ABSTRACT

SIGMON, JAMIE DANIELLE. Development of High Cell Density 30 L Fed-Batch Pichia pastoris Fermentation and Optimization of in situ Methanol Probe Response for Automated Control of Recombinant Protein Expression. (Under the direction of Dr. Michael C. Flickinger.)

Over 500 recombinant proteins have been expressed using the methylotrophic yeast Pichia pastoris. The strong methanol inducible alcohol oxidase promoter (pAOX1) has the ability to express recombinant proteins with reported yields up to 10 g/L. P. pastoris has the capacity to grow rapidly to high cell densities on the order of prokaryotic organisms, yet retains advantages of higher eukaryotes for protein processing.

A robust, 30 L fed-batch Pichia pastoris fermentation process has been developed on defined media with biomass densities exceeding 150 g/liter WCW achieved by glycerol feeding. This project involves optimization of an in situ dissolved methanol sensing system to control gene expression which includes significantly improving sensor response time from 12 minutes to 2.8 minutes. Project outcomes include startup and calibration of the bioreactor system for oxygen transfer, selection of process control parameters, scale-up from shake flask cultures, rapid inoculation strategy, and implementation of on-line residual glycerol monitoring using an automated sampling system integrated with a YSI analyzer. In addition, draft SOPs and BRs were prepared for many of the operational systems of this 30 liter fed-batch bioreactor process.
Development of High Cell Density 30 L Fed-Batch *Pichia pastoris* Fermentation and Optimization of *in situ* Methanol Probe Response for Automated Control of Recombinant Protein Expression

by
Jamie Danielle Sigmon

A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Master of Science

Biomanufacturing

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APPROVED BY:

Dr. Driss Elhanafi

Dr. Gisele Passador-Gurgel

Dr. Michael C. Flickinger
Chair of Advisory Committee
DEDICATION

I would like to dedicate this thesis to my family. They have always supported my endeavors no matter the circumstance. Mom, Dad, Grandma and Kristen you are forever in my heart.
BIOGRAPHY

Jamie Sigmon is a National Science Foundation fellowship recipient for her graduate studies in the Biomanufacturing Master’s of Science program at North Carolina State University. She received her Bachelor’s of Science degree in Biochemistry from North Carolina State University in May 2011. Subsequently, her experience working as a chemical analyst at Novozymes (Franklinton, NC) inspired her to further her education in biomanufacturing. While continuing part-time work and pursuing her thesis research, she studied both upstream and downstream biomanufacturing tracts within her Master’s program. In addition, during her graduate studies Jamie was involved with a program funded by BARDA (Biomedical Advanced Research and Development Authority) gaining research experience in viral inactivation and purification for influenza vaccine manufacturing at Golden Leaf BTEC (Biomanufacturing Training and Education Center). She also worked on a biopharmaceutical technology transfer project as an engineering intern within Manufacturing Technical Sciences at Shire (La Jolla, CA). She is an active member of the International Society for Pharmaceutical Engineering (ISPE) and currently holds the position of Public Relations Director for the NCSU student chapter. In January 2014, Jamie will begin her career in the biopharmaceutical industry working as a scientist in Global Development at Novartis (Holly Springs, NC). Jamie is personally dedicated to applying her skill set to help improve the lives of patients at-risk or suffering from life-altering diseases.
ACKNOWLEDGEMENTS

There were a number of individuals who contributed greatly to the success of this project. I would first like to acknowledge Dr. Michael C. Flickinger, research advisor and thesis committee chair, for his guidance and support over the course of this research project. I would further like to mention thesis committee members Dr. Driss Elhanafi and Dr. Gisele Passador-Gurgel for their contributions. Several student researchers were integral to this Pichia pastoris research project, including visiting graduate students Francesc Padrès-Angelats and Adrià Colomina from IQS School of Engineering (Barcelona, Spain), NCSU Chemical and Biomolecular Engineering undergraduates Caitlin Kurtz and Branson Kinsey, and NCSU Biomanufacturing graduate student Andrew Omonde. There were many additional supporters from BTEC staff and faculty, including Michele Ray, Haiwei Zhang, Michael Ray, and Rob Niklas. Finally, I would like to send my thanks to the National Science Foundation for the fellowship funding my graduate studies in the Master’s of Science in Biomanufacturing graduate program.
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<thead>
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<th>Description</th>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOX</td>
<td>Alcohol oxidase</td>
</tr>
<tr>
<td>CCA</td>
<td>Clean compressed air</td>
</tr>
<tr>
<td>CU</td>
<td>Concentration units</td>
</tr>
<tr>
<td>DCU</td>
<td>Digital control unit</td>
</tr>
<tr>
<td>DCW</td>
<td>Dry cell weight</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td>DOT</td>
<td>Dissolved oxygen tension</td>
</tr>
<tr>
<td>GBP</td>
<td>Glycerol biomass generation phase</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GFRC</td>
<td>Gas flow ratio control</td>
</tr>
<tr>
<td>HPW</td>
<td>High purity water</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>k&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Volumetric oxygen mass transfer coefficient (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MeOHUR</td>
<td>Methanol uptake rate</td>
</tr>
<tr>
<td>MIP</td>
<td>Methanol induction phase</td>
</tr>
<tr>
<td>Mut</td>
<td>Methanol utilization</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical density at 600 nm</td>
</tr>
<tr>
<td>OTR</td>
<td>Oxygen transfer rate</td>
</tr>
<tr>
<td>OUR</td>
<td>Oxygen uptake rate</td>
</tr>
<tr>
<td>PI</td>
<td>Proportional integral control</td>
</tr>
<tr>
<td>PLC</td>
<td>Programmable logic controller</td>
</tr>
<tr>
<td>PTM</td>
<td><em>Pichia</em> trace minerals</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SIP</td>
<td>Sterilization-in-place</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>slpm</td>
<td>Standard liters per minute</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard operating procedure</td>
</tr>
<tr>
<td>TP</td>
<td>Transition phase</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td>$\mu_{\text{max}}$</td>
<td>Maximum specific growth rate (h$^{-1}$)</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>vvm</td>
<td>Vessel volumes per minute</td>
</tr>
<tr>
<td>WCW</td>
<td>Wet cell weight</td>
</tr>
<tr>
<td>X</td>
<td>Biomass concentration (g/L)</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast extract peptone dextrose</td>
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1. OBJECTIVES

*Pichia pastoris* is widely used as a fed-batch protein expression platform. However, many research and industrial fermentation applications do not directly control methanol feeding for heterologous protein induction. The bioreactor system developed in this thesis contains an *in situ* dissolved methanol probe that can be used for tight control of residual methanol concentration resulting in optimized culture viability and recombinant protein expression. This thesis outlines the development of a 30 L bioreactor *Pichia pastoris* fermentation process and improvements to the methanol sensing system.

Heterologous protein expression in *Pichia pastoris* is directly proportional to cell density. In order to maximize productivity, induction of the strong AOX1 promoter should occur at cell densities greater than 100 dry cell weight (DCW). Fermentation equipment, media, and procedures must accommodate biomass generation on this scale. Furthermore, the methanol sensing system must have the ability to rapidly respond to changes in dissolved methanol.

The goals of this thesis to achieve high biomass density and controlled methanol induction include:

- Selection of bioreactor operating conditions
- Equipment calibration methods
- Scale-up of shake flask cultures to 30 L bioreactor system
- Development of glycerol biomass production phase on defined media
- Implementation of process monitoring technology
- Improvements to methanol sensing and control system
- Draft Standard Operating Procedure (SOP) and Batch Records (BR) for routine fed-batch recombinant protein production using this process
Additional overall project goals include:

- Total process automation
- Defined media development
- Scale-down process improvement studies in micro-reactors
- Development of transition and methanol induction phase feeding strategies
- Routine, automated expression of recombinant proteins
2. **PICHIA PASTORIS**

2.1. History of *Pichia pastoris* Expression System

The methylotrophic yeast *Pichia pastoris* (*P. pastoris*) was discovered in 1969 by Koichi Ogata for its ability to utilize methanol as a sole carbon source for growth. Subsequently, in the 1970s the Phillips Petroleum Company developed *P. pastoris* fermentation processes for bioconversion of methanol (from methane gas) to both yeast biomass and single cell protein for supplementation of animal feed. As a result of the oil crisis, the cost of methane gas rose significantly and Phillips Petroleum Company partnered with the Salk Institute of Biotechnology/Industrial Associates Inc. (SIBIA, La Jolla, CA) to develop *P. pastoris* as a recombinant protein expression system. Phillips Petroleum Company sold the *P. pastoris* expression system to Research Corporation Technologies (Tucson, AZ) in 1993 and at the same time licensed Invitrogen Corporation (Carlsbad, CA) to distribute *Pichia* technology globally (Sreekrishna, 2010; Macauley-Patrick et al., 2005).

2.2. Market Demands for Biotherapeutics

Global demand for heterologous proteins and bioproducts is rapidly increasing. In 2011, the protein therapeutic market reportedly reached $105 billion and is projected to reach $143.4 billion by the end of 2015 (RNCOS, 2012). In order to meet these market demands it is important to have production systems that are robust, high-yield, and economically viable (Potvin et al., 2012).

2.3. *Pichia pastoris* as a Protein Expression Platform

*P. pastoris* is an advantageous system for recombinant protein expression due to (1) ease of genetic manipulation, (2) controlled expression of recombinant proteins, (3) ability to
secrete large quantities of foreign proteins, (4) rapid growth to high cell densities, and (5) capacity for post-translational modifications. *P. pastoris* is as simple to genetically manipulate as *Escherichia coli* (*E. coli*) or *Saccharomyces cerevisiae* (*S. cerevisiae*) and, additionally, many *Saccharomyces* genetic modification techniques can be directly applied to *P. pastoris* (Invitrogen Co., 2010). The strong inducible alcohol oxidase 1 promoter (pAOX1) allows for controlled expression of recombinant proteins by induction with low concentrations of methanol. *P. pastoris* is one of the most productive eukaryotic protein expression systems (Tolner et al., 2006), with reported expression 10- to 100- times greater than that of *S. cerevisiae* (Invitrogen Co., 2010)(Table 2.3.). *Pichia pastoris* boasts expression of greater than 500 recombinant proteins to date with expression levels as high as 80% of total secreted protein (Potvin et al., 2012).

*Pichia pastoris* also has the ability to grow rapidly to high cell densities on the order of prokaryotic organisms, yet retains advantages of higher eukaryotes for protein processing. One of the greatest advantages to using this system is its capability for post-translational modifications, including polypeptide folding, glycosylation, methylation, acylation, and proteolytic adjustment (Li et al., 2007).

Disadvantages to using the *P. pastoris* expression system include high expression of protease, sensitivity to growth-inhibition by methanol concentration, potential for nutrient deficiency when cultivated on defined media, safety and toxicity concerns with bulk methanol storage, and potential for complex control strategies (Potvin et al., 2012).
Table 2.3. Examples of recombinant proteins produced by *Pichia pastoris* (Adapted from Invitrogen Co., 2010)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Expression Levels (g/L)</th>
<th>Intracellular or Secreted</th>
<th>Phenotype</th>
</tr>
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<tbody>
<tr>
<td>Enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invertase</td>
<td>2.3</td>
<td>Secreted</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Bovine Lysozyme c2</td>
<td>0.55</td>
<td>Secreted</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Streptokinase (active)</td>
<td>0.08</td>
<td>Intracellular</td>
<td>--</td>
</tr>
<tr>
<td>Alpha amylase</td>
<td>2.5</td>
<td>Secreted</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Pectate Lyase</td>
<td>0.004</td>
<td>Secreted</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Spinach Phosphoribulokinase</td>
<td>0.1</td>
<td>Intracellular</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Antigens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis B surface antigen</td>
<td>0.4</td>
<td>Intracellular</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Pertussis Antigen P69</td>
<td>3</td>
<td>Intracellular</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Tetanus Toxin Fragment C</td>
<td>12</td>
<td>Intracellular</td>
<td>Mut⁺/Mut⁺</td>
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<td>HIV-1 gp120</td>
<td>1.25</td>
<td>Intracellular</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Tick Anticoagulant protein</td>
<td>1.7</td>
<td>Secreted</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Bm86 Tick Gut Glycoprotein</td>
<td>1.5</td>
<td>Secreted</td>
<td>--</td>
</tr>
<tr>
<td>Regulatory Proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor Necrosis Factor (TNF)</td>
<td>10</td>
<td>Intracellular</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Mouse Epidermal Growth Factor (EGF)</td>
<td>0.45</td>
<td>Secreted</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Human Interferon (IFN) α2b</td>
<td>0.4</td>
<td>Intracellular</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Membrane Proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human CD38 (soluble portion)</td>
<td>0.05</td>
<td>Secreted</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Mouse Serotonin Receptor</td>
<td>0.001</td>
<td>Secreted</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Proteases and Protease Inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxypeptidase B</td>
<td>0.8</td>
<td>Secreted</td>
<td>Mut⁺/Mut⁺</td>
</tr>
<tr>
<td>Enterokinase</td>
<td>0.021</td>
<td>Secreted</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Ghilanten</td>
<td>0.01</td>
<td>Secreted</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Kunitz protease inhibitor</td>
<td>1</td>
<td>Secreted</td>
<td>--</td>
</tr>
<tr>
<td>Human Proteasine Inhibitor 6</td>
<td>0.05</td>
<td>Intracellular</td>
<td>Mut⁺</td>
</tr>
<tr>
<td>Antibodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit Single Chain Antibody</td>
<td>&gt;0.1</td>
<td>Secreted</td>
<td>Mut⁺</td>
</tr>
</tbody>
</table>

2.4. Host strains

All *Pichia pastoris* expression strains originated from NRRL-Y 11430 (Northern Regional Research Laboratories, Peoria, IL) and most have a genetic mutation in the histidinol dehydrogenase gene (*HIS4*) (Li *et al.*, 2007). Histidinol dehydrogenase is an enzyme that catalyzes the terminal step in histidine biosynthesis in yeast (Equation 2.4.). This mutation allows for selection of transformants from expression vectors containing the *HIS4* gene.
Untransformed strains require supplementation of histidine in the growth media.

\[ L - \text{histidinol} + 2 \text{NAD}^+ \xrightleftharpoons{\text{histidinol dehydrogenase}} L - \text{histidine} + 2 \text{NADH} + 2 \text{H}^+ \]

**Equation 2.4.** Conversion of L-histidinol to L-histidine by histidinol dehydrogenase

2.5. Methanol Utilization Phenotypes

Alcohol oxidase is the enzyme expressed by *Pichia pastoris* for the metabolism of methanol. Two genes are responsible for expression of alcohol oxidase, *AOX1* and *AOX2*. The *AOX1* gene accounts for nearly 90% of alcohol oxidase expression while *AOX2* is responsible for approximately 10% (Bawa and Darby, 2012). The two alcohol oxidase genes may be deleted to produce methanol utilization phenotypes different from the wild type. The three possible methanol utilization (Mut) phenotypes are (1) methanol utilization plus (Mut\(^+\)), (2) methanol utilization slow (Mut\(^s\)), and (3) methanol utilization minus (Mut\(^-\)). The Mut\(^+\) strain retains both *AOX1* and *AOX2* genes and metabolizes methanol at the wild type rate. GS115 *his4* Mut\(^+\) is the most commonly used *Pichia pastoris* expression strain. Mut\(^-\) strains are characterized by an *AOX1* gene deletion. In this situation, the *AOX2* gene is responsible for alcohol oxidase synthesis (Li et al., 2007). Mut\(^-\) strains have deletions in both *AOX1* and *AOX2* genes. They are not capable of growing on methanol, but are still able to induce high expression of recombinant proteins from the *AOX1* promoter.

Deletion of one or both *AOX* genes reduces or eliminates the ability of *Pichia pastoris* to grow on methanol, but in some cases results in higher expression of heterologous proteins (Li et al., 2007). Furthermore, these strains require lower methanol concentrations for induction, which is advantageous at large scale due to reduced requirements for bulk methanol (Table 2.5.).
There are also several protease-deficient strains available with *pep4* and *prb1* gene deletions. The *pep4* gene encodes for proteinase A, a vacuolar aspartyl protease responsible for activating other vacuolar proteases. *Prb1* encodes for proteinase B. Available protease-deficient strains include SMB1163 (*his4 pep4 prb1*), SMB1165 (*his4 prb1*) and SMB1168 (*his4 pep4*) (Li et al., 2007).

### 2.6. Methanol Metabolism in *Pichia pastoris*

Regulation of the *AOX1* promoter is accomplished by repression, derepression and induction mechanisms (Invitrogen Co., 2010). The promoter is repressed by the presence of carbon sources such as glucose and de-repressed by glycerol or the absence of another carbon source. Induction occurs under de-repressed conditions in the presence of methanol. Alcohol oxidase generated by *AOX1* and/or *AOX2* gene expression oxidizes methanol to formaldehyde. Due to the toxicity of methanol, this reaction occurs in cellular organelles called peroxisomes (Figure 2.6.). Hydrogen peroxidase in the peroxisomes catalyzes the conversion of the by-product hydrogen peroxide to water and oxygen.
Formaldehyde enters two pathways, a cytosolic dissimilatory pathway producing cellular energy and a peroxisomal assimilatory pathway resulting in biomass production (Bawa and Darby, 2012). Within the assimilatory pathway, formaldehyde reacts with xylulose 5-monophosphate in the presence of dihydroxyacetone synthase to produce dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate (GAP). These products can be used to regenerate xylulose 5-phosphate and also result in biomass production (Bawa and Darby, 2012).

2.7. Transformation Methods

Stable transformation by homologous recombination is used to integrate a gene of interest into the \textit{P. pastoris} genome. The recombinant vector is linearized in the 5’ \textit{AOX1} region by digestion with restriction enzymes (Invitrogen Co., 2010) and a single crossover event occurs with homologous regions (Figure 2.7.). Expression yields can be increased by multicopy transformation (Li \textit{et al.}, 2007). Secretion products can be cloned into Invitrogen’s pPICZa vector downstream of a signal sequence (Tolner \textit{et al.}, 2006).
Figure 2.7. Stable transformation of gene of interest into *P. pastoris* genome by homologous recombination (Invitrogen Co., 2010)

2.8. Nutrient Requirements

*Pichia pastoris* is reasonably robust in its ability to grow on relatively simple mineral salts media (Jahic *et al.*, 2006). Complex medium such as Yeast Extract Peptone Dextrose (YE PD or YPD) is sufficient for supporting growth requirements; however, complex components in such media result in batch-to-batch variability. In order to standardize the fermentation process and further optimize growth and expression, chemically defined media is preferred. The basic media requirements for growth include carbon, nitrogen, biotin, salts, and trace minerals.

Glycerol is the preferred carbon source since it is a fermentative carbon source thus allowing derepression of pAOX1 yet inhibiting expression of genes controlled by the AOX1 promoter. Alternatively, glucose is a non-fermentative carbon source and results in the by-product ethanol which represses pAOX1 even in the presence of the inducer, methanol (Bawa and Darby, 2012; Macauley-Patrick *et al.*, 2005). Glycerol has additionally
demonstrated higher biomass yields compared to glucose or ethanol (Bawa and Darby, 2012). Alanine, sorbitol and mannitol have also been examined as alternative non-repressing carbon sources (Macauley-Patrick et al., 2005). These have shown to have a positive impact on heterologous protein expression in Mut′ P. pastoris strains in comparison to glycerol or glucose (Inan and Meagher, 2001).

Ammonium hydroxide is commonly supplied both as a nitrogen source and for pH control (Macauley-Patrick et al., 2005). The disadvantage to this method is that nitrogen content in the media is dictated by fluctuations in pH. Furthermore, increased concentration of ammonium in the medium has been reported to inhibit growth and prolong the lag phase (Macauley-Patrick et al., 2005).

2.9. Bioreactor Cultivation of Pichia pastoris

Due to the high cell density, oxygen-enriched aeration is often required for glycerol and methanol metabolism. The dissolved oxygen tension (DOT) should be controlled at levels greater than 20% dissolved oxygen (DO) in solution with recommended control at 30-35% (Invitrogen Co., 2002; Li et al., 2007). A common control temperature for cell growth is 30°C. Temperatures greater than 32°C can result in cell death, but lower temperatures may be advantageous for heterologous protein yields. Reduced temperature has the effect of increased mRNA and protein stability (lessened thermal denaturation) and minimized proteolysis (Macauley-Patrick et al., 2005).

P. pastoris has the ability to grow within a broad pH range of 3.0-7.0 with an optimum pH selected based on recombinant protein stability (Tolner et al., 2006). Addition of an antifoam agent is necessary to eliminate foam in the headspace. Aeration rates up to 1.0 vvm are recommended for 15 L bioreactor cultivation (Invitrogen Co., 2002). Actual
Aeration operating rate should be determined based on the oxygen transfer capabilities of the vessel and the oxygen demands of the culture.

### 2.10. Fed-Batch Fermentation

A three-phase fermentation process typically characterizes *Pichia* cultivation (Figure 2.10.). These consist of (1) glycerol biomass generation phase, (2) transition phase, and (3) methanol induction phase. The glycerol biomass generation phase (GBP) is approximately 22-24 hours of rapid biomass production with specific growth rates around $0.26 \text{ hr}^{-1}$ (Tolner *et al.*, 2006; Jahic *et al.*, 2006). Glycerol concentration in the media during this phase is recommended to be 40 g/L with concentrations exceeding 4% v/v inhibiting growth (Jahic *et al.*, 2006; Invitrogen Co., 2002). Final cell densities at the end of the GBP can reach 220 g/L WCW (Invitrogen Co., 2002).

![Figure 2.10. Typical three-phase cultivation of *P. pastoris* with glycerol biomass generation phase (GBP), transition phase (TP), and methanol induction phase (MIP). This figure describes 5 L bioreactor Mut<sup>+</sup> biomass concentration, methanol concentration, and recombinant lipolytic activity. Methanol induction controlled by predictive algorithm with PI controller and concentration analyzed offline with gas chromatography and online with MC-168 (PTI Instruments) (Cos *et al.*, 2006)](image-url)
The transition phase (TP) is typically achieved by a slow introduction of methanol feed and cessation of glycerol feed. Pure methanol is supplemented with *Pichia* trace minerals (PTM) solution and fed to the fermentation culture (Invitrogen Co., 2002). Adaptation to growth on methanol occurs after approximately 2-4 hours and is indicated by a steady reading from the dissolved oxygen signal. The transition phase can last up to 10 hours (Tolner *et al.*, 2006).

The methanol induction phase (MIP) is designed based on the methanol utilization phenotype and optimal protein expression levels. Methanol feed rates for Mut⁺ strains can be doubled from that of the transition phase, while the 3-4 times slower growth on methanol of Mut⁻ requires much lower feed rates (Invitrogen Co., 2010). The goal of MIP is to provide sufficient concentrations of methanol to induce the promoter while avoiding toxic accumulation (Bawa and Darby, 2012). Glycerol is also fed during this phase as a carbon and energy source to support protein expression. Methanol concentrations should be maintained below 0.5% (v/v). Other methanol induction strategies include variable ratios of mixed glycerol-methanol feeding for sustained biomass growth and increased protein expression yields (Bawa and Darby, 2012).
3. BIOREACTOR SYSTEM

3.1. System Overview

The 30 L stainless steel bioreactor (Biostat® C-DCU3) is integrated with a dissolved methanol probe, an autosampler (Flonamics Fisp®, Seg-Flow®), a bioanalyzer (YSI 7100 MBS™), and sensors on the inlet and exhaust gas lines (BlueSens BCpreFerm)(Figure 3.1.).

![Figure 3.1. Bioreactor system overview](image)

3.2. Biostat® C-DCU3 30 L Bioreactor

The stainless steel Biostat® C-DCU3 (Sartorius BBI Systems, Bethlehem, PA) bioreactor is shown in Figure 3.2(a) and (b). This vessel has a 2.2:1 height-to-diameter ratio with a maximum working volume of 30 L (See Table 3.2. for vessel geometry). Internally are two 11.4 cm diameter Rushton disc turbine impellers, four baffles, and a ring sparger at the base.
Figure 3.2. Biostat® C-DCU3 30 L Bioreactor (a) Photo (b) Schematic (Cueva Tello, 2012)

Table 3.2. Bioreactor Geometry (Adapted from Cueva Tello, 2012)

<table>
<thead>
<tr>
<th>Vessel Characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Height:Diameter Ratio</td>
<td>2.2:1</td>
</tr>
<tr>
<td>Total volume (L)</td>
<td>42</td>
</tr>
<tr>
<td>Working volume (L)</td>
<td>30</td>
</tr>
<tr>
<td>Vessel Internal Diameter (cm)</td>
<td>29.2</td>
</tr>
<tr>
<td>Vessel Height (cm)</td>
<td>63.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Impeller and Baffle Characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Impeller number</td>
<td>2</td>
</tr>
<tr>
<td>Distance between impellers (cm)</td>
<td>14.7</td>
</tr>
<tr>
<td>Impeller diameter (cm)</td>
<td>11.4</td>
</tr>
<tr>
<td>Impeller type</td>
<td>Rushton</td>
</tr>
<tr>
<td>Baffle number</td>
<td>4</td>
</tr>
</tbody>
</table>
Probes incorporated into this unit include standard antifoam, high foam, temperature, dissolved oxygen, and pH. In addition, this system has a custom probe in-line for detection of dissolved methanol.

3.3. *In situ* Dissolved Methanol Probe

The dissolved methanol probe constructed by previous researchers (Cueva Tello, 2012; Berry, 2012) is shown in Figure 3.3(a) and (b). The probe is fabricated of a hollow stainless steel tube (650 mm long, 14 mm diameter) with a threaded plug fitting a port in the bioreactor head plate. Silicone tubing is coiled around the outside tip of the stainless steel rod and filtered dry nitrogen flows within the tubing lumen. Methanol diffusion occurs through the silicone tubing wall and is carried by the nitrogen gas to a hydrocarbon sensor located exterior to the reactor. Valves at the top of the probe can be closed during *in-situ* sterilization of the bioreactor. Two sizes of silicone tubing were investigated in this thesis. These tubing geometries of are listed in Table 3.3.

A tin dioxide semiconductor gas sensor, Taguchi Gas Sensor (TGS) model 822 (Figaro, Inc., Japan), is used for detection of gas-phase methanol. Metal oxide detectors have low conductivity in clean air (e.g. dry nitrogen). Conductivity detected by TGS-822 increases with increased concentration of methanol in the carrier gas. The output signal from the sensor is acquired by LabVIEW™ (National Instruments Corporation, Austin, TX) data acquisition software on the laboratory computer.
Figure 3.3. *In situ* methanol probe (a) Photo (Padrès-Angelats, 2012) (b) Schematic (Cueva Tello, 2012)

Table 3.3. Silicone tubing geometry (Adapted from Padrès-Angelats, 2012)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tube 1</th>
<th>Tube 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wall Thickness (mm)</td>
<td>0.42</td>
<td>0.28</td>
</tr>
<tr>
<td>Outer Diameter (mm)</td>
<td>2.41</td>
<td>1.19</td>
</tr>
<tr>
<td>Inner Diameter (mm)</td>
<td>1.57</td>
<td>0.64</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>258.2</td>
<td>497.5</td>
</tr>
<tr>
<td>Exposed Surface Area (cm²)</td>
<td>97.8</td>
<td>93.0</td>
</tr>
</tbody>
</table>

3.4. Glycerol monitoring by Seg-Flow® integrated with YSI 7100 MBS™

Samples are collected at a programmed interval by an *in-situ* sampling probe (Fisp®,
Flownamics, Madison, WI) that contains a porous frit to retain the cells in the bioreactor for cell-free sampling. The Fisp® probe is integrated with Seg-Flow® (Flownamics, Madison, WI) to divert samples to the YSI 7100 MBS™ (YSI Inc., Yellow Springs, OH) biochemistry analyzer. The YSI is capable of detecting analytes in solution using enzymatic reactions. The assay kit currently installed provides data on residual glycerol concentration in the fermentation broth, however this YSI analyzer system can be modified in the future to detect a variety of other analytes in the culture broth.

![Figure 3.4. Seg-Flow® module (left) YSI 7100 MBS™ (right)](image)

### 3.5. BlueSens CO₂ and O₂ Gas Analyzers

BCpreFerm (BlueSens, Herten, Germany) carbon dioxide (CO₂) and oxygen (O₂) gas analyzers are installed on the inlet and exhaust gas lines. These are coupled with mass flow controllers and FermVis (BlueSens, Herten, Germany) data acquisition software for future vent gas analysis of *Pichia pastoris* fermentation.
4. METHANOL MONITORING AND CONTROL SYSTEM

4.1. Introduction

Methanol concentration must be tightly controlled to effectively induce the AOX1 promoter yet avoid cytotoxic levels of dissolved methanol. Methanol feed control can be achieved by indirect or direct methods. Figure 4.1. shows oscillations around the desired dissolved methanol set point using three different control methods. Cos and colleagues (2006) demonstrated that when controlled manually based on a discontinuous signal, large fluctuations in residual methanol concentration occur. These types of fluctuations can lead to cell stress, inadequate induction of pAOX1, and overfeeding. Adding proportional integral control greatly reduces these oscillations. Cos and co-workers (2006) further refined the process control using a model to control the set point, allowing tight control of methanol feeding at the set point.

Figure 4.1. Comparison of methanol control methods. Methanol concentration controlled manually (⋯), PI feedback control (—), and model-based control (−−−) (Cos et al., 2006)
4.1.1. Indirect Methanol Feed Control

A commonly used indirect method is the DO spike method. When a carbon source becomes limiting, consumption of oxygen decreases and thus there is a spike in dissolved oxygen in the culture. This method is used both to determine whether glycerol is consumed prior to entering the methanol induction phase and feeding intervals for methanol during the induction phase (Invitrogen Co., 2002).

A model-based indirect method for controlling methanol fed-batch cultures is based on the specific growth rate, $\mu$, during the induction phase. This method requires complex models that are exclusive to a specific $P. pastoris$ strain and expressed protein. Enzymatic analysis of alcohol oxidase production may also be used to indirectly detect residual methanol concentration based on induction of the AOX1 promoter (Puhar et al., 1980). However, enzymatic analysis is discontinuous, requiring time for sample collection, sample preparation, and analysis. Furthermore, this option does not provide direct information about methanol concentration in solution.

4.1.2. Methanol Sensing Methods

Methanol can alternatively be controlled directly by sensing systems. At-line and in-line options for methanol sensing include: (1) gas chromatography, (2) off-gas flame ionization detection, (3) commercial gas-phase methanol sensing systems and (4) tubing coupled to a semiconductor sensor. Gas chromatography is expensive and slow. Similar to enzymatic analysis, it does not provide a continuous signal and dilution of the sample may occur prior to reaching the GC analyzer. Researchers have investigated the use of an off-gas flame ionization detector, concluding that results were not robust, being influenced by agitation, temperature, and uneven flow rates (Austin et al., 1992). Additionally, this method can cause condensation buildup in tubing.
Commercial gas-phase methanol sensing systems are available for small-scale fermentations. Two examples include the in-line Raven Biotech 2.1 probe and PTI Instruments MC-168 for detection of methanol in the exhaust gas. However, a simple dissolved methanol probe can be constructed inexpensively using a hydrocarbon sensor (e.g. Figaro TGS-822) coupled to an in situ stainless steel rod supporting methanol permeable tubing (e.g. silicone) (Lee et al., 1996; Austin et al., 1992; Cueva Tello, 2012). This tubing method involves diffusion of dissolved gases and volatiles through the semipermeable tubing wall and subsequent gas flow through a detector using inert carrier gas such as N₂ (Austin et al., 1992).

4.1.3. Dissolved Methanol Sensing

The dissolved methanol probe constructed by previous researchers (Berry, 2012; Cueva Tello, 2012) is a hollow stainless steel rod with central dry nitrogen feed and return lines, external silicone tubing for diffusion, and a threaded plug fitting the bioreactor headplate (Figure 3.3.).

Fick’s First Law of Diffusion can be used describe diffusion of methanol across the silicone membrane:

$$J = -D \frac{\Delta C}{\Delta x} = -D \frac{c_i - c_o}{\Delta x}$$

Equation 4.1.3.(a)  Fick’s First Law of Diffusion

In this equation J is flux, D is the diffusion coefficient, cₖ is the concentration of methanol in the gas phase, cₒ is the concentration of methanol in the liquid phase, and Δx is the tubing wall thickness. This relationship assumes that the gas and liquid phases are homogenous
and therefore the concentration of methanol is constant in each. Diffusion across the membrane must also account for impedance from both the liquid-silicone boundary layer and the silicone-gas boundary layer (Figure 4.1.3.). The liquid-silicone boundary layer thickness is influenced by agitation within the vessel. Similarly, the silicone-gas phase boundary layer thickness is influenced by the carrier gas flow rate inside the tubing.

![Figure 4.1.3. Methanol diffusion across silicone tubing wall (Padrés-Angelats, 2012)](image)

Signal output from the hydrocarbon sensor is proportional to the exposed tubing area (A), the permeability of the silicone tubing for methanol (P), the concentration of methanol in the liquid phase, and the inverse of the tubing wall thickness (∆x⁻¹):

$$\Delta\text{Output} \propto A \cdot \left(\frac{P}{\Delta x}\right) \cdot [\text{MeOH}]$$

Equation 4.1.3.(b). Relationship of methanol probe signal output to tubing area, tubing permeability, wall thickness, and methanol concentration
The probe construction allows for a specified length over which the silicone tubing surface can be coiled and therefore this parameter can be considered constant. The permeability coefficient of methanol in silicone is also constant as long as silicone tubing is used. Tubing wall thickness and methanol concentration impact the probe signal.

### 4.1.4. Methanol Feed Rate

Methanol feed rate must consider not only dissolved methanol, but also cellular uptake rate and loss of methanol to evaporation (stripping rate). The mass balance for methanol feed rate is shown in Equation 4.1.4.(a):

\[
MeOH_{Feed} = MeOH_{Consumed} + MeOH_{Stripped} + MeOH_{Dissolved}
\]

**Equation 4.1.4.(a) Methanol mass balance**

The methanol uptake rate (MeOHUR) is related to the maximum specific growth rate \( \mu_{max} \) on methanol, biomass concentration \( X \) and biomass yield on methanol \( Y_{X/MeOH} \). This relationship is described by the equation:

\[
MeOHUR = \frac{\mu_{max} \cdot X}{Y_{X/MeOH}}
\]

**Equation 4.1.4.(b) Methanol uptake rate**

During the methanol induction phase \( \mu_{max} \) decreases to \( \leq 0.14 \text{h}^{-1} \) (Table 2.5) and methanol uptake becomes additionally dependent upon protein productivity. The yield on methanol will then include both biomass and protein product. Therefore, MeOHUR for *P. pastoris* will depend on the strain, the protein expression product, media formulation, and fermentation
conditions which control stripping rate. The yield on methanol and specific growth rate should be determined experimentally for the process of interest.

4.2. Purpose

The primary purpose of this investigation was to optimize methanol probe sensitivity and response for tight control of dissolved methanol. Parameters investigated include carrier gas flow rate, tubing wall thickness, agitation speed, and methanol concentration. Probe durability following sterilization-in-place (SIP) and methanol stripping rate under operating conditions were also considered.

4.3. Experimental Methods

4.3.1. Carrier Gas Rotameter Calibration

In-house dry nitrogen was used as a carrier gas through the lumen of the silicone tubing in the methanol probe. The rotameter on the N₂ gas line was calibrated by measuring the time required to displace 100 or 200 ml of water at room temperature into a graduated cylinder. This experiment was performed in duplicate for rotamerter settings of 30, 60, 100, 200, and 260 ccm (Padrés-Angelats, 2012). A calibration curve was generated for conversion of N₂ ccm to flow rate (ml/min) at 25°C (Figure 4.4.1.).

4.3.2. Nitrogen Flow Rates

Response curves were created by placing the methanol probe (0.4 mm wall thickness tubing) in a 2 L stirred bottle of high purity water at 25°C. A 17% v/v methanol in water solution was used to make volumetric additions to the bottle with final methanol concentrations of 10.2, 20.3, 30.2, 40.0, 59.4, 78.3, 134.4, and 206.2 mM (Padrés-Angelats,
2012). The methanol concentrations were chosen to target the control range for the probe, < 0.5% v/v dissolved methanol (< 124 mM). Nitrogen flow rates tested were 136, 246, 300, 411, 522, and 632 ml/min. The response signal was determined when the output signal (V) from the probe stabilized. Response time was recorded at 90% of the stabilization signal.

4.3.3. Agitation Rate and Tubing Wall Thickness

Effects of agitation speed on the probe response signal were evaluated in the bioreactor at 30°C, 30 L volume, 411 ml/min nitrogen flow, and 0.4 mm wall thickness silicone tubing. Pure methanol was measured volumetrically and added to the bioreactor through an open port in the headplate. Accounting for dilution in the reactor, final methanol concentrations tested were 8.1, 16.2, 24.3, 40.5, 64.8, 97.0, 129.1, and 161.1 mM (Padrés-Angelats, 2012). Agitation rates investigated were 300, 400, 500, 600, and 800 rpm controlled by the bioreactor digital control unit (DCU). Signal and time were determined in the same manner as described in 4.3.2.

The 0.4 mm wall thickness tubing was replaced with thinner walled tubing (0.28 mm silicone tubing) of the same material (Dow Corning Corp. Silastic). The agitation rate experiment was reproduced with an additional point at 1000 rpm.

4.3.4. Sterilization-In-Place (SIP) Durability

The probe was subjected to sterilization cycles that exceed 1 bar in pressure and maintain temperature at 121°C for 30 minutes. Inlet and outlet carrier gas valves were closed during this procedure. The N₂ gas line was redirected to bypass the probe and flow directly through the hydrocarbon sensor. Following each SIP cycle the probe response was measured at 600, 800 and 1000 rpm. This experiment was executed using 0.28 mm wall thickness tubing.
4.3.5. Effect of Methanol Concentration on Response Time

Concentrations of 0.4%, 0.6%, 0.8%, and 1% v/v methanol in water were studied to determine the effect of concentration of response time. This data was combined with data generated from the SIP durability study (Padrés-Angelats, 2012).

4.3.6. Methanol Stripping

Methanol stripping experiments were performed at 30°C, 30 L of water, 1 vvm airflow (clean compressed air), and methanol concentration of 0.5%. Agitation speeds of 600, 800, and 900 rpm were tested. The initial experiment was performed overnight (Figure 4.4.6.(a)). Stripping rate was determined by selecting a representative 70 minute window over which stripping was greatest and the slope determined by linear regression.

4.4. Results

4.4.1. Carrier Gas Rotameter Calibration

The point (0, 0) was added to fit the data through zero. The flow rate (ml/min) of house dry nitrogen is related to rotameter setting (ccm) by the linear equation $y = 2.7548x + 25.7638$ with a coefficient of determination, $R^2$, of 0.992 (Figure 4.4.1.) (Padrés-Angelats, 2012).
4.4.2. Nitrogen Flow Rate

Figure 4.4.2.(a) shows the linear relationship between methanol concentration and response signal for nitrogen flow rates investigated. The slopes of these regression lines are plotted as a function of flow rate in Figure 4.4.2.(b). The slopes stabilize at flow rates equal and greater than 300 ml/min. Figure 4.4.2.(b) also shows that the time of response (ToR) in seconds for flow rates tested stabilizes less than or equal to 411 ml/min. The calibration curve relating methanol concentration to output signal at 411 ml/min is provided in Figure 4.4.2.(c).
Figure 4.4.2. (a) Methanol probe response signal to \( \ln[\text{MeOH}] \) over \( \text{N}_2 \) flow rates 136-632 ml/min (b) Slope of response and time of response for \( \text{N}_2 \) flow rates 136-632 ml/min (c) Calibration curve for 411 ml \( \text{N}_2 \)/min and 25°C

4.4.3. Agitation Rate and Tubing Wall Thickness

Figures 4.4.3.(a),(b) and (c), show that for both 0.42 mm and 0.28 mm tubing the slope of the response increases with respect to agitation speed. The time of response was standardized with tubing surface area in Figure 4.4.3.(d). In both experiments, an agitation rate of greater than 600 rpm minimizes the response time from the probe. Additionally, the data shows that the 0.28 mm wall thickness tubing has a significantly faster response time when compared to 0.4 mm, 1.82 s cm\(^{-2}\) and 3.62 s cm\(^{-2}\), respectively.
Figure 4.4.3. Probe response to agitation range 300-1000 rpm (a) Response signal for 0.42 mm tubing (b) Response signal for 0.28 mm tubing (c) Slope of response for 0.42 mm and 0.28 mm tubing (d) Time of response over tubing area (ToR/A) at increasing agitation speed.
4.4.4. Sterilization-in-Place (SIP) Durability

Results from the SIP durability study are overlaid with previous response time data in Figure 4.4.4. The data was subjected to ANOVA analysis using response time as analyzed response to the SIP factor (Padrés-Angelats, 2012). Using a critical P-value of 0.05, the ANOVA analysis determined that SIP has no influence on time of response (P = 0.46).

Figure 4.4.4. Effect of SIP on methanol probe response as a function of tubing wall thickness and agitation speed

4.4.5. Effect of Methanol Concentration on Response Time

ANOVA analysis of the effects of methanol concentration on response time suggests that concentration is a significant factor in response time (P = 1.72*10^{-12})(Padrés-Angelats, 2012). The figure below shows the distribution of response over the course of
concentration values tested (Figure 4.4.5.). Above 0.4% methanol, time of response stabilizes. Below 0.4% response time increases with decreased concentration.

![Graph](image)

**Figure 4.4.5.** Effect of methanol concentration on probe response time

### 4.4.6. Methanol Stripping

Figures 4.4.6.(a), (b), and (c) show data for methanol stripping over a 14.5-hour time course. Greatest stripping occurred within the first two hours of the experiment and a representative sample of the stripping rate was selected for the first 70 minutes (Figure 4.4.6.(b)). The slopes from all stripping experiments are shown in Figure 4.4.6.(c). Stripping rate was relatively constant for the conditions tested with an overall average stripping rate of $0.16 \pm 0.04$ mmol MeOH L$^{-1}$ min$^{-1}$.
Figure 4.4.6. Methanol stripping from liquid to gas phase (a) Stripping over 14.5 hour time course (b) Determination of stripping rate over 70 minute period following addition (c) Effect of agitation speed on stripping rate at 30°C and 400 mbar

4.5. Discussion

Nitrogen flow rate parameters that result in a higher voltage signal in response to methanol in solution are desirable because increased signal demonstrates sensitivity of the probe to detect methanol. Since the probe will be used to control methanol at low concentrations <0.5% v/v methanol (<124 mM), sensitivity should be maximized for this range. The
stability of the linear regression slopes demonstrates that the slope is independent of nitrogen flow rate in the tubing lumen at greater than 300 ml/min (Figure 4.4.2(b)).

Fermentation cultures are dynamic environments especially at high cell densities and the environment can change dramatically in very short periods of time. For this reason, the response time of the probe is critical to maintaining culture viability and to optimize induction. The results of this study indicate that the time of probe response to changing methanol concentration is independent of nitrogen flow rate at \(\leq 411\) ml/min. Based on this data for probe response time (in combination with the results for slope independent of flow rate), a nitrogen flow rate of 400 ml/min was chosen as the optimal signal and response time from the probe.

Fick’s First Law of Diffusion (Equation 4.1.3.(a)) can describe flux across a membrane as being directly proportional to the concentration gradient. This flow is impeded by boundary layers across the tubing surface (Figure 4.1.3.). Minimizing the boundary layer results in decreased resistance to diffusion and therefore better flux across the membrane. Agitation rate affects the liquid-silicone tubing boundary layer. Furthermore, increased agitation results in better mass transfer. As expected, response time decreased with increased agitation speed. Agitation rate experiments for both tubing sizes confirmed that agitation rates of 600 rpm or greater are optimal for the most rapid probe response time. For this reason, a minimum agitation speed of 600 rpm was chosen for methanol sensing in the 30 L reactor at 30°C without cells.

Tubing wall thickness inversely hinders diffusion across the silicone membrane (Equation 4.1.3.(a)). Replacing the 0.4 mm wall thickness tubing with 0.28 mm wall thickness tubing decreased the response time from 3.62 s cm\(^{-2}\) to 1.82 s cm\(^{-2}\) at 600 rpm. With tubing surface area of 97.8 cm\(^2\) and 93.0 cm\(^2\) for the 0.4 mm and 0.28 mm tubing, respectively, the response time was reduced from 5.6 minutes to 2.8 minutes. Both response times were
further improved from previously reported values for this probe of 10-12.5 minutes (Cueva Tello, 2012). It is important to note that the 0.28 mm wall thickness tubing is the smallest tubing that can be used with the current probe design due to the large diameter connections to the stainless steel tubing within the stainless steel rod. A next generation probe should consider these connections to accommodate thinner walled tubing.

The sterilization-in-place procedure has the ability to damage the silicone tubing as a result of high temperature and pressure. However, the study demonstrated that the current probe design was sufficiently robust such that the response time was not affected by several SIP cycles.

The time of probe response under low concentrations of methanol is a critical consideration for this process. It was shown that response time is constant at methanol concentrations of 0.4-1.0%. Concentrations lower than 0.4% had a strong influence on response time (P = 1.72*10^{-12}). This effect is expected since lower concentration will result in a smaller concentration gradient for diffusion of methanol across the silicone membrane. Concentrations greater than 0.4% may be limited by the permeability of the membrane for methanol resulting in a stable response time.

The maximum methanol stripping rate measured at 0.5% initial methanol concentration did not vary much over the agitation rates tested with an average stripping rate of 0.16 ± 0.04 mmol MeOH L^{-1} min^{-1}. In practice, it is likely that this value will be much lower due to the effect of the rapid methanol uptake rate of the cells resulting in a very low residual liquid-phase methanol concentration.
4.6. Conclusion

A minimum nitrogen flow rate of 411 ml/min was selected for methanol probe operation. It is important to note that this data was obtained in a stirred 2 L bottle at 25°C rather than in the reactor with air sparging. The effect of air sparging was not investigated in this study. Sparging can lead to stripping of methanol in solution and also affect methanol mass transfer rate across the liquid-silicone boundary layer, especially at low agitation rates.

The minimum agitation speed for fast probe response in 30 L of water at 30°C was 600 rpm. Silicone tubing with wall thickness of 0.28 mm reduced the response time by half compared to 0.4 mm wall thickness tubing. For this reason the 0.28 mm tubing was selected as the optimal tubing for the current probe.

Methanol induction will occur at high cell densities (>150 OD₆₀₀) and oxygen-enriched aeration conditions with airflow rates up to 30 slpm (standard liters per minute). Furthermore, protein induction may be optimal at lower temperatures, so the temperature set point during this phase could be less than 30°C. These factors are likely to influence probe response time. When the GBP for the expression strain is developed, the probe response conditions should be monitored along with induction phase optimization experiments.

The current design of the dissolved methanol probe is sufficiently durable after repeated sterilization with results showing SIP has no significant effect on response time. Low concentrations of methanol will reduce probe response time, however even at 0.2% methanol the response time of the improved probe was shown to be less than 3 minutes. Furthermore, future considerations during methanol feeding should minimally consider loss of methanol due to stripping, with an average methanol stripping rate of 0.16 ± 0.04 mmol MeOH L⁻¹ min⁻¹ in the cell-free bioreactor.
5. BIOREACTOR STARTUP AND CALIBRATION

5.1. Introduction

Bioreactor preparation for routine processing involves calibration, labeling, controller setup and testing, and drafting SOPs for operation.

5.1.1. Bioreactor Sterilization Procedure

Bioreactors must provide an aseptic environment for uncontaminated cultivation. A sterilization process is used to kill any adventitious organisms in the reactor. Stainless steel reusable bioprocess equipment must be sterilized-in-place using clean steam and high pressure over a prescribed time and temperature to ensure several log reduction of difficult to kill microorganisms.

5.1.2. Oxygen Mass Transfer

A well-mixed fermentation system will provide aeration and agitation for homogeneity of chemical nutrients, dissolved gases, and temperature to the growing cells. The ability of a vessel to supply oxygen is a critical parameter to sustain growth, maintenance, and bioproduct production in high-density fermentation cultures (Garcia-Ochoa and Gomez, 2010).

The maximum OUR (OUR_max) of an exponentially growing cells can be calculated using the maximum specific growth rate (μ_max, hr⁻¹), maximum biomass concentration (X_max, g/L), and biomass yield on oxygen (Y_X/O2, g/L-hr).
\[
OUR_{\text{max}} = \frac{H_{\text{max}} \cdot X_{\text{max}}}{Y_{X/02}}
\]

**Equation 5.1.2.(a)** Maximum oxygen uptake rate

The rate of transfer of a gas to the liquid phase is described by Equation 5.1.2.(b)(Flickinger et al.):

\[
Rate = \frac{\text{Driving Force}}{\text{Resistance}}
\]

**Equation 5.1.2.(b)** Rate of gas transfer to liquid phase

This relationship can be defined for oxygen transfer in a stirred bioreactor. Transport of oxygen from the gas phase to the cell is impeded by a number of mass transfer resistances (\(K_i\)). These resistances include transport within the bubble to the gas-liquid film, movement across the gas-liquid interface, diffusion through the liquid film surrounding the bubble, transport through the bulk liquid, diffusion over the liquid film surrounding the cell, and movement across the liquid-solid interface of the cell (Garcia-Ochoa and Gomez, 2010). However, since oxygen has low solubility in water, impedance to oxygen mass transfer is primarily due to the resistance through the boundary layer surrounding the bubble. Oxygen transfer rate can therefore be written as oxygen flux \(J_0\) per gas-liquid interfacial area \(a\), with the major driving force being the concentration gradient of oxygen in the gas phase \((C^*)\) to oxygen in the liquid phase \((C_L)\) (Garcia-Ochoa and Gomez, 2010):
\[ OTR = j_o a = k_L a(C^* - C_L) \]

**Equation 5.1.2.(c)** Oxygen transfer rate from gas to liquid phase in a stirred bioreactor

The oxygen transfer rate (OTR) of the bioreactor must exceed the cellular oxygen uptake rate (OUR):

\[ k_L a(C^* - C_L) > \frac{\mu_{\text{max}} \cdot X_{\text{max}}}{Y_{X/O_2}} \]

**Equation 5.1.2.(d)** Relationship between oxygen transfer requirements and a microbial culture’s oxygen uptake rate to maintain high biomass densities

It is important to note that volumetric mass transfer can be reduced by a factor of 4 in high cell density yeast broths (Galaction et al., 2010). In addition, the solubility of oxygen in water is reduced with increased temperature and salt concentration, thus further affecting oxygen transfer during fermentation processes (Flickinger et al.).

**5.1.3. Sulfite Oxidation for Determination of Oxygen Transfer Rate**

The volumetric oxygen mass transfer coefficient of a stirred tank reactor can be determined using a zero order chemical reaction that depletes oxygen from the bulk liquid. The sulfite oxidation method is a chemical determination based on the reaction:
\[ 2Na_2SO_3 + O_2 \xrightarrow{Ca^{2+}} 2Na_2SO_4 \]

**Equation 5.1.3.** Sulfite oxidation chemistry

This oxygen-consuming reaction allows for calculation of cell-free OTR or \( k_{La} \) based upon the time that it takes for oxygen to replenish within the system.

### 5.2. Purpose

The purpose of these procedures was to perform startup operations for a formerly idle 30 L bioreactor. Operating procedures, calibration, and equipment labeling were included in these operations.

### 5.3. Experimental Methods

#### 5.3.1. SIP and Sterility Test

The SIP procedure for the 30 L bioreactor (BTEC equipment number BR-2180) was previously programmed into the DCU. The procedure contains heating, sterilization hold at 121°C for 30 minutes, and cooling phases. This process requires approximately 3 hours to complete. As part of the operation portfolio for this equipment, an SOP was written for the execution of this step (Appendix 9.1.). This procedure was tailored to the *Pichia pastoris* process, including effects of SIP on methanol probe tubing durability and acceptance criteria for sterility. Triplicate sterility tests were performed using 600 g tryptic soy broth (TSB) with 4 mL Antifoam 204 at a total volume of 20 L. The rich media was sterilized using the SIP protocol and subsequently sampled and plated in triplicate on YPD. The plates were incubated at 37°C and checked for microbial growth after 24 hours.
Furthermore, media holds in the bioreactor were performed at 12 slpm airflow, 30°C, and fixed agitation at 400 rpm. These media holds were monitored with the batch recording software MFCS/win over time for changes in DO%.

5.3.2. DO Probe Response Time

DO probe (InPro® 6800 series, Mettler Toledo) response time was measured experimentally by placing the probe in a stirred 2 L bottle of high purity water at 23°C (Padrés-Angelats, 2012). Water was degassed with nitrogen followed by injection with air. The time for the signal to reach 90% of the stationary value was selected as response time.

5.3.3. Determination of Oxygen Transfer Rate

OTR was determined using the sulfite oxidation chemical method. The bioreactor was filled with 20 L HPW at 30°C. Airflow was set to 1 vvm CCA through the sparger. The DO probe was calibrated (2-point) under these conditions. Cobalt (II) chloride, 0.01 M, was added to the bioreactor at a concentration of 0.1% v/v and allowed to mix for 1 minute. Sodium sulfite was subsequently added at 3000 mg/L. The period of time when the DO was below 10% was timed and used as the reaction time for OTR calculation. This experiment was performed at both 20 L and 30 L and fixed agitation rates of 400, 600, 675, 750, 825, 900, and 1000 rpm.

5.3.4. DO Controller Test Method

Cascade control of agitation, aeration, and oxygen-enriched aeration was selected as the DO control scheme for the P. pastoris 30 L fermentation process. Agitation was designated to ramp up from the minimum rate of 600 rpm to a maximum of 850 rpm, followed by a ramp up of aeration from 12 to 25 slpm, and oxygen enriched aeration of 0-50% GFRC (gas
flow ratio control) to maintain dissolved oxygen tension at or above 30%. The controller was tested to ensure proper operation using sulfite oxidation to deplete oxygen from the liquid phase and monitor the response to deviations from the set point.

5.3.5. Comparison of Optek® and OD$_{600}$ Measurements

The Optek® in-line turbidity probe (ASD25-BT-N) was calibrated with offline OD$_{600}$ values against concentration units (CU) from a 20 L batch _Pichia pastoris_ fermentation using defined media. The CU output was compared with offline OD$_{600}$ values over time from a subsequent fermentation process and plotted together for comparison.

5.4. Results

5.4.1. SIP and Sterility Test

Of the triplicate sterility test runs, none of the YPD plates contained microbial growth after incubation at 37°C for 24 hours. Output from MFCS/win for one of the media holds is shown in Figure 5.4.1. Temperature remained constant at 30°C and DO% was maintained above 100%. The increased readings from the DO probe indicate drift in the signal from the probe. There is a drop to around 94% in the DO reading at 22 hours in response to a corresponding drop in aeration rate. Overall, dissolved oxygen was not depleted from the system during the media hold.
Figure 5.4.1. Media hold for sterility assurance. Fixed agitation at 400 rpm, fixed airflow at 12 slpm, and calibrated DO probe at 30°C. Depletion of oxygen over time would indicate microbial growth in the reactor.

5.4.2. DO Probe Response Time

Times required to reach 90% of the stationary value were 72, 69 and 96 s, with an average response time of 79 ± 14.8 s (Padrés-Angelats, 2012).
5.4.3. Determination of Oxygen Transfer Rate

OTR was calculated using Equation 5.4.3.:

\[
OTR = \frac{[Na_2SO_3] \text{ g/L} \cdot \left( \frac{16 \text{ g O}_2}{126 \text{ g Na}_2\text{SO}_3} \right)}{\text{Reaction Time (h)}}
\]

Equation 5.4.3. Calculation of oxygen transfer rate by timed sulfite oxidation method

Figure 5.4.3. shows OTR values obtained over agitation range tested at 30°C and atmospheric pressure. The OTR values for 20 L volume were consistently higher than those at 30 L with aeration with CCA (21% O\textsubscript{2}). Both volumes tested indicate a plateau in the OTR values obtained over 900 rpm. The relationship between OTR (g O\textsubscript{2}/L-h) and agitation speed at 20 L and 30°C is expressed by the equation \( y = 0.0105x - 1.4781 \). The 30 L relationship is described by the equation \( y = 0.00756x - 0.4984 \). Addition of 20% pure oxygen by GFRC (36.8% oxygenated aeration) further demonstrated an increase in oxygen transfer over non-enriched aeration, \( y = 0.0127x + 0.1298 \).
Figure 5.4.3. OTR for 30 L bioreactor with 20 L and 30 L of water at 30°C and atmospheric pressure over agitation speeds of 400-1000 rpm

5.4.4. DO Controller Test Method

The DO control cascade responded to the drop in dissolved oxygen below the 30% set point by staggering a ramp up in agitation, aeration, and oxygen-enriched aeration (GFRC). When the chemical reaction was complete dissolved oxygen rose above the 30% set point, the agitation, aeration, and GFRC inputs dropped back to the minimum established values. Figure 5.4.4. shows a simulated version of the cascade response.
5.4.5. Comparison of Optek® and OD<sub>600</sub> Measurements

The plot of OD<sub>600</sub> and Optek® turbidity (CU) measurements for the same P. pastoris fermentation run is shown in Figure 5.4.5. The CU response from the optek® probe breaks from the base about 5 hours into the fermentation and begins to plateau near 30 hours. Offline OD<sub>600</sub> values are delayed in response by about 10 hours from Optek® measurements. The slope of each line appears linear within the range of 17-25 batch
hours, but a plateau of Optek\textsuperscript{®} response follows as offline OD\textsubscript{600} readings continued increasing exponentially (indicated by red line). Figure 5.4.5. demonstrates that the correlation between the two is no longer linear at OD\textsubscript{600}≥10.

![Graph showing OD\textsubscript{600} and Turbidity measurements over time.](image)

**Figure 5.4.5.** Comparison of Optek\textsuperscript{®} in-line turbidity probe measurements to offline OD\textsubscript{600}.

5.5. Discussion

The sterility protocol was tested using both rich media plating and media hold monitoring. These experiments were performed in triplicate. There was no microbial growth on any of the triplicate YPD plates after incubation at 37°C for 24 hours. Additionally, the 24-hour TSB media hold experiments indicated no depletion of dissolved oxygen from the bioreactor at 30°C with fixed agitation and aeration. There was some upward drift in the DO reading from the probe over time, but it maximized at 104% dissolved oxygen. Probe drift can be a common problem in biomanufacturing systems and it is advisable to monitor drift from in-line probes over time, performing maintenance or replacement when drift exceeds a pre-established range.
Since the doubling time for yeast is typically 2-2.5 hours, a DO probe response time of 79 ± 14.8 seconds is appropriate for controlling dissolved oxygen in *Pichia pastoris* fermentation processes. Additionally, the control set point for dissolved oxygen in this process is 30%, which is above the critical range of <20% that could result in cell death. The response experiment was performed in a stirred bottle rather than in the bioreactor itself. The actual response time may be improved as a result of better mixing within the vessel. It is possible that the response time could affect oxygen replenishment time for sulfite oxidation OTR calculations, but this is unlikely since timing in this method is initiated and terminated at a specific depletion level of 10% dissolved oxygen.

The OTR experiments demonstrated that oxygen transfer is reduced with increased volume within the bioreactor. Greater oxygen transfer rates can be achieved by enriching air sparging with pure oxygen. This increases the concentration of oxygen in the gas phase (C*) thus increasing the driving force for oxygen transfer to the liquid phase (C_L). The *P. pastoris* fermentation increases in volume over time due to nutrient feeding. Therefore, oxygen transfer can be expected to decrease as culture volume increases from 20 to 30 L. In addition, biomass densities on the order of 200 OD_600 near the end of the glycerol fed-batch phase will increase the viscosity of the fermentation broth. This effect will further reduce volumetric oxygen mass transfer and require higher agitation rates supplemented with oxygen-enriched aeration to obtain oxygen transfer rates exceeding culture oxygen uptake rate. Since stabilization of OTR occurs at agitation rates greater than 900 rpm, 850 rpm was selected to be the maximum agitation rate for operation with a minimum of 600 rpm.

Dissolved oxygen control was selected at a minimum value of 30% based on recommendations from literature. Control was established using a cascade of agitation, aeration, and oxygen enriched aeration with GFRC. Agitation rates of 600-850 rpm were selected based on methanol probe response and OTR experiments. Aeration ramp up from
12-25 slpm was selected based on typical reported literature aeration rates of 0.5-1 vvm and up to 50% pure oxygen sparging with GFRC. Once the parameters were properly entered into the DCU, the cascade performed as expected.

Response from the Optek® turbidity probe was determined not robust for the *Pichia pastoris* fermentation process. Even at low concentrations of OD$_{600}$ = 10, the Optek® response to offline OD$_{600}$ measurements was no longer linear. This would greatly magnify error in the readings when cell densities reach OD$_{600}$ = 200 at the end of the glycerol biomass generation phase. It is important to have accurate measurement of cell density since growth modeling can be used to automatically trigger nutrient feeding and phase changeover. The Optek® probe used for this experiment uses NIR in the range of 840-910 nm for detection. This particular probe has a detection path length 10 mm. This path length is too long for high density microbial or yeast cultures and is much more appropriate for a mammalian cell culture application. Alternatively, a similar turbidity probe with a smaller (1 mm) path length would be more appropriate for the *P. pastoris* application and may have generated more reliable results in comparison with the offline OD$_{600}$ measurements.

### 5.6. Conclusion

The SIP procedure along with the sterility test method resulted in no microbial growth and these were documented in the form of an SOP for BR-2180 (Appendix 9.1.). The DO probe response time was measured to be 79 ± 14.8 seconds. This response time of less than 2 minutes should be appropriate for *P. pastoris* cultivation. Calibration of the bioreactor for oxygen transfer demonstrated lower OTR for 30 L volume compared with 20 L, but oxygen-enriched aeration at 30 L increased OTR over both non-enriched volumes tested. Operating conditions for effective oxygen control were selected and the DO controller was tested for proper operation using a chemical, oxygen-consuming reaction. Offline OD$_{600}$ and Optek®
turbidity probe measurements were not linear for *P. pastoris* fermentation even at low cell densities. For automated processing, there is need for new method for determining biomass density on-line such as a smaller path length in-line turbidity probe or a flow-through spectrophotometer.
6. BIOREACTOR GBP FERMENTATION DEVELOPMENT

6.1. Introduction

As previously described, *P. pastoris* fermentation commonly involves a three-phase fed-batch strategy. The glycerol biomass generation phase (GBP) is important for increasing cell densities prior to induction of protein expression (Figure 6.1.). Recombinant protein productivity is proportional to cell density; therefore it is important to have a large number of cells for high product yield. GBP is also referred to as the glycerol batch phase, however in order to achieve high biomass densities glycerol feeding is necessary. For this reason GBP is commonly executed as a batch followed by fed-batch process.

![Typical three-phase P. pastoris fermentation highlighting the glycerol biomass generation phase (GBP) (Adapted from Cos et al., 2006)](image)

**Figure 6.1.** Typical three-phase *P. pastoris* fermentation highlighting the glycerol biomass generation phase (GBP) (Adapted from Cos et al., 2006)

Once the GBP has been developed and optimized, a transition into the methanol induction phase is implemented. The MIP can be developed at smaller scale concurrently with GBP development. Selection of MIP strategy involves screening methanol induction concentrations for optimal expression of recombinant proteins. The purpose of a transition
is to sustain cell viability while derepressing the AOX1 promoter. When derepression occurs in the presence of low concentrations of methanol, basal expression of alcohol oxidase allows cells to adapt to methanol feeding in preparation for MIP protein induction.

6.2. Purpose

The purpose of these experiments was to develop an environment for optimal biomass generation during the GBP in the 30 L bioreactor. Considerations include bioreactor cultivation temperature, pH, and DO control along with media composition, nutrient feeding and inoculum viability.

6.3. Experimental Methods

6.3.1. Media

Defined media for P. pastoris cultivation was referenced from literature and adapted for cultivation of GS115 His' Mut+. These include a salts media (BFM21*) and Pichia Trace Minerals (PTM). Sodium citrate was added to BFM21* as a chelating agent to prevent precipitation of media components. In addition, shake flask studies were performed to calculate biomass yield on key media components glycerol, ammonium sulfate, and histidine. Fed-batch media was developed using a concentrated formulation of the batch media. The optimized batch and fed-batch media composition is displayed in Table 6.3.1.
Table 6.3.1. *P. pastoris* defined media composition (a) Batch media (b) PTM₄ (c) Fed-batch media

(a) **Batch Media**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₃PO₄, 85%</td>
<td>3.5 mL/L</td>
</tr>
<tr>
<td>CaSO₄</td>
<td>0.12 g/L</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>2.4 g/L</td>
</tr>
<tr>
<td>MgSO₄•7H₂O</td>
<td>1.95 g/L</td>
</tr>
<tr>
<td>KOH</td>
<td>0.655 g/L</td>
</tr>
<tr>
<td>Sodium Citrate</td>
<td>2.94 g/L</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>6.00 g/L</td>
</tr>
<tr>
<td>NaOH</td>
<td>1.54 g/L</td>
</tr>
<tr>
<td>Acetate Buffer, 1 M</td>
<td>40 mL/L</td>
</tr>
<tr>
<td>Glycerol</td>
<td>40.0 g/L</td>
</tr>
<tr>
<td>PTM₄</td>
<td>87 mL</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>0.400 g</td>
</tr>
<tr>
<td>Antifoam 204</td>
<td>0.2 mL/L</td>
</tr>
</tbody>
</table>

(b) **Pichia Trace Minerals 4 (PTM₄ Composition)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂SO₄</td>
<td>1 mL/L</td>
</tr>
<tr>
<td>CuSO₄•5H₂O</td>
<td>2 g/L</td>
</tr>
<tr>
<td>NaI</td>
<td>0.08 g/L</td>
</tr>
<tr>
<td>MnSO₄•H₂O</td>
<td>3 g/L</td>
</tr>
<tr>
<td>Na₂MoO₄•2H₂O</td>
<td>0.2 g/L</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.02 g/L</td>
</tr>
<tr>
<td>CaSO₄•2H₂O</td>
<td>0.5 g/L</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>0.5 g/L</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>7 g/L</td>
</tr>
<tr>
<td>FeSO₄•7H₂O</td>
<td>22 g/L</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.2 g/L</td>
</tr>
</tbody>
</table>

(c) **Feed Media**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>40 g/L</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>120 g/L</td>
</tr>
<tr>
<td>PTM₄</td>
<td>87 mL</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>0.400 g</td>
</tr>
</tbody>
</table>
6.3.2. Bioreactor Batch Fermentation

Shake flask media was scaled up to 20 L in the bioreactor and autoclaved in the vessel using the SIP procedure. Two, 2 L shake flasks with 200 ml media were inoculated with 1 mL cryopreserved *P. pastoris* and incubated at 30°C and 400 rpm for approximately 18 hours. Inoculum was transferred in a biosafety cabinet into an autoclaved 1 L glass bottle with transfer cap. The initial inoculation procedure involved using a diaphragm valve connected to the transfer assembly on the glass bottle, steaming the valve onto the bioreactor feed port and allowing the connection to cool. Upon cooling, a peristaltic pump was used to transfer the inoculum into the bioreactor. The first bioreactor batch fermentations were carried out at 30°C, fixed aeration at 1 vvm CCA, fixed agitation at 600 rpm and pH control via buffered medium (no automatic pH control). These fermentations were allowed to proceed until stationary phase occurred, approximately 22 hours following inoculation.

6.3.3. Rapid (5-6 Minute) Inoculation Procedure

Subsequent batch runs were performed using a rapid inoculation procedure to improve inoculum health (minimize O₂ starvation). The inoculum transfer bottle was prepared with weld-able tubing (Cole Parmer 06422-05) on the transfer cap. A diaphragm valve was prepared with a transfer line of weld-able tubing and steamed onto feed port. This connection was allowed to cool prior to removal of inoculum flasks from the incubated shaker. During inoculum transfer, the inoculum was placed on heated stir plate at 30°C and agitated manually agitated during the tube welding and inoculation processes.

6.3.4. Automatic pH Control at 5.0

Buffered medium is not sufficient to maintain pH as cell density increases. Automatic control of pH at 5.0 was implemented using 5 M NaOH. Sodium hydroxide was selected
over ammonium hydroxide in order to maintain defined concentration of nitrogen in the fermentation broth. The 5 M concentration was selected in order to minimize dilution from pH control.

6.3.5. Fed-Batch Fermentation

The fed-batch fermentations were initiated the same as the batch fermentations with the addition of bolus feeding with concentrated fed-batch media (Table 6.3.1.(c)). Residual glycerol was monitored during the fermentation process using a Seg-Flow® autosampler integrated with YSI 7100 MBS™ technology. Feeding occurred manually in response to YSI measurement <30 g glycerol/L.

6.3.6. DO Control Cascade

DO control at 30% DOT was implemented by cascade control of agitation (600-850 rpm), aeration (12.5-25 slpm), and oxygen-enriched aeration (0-50% pure oxygen by GFRC).

6.3.7. Process Repeatability

To investigate the repeatability of the process, three identical fed-batch runs were executed using the developed methods: rapid inoculum transfer, automatic pH control at 5.0, bolus feeding, and DO control cascade at 30%. Samples were collected every two hours for OD₆₀₀ measurement.
6.4. Results

6.4.1. Bioreactor Batch Fermentation

Batch phase fermentations (n=4) showed consistent final $OD_{600} = 13.0 \pm 5.1$ over $36.5 \pm 6.9$ recorded batch hours (Figure 6.4.1.). Stationary phase was reached after 22 hours of batch fermentation on defined media.

![Graph showing batch fermentation runs achieving stationary phase](image)

**Figure 6.4.1.** Batch fermentation runs achieving stationary phase after 22 hours and with average final $OD_{600} = 13.0 \pm 5.1$

6.4.2. Rapid Inoculation Procedure

Figure 6.4.2. compares growth of batch cultures with differing inoculation times (>10 minutes, 9.7 minutes, 6.8 minutes). Transfer time was decreased from >10 minutes to minimum of 3 min 40 s, with an average of 5-6 minutes (not pictured). Growth lag was
minimized with faster transfer times. In addition, the maximum specific growth rate ($\mu_{\text{max}}$) was increased from 0.228 hr$^{-1}$ to 0.261 hr$^{-1}$ when implementing the faster inoculum transfer method. This led to a maximum increase in specific growth rate by 12.6%.

![Figure 6.4.2. Effects of inoculum transfer on growth lag and maximum specific growth rate ($\mu_{\text{max}}$)](image)

6.4.3. Automatic pH Control at 5.0

Figure 6.4.3.(a) demonstrates that buffered medium alone was not sufficient to control pH at 5.0 with OD$_{600} > 9.3$. Addition of automatic pH control with 5 M NaOH overall maintained pH up to OD$_{600} < 100$ in fed-batch cultures (Figure 6.4.3.(b)). However, two of the three fermentation runs represented in Figure 6.4.3.(b) show a dip in pH control at 15 hours. A spike in the pH is also present at the end of two of the three fermentations.
Figure 6.4.3. pH Control (a) Batch fermentation with control using buffered media only (b) Triplicate fed-batch fermentations with automatic control at 5.0 by 5 M NaOH addition
6.4.4. Fed-Batch Fermentation

Bolus fed-batch feeding of glycerol, PTM₄, and histidine increased OD₆₀₀ from an average of 13 up to 176 using residual glycerol monitoring. Figure 6.4.4. shows progression of a fed-batch fermentation run with stepwise addition of feed media in response to depletion of glycerol. Even though the dissolved oxygen became limiting, the final cell density achieved was OD₆₀₀ = 176.

![Graph showing fed-batch fermentation run with residual glycerol monitoring](image)

**Figure 6.4.4.** Fed-batch bioreactor run with residual glycerol monitoring
6.4.5. DO Control Cascade

Addition of DO control at 30% DOT by supplementing the agitation and aeration control cascade with oxygen-enriched aeration further increased OD\textsubscript{600} in fed-batch cultures when compared with fixed agitation and aeration (Figure 6.4.5.). Fermentations with fixed aeration at 1 vvm (25 slpm) and fixed agitation of either 600 or 800 rpm resulted in oxygen limitation after 20 hours of fed-batch fermentation. Maximum OD\textsubscript{600} of each were 137 and 121, respectively. Addition of oxygen-enriched aeration maintained DO at 30% and achieved final cell densities exceeding 193 OD\textsubscript{600}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.4.5}
\caption{Comparison of fixed DO control to cascade control at 30% DOT}
\end{figure}


6.4.6. Process Repeatability

Figure 6.4.6.(a) shows the combined data from the triplicate runs. All ln(OD$_{600}$) measurements were averaged based on sample time and plotted together to show error in the measurement. Using this comparison method, the sample points have an average maximum specific growth rate of 0.2537 h$^{-1}$ with a coefficient of determination of 0.9842. The separate data points from each run are plotted in Figure 6.4.6.(b). These points all fall along the same line, with an average slope of 0.252 ± 0.002 h$^{-1}$, consistent with the averaged data. The final OD$_{600}$ for these runs was 192.0 ± 42.0 over 29.8 ± 0.8 hours.
Figure 6.4.6. Process repeatability triplicate fermentations (a) Averaged data points (b) Individual data points
6.4.7. Batch Evolution

The combination of offline OD<sub>600</sub> measurements over time for <i>P. pastoris</i> bioreactor runs is shown in Figure 6.4.7. Batch runs ended in OD<sub>600</sub> = 13.0 ± 5.1, fed-batch without DO control maintaining 30% achieved a maximum of OD<sub>600</sub> = 176, and fed-batch with DO control 192.0 ± 42.0.

![Graph showing batch evolution](image)
6.5. Discussion

Inoculum viability is critical for an optimized, robust fermentation process. The implementation of a faster inoculum transfer method minimized the growth lag after inoculation, thus increasing the maximum specific growth rate ($\mu_{\text{max}}$) was from $0.228 \text{ hr}^{-1}$ to $0.261 \text{ hr}^{-1}$.

Automatic pH control with 5 M NaOH was overall successful in maintaining pH over the course of the fed-batch fermentation. The dip in pH control shown in Figure 6.4.3.(b) was possibly the result of exceeding the buffering capacity of the media and insufficient priming of the base addition line. The increased pH near end of fermentation could be due to over-titrating or a misreading from the pH probe due to a failure in temperature control system during these runs.

The growth plateau at $\text{OD}_{600} = 13.0 \pm 5.1$ for the four batch fermentation runs was likely due to nutrient exhaustion in the batch media. Subsequent addition of bolus nutrient feeding significantly increased the final cell density to $\text{OD}_{600} = 176$. Maintenance of dissolved oxygen at 30% allowed biomass densities even greater at $192.0 \pm 42.0 \text{ OD}_{600}$.

6.6. Conclusion

Addition of faster inoculum transfer, bolus feeding, DO control cascade at 30% DOT, automatic pH control at 5.0 with 5 M NaOH achieved minimized growth lag and cell densities exceeding $\text{OD}_{600}$ of 190. Furthermore, maximum specific growth rates near $0.26 \text{ h}^{-1}$ were regularly achieved and are comparable to values reported in literature for $P. \textit{pastoris}$ during the GBP (Jahic $et \ al.$, 2006).
7. FUTURE WORK

7.1. Recombinant Protein Expression

This project recently acquired a strain GS115 His\(^+\) Mut\(^s\) that secretes human serum albumin (67 kDa) (Invitrogen Co., 2010). It is said to express at levels greater than 1 g/L (Invitrogen Co., 2010). The next researchers will investigate growth of this model strain on the defined media (Table 6.3.1.) in shake flask cultures during the GBP and develop the MIP using shake flask fermentation. Further MIP investigations can be scaled down to microreactor cultivation (Micro-24, Pall Corp.). Consideration for this development will include the difference in doubling time on methanol for Mut\(^+\) compared with Mut\(^s\) strains, reported to be approximately 5 hours compared to 18, respectively (Invitrogen Co. 2010). In addition, development optimized bioreactor operating conditions can be selected for specific protein expression by considering induction temperature, pH, and methanol induction levels at the microreactor scale. This type of high-throughput screening can be useful for optimization of fermentation processes and protein expression of new recombinant *P. pastoris* strains by simply modifying the process developed in this thesis.

7.2. Closed-Loop Methanol Control

The current methanol control system for the bioreactor has the capability of sensing and data acquisition of methanol concentration, but is missing the feedback control function. Future researchers will write a logic control algorithm for methanol induction that will maintain dissolved methanol concentration at specific induction levels as determined by MIP optimization on the expressing strain (e.g. 0.4% v/v). The probe signal should trigger a programmable logic controller (PLC) to actuate a peristaltic pump to turn on/off methanol feed to maintain the control set point.
7.3. At-line OD$_{600}$ Detection using PendoTECH Turbidity Sensor

This project will soon acquire a custom at-line turbidity sensor that has a 1 cm path length spectrophotometer set at 600 nm (PendoTECH). This sensor will need to be implemented into the _P. pastoris_ fermentation system using a sampling probe withdrawing cells and media (e.g. Fisp®) and calibrated against offline OD$_{600}$ measurements. The limit of quantification for the detector will need to be determined by measuring the maximum cell concentrations by which the absorbance is no longer linear with cell density. The unit will very likely need to be coupled to a dilution module to ensure cell density remains below the maximum limit of quantification of the detector. Data acquired from this sensor will be used to model _P. pastoris_ growth and implemented for controlled feeding, transition to new phases, and determination of harvest time.

7.4. Exhaust Gas Monitoring

The BlueSens gas monitoring system that is currently installed on the inlet and exhaust gas lines needs to be calibrated and implemented into the process data acquisition system. Exhaust gas monitoring using BlueSens technology can be used to collect additional process information such as carbon evolution (CER), oxygen uptake rate (OUR), and respiratory quotient (RQ).
8. REFERENCES CITED


APPENDIX A: BR-2180 Sterilization-In-Place for *Pichia pastoris* Fermentation
with Dissolved Methanol Probe
1. PURPOSE:

1.1. Protocol for performing SIP and sterility testing on BR-2180, BIOSTAT C-DCU3 30 L bioreactor with dissolved methanol probe. Procedure includes media addition, pressure hold test, sterilization-in-place (SIP), media hold, and sterility analysis.

2. SCOPE:

2.1. This procedure applies to all operations staff, faculty, students and other BTEC staff utilizing BR-2180, BIOSTAT C-DCU3 30 L bioreactor, for performing any of the following: sterility testing, sterilization-in-place, and pressure hold procedures.

3. DEFINITIONS:

3.1. CF – Chilled Fluid
3.2. DCU – Digital Control Unit
3.3. DO – Dissolved Oxygen (e.g. DO probe)
3.4. HPW – High Purity Water
3.5. HV – Hand Valve
3.6. IA – Instrument Air
3.7. Media Hold – Process by which all media components required for fermentation are heat and/or filter sterilized and subjected to a defined hold time at fermentation operating conditions
3.8. SIP – Sterilization-in-Place
3.9. Sterile – Absence of biological material
   3.9.1. Pass – No colony formation on rich media agar plates after 24 hours
   3.9.2. Fail – Colony formation on 2 of 3 rich media agar plates
   3.9.3. Re-sample - Colony formation on 1 of 3 rich media agar plates

4. RESPONSIBILITIES:

4.1. All operations staff, faculty, students, and other BTEC staff utilizing BR-2180 for P. pastoris fermentation are responsible for following procedures as described by this SOP.

5. SAFETY:

5.1. Follow all applicable safety practices and MSDS when executing this procedure
5.2. Use caution when working in and around steam lines or reactor, outside surfaces may be very hot
5.3. Use caution when working with reactors, vessel may be under pressure
5.4. Ensure vessel assemblies are in correct position and all valves are closed
5.5. Ensure proper documentation and equipment status tag guidelines are followed

6. MATERIALS:

6.1. BR-2180, Sartorius BIOSTAT C-DCU3 30 L Bioreactor
6.2. Media Components, including antifoam
6.3. Probes
   6.3.1. Antifoam
   6.3.2. Methanol
   6.3.3. pH
   6.3.4. DO
   6.3.5. Temperature
6.4. Utilities
   6.4.1. CCA
   6.4.2. Clean steam
   6.4.3. Nitrogen gas
   6.4.4. City water
   6.4.5. HPW
6.5. Filters
   6.5.1. Overlay (optional)
   6.5.2. Sparge (part number: 39236048)
   6.5.3. Exhaust (part number: 39236056)
6.6. CF
6.7. Chiller
6.8. Heat resistant insulated gloves
7. **PROCEDURE:**

![Figure 1: BR-2180, BIOSTAT C-DCU3](image)

#### Figure 1

A. Hydrocarbon Sensor  
B. Agitator drive motor  
C. High foam probe  
D. Exhaust air filter housing  
E. Feed port  
F. Inlet air filter housing  
G. Foam probe  
H. Methanol probe  
I. Digital Control Unit (DCU)  
J. Temperature probe  
K. Sample port  
L. Harvest drain  
M. OPTEK drain  
N. DO probe  
O. pH probe

7.1. Ensure inlet air CCA is open and N\textsubscript{2} is closed.
7.2. Clean bioreactor BR-2180  
7.2.1. Wipe down exterior  
7.2.2. Rinse interior with HPW  
7.3. Calibrate pH probe using a 2-point procedure  
7.3.1. If the probe is stored *in situ*, ensure the bioreactor is empty by opening the drain valve
7.3.2. Unscrew the probe from the bioreactor entry port
7.3.3. On the DCU control screen, select CALIBRATION
7.3.4. Select pH PROBE
7.3.5. Select CALIB
7.3.6. Select START CALIB
7.3.7. Select MANU TEMP COMP
7.3.8. Select TMP CO
7.3.9. ENTER current ambient temperature value
7.3.10. Buffer 7.0 ENTER
7.3.11. Place the pH probe in a beaker or 50 ml tube with pH 7.0 buffer and gently swirl
7.3.12. When mV reading is stable, press ENTER
7.3.13. Rinse the probe with deionized water
7.3.14. Buffer 4.0 ENTER
7.3.15. Place probe in a beaker or 50 ml tube with pH 4.0 buffer and gently swirl
7.3.16. When mV reading is stable, press ENTER
7.3.17. Press MAIN to return to the main screen
7.3.18. Insert the probe into the aseptic housing on the sidewall of the reactor
7.4. Ensure DO, Temperature, and Methanol probes are installed
7.5. Ensure DO, pH, probes are disconnected from control unit
7.6. Ensure temperature probe and high foam sensors are connected to control unit
7.7. Soft Parts
7.7.1. Check the integrity of all soft parts, ensure they are in place, and all connections are tight
7.7.2. Septa may undergo sterilization 5 times prior to replacement
7.7.2.1. Track SIP cycle count by marking a circle on top of septa using permanent marker. Circles should be added in a clockwise manner. New septa will be marked with a line upon installation
7.7.3. Any septa that are visually damaged from frequent use or punctured with a needle must be replaced
Title: BR-2180 Sterilization-In-Place for *Pichia pastoris* Fermentation with Dissolved Methanol Probe

Revision: 3
Effective Date: 12-09-13

Figure 2: Probe setup for fermentation

A. Outlet N₂ (to hydrocarbon sensor)
B. Liquid trap
C. Inlet N₂ flow (from in-house N₂)
D. Inlet filter
E. Inlet and outlet valves (open)

Figure 3: Probe setup for SIP

A. Hydrocarbon sensor
B. Inlet N₂ line (connected to hydrocarbon sensor)
C. Inlet and outlet valves (closed position)
7.8. Prepare Methanol Probe for SIP

7.8.1. Disconnect N₂ inlet and reconnect to hydrocarbon sensor

7.8.1.1. NOTE: Hydrocarbon sensor should have N₂ gas flowing through at all times

7.8.2. Close inlet and exit valves on top of probe

7.8.3. Disconnect probe condensate trap

7.9. Open computer batch record program

7.9.1. MFCS Shell

7.9.2. Run

7.9.3. Operator Service

7.9.4. Right click BIOSTAT C

7.9.5. Start Batch

7.9.6. Name batch (e.g. Pichia_MMDDYY_RunXX)

7.10. Perform pressure hold test

7.10.1. Ensure air supply (CCA) is open. If not, turn the HV located on the wall to “On” position (parallel to piping)

7.10.2. Ensure all valves are closed on the vessel

7.10.3. Place Sparge Filter into Sterilization Mode

7.10.4. At the PLC unit, ensure Main Menu is displayed

7.10.5. From Main Menu select Airflow 1. Set flow rate at 10 L/min. Next select Mode and select auto.

7.10.6. From Main Menu select Maintenance, then Manual Operation. Enter passcode “9”. Select Dig. Out., scroll down to V501, change Mode to Manual, select OFF. This will close the exhaust solenoid valve. Return to main screen. Pressure will start to build up in the vessel

7.10.7. Once vessel pressure from main screen reaches above 1000 mbar, go to Airflow 1 and change the mode to OFF. This will stop the air supply to the vessel. Perform a visual check on the pressure gauge located off the exhaust line. Pressure gauge should read 1 bar

7.10.8. Go back to main menu screen and wait for pressure to stabilize. Once pressure has stabilized, pressure hold time is started

7.10.8.1. NOTE: Minimum hold time on pressure hold is 30 minutes. If pressure decreases more than 17 mbar, the test has failed. Check reactor to ensure all ports are secure and perform pressure hold again. If problem continues consult the lab manager

7.10.9. To depressurize vessel from main menu, select Maintenance, select Manual Operation. Select “9” when prompted for password. Select Dig. Out., scroll down to V501, change Mode to AUTO. This opens the exhaust solenoid valve and allows the vessel to depressurize
7.11. Chiller unit preparation

7.11.1. Drain condensate from chiller using screwdriver to open drainage port
7.11.2. Water and CF will separate in collection vessel. Water (bottom layer) and CF (top layer)
7.11.3. Drain until all condensate is coolant (no bubble formation)
7.11.4. Turn on chiller unit by selecting both power switches and pressing OK on chiller control screen. Set to -20°C
7.11.5. Allow approximately 20 minutes to reach set point temperature

7.12. Exhaust air cooler
7.12.1. Close exhaust coolant valve (HV512)
7.12.2. Disconnect exhaust coolant lines and connect together
7.12.3. Drain exhaust cooler jacket by connecting tubing with a male adaptor to female connector into collection vessel
A. Heat exchangers
B. Jacket overflow valve

A. Jacket Overflow
B. Jacket Pressure Gauge
7.13. Rinse Jacket
7.13.1. Open top jacket overflow valve (HV323)
7.13.2. Open inlet jacket water valve (HV326)
7.13.3. Open cool water inlet from sink
7.13.4. Run a few minutes to drive out any trapped air
7.13.5. Close overflow valve (HV323)
7.13.6. When jacket pressure reaches just above 0.5 bar, close inlet valve (HV326)
7.13.7. Shut water off
7.13.8. Open overflow slowly until pressure reaches 0.5 bar (HV323)

7.14. Tare bioreactor
7.14.1. Ensure harvest valve and sample port are closed
7.14.2. From Main Menu, select CALIBRATION
7.14.3. Select WEIGHT
7.14.4. Select START TARING
7.14.5. Add heat sterilizable media components and fill to desired volume
7.14.5.1. NOTE: Dissolve media in several (3-5) liters of HPW prior to addition

7.15. Equipment Status Tags
7.15.1. Update BR-2180 and Methanol probe status tags for “SIP IN Progress”
7.15.2. Complete any additional forms as described by area Lab Manager

7.16. Sterilization-in-place
7.16.1. Open clean steam valve and verify that pressure gauge is reading 40 to 45 psi.
7.16.2. Check agitator seal sight glass to ensure level is half full before starting agitator. If level needs to be filled open nupro valve (NPAS001) of the clean steam reservoir for seal housing lubrication
7.16.3. Ensure sparge filter housing into sterilization mode
7.16.4. Ensure instrument air and sparging air supplies are on. If not, turn on the air supplies at the hand valves on the wall
7.16.5. From Main Menu, select Temp. Next select Mode and place into Auto. Select Temp. and set temperature to 30°C
7.16.6. From Main Menu, select
7.16.6.1. Batches
7.16.6.2. Vessel
7.16.6.3. State
7.16.6.4. Start
7.16.6.5. NOTE: Monitor jacket and vessel temperatures during SIP
7.16.7. Vessel phases are displayed and heating starts. Once the vessel has reached the set-point of 121°C (approx 45 min after start) the following valves need to be opened:
7.16.7.1. Steam supply to sample port (HV106)
7.16.7.2. NOTE: Use caution when working near hot bioreactor vessel and steam lines
7.16.7.3. NOTE: Ensure valve SV25 (hand valve to vessel) is closed and steam trap is in place on sample port
7.16.7.4. Steam supply to Inoc./Transfer addition port (HV191)
7.16.7.5. Inoc./Transfer addition condensate valve (HV192)
7.16.7.6. Steam supply to harvest/bottom valve (HV102)
7.16.7.7. NOTE: Ensure bottom valve is closed and steam trap is in place
7.16.7.8. Perform temperature checks on vessel using temp stick or viewing trend off local workstation every 15 minutes
7.16.7.9. NOTE: If melts are not attainable, troubleshoot area to ensure condensate/steam traps are functioning correctly. If problem continues, contact lab manager
7.16.10. Upon successful completion of sterilization (30 min), close the following valves:
7.16.10.1. Steam supply to harvest/bottom valve (HV102)
7.16.10.2. Inoc./Transfer addition condensate valve (HV192)
7.16.10.3. Steam supply to harvest/bottom valve (HV102)
7.16.10.4. Steam supply to sample port (HV106)
7.16.11. COOL1
  7.16.11.1. Once vessel reaches COOL1, from the control panel Main Menu select AIRFL1
  7.16.11.2. Set airflow to 6 slpm (compensates pressure drop from cooling)
7.16.12. COOL2
  7.16.12.1. Once vessel reaches COOL2 and temperature is ≤80°C, reconnect coolant lines on the exhaust cooler
  7.16.12.2. Open coolant valve (HV512)
7.16.13. End of SIP – Acknowledge by selecting ACK
  7.16.13.1. Once the vessel has reached fermentation temperature (30°C), change the Julabo chiller temperature to -18°C.
  7.16.13.2. Update Status Tag to “SIP Complete” and complete any other required forms
7.17. Aseptically transfer any filter sterilized (heat sensitive) media components
  7.17.1. Use latex or neoprene gloves rinsed with an alcohol solution (e.g. 70% ethanol)
  7.17.2. Remove tri-clamp from the valve cap of the feed port, keeping the cap and gasket in place
  7.17.3. Remove the protective covering from the connection on the end of the media tubing
7.17.4. Remove the valve cap and replace with the connection on the media tubing
7.17.5. Ensure that the gasket is aligned and replace the tri-clamp
7.17.6. Open the steam and condensate return hand valves to steam the connection
7.17.7. Steam connection for 20 minutes
7.17.8. Close steam and condensate hand valves and allow to cool
7.17.9. Open both inoculum/transfer hand valves
7.17.10. Feed media using peristaltic pump
7.17.11. Once media bottle is empty, close the valve on the tubing and the inoculum/transfer hand valves
7.17.12. Undo the tri-clamp, remove the tubing connection valve and replace the end cap
7.17.13. Open the steam and condensate return hand valves to steam the connection
7.17.14. Steam connection for 20 minutes
7.17.15. Close steam and condensate hand valves and allow to cool

7.18. Connect DO and pH probes to control unit
7.19. Calibrate DO probe
7.20. Prepare base for pH control
7.20.1. Connect output line from base transfer bottle into peristaltic pump on control unit
7.20.2. Press pump on manual to prime the line to right before the vessel
7.20.3. Set base bottle on a scale to keep track of weight loss
7.20.4. Turn on pH control to Auto
7.20.5. Turn pump control to Auto

7.21. Sterility Test
7.21.1. Set air inlet to Fermentation mode
7.21.2. Select fermentation operating parameters (e.g. 400 mbar, 1 vvm, 600 rpm, 30°C, pO2 Setpoint = 30 % (“Auto” Mode)
7.21.3. Hold for 24 hours
7.21.4. Sampling
7.21.4.1. Steam sample port 1 minute
7.21.4.2. Open plunger to sample. Discard first 50 ml. Sample another 50 ml. Use care to minimize contamination from air into sample
7.21.4.3. Steam sample port 5 minutes after sampling
7.21.4.4. NOTE: It is best to sample using a sterile sample assembly
7.21.5. Plate in triplicate using biosafety cabinet on rich media (e.g. YPD)
7.21.6. Incubate plates at 37°C for 24 hours
7.21.7. If no colony formation occurs, sterility test has passed
7.21.8. If colony formation occurs on only one plate, re-sample and plate for 12 hours to ensure sterility
7.21.9. If colony formation occurs on two or three plates, the pressure hold and SIP procedures must be repeated
7.21.10. Batch history plots acquired through MFCS Shell may also be used to determine contamination, where pH, DO, and Turbidity drifts may suggest biological growth.

7.22. Prepare methanol probe for fermentation

7.22.1. Re-direct \( \text{N}_2 \) gas line through Methanol probe

7.22.2. Open inlet and exit valves

7.22.3. Allow \( \text{N}_2 \) to flow through probe for 10 minutes

7.22.4. Close inlet and exit valves

7.22.5. Disconnect and re-weigh liquid trap

7.22.5.1. If liquid trap weight increases by more than 1.6 g (internal tubing volume), the tubing must be replaced prior to use of probe

7.22.5.2. Common gain is 0.2-0.3 g

7.22.5.3. Note weight increase on probe status tag next to SIP cycle count

7.22.6. Re-connect liquid trap

7.22.7. Open inlet and exit valves

7.23. Media Hold

7.23.1. A media hold at fermentation conditions may be necessary while preparing inoculum

7.23.2. Media must remain at fermentation conditions during the hold time

7.23.3. Maximum hold time is 72 hours post-sterilization

7.23.4. If hold time exceeds 72 hours, drain the vessel, rinse well and repeat the sterility test procedure with fresh media

8. REFERENCES:

- Sartorius Biostat® C-DCU3 Bioreactor Operating Manual
- Methanol Probe Care SOP
- Preparation of \textit{P. pastoris} glycerol batch media for 30 L Fermentation SOP
APPENDIX B: *Pichia pastoris* 30 L Bioreactor BR-2180 Inoculum Transfer Procedure
1. PURPOSE:
   1.1. Protocol for performing *Pichia pastoris* inoculum transfer from 2 L shaker flask to 30 L fermentor BR-2180.

2. SCOPE:
   2.1. This procedure applies to all operations staff, faculty, students and other BTEC staff utilizing BR-2180, BIOSTAT C-DGU 30 L bioreactor for performing *Pichia pastoris* fermentation.

3. DEFINITIONS:
   3.1. N/A

4. RESPONSIBILITIES:
   4.1. All operations staff, faculty, students, and other BTEC staff utilizing BR-2180 for *P. pastoris* fermentation are responsible for following procedures as described by this SOP.

5. SAFETY:
   5.1. Follow all applicable lab safety practices when executing this procedure.
   5.2. Wear appropriate PPE at all times when in the lab.
   5.3. Use care when working with vessels under pressure.

6. MATERIALS:
   6.1. 500 mL glass bottle
   6.2. Weld-able Tubing (Cole Parmer 06422-05), 3 feet
   6.3. ½” diaphragm valve
   6.4. Zip ties
   6.5. Zip tie fastener
   6.6. Sanitary clamp
   6.7. ½” hose barb fitting
   6.8. ½” gasket
   6.9. Autoclave paper, 2, 4”x4” squares
   6.10. Autoclave tape
   6.11. Rubber band
   6.12. Transfer cap with rubber gasket
   6.13. Tubing welder
   6.14. ¼” tubing welder cassettes
   6.15. Blade
   6.16. Peristaltic pump
7. PROCEDURE:

7.1. Prepare transfer valve by connecting 3 feet of weld-able tubing to ½” hose barb fitting and fixing tightly with zip tie

7.2. Attach fitting to ½” diaphragm valve with ½” gasket and sanitary clamp

7.3. Cover valve opening with 4”x4” square and fix with rubber band

7.4. Leave valve open during autoclave cycle

7.5. Put clamp on tubing end (leave open)

7.6. Cover tubing end tightly and wrap with autoclave tape

7.6.1. Leave end of tubing open

7.6.2. Cover tubing

7.6.3. 20 minutes after autoclave cycle completion close clamp and valve

7.7. Prepare glass transfer bottle by attaching weld-able tubing:

7.7.1. Dip tube inside bottle, cut ½-1” above bottle lid

7.7.1.1. Cut dip tube opening at angle

7.7.2. Attach 3” weld-able transfer tubing to hose barb opposite dip tube opening

7.7.2.1. Fix with zip tie

7.7.2.2. Tape and clamp open tubing end

7.7.3. Attach vent filter to remaining external hose barb

7.7.3.1. Ensure vent is not attached to dip tube

7.7.3.2. Filter should be oriented with writing faced up
7.8. Autoclave with clamp open on dry goods cycle
7.9. After ~20 minutes, close clamp
7.10. Steam autoclaved transfer fitting to feed port of fermentor
7.11. Allow to cool for 10 minutes
7.12. Prepare tubing welder by turning on and closing cover to ensure alignment of tube cassettes
7.13. Ensure right cassette for tubing size is installed (e.g. ¼”)
    7.13.1. Always remove bottom 1st then top and vice versa
7.14. Transfer inoculum into prepared transfer bottle in biosafety cabinet
7.15. Use stir plate set to 30 C and agitate transfer bottle with hand during transfer procedure
7.16. Fit tubing from prepared transfer bottle and feed tube in correct order
7.17. Add blade with sensor in correct position (as shown in picture)
7.18. Fit snugly
7.19. Close lid all the way. Screen will display “depyrogenation”
7.20. Fit blade removal tool on left
   7.20.1. Force blade up and to the right
   7.20.2. Peel off tubing to re-use blade
7.21. Pop weld open
7.22. Use peristaltic pump to quickly transfer inoculum into fermentor
7.23. Reset welder cassettes
7.24. Close then and close lid
7.25. NOTE: Don’t use bent or stained blades

8. REFERENCES:
   N/A
APPENDIX C: Determination of $k_L a$ in 30 L Bioreactor using Modified Sulfite Oxidation Method
1. PURPOSE:

1.1. The purpose is this protocol is for determination of the oxygen mass transfer coefficient, $k_La$, for the 30 L bioreactor BR-2180 using a modified sulfite oxidation chemical method.

2. SCOPE:

2.1. This procedure applies to all operations staff, faculty, students, researchers and other BTEC users of BR-2180 for performing $k_La$ studies.

3. DEFINITIONS:

3.1. CCA: Clean compressed air
3.2. DCU: Digital control unit
3.3. DO: Dissolved oxygen
3.4. $k_La$: Oxygen mass transfer coefficient; a product of the liquid film mass transfer coefficient, $k_L$, and the gas-liquid interfacial area, $a$
3.5. Sulfite Oxidation: Zero order chemical reaction between sodium sulfite and cobalt chloride. This method can be used to determine oxygen transfer capabilities in a stirred tank reactor.
3.6. Modified Sulfite Oxidation: Sulfite oxidation method where reaction is timed based on oxygen depletion.

4. RESPONSIBILITIES:

4.1. All operations staff, faculty, students, and other BTEC staff utilizing BTEC 30 L bioreactors for modified sulfite oxidation $k_La$ measurement are responsible for following procedures as described by this SOP.

5. SAFETY:

5.1. Wear appropriate PPE when handling chemicals and working in the lab. These include lab coat, gloves, and safety glasses.
5.2. Use care when working around steam lines.
5.3. Consult MSDS for material hazards.
5.4. WARNING: Cobalt (II) chloride is a hazardous material.
6. MATERIALS:
   6.1. Cobalt (II) chloride, anhydrous, purity ≥ 97% (e.g. Sigma Aldrich part # 232696)
   6.2. 0.01 M Cobalt (II) chloride in glass transfer bottle
   6.3. Sodium sulfite
   6.4. 50 ml Syringe
   6.5. 100 ml graduated cylinder
   6.6. Deionized water wash bottle
   6.7. Timer
   6.8. DO probe (e.g. Mettler Toledo InPro® 6800)

7. PROCEDURE:
   7.1. Turn on DCU, CCA, clean steam, and chiller (4°C)
   7.2. Drain and refill jacket water
   7.3. Rinse reactor with 20 L water for 2 minutes. Drain.
   7.4. Tare weight on reactor
   7.5. Fill to desired volume ± 0.15 kg (e.g. 20.03 kg for 20 L)
       7.5.1. Note fill volume
   7.6. Turn on temperature control to 30°C
   7.7. Turn on airflow to 1 vvm
       7.7.1. Ensure sparge filter housing is in fermentation mode
   7.8. Turn on agitation to desired setpoint
   7.9. Calibrate DO probe zero point calibration by disconnecting probe
   7.10. Calibrate DO probe for slope at 30°C and 1 vvm
   7.11. Turn off airflow
   7.12. Add 1 ml/L of 0.01 M Cobalt (II) chloride using a syringe through empty port in headplate
       7.12.1. E.g. 20 ml for 20 L condition
   7.13. Allow to mix for 1 minute
   7.14. Add 3 mg/L Na$_2$SO$_3$ through empty port in headplate
       7.14.1. E.g. 60 mg/20 L
       7.14.2. Suggestion: Use 100 ml graduated cylinder for transfer
   7.15. Quickly rinse any residual sodium sulfite into reactor with water
   7.16. Turn airflow on
   7.17. Begin timing when DO drops to 10%
   7.18. Stop timing when DO rises back to 10%
   7.19. Calculate $k_{L,a}$ using the equations:
       7.19.1. $QO_2 = k_{L,a}(C^*-C_L)$
       7.19.2. $C_L = 0$ mg/L, $C^* = 7.6$ mg/L at atmospheric pressure
7.19.3. \( \text{QO}_2 = ([\text{Na}_2\text{SO}_3] \text{ mg/L} \times (16 \text{ g Oxygen} / 126 \text{ g Na}_2\text{SO}_3)) / \text{Reaction time (s)} \)

7.19.4. \( k_L a \ (s^{-1}) = (3000 \text{ mg/L Na}_2\text{SO}_3 \times 0.127 \text{ g Oxygen/g Na}_2\text{SO}_3) / (\text{Reaction time (s)} \times 7.6 \text{ mg Oxygen/L}) \)

7.19.5. Example calculation:

If time = 214 s, and \([\text{Na}_2\text{SO}_3] = 3000 \text{ mg/L}, \)
\( k_L a = (3000 \times 0.127) / (214 \times 7.6) = 0.231 \text{ s}^{-1} \)

7.20. Between replicate experiments be sure drain, rinse, drain tare reactor
7.21. Recalibrate DO probe if necessary
7.22. Note: Calibrate DO probe when vessel temperature is at the desired set point

8. REFERENCES:
BBS 426/526 Modified Sulfite Oxidation Lab Protocol
APPENDIX D: BR-2180 *in-situ* Methanol Probe Care and Maintenance
1. PURPOSE:
   1.1. Protocol for silicone tubing change and preparation for SIP for BR-2180 In situ dissolved methanol probe.

2. SCOPE:
   2.1. This procedure applies to all operations staff, faculty, students and other BTEC staff utilizing BR-2180, BIOSTAT C-DCU3 30 L bioreactor for care and maintenance of the in situ dissolved methanol probe.

3. DEFINITIONS:
   3.1. In situ – Inline probe
   3.2. RTV Silicone – Room Temperature Vulcanizing silicone
   3.3. SIP – Sterilization-in-Place

4. RESPONSIBILITIES:
   4.1. All operations staff, faculty, students, and other BTEC staff utilizing BR-2180 for the dissolved methanol probe and/or P. pastoris fermentation are responsible for following procedures as described by this SOP

5. SAFETY:
   5.1. Use proper PPE when working in the lab
   5.2. Consult MSDS for material hazards
   5.3. NOTE: Chloroform is considered a hazardous material and should be handled in a chemical hood

6. MATERIALS:
   6.1. Methanol probe
   6.2. Dow Corning Silastic® tubing (0.062x0.095")
   6.3. 100% RTV Silicone Sealant
   6.4. Razor blade
   6.5. Chloroform (if needed)

7. PROCEDURE:
   7.1. Tubing Change
       7.1.1. Remove old tubing and RTV using razor blade
       7.1.2. Attach one end of new silicone tubing to steel port on the probe
       7.1.2.1. NOTE: If needed, tubing ends may be swelled by soaking in chloroform
       7.1.3. Apply thin layer of fresh RTV sealant to probe surface
7.1.4. Tightly coil new silicone tubing around probe until probe surface is covered
7.1.5. Attach opposite end of tubing to steel port on the bottom of the probe
7.1.6. Allow to dry at least 24 hours
7.1.7. Check for tubing leaks by placing probe in 2 L bottle of water
7.1.8. If leaks occur, apply thin layer of RTV to leak area and allow to dry at least 24 hours and repeat leak check
7.1.9. Check probe response time to methanol additions up to 1% v/v
7.1.10. Response time should be less than 4 minutes

7.2. Preparation for SIP
7.2.1. Prepare probe for SIP by closing both valves allowing N₂ flow in and out of the probe
7.2.2. Redirect N₂ flow directly through hydrocarbon sensor
7.2.3. Upon completion of SIP cycle, open both inlet and exit valves
7.2.4. Check tubing integrity by attaching liquid trap upstream of N₂ flow to sensor
7.2.5. Prior to inoculation, weigh liquid trap to ensure weight gain <1.6 g (internal tubing volume)
   7.2.5.1. Typical gain is 0.2-0.3g
   7.2.5.2. Note weight gain on probe status tag next to SIP cycle count

8. REFERENCES:
   N/A
APPENDIX E: *Pichia pastoris* Fermentation Batch Record
### Pichia pastoris Fermentation Batch Record

<table>
<thead>
<tr>
<th>Step #</th>
<th>Task</th>
<th>Item</th>
<th>Data</th>
<th>Executed By/Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Bioreactor Preparation for SIP</td>
<td>Ensure inlet air CCA is open and N₂ is closed</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Clean bioreactor BR-2180 and rinse with HPW</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Calibrate pH probe using pH 4 and pH 7 buffers</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ensure DO, Temp, Methanol, pH probes installed</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Check integrity of soft parts: replace damaged septa</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Close valves on methanol probe. Remove liquid trap and connect inlet N₂ line to hydrocarbon sensor</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Start Batch Record</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Ensure air supply (CCA) is open</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Ensure all valves are closed on the vessel</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Place Sparge filter into Sterilization Mode</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Main Menu&gt;Airflow1&gt;Flow rate&gt;10 L/min</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Mode&gt;Auto</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Once Pressure is above 1000 mbar&gt; Airflow 1&gt; Mode&gt;off</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Update equipment tags to “Pressure Hold”</td>
<td>(45)</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Perform pressure hold on vessel for 30 minutes once pressure stabilizes</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Pressure hold passing conditions result in a loss of no more than 17 mbar in 30 minutes**

<table>
<thead>
<tr>
<th>Start Time</th>
<th>Final press. (mbar)</th>
<th>Finish Time</th>
<th>ΔP (mbar)</th>
<th>ΔTime (min)</th>
<th>Pass/Fail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial press. (mbar)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Step # | Task | Item | Data | Executed By/Date |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>To Depressurize: Main Menu&gt;Maintenance&gt; Manual Operation&gt;Passcode 9&gt; Dig Out&gt; VS01&gt; Mode&gt; Auto</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Disconnect DO, pH probes from DCU</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Ensure Temp probe &amp; High Foam sensor connected to DCU</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Drain condensate from chiller</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Ensure chiller has enough coolant (see chiller level indicator)</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Turn on chiller to -20°C for 5P</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Close exhaust cooler coolant valve (HV512)</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Disconnect exhaust cooler coolant line and connect together</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Drain exhaust cooler jacket</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Open top jacket Overflow valve (HV323)</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Open Inlet Jacket water valve (HV326)</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Open cool water inlet from sink</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Run few minutes to drive out trapped air</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Close overflow valve (HV323)</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>When jacket pressure just above 0.5 bar, close inlet valve (HV326)</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>Shut water off</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>Open overflow slowly until pressure 0.5 bar</td>
<td>Press. (bar)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Ensure harvest valve closed and tare weight on bioreactor</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>Continue on to add heat sterilizable media components</td>
<td>(B)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Pichia pastoris Fermentation Batch Record #*
### 2. Glycerol Batch Medium Preparation (20 L)

<table>
<thead>
<tr>
<th>Step #</th>
<th>Task</th>
<th>Item</th>
<th>Data</th>
<th>Executed By/Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Obtain clean 5 L bucket, large stir bar, spatula, 1 L graduated cylinder, large scale</td>
<td>[66]</td>
<td>☐</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Dissolve glycerol batch media components in approx. 3 L HPW and stir for a minimum of 20 min</td>
<td>[66]</td>
<td>☐</td>
<td></td>
</tr>
</tbody>
</table>

- **70 ml** H3PO4
- **2.374 g** CaSO4 2H2O
- **48.0 g** K2SO4
- **39.0 g** MgSO4•7H2O
- **13.1 g** KOH
- **58.8 g** Sodium Citrate
  - (Na3C6H5O7•2H2O)
- **120.0 g** (NH4)2SO4
- **30.7 g** NaOH
- **800 ml** 1 M Acetate Buffer

Prepare it from mixing 1.280 L of a sodium acetate solution (164 g/2L HPW) and 0.720 L of a glacial acetic acid solution (57.67 ml/1L HPW).

- **635 ml (800g)** Glycerol
- **4.0 ml** Antifoam 204

<table>
<thead>
<tr>
<th>Vol (ml)</th>
<th>Lot #</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amount (g or ml)</th>
<th>Lot #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Transfer heat-sterilizable media components to clean, tared bioreactor and fill to 20 kg.

<table>
<thead>
<tr>
<th>Weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

4. Mix for ~5 minutes at 600 rpm, and remove media until there are around 17.5 kg total.

<table>
<thead>
<tr>
<th>Weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

5. Continue on to SIP on BR-2180 by BTEC SOP XXXX | ☐    |

96
<table>
<thead>
<tr>
<th>Step #</th>
<th>Task</th>
<th>Item</th>
<th>Data</th>
<th>Executed By/Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.</td>
<td><strong>Sterilization-in-place</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Turn on clean steam and CCA100</td>
<td>Clean steam press. (psi)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Check agitator lubricant level through sightglass. Ensure level at</td>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>least half-full of condensate; otherwise, open valve NPAS001 of the</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>clean steam reservoir for seal housing lubrication.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Follow BTEC SOP XXXX for sterilization-in-place procedure.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ensure Batch Record program is recording</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Ensure CCA sparge filter housing in sterilization mode</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Auto temperature control to 30°C</td>
<td>Temp (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Initiate sterilization-in-place. Update equipment status tag to read</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot;sterilization in progress&quot;. Check for temperature regularly.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>During &quot;sterilization&quot; phase (Temp=121°C), Steam ports (HV106,</td>
<td>Time Start Steaming</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HV191, HV192, HV 102), Shut when done</td>
<td>Time Stop Steaming</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>~30m When COOL1 begins, turn on airflow 1 to 6 slpm</td>
<td>Cool1 start time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>~20m When COOL2 begins, connect coolant lines to exhaust condenser</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>and turn on coolant valve (HV512)</td>
<td>Cool2 start time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>~45m Acknowledge completion of SIP (ACK)</td>
<td>Finish Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Update Tag to &quot;SIP Complete&quot; &amp; the Date</td>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Reconnect pH &amp; DO Probes to the DCU</td>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Set operating parameters</td>
<td>Power (mbar)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Airflow = 18 slpm (1vvm) (&quot;Ratio&quot; control mode)</td>
<td>Airflow (slpm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stir = 600 rpm</td>
<td>Agitation (rpm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pO2 Set Point = 30 % (&quot;Auto&quot; mode)</td>
<td>Setpoint</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chiller Set Point = 18 °C</td>
<td>Temp (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Set air inlet to Fermentation mode</td>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Continue on to other bioreactor preparations as necessary</td>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step #</td>
<td>Task</td>
<td>Item</td>
<td>Data</td>
<td>Executed By/Date</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------------------</td>
</tr>
<tr>
<td>1</td>
<td>Autoclave following prepped items:</td>
<td>(a)</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
<tr>
<td></td>
<td>100mL bottle for PTM 4</td>
<td>(a)</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
<tr>
<td></td>
<td>500mL bottle for inoculum</td>
<td>(a)</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
<tr>
<td></td>
<td>1L bottle for L-Histidine</td>
<td>(a)</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
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<td></td>
<td>Tri-punch</td>
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<td></td>
<td>Inoculation valve</td>
<td>(a)</td>
<td>[ ]</td>
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<td></td>
<td>Recycle + inline spectrophotometer</td>
<td>(a)</td>
<td>[ ]</td>
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<td></td>
<td>2L bottle for Base (autoclave not necessary)</td>
<td>(a)</td>
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<td></td>
<td>10L bottle for glycerol feed (autoclave not necessary)</td>
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<td>2</td>
<td>Prepare ≥530 mL PTM 4 (BTEC SOP XXXX)</td>
<td>Prep date</td>
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<td>3</td>
<td>Aseptically transfer 87 mL PTM 4 to bioreactor</td>
<td>Vol added (ml)</td>
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<td>4</td>
<td>Prepare approximately 700 mL of histidine sol: 35g/L, filter sterilized</td>
<td>Prep Date</td>
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<td>5</td>
<td>Transfer 700 mL histidine aseptically to 1L prepped bottle</td>
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<td>6</td>
<td>Connect 1L histidine bottle to bioreactor aseptically</td>
<td>(a)</td>
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<td>7</td>
<td>Aseptically transfer 100 mL L-Histidine to bioreactor</td>
<td>Prep date (mm/dd/yy)</td>
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<td>8</td>
<td>Hold vessel for 24 hours, then Sample &amp; plate triplicates on YPD for 24 hrs at 37 °C. No growth = sterility</td>
<td>Hold Start Time</td>
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<td>Plates with growth</td>
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<td>9</td>
<td>Prepare glycerol feed: ~ 5.5L total</td>
<td>Vol (L)</td>
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<td>2L DI Water</td>
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<td>600 g (NH₄)₂SO₄</td>
<td>Lot</td>
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<td>4 kg (3.175L) glycerol</td>
<td>Lot</td>
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<td></td>
<td>Bring to volume of 5 L with DI water</td>
<td>Volume (L)</td>
<td>[ ]</td>
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<tr>
<td></td>
<td>Transfer to 10L bottle setup &amp; autoclave</td>
<td>(a)</td>
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<tr>
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<td>Add 435 mL PTM 4 to feed bottle aseptically</td>
<td>Prep Date</td>
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<td></td>
<td>Vol added (ml)</td>
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<tr>
<td>Step #</td>
<td>Task</td>
<td>Item</td>
<td>Data</td>
<td>Executed By/Date</td>
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<td>----------------------------------------------------------------------</td>
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<tr>
<td>10</td>
<td>Connect glycerol feed bottle aseptically to reactor</td>
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<tr>
<td>11</td>
<td>Prepare 2L 5M NaOH base (200g pellets/L)</td>
<td>Date Prepped</td>
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<tr>
<td>12</td>
<td>Transfer base to 2L bottle &amp; connect aseptically to bioreactor</td>
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<tr>
<td>13</td>
<td>Change pH control to Auto with set point of 5.0</td>
<td>Set point</td>
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<td>14</td>
<td>Clean liquid trap</td>
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<td>15</td>
<td>Add DI water to bottom of the liquid trap</td>
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<td>16</td>
<td>Weigh liquid trap + water</td>
<td>Weight (g)</td>
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<tr>
<td>17</td>
<td>Re-connect liquid trap to methanol probe. Open probe valves</td>
<td>Start Time</td>
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<tr>
<td>18</td>
<td>Close probe valves and disconnect trap after 10 min</td>
<td>Stop Time</td>
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<tr>
<td>19</td>
<td>Re-weight trap + condensate</td>
<td>Weight (g)</td>
<td></td>
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<tr>
<td>20</td>
<td>Determine integrity of probe tubing. Liquid trap gain &gt; 1.6 g results in failure</td>
<td>ΔWeight (g)</td>
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<tr>
<td>21</td>
<td>Re-connect liquid trap to methanol probe. Open probe valves</td>
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<tr>
<td>22</td>
<td>Ensure waste is empty in YSI</td>
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<tr>
<td>23</td>
<td>Make sure YSI Buffer 2705 is full</td>
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<td>24</td>
<td>Throw away old calibration standard and add new</td>
<td></td>
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<tr>
<td>25</td>
<td>Renew Membrane if necessary</td>
<td>Membrane info</td>
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<tr>
<td>26</td>
<td>Flush YSI probe 2</td>
<td></td>
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<tr>
<td>27</td>
<td>Recalibrate the YSI, when probe 2A is below 5mA</td>
<td>PI Current</td>
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<td>28</td>
<td>If YSI is working, change service to Remote</td>
<td></td>
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<tr>
<td>29</td>
<td>Set Seg:Flow to measure glycerol concentration every 30 min</td>
<td>Interval (min)</td>
<td></td>
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<td>30</td>
<td>30 min prior to inoculation start Seg:Flow</td>
<td>Start Time</td>
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<tr>
<td>31</td>
<td>Harvest 100 mL of blank (media from bioreactor)</td>
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<tr>
<td>Step #</td>
<td>Task</td>
<td>Item</td>
<td>Data</td>
<td>Executed By/Date</td>
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<td>5.</td>
<td>Inoculum Preparation</td>
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<tr>
<td>1</td>
<td>Autoclave two 2 L Baffled Shake Flasks</td>
<td>[5]</td>
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<tr>
<td>2</td>
<td>Fill each flask with 200 ml of media previously prepared</td>
<td>[5]</td>
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<tr>
<td>3</td>
<td>Remove two cryovials from freezer and thaw in hand</td>
<td>Cryovial Batch (mmddyyyy)</td>
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<td>4</td>
<td>Pipet 1 ml cryovial contents into each prepared 2 L shake flask</td>
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<td></td>
<td>Flask 1</td>
<td>Volume added (ml)</td>
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<td></td>
<td>Flask 2</td>
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<td></td>
<td>Flask 1</td>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
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<td></td>
<td>Flask 2</td>
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<tr>
<td>5</td>
<td>Record inoculum start time</td>
<td>Inoculum Start Time</td>
<td>Date</td>
<td></td>
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<tr>
<td>6</td>
<td>Connect and steam inoculation valve for 15-20 minutes. Allow connection to cool to room temperature prior to inoculation</td>
<td>Steam time (min)</td>
<td></td>
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<tr>
<td>7</td>
<td>Incubate flasks at 30 °C and 400 rpm for approximately 18 hours to an OD&lt;sub&gt;600&lt;/sub&gt; of about 6 or higher (maximum accepted value around 14). Record finish time.</td>
<td>Inoculum Finish Time</td>
<td>Date</td>
<td>Duration (hr)</td>
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<tr>
<td></td>
<td>Flask 1</td>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
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<td></td>
<td>Flask 2</td>
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<tr>
<td>8</td>
<td>Transfer inoculum to bioreactor while shaking on hot plate at 40%</td>
<td>Transfer Time (min:sec)</td>
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<tr>
<td></td>
<td>following the steps from the Inoculation procedure (BTEC SOP XXXX)</td>
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<tr>
<td>9</td>
<td>Start new Batch Record after inoculation</td>
<td>Title</td>
<td></td>
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<tr>
<td>10</td>
<td>Take first Sample &amp; record data</td>
<td>[5]</td>
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</tbody>
</table>
5. Fermentation Run

Remember

- Feed ~1L of glycerol feed media when the glycerol concentration drops below 30g/L
- Feed ~100mL of histidine solution at each glycerol feeding
- Ensure there is adequate amount of NaOH base buffer to maintain the pH
- Note events in the comments by the batch time when they occurred.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initials</th>
<th>Date</th>
<th>Time</th>
<th>Batch Time (hrs)</th>
<th>OD1 (dil)</th>
<th>OD2 (dil)</th>
<th>OD3 (dil)</th>
<th>DI</th>
<th>Total Weight (kg)</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td>Blank</td>
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</table>
### 7. Post Fermentation Clean up

#### Tips
- Rotor should be off when there is less than 20L of liquid in it
- Backpressure helps empty bioreactor quicker
- When filling, make sure to open a port to prevent backpressure

<table>
<thead>
<tr>
<th>Step #</th>
<th>Task</th>
<th>Item</th>
<th>Data</th>
<th>Executed By/Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stop Batch Record</td>
<td>$\text{(5) } \square$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Stop Seg Flow</td>
<td>$\text{(5) } \square$</td>
<td></td>
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<tr>
<td>3</td>
<td>Once Seg Flow is in idle mode, change YSI to normal mode</td>
<td>$\text{(5) } \square$</td>
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<tr>
<td>4</td>
<td>Empty Seg Flow waste</td>
<td>$\text{(5) } \square$</td>
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<tr>
<td>5</td>
<td>Empty YSI waste</td>
<td>$\text{(5) } \square$</td>
<td></td>
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<tr>
<td>6</td>
<td>Stop all auto control on bioreactor</td>
<td>$\text{(5) } \square$</td>
<td></td>
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<tr>
<td>7</td>
<td>Open harvest valve &amp; empty bioreactor</td>
<td>$\text{(5) } \square$</td>
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<tr>
<td>8</td>
<td>Turn off chiller</td>
<td>$\text{(5) } \square$</td>
<td></td>
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<tr>
<td>9</td>
<td>Rinse bioreactor 3 times with 30L of DI water</td>
<td>$\text{rinses}$</td>
<td></td>
<td>$\text{rinses}$</td>
</tr>
<tr>
<td>10</td>
<td>Disconnect tri-punch &amp; associated bottles</td>
<td>$\text{(5) } \square$</td>
<td></td>
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<tr>
<td>11</td>
<td>Replace punctured septa</td>
<td>$\text{(5) } \square$</td>
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</tr>
<tr>
<td>12</td>
<td>Disconnect inline spec &amp; recycle, any valves, or other bottles</td>
<td>$\text{(5) } \square$</td>
<td></td>
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</tr>
<tr>
<td>13</td>
<td>Leave reactor filled with 25L of DI water</td>
<td>$\text{(5) } \square$</td>
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<tr>
<td>14</td>
<td>Turn off bioreactor (green button then red switch)</td>
<td>$\text{(5) } \square$</td>
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<tr>
<td>15</td>
<td>Turn off CCA &amp; Steam</td>
<td>$\text{(5) } \square$</td>
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<tr>
<td>16</td>
<td>Store any reusable materials. Make sure labeled</td>
<td>$\text{(4) } \square$</td>
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<tr>
<td>17</td>
<td>Discard waste appropriately. Make sure liquids are labeled</td>
<td>$\text{(4) } \square$</td>
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<tr>
<td>18</td>
<td>Wipe down &amp; clean all equipment</td>
<td>$\text{(5) } \square$</td>
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<tr>
<td>19</td>
<td>Return borrowed equipment (bottles, valves, etc.)</td>
<td>$\text{(5) } \square$</td>
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<td>20</td>
<td>Put away equipment</td>
<td>$\text{(5) } \square$</td>
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<tr>
<td>21</td>
<td>Organize and clean off lab bench</td>
<td>$\text{(5) } \square$</td>
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<tr>
<td>22</td>
<td>Wipe down lab bench</td>
<td>$\text{(4) } \square$</td>
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<td>23</td>
<td>Save all data &amp; upload to Dropbox</td>
<td>$\text{(4) } \square$</td>
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</table>