic stirrer

13.2.2. Put 5L distilled water and put a stirring rod

13.2.3. Start stirring

13.2.4. Put in 1.64 kg of sodium acetate

13.2.5. Wait at least 10 minutes to completely dissolve the salts
13.2.6. Turn off stirring to confirm no remaining salts in Carboy
13.2.7. Fill carboy until 20L scale
13.2.8. Stir 5 more minutes and remove the stirring rod

13.3. Preparing sample dilution and drain circuit

13.3.1. Put a 7 inch C-Flex tubing on the pump head of the Watson-Marlow 323du pumps.
13.3.2. Connect both ends of the tube piece with two stepped tubing connector
13.3.3. Fasten zip tie on the both side of the C-Flex tubing enough to hold the tubing piece at place while the pump runs
13.3.4. Use pen marking to check the change of C-Flex tubing position after 2 minutes of continuous running. If C-Flex moves with pumping, tighten the zip tie more.
13.3.5. Tubing piece should be replaced daily during operation.

13.3.6. Connect the C-Flex tube attached to tip #1 prepared in step 14.1.11 through valve #3 and to T-connector. Tightly zip-tie the tube to the T-connector.
13.3.7. C-Flex tube connected to tip #2 connects to one end of the drain pump (323du pump #1). Connect the other end of the pump with 5 feet C-Flex.

13.3.8. Attach an anchor to the other end of the tube to keep the tube submerged to the bottom of the carboy.

13.3.9. Connect the C-Flex tube attached to tip #3 to one end of the recirculation/drain pump (323du pump #2).

13.3.10. Connect a 1 feet C-Flex tube to the other end of the pump #2 and connect to the T-connector.

13.3.11. Connect a 6 feet C-Flex tube to the remaining end of the T-Connector, and have the tube pass through valve # 4.

13.3.12. Attach an anchor to the other end of the tube to keep the tube inside the carboy containing at least 500ml bleach.

13.3.13. Confirm the direction of rotation of the 323du pumps is correct. Press the rotation direction switch button accordingly, and re-confirm ANA button is pressed.
14. References

14.1. Oomlout ARDX CIRC11: Relays
Appendix B: Assembly of Glycerol Fed-Batch Control System and Methanol Control System
1. PURPOSE:

1.1. This procedure provides directions on how to create glycerol Fed-Batch control system and methanol control for 30L scale *Pichia pastoris* fermentation.

2. SCOPE:

2.1. This procedure applies to all staff, faculty, and students of BTEC.

3. RESPONSIBILITIES:

3.1. All students or BTEC staff attempting to build glycerol fed-batch control system culturing *Pichia pastoris* in the 30L BIOSTAT-C bioreactor of lab 218 are responsible for following the established procedures.

4. SAFETY:

4.1. Follow all general lab safety procedures
4.2. Follow all safety procedures listed in SOPs related to a material, tools or equipment in question.

5. MATERIALS:

5.1. SILASTIC® laboratory tubing (Dow Corning part # 508-007 )
5.2. Platform scale (Sartorius EB60EDE-L)
5.3. Peristaltic pump (Watson-Marlow 520Du)
5.4. 2 of DB25 male breakout board (mdfly.com model # MOL-DB2501)
5.5. 2 of DB25 male to female serial cable
5.6. 1 of vinyl coated wire, each containing two smaller wires with > 1 watt capacity
5.7. 1 of A-to-B USB cable
5.8. 1 of female DB9 to 8-Pin Mini-DIN cable
5.9. USB Hub (D-Link part # DUB-H7)
5.10. 2 of A-to-A USB cable
5.11. Micro850 Programmable Logic Controller (Allen Bradley model # 2080-LC50-24QBB)
5.12. Peristaltic pump (Autotrol model # 550-0213)
5.13. Electronic protector (Eaton model # WMZS1C02)
5.14. Relay module (Weidmuller model # 13366)
5.15. Power outlet (Phoenix contact # 5600462)
5.16. 120V power cord
5.17. 30 feet 120V power cord
5.18. Insulated white 16 gauge stranded wire
5.19. Insulated black 16 gauge stranded wire
5.20. Insulated black 16 gauge solid copper wire
5.21. 1 male to female DB9 serial cable
5.22. USB to Serial Adapter (Startech part # ICUSB2321F)
5.23. PLC power contact terminal
5.24. Mini screwdriver set
5.25. Wire stripper
5.26. Stirring plate
5.27. 1 capacitor (0.68 µf)
5.28. 800 point solderable breadboard
5.29. Soldering iron
5.30. Electrical multimeter
5.31. Large stirring plate
5.32. Wall adapter power supply 5V DC (e.g. Sparkfun model # TOL-08269)
5.33. Connected Components Workbench Software (Version 6.01.00, Standard Edition)

6. Making glycerol fed batch media supply and methanol supply.
   6.1. For making glycerol fed-batch media and connecting into BIOSTAT-C bioreactor, follow Appendix B and Append E of the thesis of Jamie Sigmon (Sigmon, 2013). During the process, additionally, connect a 5L bottle to the glycerol fed batch media tube at the midpoint between the Watson Marlow 520Du pump and the tri-punch through a T splitter. The tube and the bottle should be prepared and autoclaved with the rest of the system using the same methods in the protocol. Additionally, in the glycerol feed bottle, there should be a large stir bar.
   6.2. Zip-tie the tubes to the T-splitter.
   6.3. In a sterile biosafety hood, add 3L of filter-sterilized methanol to the 5L bottle.
6.4. Add 21ml of filter-sterilized PMT4 to the methanol bottle and mix thoroughly by shaking the bottle. Connect the liquid circuit to the bioreactor following the SOP.

7. Setting up the platform scale

7.1. Set the Satorius platform scale on a flat surface
7.2. Set the large stirring plate on the scale
7.3. Set the glycerol fed-batch media bottle on top of the scale
7.4. Turn on the stirring plate to keep the fed-batch media agitated and homogenized
7.5. Connect the DB25 serial cable to the scale
7.6. Connect the cable to DB25 breakout board
7.7. Connect the DB9 cable to the serial to USB to Serial Adapter (Startech part # ICUSB2321F)
7.8. Strip the other end of the DB9 cable, exposing color-coded smaller wires.
7.9. Connect individual wire to the breakout board connected to the scale following the EB60EDE-L manual.

8. Setting up Programmable Logic Controller

8.1. Mount the PLC, relay, electric protector, power outlet, power contact terminal device on a rail installed on a wall.

8.2. Strip a 120V power cord and install its black, white, and green wire on the power inputs of the PLC power supply. Strongly tighten the PLC pin using a screwdriver. Do not power on the PLC yet.
8.3. Connect +24V end to one set of power connector and connect ground to the other sets of power connector.

8.4. Strip the wire of the 5V wall adapter power supply exposing 5V positive end and ground. Use digital multimeter to confirm the polarity of the wires.

8.5. Connect the +5V end to +CM1 and 5V ground to –CM1 pin of the output terminal block of the PLC. Strongly tighten the pin using the mini screwdriver.

8.6. Strip the both ends of vinyl coated wire, exposing two wires inside.

8.7. Connect one wire to O-09 pin and one wire to –CM1 pin. Strongly tighten the pin using a mini screwdriver.

8.8. Strip the wire of the 24V wall adapter power supply exposing 24V positive end and ground. Use digital multimeter to confirm the polarity of the wires.

8.9. Connect the +24V end to +CM0 and 24V ground to –CM0 pin of the output terminal block of the PLC. Strongly tighten the pin using mini screwdriver.

8.10. Connect 16 gauge white wire to the O-01 pin of CM0 output block and connect 16 gauge black wire to the –CM0 pin of CM0 output block.

8.11. Connect the DB9 to 8-Pin Mini-DIN cable to the serial port of the PLC, and plug in the other end of the cable directly into the DB9 serial port of the PC.

8.12. Connect the A-to-B USB cable to the USB port of the PLC, and plug in the other end of the cable to the USB hub.
8.13. Connect the USB hub to the PC through A-to-A USB cable.
8.14. Power on the PLC by plugging in the 120V cord connected to the PLC power supply.
8.15. Open the Connected Components Workbench software.
8.16. Go to Device Toolbox panel > Catalog > Controllers and choose 2080-LC50-24QBB.
8.17. Load the ladder logic program Project34_Copy_1
8.18. Right-click on the program icon and choose Build
8.19. Now go to the Device Toolbox panel and click Discover button
8.20. Within the Connection Browser, open the USB section.
8.21. Check the 2080-LC50-24QBB listed in the USB section
8.22. Click 2080-LC50-24QBB and press OK
8.23. In the Project Organizer window, right-click Micro850 controller, and click Upload
8.24. Confirm the software upload in the output window

9. Setting up peristaltic pump for glycerol fed batch (Watson-Marlow 520Du)

9.1. Connect a DB25 cable to the pump
9.2. Connect the other end of the cable to DB25 breakout board
9.3. Connect the wire connected to pin O-9 of the PLC to pin #4 of the breakout board, and connect the wire connected to pin –CM1 of the PLC to pin # of the breakout board
9.4. Install the glycerol fed-batch supply tube to the pump
9.5. Adjust the direction of the pump accordingly by pressing the rotation switch

10. Setting Segflow pump sampling system

10.1. Follow the ‘SegFlow 4800 Automated Online Sampling System User Guide’ provided by Flownamics®
10.2. Make sure the SegFlow 4800 is connected to the Internet by connecting the Ethernet cable into the networking port
   10.2.1. To setup internet connectivity, follow ‘FlowWeb™ Software Administrator Guide’ provided by Flownamics®
   10.2.2. Make sure the IP address is accessible only through on-campus network. For this, inquire network administrator
11. Setting up YSI integration with SegFlow pump

11.1. Follow the ‘SegFlow 4800 User Guide Supplement: YSI Biochemistry Analyzer Integration’ provided by Flownamics®

12. Setting up methanol control system

12.1. Setting up PLC control of methanol peristaltic pump

12.1.1. Turn off PLC.

12.1.2. Using 16 gauge wires, connect the +24V power contact terminal to +CM0 pin and 24V ground power contact terminal to –CM0 pin of the output terminal block of the PLC. Strongly tighten the pin using mini screwdriver.

12.1.3. Connect 16 gauge white wire to the O-01 pin of CM0 output block and connect 16 gauge black wire to the –CM0 pin of CM0 output block.

12.1.4. Strip a 120V power cord exposing green, white, and black wire.
12.1.5. Connect power outlet, power protector, power cord, 24V relay, and PLC following the wiring diagram below. All of the components should already be mounted on a rail on a wall before working. Strongly tighten all pins with a screwdriver.

12.2. Setting up a peristaltic pump for methanol delivery

12.2.1. On a breadboard, build the following electrical circuit incorporating a 0.68 µf capacitor. Through the same row in a breadboard, the black wire connected to the Autotrol pump should connect to both the capacitor and +120V AC from the power outlet controlled by the PLC. In the same way, -120V AC from the outlet should connect to both of the white wires on the pump.
12.2.2. Mount the circuit and the pump on an electrical box, and connect the methanol circuit through the pump head.

12.3. Building the methanol probe

12.3.1. Follow Appendix D of the thesis of Jamie Sigmon (Sigmon, 2013) to build methanol probe. Additional details for performance improvement are:

12.3.1.1. Air flow in the probe, instead of nitrogen gas flow, results in higher stability in voltage reading.

12.3.1.2. Not all surface between the two steel ports should be covered with silicone tube: there should be a half inch distance between the steel port and the silicone tubing. Silicone tube extends after sterilization and cell culture.

12.3.1.3. To maximize adhesion between the silicone tube and the steel port, apply superglue at the tip of the silicone tube. Also, wrap 1/3 inch of side-cut C-Flex® tubing around the contact point between the silicone tube and the steel port and fasten it with a mini zip tie around the setup. Finally, to make sure the probe
passes through the bioreactor headplate, rotate the head of the zip tie around the tube to the surface of the steel rod.

13. References
   13.1. Autotrol 550-0213 electrical wiring diagram
        http://www.autotrol.com/Products/Permanent-Magnet-Synchronous-AC-Gear-Motors/Model-550-AC-SYNCHRONOUS-ELECTRICALLY-REVERSIBLE-MOTOR
Appendix C: Launching and Initiating SCADA Server for Cell Density Sensor and Glycerol Fed-Batch Control
1. PURPOSE:

1.1. This procedure provides directions on how to setup the software system for the automated cell density measurement and glycerol feedback control system for 30L scale cell culture of *Pichia pastoris*.

2. SCOPE:

2.1. This procedure applies to all staff, faculty, and students of BTEC.

3. RESPONSIBILITIES:

3.1. All students or BTEC staffs attempting to install and run the SCADA server for *Pichia pastoris* fermentation automation in the 30L BIOSTAT-C bioreactor of lab 218.

4. SAFETY:

4.1. Follow all general lab safety procedures
4.2. Follow all safety procedures listed in SOPs related to a material, tools or equipment in question.

5. MATERIALS:

5.1. PC with Windows 7

6. Obtaining the control server

6.1. Obtain the software in the Pichia_Control_System.zip file from web addresses provided at Appendix G. Follow the instructions in the ‘Notes.docx’ file to initiate the control server. If using the server already installed in the desktop of lab 218 in BTEC, follow up to step 6.7 to initiate the server.
6.2. Open the SCADA server folder in the C:\Users\BTEC\Desktop\Python\mysite
6.3. Launch SCADA user interface by double clicking the SCADA_Panel.bat file
6.4. Open the Command Prompt program from the Start menu
6.5. Within the Command Prompt type and enter the following:
   cd C:\Users\BTEC\Desktop\Python\mysite
6.6. Launch SCADA server by typing and enter the following:
   python SCADA_Server.py
6.7. Next, open Chrome web browser and enter:
   http://127.0.0.1:8000/SCADA/

7. Obtaining configurations for hardware networking

7.1. Connect USB hubs to the PC
7.2. Open the Command Prompt program
7.3. Type in the followings and record the output:
   python -m serial.tools.list_ports
7.4. Connect a single hardware to the USB hub through type A to type A USB cable
7.5. Type in the followings and record the output:
   python -m serial.tools.list_ports
7.6. The additional entry appearing in the output is the communication configuration for the device. Record the value.
7.7. Repeat this procedure to all of the devices that will be controlled by the SCADA server. PLC will be assigned to COM1 always, and no need to perform this procedure.

8. Initiating networking with hardware

8.1. Open the web browser interface to the SCADA server
8.2. Navigate to the Hardware Setup section of the panel
8.3. Enter in the port numbers obtained in the previous steps into the input boxes. Port number is the integer number following ‘COM.’ For example, port number for ‘COM8’ is 8.

8.4. Use default values for Segflow URL and Labview output file directory

8.5. Click the Submit button

8.6. In the Submit Output window, check for the connection status of the network initiation

8.7. If error report occurs, read the outputs in the Command Prompt of the SCADA control system. Troubleshooting methods follow

8.7.1. Check if the correct port # is entered.
8.7.2. Troubleshoot using the outputs of Submit Output panel
8.7.3. Troubleshoot using the outputs of the command window for the SCADA_Server.py program
8.7.4. Navigate to Start, search for ‘device manager’, find the problematic port in Ports section, and right click the icon for disabling and enabling.
8.7.5. Press Ctrl + Alt + Delete key to open task manager and disable all Python processes. In this case, server initiation has to be performed in this case.
8.7.6. Turn on/off computer. Server initiation has to be performed in this case.
9. Testing networking with hardware

9.1. Navigate to the ‘Hardware Control’ section.

9.2. Click the on/off switch or enter pumping duration for one hardware to be tested, and click the submit button. For cell density module, note that the entire sequence of electromechanical steps will be performed. Also, testing should be performed for one hardware at a time.

9.3. Check the result of networking with the hardware on the Submit Output section. For pumping, actual activation of the peristaltic pumps should be observed.

10. Setting up Autohotkey control of the Biostat-C Bireactor

10.1. Open MFCS 3.0 software. Keep the MFCS Shell in the desktop throughout the cell culture run.

10.2. Open Group Display window titled ‘Group 18’, which shows control panel for adjusting pH, pO%, and temperature. Keep the window opened throughout the cell culture run.

10.3. On the digital control unit of the bioreactor, press the Remote button to initiate real-time data transfer.

10.4. Confirm the interaction between the PC and the bioreactor by the real time update of the values in the pH, pO%, and temperature within the Group Display window.

10.5. Keep the Group Display open
11. Configuring cell culture process parameters

11.1. Navigate to the process parameter input fields.

11.2. Input process parameters for each control system, and press the submit button that the input field belongs to. The explanation of the process parameters are explained in the table below

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<tr>
<th>Fed Batch Control Process Parameter</th>
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<td><strong>Gain</strong></td>
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<td><strong>Wait after Start</strong></td>
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<td><strong>Set point</strong></td>
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<td><strong>Fed-Batch Gly. Concentration</strong></td>
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<td><strong>Pumping Duration (Sec)</strong></td>
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<tr>
<td>Parameter</td>
</tr>
<tr>
<td>----------------------------</td>
</tr>
<tr>
<td>Wait after depletion (hrs)</td>
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<td>Set point (V)</td>
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**MFCS Control**

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<th>Description</th>
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<td>Perform?</td>
<td>Perform dynamic adjustment of the BIOSTAT-C process parameters</td>
</tr>
<tr>
<td>Parameter</td>
<td>BIOSTAT-C process parameters to be controlled, pH, pO2, and temperature can be controlled</td>
</tr>
<tr>
<td>Set point</td>
<td>Set point for the selected parameter</td>
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</tbody>
</table>

11.3. All of the parameters inputted to the system, including communication configuration, are listed in the Current Control Parameters section of the panel.

12. **Initiating cell culture and real-time process report**

12.1. After completing setup for glycerol feedback control, methanol feedback control, cell density sensor module, SCADA server setup, navigate to the Master Control section.

12.2. Click the Run button and press the submit button. Check the initiation status of the Run Status.

12.3. Real time update of process status and process parameters are done through the command prompt output of the SCADA server, Status section, and Process Parameters section of the web browser interface.
12.4. To enable real-time plotting of process parameters, click the plot button of the Process Parameters section.
Appendix D: ELISA for Human Serum Albumin (HSA) Quantification.
1. **PURPOSE:**
   1.1. Protocol to quantify extracellular Human Serum Albumin concentration.

2. **SCOPE:**
   2.1. N/A

3. **DEFINITIONS:**
   3.1. N/A

4. **RESPONSIBILITIES:**
   4.1. N/A

5. **SAFETY:**
   5.1. Use corresponding security measures when working with sulfuric acid

6. **MATERIALS:**
   6.1. 300µl multichannel pipette
   6.2. 50ml reagent reservoir for multichannel pipette
   6.3. 1-step ultra TMB-elisa (Fridge 4°C)
   6.4. Non-woven wipers
   6.5. Normal 96 wells plates
   6.6. Coating antibody (Catalog # 15-288-10067F) Affinity purified chicken IgY against HSA
   6.7. Protein Calibrator (Catalog # 10-288-10067F) Purified HSA antigen
   6.8. Detection Antibody (Catalog # 27-288-10067F) Affinity purified Chicken IgY against Human HSA- Horseradish Peroxidase (HRP) Conjugate
   6.9. Costar 96 well EIA/RIA high binding plate (Cornig, Inc. Cat # 3590)
   6.10. Sulfuric acid 4.0N
   6.11. Sodium Bicarbonate
   6.12. Tris Hydroxymethyl Aminomethanol Hydrochloride
   6.13. Tris Crystallized free base
   6.15. Tween 20
   6.16. BSA
   6.17. **Coating Buffer:** 0.05M Carbonate-Bicarbonate, pH 9.6
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<th><strong>SOP</strong></th>
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<td><strong>Revision:</strong></td>
<td><strong>KC-4</strong></td>
<td><strong>Effective Date:</strong> 03-27-15</td>
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</tbody>
</table>

6.18. **Wash Solution:** 0.05% Tween 20 in PBS, pH 7.4 (Pouches)
6.19. **Blocking Solution:** 50mM Tris, 0.14M NaCl, 1% BSA, pH 8.0
6.20. **Sample/Conjugate Diluents:** 50mM Tris, 0.14M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0
6.21. Microplate Reader ELx808 from BIO-TEK INSTRUMENT, INC.

7. **PROCEDURE:**

7.1. **Coating**

7.1.1. Take the microplate wells of high-binding capacity that you are going to use. You can tag them if you are going to use more than one.
7.1.2. Select the number of wells that you are going to use. Keep in mind that the standards (8) are usually used in triplicated (3x8=24). Also remember to use blanks.
7.1.3. Dilute capture antibody to a concentration of 1.8 ug/ml in Coating Buffer
7.1.4. With the multichannel pipette add 100ul of coating antibody dilution in each well.
7.1.5. Cover the microplate with 96-well plate sealing tape and incubate it at room temperature for an hour.
7.1.5.1. When there are 10 minutes left, fill, in the sterile cabinet, a vial with the volume that you are going to use of blocking buffer (Stored at 4°C) during the next step (Blocking). Keep in mind that each well will be filled with 200ul.
7.1.6. When the incubation is finished, empty the microplate in a bucket, and hit it against some nonwoven wipers to dry them. Then, to rinse them, fill the wells two times with 175ul (350ul in total), empty them in the bucket and the plate again against the wipers. Rinse them in this way three times.

7.2. **Blocking**

7.2.1. Add 200ul of blocking buffer to each well.
7.2.2. Cover the microplate with 96-well plate sealing tape and incubate it at room temperature for an hour.
7.2.2.1. While waiting, prepare the 8 standards: Dilute the standards (calibrator) in Sample Diluent to a concentration of 3000 ng/ml, then do a 1/3 serial dilutions down to 4.11 ng/ml, the last standard is the blank.
7.2.2.2. Dilute the samples in Sample Diluent. Use a non-coated 96-well plate to dilute and mix. Dilute them up to a volume of 200µl.

7.2.3. When the incubation is finished, empty the microplate in a bucket, and hit it against some nonwoven wipers to dry them. Then, to rinse them, fill the wells two times with 175ul (350ul in total), empty them in the bucket and it the plate again against the wipers. Rinse them in this way three times.

7.3. Standards and Samples
7.3.1. Transfer 100ul of each standard or sample to the assigned well.
7.3.2. Cover the microplate with 96-well plate sealing tape and incubate it at room temperature for an hour.
7.3.2.1. 10-15 minutes before the incubation ends, dilute the HRP conjugate in dilution buffer to a concentration of 200ng/ml
7.3.3. When the incubation is finished, empty the microplate in a bucket, and hit it against some nonwoven wipers to dry them. Then, to rinse them, fill the wells two times with 175ul (350ul in total), empty them in the bucket and it the plate again against the wipers. Rinse them in this way five times.

7.4. HRP Detection Antibody
7.4.1. Transfer 100ul of HRP conjugate 200ng/ml dilution to each well.
7.4.2. Cover the microplate with 96-well plate sealing tape and incubate it at room temperature for an hour.
7.4.3. When the incubation is finished, empty the microplate in a bucket, and hit it against some nonwoven wipers to dry them. Then, to rinse them, fill the wells two times with 175ul (350ul in total), empty them in the bucket and it the plate again against the wipers. Rinse them in this way five times.

7.5. Substrate Reaction
7.5.1. Transfer 100ul of 1-step ultra TMB-Elisa at room temperature to each well.
7.5.2. Incubate the plate for 5-25 minutes (Until the color blue is clear)
7.5.3. Stop the reaction with 100ul of 2.0N Sulfuric acid.

7.6. Plate Reading a Analysis
7.6.1. Analyze the plate using the microplate reader ELx808 from BIO-TEK INSTRUMENT, INC. at lab 110.
7.6.2. Open the program Gen5 1.11 and select the protocol “Elisa_450.prt”.
7.6.3. Select File > New experiment > Elisa_450.prt > Read plate> Read > Ok.
7.6.4. Save the data in an excel file.
7.6.5. Average the results of standards, control and triplicates.
7.6.6. Subtract the zero value from every value.
7.6.7. Create a standard curve using a five-parameter logistic and use it to interpolate the values.

8. REFERENCES:
8.1. Human Serum Albumin (HSA) ELISA Quantitation Kit Manual Catalog number: 40-288-10067F
Appendix E: Overview of the SCADA Software
Figure 13: Overview of the SCADA software. The control software consists of two parts: Django web server and PAT control server. Django web server is an interface between the user and the PAT control system. First, a user submits an input related to fermentation control through a web page. The set point changes are collected by the Django web server which relays the information to the PAT control server. The bioprocess change takes effect by controlling the bioprocess device that enables the set point change. The PAT control server performs real-time plotting of multiple process parameters which are visualized on the web page by the web server. Most of the source codes of PAT control server is run as text files under ‘exec’ Python command, which enables flexible source code change while the PAT control program runs. The Django web server can be controlled remotely over the Internet, if a static IP address, gateway, and subnet mask are assigned to the server. The PAT server controls another SCADA software (MFCS/win) that runs the BIOSTAT C-DCU bioreactor, enabling dynamic control of the bioreactor. The SCADA software is built on open source programs.
Communication_setup.py applies communication protocols like serial communication and Ethernet to perform real-time monitoring and control of diverse bioprocess devices. The control program sends commands in RS232 serial data to PLC and Arduino that activate/deactivate devices through analog voltage signals. Similarly, serial communication was used to receive readings from electronic scales and spectrophotometer. pySerial was used for serial communication. To control and monitor bioprocess parameters of the BIOSTAT C-DCU bioreactor unit, MFCS_COMM directs a software called AutoHotkey which utilizes macro to control the MFCS/win which is a control software for the BIOSTAT C-DCU bioreactor system.

**Figure 14:** Structure of the SCADA software and the function of the components. Blue boxes are programs of the PAT control server, yellow boxes are the user interface to the PAT control server, and violet hexagons are bioprocess devices that are controlled by the software.
Figure 15: Bioprocess control systems managed by the SCADA software and their activation sequence. The SCADA programs and the bioprocess devices are grouped by for which *Pichia pastoris* fermentation step they are for: glycerol fed-batch control, methanol control, cell density monitoring, and BIOSTAT C-DCU control. Each bioprocess control system is activated in cascade as *Pichia pastoris* fermentation progresses: 1) BIOSTAT C-DCU control automation, 2) cell density sensing, 3) glycerol fed-batch control, and 4) methanol level control. As an example, color-coded activity schedules of the control systems are matched to typical fermentation steps of an industrial upstream process (Cos et al., 2006).
Figure 16: An excerpt from a bioprocess log file generated by the SCADA software. Each line includes time stamps and a set of lines include source of the event data. This section contains the details of one automated measurement of the cell density sensing device. This reports the precise amount of bioreactor sample added into the dilution bottle to be 0.5 gram and the dilution factor of the sample dilution to be 250.84.
Appendix F: Overview of the Glycerol Fed-Batch Control System
Figure 17: Overview of automated glycerol fed-batch control system. (1) Satorius platform scale EB60EDE-L, (2) control PC, (3) glycerol fed-batch media, (4) cell density sensing device, (5) 30L BIOSTAT C bioreactor, (6) digital control unit for the bioreactor, (7) Seg-Flow bioreactor sampling system, (8) YSI MBS 7100 biochemistry analyzer, (9) Micro850 programmable logic controller, (10) Watson-Marlow 520Du peristaltic pump, (11) Julabo chiller, (12) sampling line for the Seg-Flow system, (13) Ethernet cable for the Seg-Flow web server, (14) RS232 cable connecting PC to PLC, (15) methanol media, and (16) Autotrol peristaltic pump 550-0213 for methanol delivery. A missing item in the figure is a stirring plate which is set between the scale and the glycerol media bottle to continuously mix the media. The glycerol fed-batch control algorithm (Equation 4) requires the concentration of glycerol, mass of the cell culture broth in the bioreactor, and mass of the glycerol added in the previous feedback cycle. Cell culture broth mass is obtained from the bioreactor control program MFCS through software automation by AutoHotkey program. At-line glycerol measurements by YSI biochemistry analyzer is obtained from the Seg-Flow web server through automated web browser interaction by Splinter program and Beautiful Soup program. This is possible because the analyzer reports glycerol data to the Seg-Flow server. Mass of the glycerol pumped is measured by performing serial communication with the EB60EDE-L scale before and after pumping to record mass change. Lastly, the SCADA software sends serial communication to the PLC which, as a response, sends voltage signal to the 520Du peristaltic pump for injecting glycerol for a duration calculated by the algorithm.
Figure 18: Ladder logic program loaded in the Micro850 PLC. The SCADA software sends serial communication containing ASCII data for the function ARL1 to interpret. The interpreted data gets saved in ARL_1.Destination. The equality comparison boxes at the branches compare the ASCII # of the ASCII data (stored in ARL_1.Destination) to their assigned integer. If equal, the pin corresponding to activating / deactivating individual pump gets activated. The PLC is connected to two pumps: Autotrol peristaltic pump 550-0213 for methanol delivery and Watson-Marlow 520Du peristaltic pump for glycerol fed-batch media delivery. Serial data corresponding to ASCII letter ‘A’ turns on the methanol pump, ‘a’ turns off the pump, ‘B’ turns on the glycerol fed-batch media pump, and ‘b’ turns off the glycerol pump. ACL_1 clears all of the buffers used for storing the ASCII data. ACB_1 counts the number of ASCII characters received from serial communication, saves the result to variable ‘Go’, ‘<>’ compares the number to variable ‘BC’, and MOV copies the values of ‘Go’ to ‘BC’. The program was made written by using the Rockwell Automation Connected Components Workbench software 6.01.00.
Appendix G: Overview of Automatic Cell Density Sensing Module
**Figure 19:** Structure of the cell density sensing device. (1) Sample waste container, (2) container for sample diluent (0.1M sodium acetate), (3) electronic scale for measuring dilution factor, (4) Watson-Marlow peristatic pump 323Du for sample mixing and waste draining, (5) Arduino Uno for machine control, (6) Rainin Dynamax RP-1 peristaltic pump for bioreactor sampling, (7) Takasago Electric pinch valve PK-0802-NC-3 for opening sample waste draining tube, (8) pinch valve of the same model for opening sample recirculation loop, (9) lab stand holding liquid inlets to the dilution bottle, (10) bioreactor sampling device, (11) measurement unit of the Pendotech 600nm UV-Vis-NIR spectrophotometer, (12) plastic container of power cables for the cell density sensing device, (13) flow cell connected to the spectrophotometer fiber optics, (14) Watson-Marlow peristatic pump 323Du for diluent delivery, (15) PressureMAT digital monitoring unit for spectrophotometer, (16) sample recirculation loop, (17) bioreactor sampling duct, (18) 250ml dilution bottle set on the electronic scale. The SCADA control software performs serial communication with the electronic scale and the PressureMAT monitor to record sample mass and turbidity data during the mass-based precision dilution of the sample. Similarly, serial data sent from the SCADA software directs Arduino Uno to control all pumps and valves to perform electromechanical steps of at-line cell density measurement.
**Figure 20:** The sequence of cell density sensing device operation. Gas purging is performed with filtered 15 psi air and cleansing of the bottle is performed with diluent (0.1M sodium acetate). Delivery of 0.5 gram of sampling is achieved by several cycles of short pumping and weighing. Precise 250-fold dilution is achieved by performing two steps of pumping. The first step is at least 30 seconds of pumping to measure pumping rate of diluent. Using the measured pumping rate, the second pumping is performed to achieve precise 250 fold mass based dilution. Running the diluted sample through the recirculation loop is done to perform mixing of the sample. See the annotated source code ‘Cell_Density_Sensing.py’ included in Appendix G for precise electromechanical steps performed by the machine.
**Figure 21:** The structure and the steps of bioreactor sampling module.
Appendix II: Source Codes
Download links for the source codes:

Annotated source codes of the SCADA software, PLC, Arduino, the cell density sensing device, and other items can be downloaded from:

https://s3.amazonaws.com/cm-6f58ce79a6d20c46c8b85b67dce24a8c/Pichia_Control_System.zip
https://drive.google.com/open?id=0B_vSzzCfIM5lZzdpdEhHV1B3VVU
https://www.dropbox.com/s/zcxi9h1zk369nid/Pichia_Control_System.zip?dl=0