This is to certify that the
thesis entitled

Optimization and Control of Recombinant Protein Expression

presented by

Casey Preston

has been accepted towards fulfillment
of the requirements for the

M.S. degree in Chemical Engineering and Materials Science

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12/16/04
Date

OPTIMIZATION AND CONTROL OF RECOMBINANT PROTEIN EXPRESSION

By

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A THESIS

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE

Department of Chemical Engineering and Materials Science

2004

ABSTRACT

OPTIMIZATION AND CONTROL OF RECOMBINANT PROTEIN EXPRESSION

By

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The skills required for the optimization and control of protein expression in recombinantly engineered microorganisms is of increasing value to chemical engineers and makes them attractive candidates for positions in the biotechnology industry. Therefore, protocols have been developed for use in the Biochemical Engineering Teaching Laboratory. Procedures for batch fermentations in shake flasks, fed-batch fermentations in the New Brunswick BioFlo IIC, SDS-PAGE, absorbance analysis, and media selection are included.

Novel and robust software has been developed for use in the control of fed-batch bioreactors. The software allows the operator of the equipment to easily develop and implement flexible, knowledge-based control schemes based on the environmental variables monitored by the control unit. A section of the operator's manual and descriptions of the controller components and concepts are presented.

A 185 residue segment of the HA2 domain of hemagglutinin has been expressed in the E. coli host Rosetta pRARE in both batch and fed-batch fermentations. Isotopically labeled amino acids have been selectively incorporated into the expressed protein for the purposes of analysis by rotational-echo double resonance (REDOR). The expression optimization procedure that has been followed utilizing the developed protocols is outlined.

ACKNOWLEDGMENTS

I would first like to thank past members of Dr. Worden's research group who supported me and taught me how to do research: Tyler Ames, Mark Widman, David Knop, and Prashant Srivistava. I also want to thank the new member's: Neeraj Kohli, Kai Wang, and Brian Hassler. It has been a pleasure working with you and I wish you all success. I also appreciate all of the help that I have been given from the faculty and office staff of the chemical engineering department throughout my time at Michigan State University. Specifically, thank you JoAnn Peterson for keeping me on track to graduate and Dr. Bruce Dale for agreeing to be on my committee.

I want to acknowledge the people who made this thesis possible. Jun Sun, I thank you for teaching me much of what I know about recombinant engineering and guiding me through my work. I also need to thank Jason Criscione for collaborating with me on the project. Your help made the work much easier, quicker, and more enjoyable. I could not have asked for a better research partner. Similarly, thank you Dr. David Weliky for being on my committee, developing the project, and giving me so much support and information. You all helped to make my return to graduate school worthwhile.

This work also would not have been possible without the support of my family. Dad, Mom, Carrie, and Ian- thank you for keeping me going. I would not have finished otherwise.

Finally, thank you Dr. Mark Worden for everything that you have done for me. You were the major force behind my education in engineering and I would not have been able to succeed with you. I cannot thank you enough.

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1. Introduction

1.1 Recombinant Engineering

Proteins represent the most abundant class of organic molecules in the human body. They function in regulating cellular responses, maintaining cell shape, transporting molecules, initiating a body's defensive mechanisms, and catalyzing reactions. The human body is believed to contain 10,000 to 20,000 different proteins and over 2000 of these are believed to be enzymes. Proteins have incredible potential as marketable products in the biotechnology and pharmaceutical industries, yet the majority of these proteins have yet to be studied. Techniques such as polymerase chain reaction have been developed that allow the genes that encode these proteins to be identified, but the proteins themselves are very difficult to create. Smaller proteins can be synthesized in a step-wise fashion, but proteins over 100 amino acids are difficult to produce synthetically. Therefore, larger proteins are more easily created using a biological host; the anabolic mechanisms of a cell are more proficient at producing complex macromolecules than an organic chemist. Even so, the natural abundance of a protein in a cell is generally very small making the purification of the protein both difficult and uneconomical.

Recombinant engineering (expression of foreign DNA in host cells) is commonly used to overcome the low yields found in native expression. In recombinant engineering, genes that produce the protein of interest are isolated from the native host and cloned into an expression vector. A host cell for the vector is selected that is easily grown and controlled. The host is then transformed by the vector and utilized in a fermentation process to produce the protein at the quantities required for study or commercial sale. In

this way, intracellular proteins can be overexpressed to such an extent that they may make up as much 50% of the final cell culture protein mass (Grabski et al., 2003; Laarge and Langosh, 2001). With the proper host and bioreactor equipment, extracellular proteins can be produced to an even greater overall concentration (Bailey and Ollis, 1986).

Recombinant engineering has become the prevalent method of producing proteins in the biotechnology and pharmaceutical industries. Sales of protein based biotechnology products are expected to be $32 billion by 2006 (Shuler and Michael, 2002). Recombinantly produced pharmaceuticals such as antibodies, human growth hormone, and insulin are used in the treatment of such diseases as cancer, arthritis, diabetes, and viral illnesses. In addition, proteins are beginning to have applications in sensors, food processing, and enzymatic catalysis. The biotechnology industry presents increasing employment opportunities for chemical engineers with the proper training.

1.2 Teaching and Research Opportunities

Many of the skills and techniques that characterize the discipline of chemical engineering are applicable to the field of biotechnology. In particular, chemical engineers are required to fulfill bioprocess purification roles such as the development of chromatography operations and crystallization and tableting procedures. Another field in which chemical engineers can be beneficial is bioreactor development. In essence, fermentation is the same as the traditional forms of reaction engineering for which chemical engineers are trained. Reactants (air and feed sources) and a catalyst (cells) are added to a reactor and a value added product (protein) is created. Also, the analytical methods of chemical engineering are essential for reliable reaction control of the 100 L to

10,000 L bioreactors found in industry. Classical reaction issues such as heat production, heat transfer, mixing, reaction stoichiometry, and reaction rate all apply. Therefore, chemical engineering students with a background in the basic laboratory techniques of recombinant engineering and fermentation are attractive candidates for bioprocessing positions.

Skills related to fermentation can also be of benefit to those pursuing academic research in other fields involving bioscience, such as proteomics and biochemistry. A primary objective of much research in biochemistry is to understand the structure and function of proteins in the body (Julka and Regnier, 2004; Zhu et al., 2002). When the proteins become too large for direct synthesis, many biochemists utilize recombinant engineering. Unfortunately, due to a lack of equipment and expertise, some researchers attempt to produce the proteins in poorly regulated and modeled environments. Batch fermentations in shake flasks are often used even though they do not allow active control of growth or protein production. The inability to monitor the environmental conditions and physiological state of their expression hosts in these batch processes can lead to many problems (Liden, 2002). Impediments to recombinant protein expression include the stringent response of the cell due to a lack of substrate (Notley and Ferenci, 1996), toxicity of the heterologous protein or its inducer (Glick, 1995), toxicity of fermentation byproducts such as acetate (Konstantinov et al., 1990; Akesson et al., 1999), catabolite repression of expression (Grossman et al., 1998), oxidative stress and protein degradation (Konz et al., 1998), oxygen starvation (Chen et al., 1999), and inhibition of growth due to high metabolite concentrations (Lee, 1996). Chemical engineering fundamentals such as process control, reaction rate calculations, and modeling of the mass transport

characteristics of the system can be used to solve many of the issues facing growth and protein production in cell cultures.

1.3 Available Resources

The Chemical Engineering and Materials Science department is especially well suited to providing learning and research opportunities for students interested in recombinant engineering and fermentation processes. The Biochemical Engineering Teaching Laboratory in rooms 3269 and 3262 of the Engineering Building houses much of the equipment required for basic experiments related to the optimization of protein expression in batch and fed-batch fermentations. This equipment includes 1 L and 10 L stirred tank bioreactors, a laminar flow hood, electrophoresis equipment, incubators, glassware, and large and small centrifuges. More importantly, expertise and research opportunities can be supplied via the Protein Expression Laboratory (PEL) and the multiple faculty members in the chemical engineering department with projects related to biochemical engineering. Students can potentially gain experience in bioreactor process development through independent research projects or assigned laboratory experiments

Some of the fermentation infrastructure in the Biochemical Engineering Teaching Laboratory is a result of the work done by David Knop (Knop, 2002). As part of his thesis project, the control system for the 1 L bioreactors (BioFlo IIc) in the teaching laboratories was substantially improved. Much of the internal controller software for the bioreactors was bypassed and a stand alone control unit was implemented. Graphical programming software and additional control modules were added to increase the control capabilities. The 1 L bioreactors now have the ability to be driven by very robust control schemes as compared to the limited control capabilities of many commercial units.

Operators can employ previously impossible control schemes for fed-batch fermentations of recombinant microorganisms.

1.4 Thesis Contents

The work done in for this thesis is intended to help students and researchers use the equipment in the Biochemical Engineering Teaching Laboratory. Appendix A of this thesis contains protocols related to the optimization of protein expression in recombinant cell cultures. These procedures were developed specifically for use with the equipment available in the teaching laboratory. Protocols are written for batch fermentations in shake flasks and test-tubes, as well as fed-batch fermentations in the BioFlo IIC. Protocols are also included for use of the autoclave, the spectrophotometer, the various centrifuges, the SDS-PAGE equipment, and for choosing and preparing media. Each protocol contains an introduction to the procedure, a list of the required equipment, and hints for students who have never before used the equipment or worked with recombinant cell cultures. It is hoped that these protocols will be the basis for laboratory experiments developed for use in the Biochemical Engineering Laboratory courses.

Appendix B consists of a portion of the manual that describes the use of Presto Control. Presto Control is the control system developed for operating the BioFlo IIc during fed-batch fermentations. It was developed in response to complaints that the previous control system was inflexible and caused controller responses that were not well understood. The previous control system was created by David Knop specifically for producing extracellular secondary metabolites in high cell density cultures (Knop, 2002). However, the controller's ability to deal with the issues encountered in cultures expressing recombinant proteins via induction was limited. In addition, the computer

interface for the controller did not make obvious to the operator the controller actions that were being implemented. Presto Control was designed specifically to address those issues.

The primary text of this thesis is an analysis of example experiments to demonstrate the use of the protocols and the control software described in the Appendices. The experiments were an attempt to optimize the expression of a segment of the influenza fusion peptide hemagglutinin that had been cloned onto a pET vector in the Escherichia coli host strain Rosetta pRARE. The experiments follow the protocols in Appendix A. Experiments were also utilized to test the ability of Presto Control to maintain optimal growth and expression conditions in the BioFlo IIc. The discussion of the results helps to illustrate the features of the software.

2. Isotopic Labeling of Hemagglutinin

2.1 Hemagglutinin

Cellular infection by viral DNA results from the fusion of the protective membranes of the virus and the cell. The union of the lipid bilayers of the virus and the cell is facilitated by a class of proteins generally identified as "fusion glycoproteins" (Hernandez et al., 1996). A hydrophobic domain of the fusion glycoprotein called the fusion peptide embeds itself in the hydrophobic lipid layer of the cellular membrane. A conformational change in the fusion glycoprotein then causes the viral membrane to fuse with the cell membrane and viral DNA to be released into the cytoplasmic space in the cell. The structure and conformation changes of the fusion glycoprotein are of interest to researchers because of their relationship to infection and disease. A full understanding virus-cell fusion may one day lead to treatments for viral illnesses.

Hemagglutinin (HA) is the fusion protein characteristic of influenza. It is a homotrimeric glycoprotein that induces fusion with an endosomal membrane in the cell when the local pH drops to 5.0 (Kim et al., 1998). It is the most widely studied of the fusion glycoprotein (Wilson et al., 1981; Wiley and Skelhel, 1987). Hemagglutinin is divided into two subunits, HA1 and HA2, by proteolytic cleavage after expression in the infected cell. HA2 is of particular interest because it houses the fusion peptide and exhibits massive, pH triggered, conformational changes (Kim et al., 1996). It is the subunit that is primarily responsible for the actual fusion event (Leikina et al., 2001; LeDuc et al., 2000; Epand et al. 1999).

Recently, specific sections of HA2 have been recombinantly expressed in E. coli and studied by spin-labeling electron paramagnetic resonance (EPS) techniques (Macosko at al., 1997; Kim et al., 1998; Kim et al., 1996) and atomic force microscopy (AFM) (Epand et al., 2001). Dr. David Weliky of the Department of Chemistry at Michigan State University believed that rotational-echo double resonance (REDOR) analysis of HA2 would provide further insight into the conformational changes of the protein (Yang et al., 2002). REDOR is a nuclear magnetic resonance (NMR) technique that utilizes the dipolar coupling between adjacent, isotopically labeled elements to filter out the magic angle spinning (MAS) signal of the labeled elements from the natural abundance signals. This allows the local conformation of the two isotopically labeled elements to be determined. REDOR has been successfully used to determine the conformation of lipid bound proteins in solution (Murphy et al., 2001; Wang et al., 1997). Its use in studying HA2 would allow a better understanding of the structure of fused HA2 before and after its pH induced conformation change.

One of the most efficient means of producing hemagglutinin is through the use of recombinant engineering techniques. A DNA construct of a segment from the N terminal end of HA2 was obtained from Yeon-Kyun Shin, a professor in the Department of Chemistry at the University of California, Berkeley. The construct encodes for the first 185 residues (HA2_185) of the peptide and it contains the 127 amino acid section studied by his research group (Kim et al., 1996). HA2_185 is characterized by a 25 amino acid fusion peptide at the N terminus and a longer chain of more hydrophilic residues at the C-terminus. The hydrophobic character of the fusion peptide makes HA2_185 both difficult to express and utilize in the soluble, active form required for REDOR analysis.

The protein can only remain in its native conformation during expression by interacting with the internal lipid membranes of the host cell (Kim et al., 1996). Therefore, the fusion peptide has a tendency to aggregate and form inclusion bodies at high expression levels and limited membrane surface areas. Inclusion bodies are dense, intracellular aggregates of denatured protein that often characterize heterologous protein expression in prokaryotes. In order to overcome these issues and produce the protein in the quantities required for REDOR analysis, optimization of the expression system was attempted.

2.2 Expression

For expression, the HA2_185 gene was cloned into the pET-24b(+) vector and used in the transformation of the E. coli host Rosetta pRARE (Novagen). The utilization of this expression system immediately placed certain constraints on media selection. First, the pET vector and Rosetta act in concert to maintain stringent control of the induced protein by way of a T7 lac promoter (Novagen, 2002). Therefore induction of protein expression occurs upon the introduction of isopropyl β –D,thiogalactopyranoside (IPTG) to the media. Kanamycin at a recommended concentration of 15 μg/mL is required in the fermentation media to prevent plasmid loss. Rosetta also contains the plasmid pRARE which expresses eukaryotic specific codons not normally produced in E. coli host strains. This plasmid is maintained by chloramphenicol at a suggested level of 34 ug/mL.

Carbon and nitrogen sources for the media were chosen based on the needs of the REDOR experiments. REDOR analysis requires that the peptide bond between specific pairs of amino acids be labeled with ^{13}C and ^{15}N atoms. The procedure for isotopically labeling amino acids in recombinantly expressed proteins is well documented (Muchmore

et al., 1989; Lian and Middleton, 2001; Goto and Kay, 2000). Generally, the labeled amino acids are added directly before induction of the protein of interest. The host strain preferentially incorporates the labeled residues into the protein rather than building the amino acid from the available nitrogen and carbon sources in the media. Therefore, complex media containing protein cannot be used because it contains unspecified amounts of unlabeled amino acids. Instead, a defined media must be employed. M9 was chosen for the experiments described in this work due to its simplicity in preparation and cost effectiveness. Glucose was utilized as the substrate because it is the preferred carbon source for the host strain. As long as glucose was present during induction, the culture was unlikely to break down the added isotopically labeled amino acids for anabolic and catabolic purposes. Metabolism of the labeled amino acids could lead to incorporation of the labeled atoms into other, non-specific amino acids. The resulting isotopic dilution might add noise or diminish the REDOR signal.

2.3 Methods

All fermentation experiments followed the protocols described in Appendix A. Times and quantities suggested in Appendix A were the values used unless otherwise stated. M9 media was used for growth and it was prepared as described in the MEDIA section of Appendix A. Chloramphenicol and kanamycin were added to each volume of fresh media to obtain 20 mg/L and 50 mg/L respectively. Initial colonies of the expression host were obtained from a single glycerol freeze source that had been inoculated onto M9 plates containing 4 g/L glucose. When the OD_{600} reached a value between 1.0 and 2.0, suspension cultures were transferred to new media at a dilution ratio ranging from 5% to 10%. Fed-batch fermentations were carried out in the BioFlo IIc

(New Brunswick) following the set-up and procedure outlined in the FED-BATCH FERMENTATIONS section of Appendix A. During the fermentation, glucose was fed at a concentration of 50% w/v utilizing Presto Control and the Standard Fed Batch control scheme described in Appendix B.

Analytical techniques also followed the protocols in Appendix A. The OD_{600} of the cultures was measured and analyzed for the specific growth rate (SGR) as described in the CELL DENSITY- OD600 section of Appendix A. SDS-PAGE analysis of cell samples was undertaken as described in the SDS-PAGE section using the FB-VE10-1 10 mL Fischer vertical electrophoresis stand (Fischer Scientific). All gels were poured with a 12% acrylimide concentration in the separating gel. When phosphate buffer was used in the cell fractionation procedure, 0.5% n-lauroyl sarkosine was added to aid in solubilization of the membrane and HA2_185. Detergent had to be added to any preparation of the purified protein in order for it to remain in solution

3. Effect of Glucose Concentration in M9 Media

3.1 Background

Experiment were conducted to determine the culture's specific growth rate (SGR) and growth limiting nutrient in M9 media. It had been assumed that the maximum cell density obtainable in batch growth in M9 would be dependent on either the glucose or the ammonium chloride concentration. Glucose is the only source of carbon in M9 and its depletion leads to a massive reduction in catabolic and anabolic activities of the cell culture. The presence of glucose in the media, however, can also cause growth inhibition as a result of the formation of byproducts related to anaerobic metabolism of the substrate (Lee, 1996). For example, acetic acid, a fermentation byproduct produced at high glucose concentrations, can cause inhibitory effects in E. coli at concentrations as low as 2 g/L (Konz et al., 1998). In bioreactors with pH control, acetic acid can also indirectly cause growth inhibition as a result of the salt formed via the base that is added to maintain the pH.

The nitrogen source in M9 media is ammonium chloride and its depletion also leads to growth inhibition. The molar ratio of carbon to nitrogen in standard M9 media is 6.2. It is on the same order of magnitude as the ratio of carbon to nitrogen in the cell, 4.0. These calculations assume a concentration of 1.0 g/L ammonium chloride, 4.0 g/L glucose, and a general cellular composition of $CH_{1.8}O_{0.5}N_{0.2}$ (Bailey and Ollis, 1988). Therefore, either component could be the growth limiting nutrient depending on the yields of the substrate.

Batch growth experiments were conducted with three 50 mL Rosetta cultures in 250 Erlenmeyer flasks in order to determine the effect of increased glucose concentration on cell growth. The initial concentration of glucose in the media was either 5 g/L, 10 g/L, or 20 g/L. The ammonium chloride concentration for each flask was increased to 5.0 g/L over the 1.0 g/L suggested for standard M9. This was done to reduce the chance of nitrogen becoming the growth limiting nutrient at the relatively high glucose concentrations utilized in this experiment. An ammonium chloride concentration of 5 g/L is just below the concentration seen to inhibit growth in E. coli (Lee, 1996). The optical density (OD_{600}) was monitored and the pH of the cultures were checked when they had reached stationary phase. Then, additional M9 buffer and salts were added to the media to discover whether it would spur growth.

3.2 Results

Figure 1 shows the OD_{600} of the culture at various glucose concentrations. Figure 2 exemplifies the method used to determine the specific growth rate (SGR) of the culture as described in the CELL DENSITY - OD600 section of Appendix A. Lines were fitted using least squares analysis with the y-axis defined as the natural log of the ratio of A and A_0, where A is the OD_{600} at the time specified on the x-axis and A_0 is the OD_{600} at time equals zero. The slope of the line is the SGR and it is listed next to the legend entry for Figure 2. Table 1 describes the overall growth of the culture at various initial glucose concentrations. It includes the pH and OD_{600} of the culture 20 hrs after inoculation.

Figure 1: Effect of Glucose on Growth

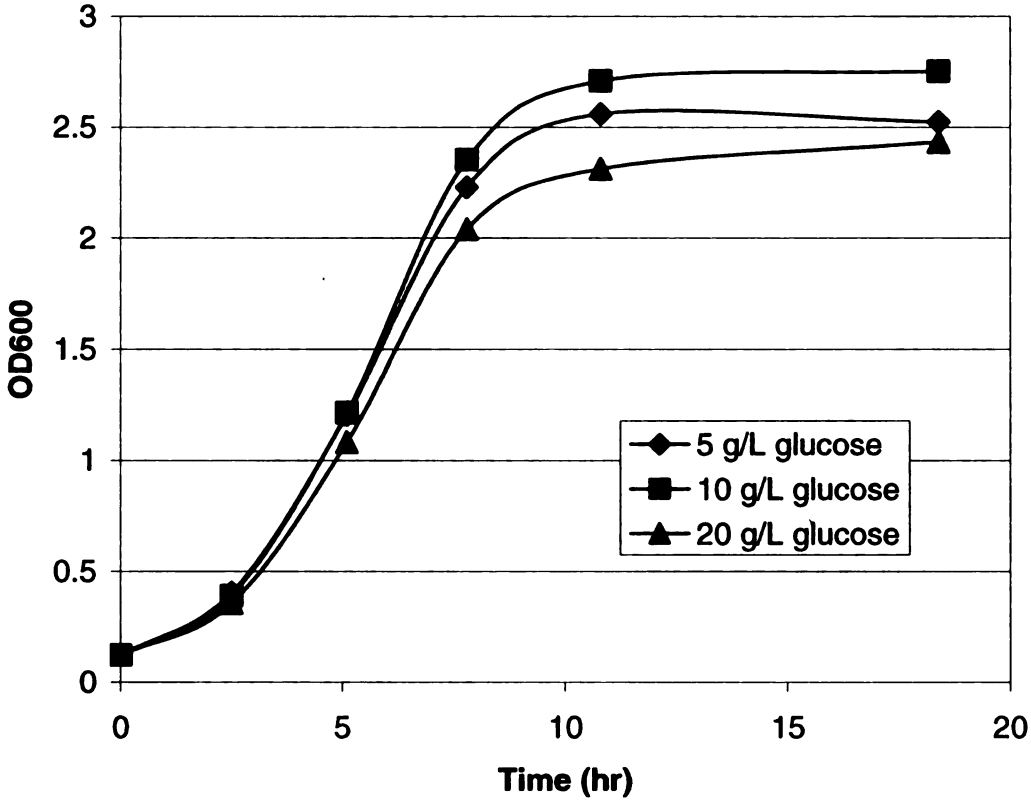


Figure 2: Effect of Glucose on Specific Growth Rate

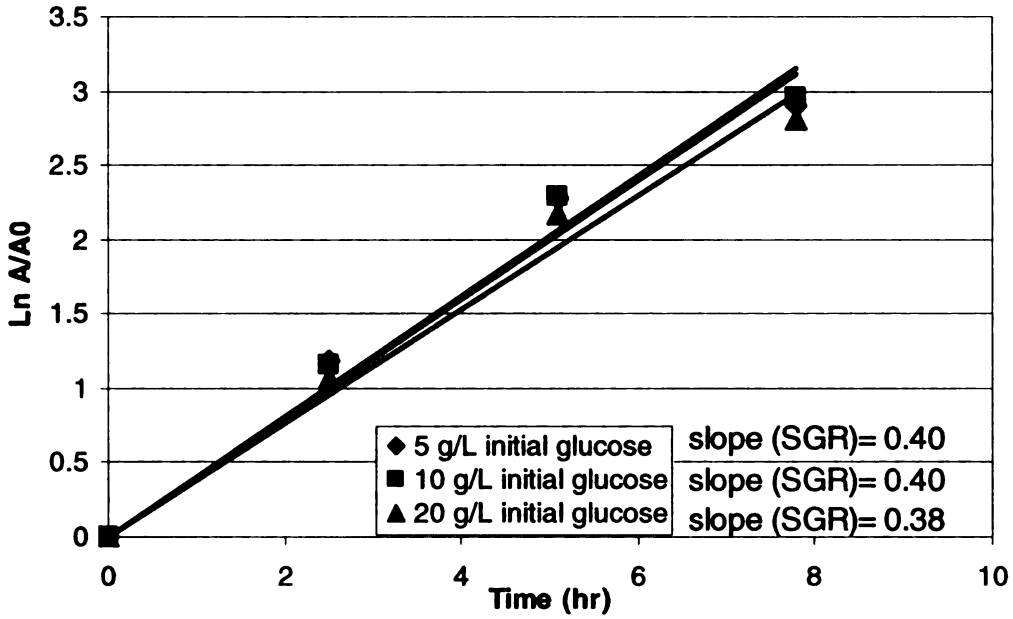


Table 1: Overall Effect of Glucose on Growth

Initial Glucose Concentration	SGR	Final OD_{600}	Final pH
5 g/L	0.40 hr^{-1}	2.472	6.21
10 g/L	0.40 hr^{-1}	2.685	4.21
20 g/L	0.38 hr^{-1}	2.325	4.28

After the culture had reached stationary phase at 20 hours, 10 mL of M9 buffer and salts were added to the 34 mL of broth still left in each shake flask. The addition of the buffer caused the pH of the cultures to rise. The additional M9 buffer had little effect on the growth of the 5 g/L culture. The cell density of the 10 g/L and 20 g/L glucose cultures, however, increased slightly over the next 2 hours until the pH again dropped to a level below 5.0 (results not shown).

3.3 Discussion

The results shown in Figure 1 and Figure 2 suggest that the growth limiting factor in these batch cultures was the buffering capacity of the media. The low pH that resulted in the high glucose concentration cultures was far outside the optimum range of 6.5 to 7.5 for cell growth (Bailey and Ollis, 1986), and may have triggered the onset of stationary phase. Increasing the pH by adding buffer allowed additional cell growth to occur. This new growth stopped when the pH once again dropped below the threshold value of 5.0. Therefore, nutrient limitations did not appear to be a growth limiting factor in these cultures.

The 5 g/L glucose culture, however, did not respond to the addition of M9 buffer. Since the primary nutrient in the buffer is ammonium chloride, it can be assumed that nitrogen had not become depleted in this culture. Also, the pH was never low enough in this culture either before or after the addition of the buffer to induce a stationary phase. Instead, the culture had apparently consumed its entire carbon source by the time it reached its final OD_{600} of 2.5.

The specific growth rate and final cell concentration of the culture were not noticeably affected by the concentration of glucose in the media. The glucose concentration itself was not expected to lead to SGR limitations since substrate inhibition is normally only found at glucose concentrations greater than 50 g/L (Lee, 1996). Acetic acid formation at high glucose concentrations, however, is a common problem in culturing E. coli. This byproduct is produced when the glucose concentration is high enough to cause its uptake rate to exceed the oxygen uptake rate required for full catabolic oxidation. A characteristic of this process is a relatively rapid growth of the

culture. In E. coli, acetate is generally only produced at an SGR above 0.35 hr^{-1} (van der Walle and Shiloach, 1998; Lee, 1996). This SGR is similar to the growth rate found here. Rosetta, however, is a derivative of the B strain and this host tends not to produce acetic acid at toxic levels irrespective of the glucose concentration (Shiloach et al., 1996).

At the low cell densities allowed by shake flasks, the growth of Rosetta is not noticeably inhibited by nutrient depletion. Instead, the growth limiting factor in batch fermentations is believed to be the buffering capacity of the media. In M9 media, the utilization of ammonium chloride as a nitrogen source causes the release of hydrogen ions into the media (Liu et al., 2001). Over time, these hydrogen ions accumulate and overcome the buffering capacity of the media, thereby causing a drop in the pH. Contributions to the hydrogen ion concentration by acidic fermentation byproducts such as acetic acid are also a possibility (Jaradat and Bhunia, 2002; Lee, 1996). These experiments show that the initial glucose charge specified in the recipe for M9 becomes completely consumed at about the same time that the buffering capacity of the media is lost. To combat the pH drop, base could be added that would moderate the pH and thus allow growth to higher potential cell densities. This type of fed-batch fermentation is much easier in a bioreactor with automated pH control, though. Furthermore, oxygen transfer limitations in a shake flask could begin to become an issue at high cell densities. Therefore, high cell density culture growth requires the use of specialized equipment such as the BioFlo IIc.

When studying protein expression in shake flask experiments, a steady state glucose concentration does not necessarily need to be maintained because the concentration of the substrate does not have a noticeable effect on the SGR. If it did have

an effect, then any testing of expression levels would need to take glucose concentration into account. The glucose levels would have to remain consistent in order to prove that a change in the SGR during induction would be due to protein expression and not to a change in the glucose concentration. This is not an issue in these optimization experiments. Instead, an E. coli culture in M9 should be induced at a cell density near 1.0 in order to maintain physiological pH.

This experiment was unable to test whether the accumulation of byproducts such as acetic acid could affect cell growth in high cell density fed batch fermentations. The relatively short amount of time allowed for culture growth in shake flasks does not allow inhibitory concentrations of byproducts to be produced. It is unclear whether the drop in pH in the high glucose concentration cultures resulted from either ammonium uptake, acetic acid production, or both. The creation of acetic acid is a possibility, however, since the specific growth rate calculated in these experiments is relatively high. A shake flask experiment was attempted in which small quantities of glucose were added at regular time intervals in order to maintain a low average glucose concentration. Even in these conditions, the pH dropped to inhibitory levels by the time an OD_{600} of 2.5 was reached (results not shown). Therefore, experiments related to glucose concentration and acid formation are best left to fed-batch experiments in the bioreactor.

4. Catabolite Inhibition of Induction

4.1 Background

The lacUV5 promoter employed to express recombinant DNA in the Rosetta/pET system is based on a lac promoter. In the presence of lactose or the non-metabolizable analog IPTG, the wild type lac promoter initiates the production of galactosidase. Galactosidase is an enzyme required by the cell for metabolism of lactose and galactose. The wild type lac promoter also requires the presence of cAMP before it allows bonding of polymerase. The culture only produces cAMP when glucose is below 1%w/v as a signal that substrates other than the preferred carbon source of glucose should be catabolized (Hirschel et al., 1980). The lacUV5 promoter includes three point mutations to the wild type lac promoter that are intended to reduce its dependence on cAMP for induction. Previous researchers have found that lacUV5 does not experience catabolite inhibition in the presence of glucose (Ochocka et al., 2003; Hirschel et al., 1980), but glucose has been found to have an effect on basal level expression (Grossman et al., 1998; Novy and Morris, 2001). Therefore, the effect of glucose on expression of HA2_185 in Rosetta was tested.

The host strain was grown in 50 mL cultures in M9 media with initial concentrations of either 2 g/L or 20 g/L glucose. Seven hours after inoculation, 5 mL of each culture were collected for use as the uninduced, control samples and transferred to 10 mL culture tubes. IPTG was added to the remaining culture in each shake flask to yield a final inducer concentration of 1 mM. Multiple samples of the induced and uninduced cultures were taken at 2 hrs after induction. A portion of the samples were

lysed and separated into soluble and insoluble fractions and analyzed by SDS-PAGE according to the protocol in Appendix A. The rest of the samples were retained as total cell protein (TCP) fractions. The TCP fraction is a solubilization of the entire culture pellet without any purification

4.2 Results

The growth curves for both the induced and uninduced cell cultures are shown in Figure 3. The SGR for the uninduced cultures were calculated to be 0.36 hr^{-1} for the culture with 2 g/L initial glucose and 0.34 hr^{-1} for the culture with 20 g/L initial glucose concentration. The induced cultures showed significant growth inhibition during protein expression.

Figure 3: Effect of Glucose on Growth Before and After Induction

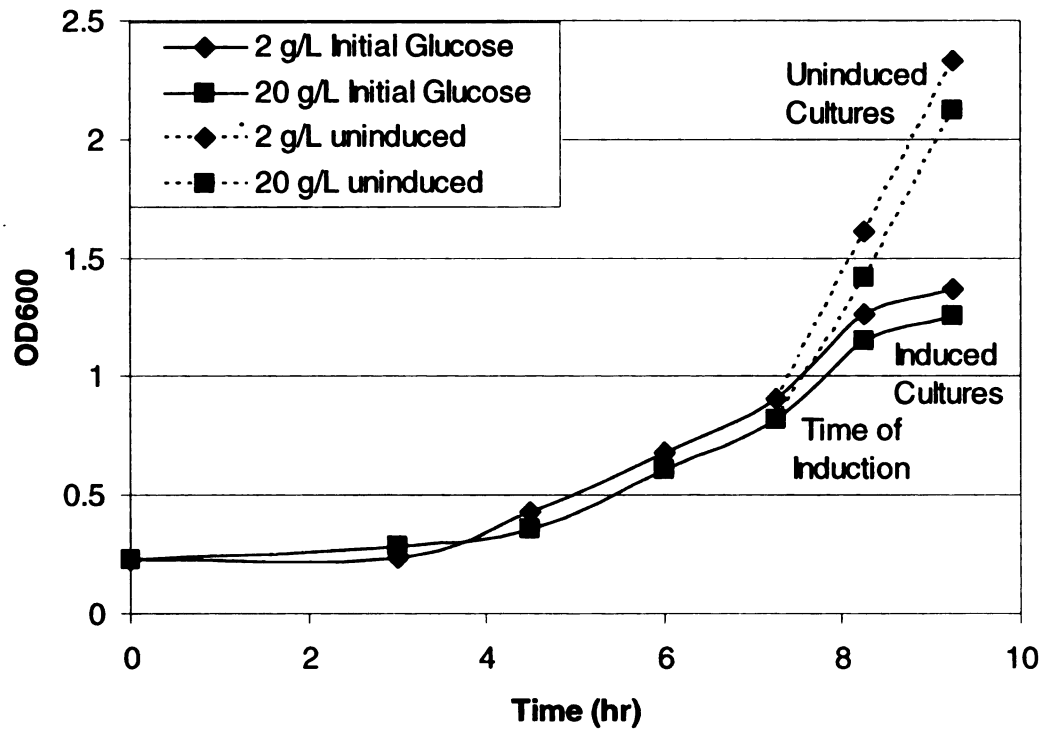
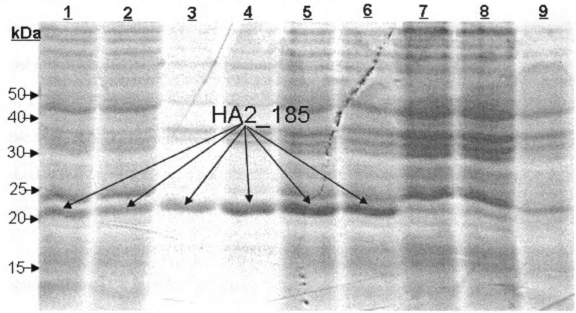


Figure 4 exhibits the results of the SDS-PAGE analysis of the culture samples. Lane 9 contained the TCP fraction of a sample taken at the time of induction. Lanes 1 through 8 contained the protein from the culture samples taken 2 hrs after the time of induction. Specifically, lanes 5 through 8 contained the TCP fractions, lanes 1 through 2 contained the soluble fractions, and lanes 3 through 4 contained the insoluble fractions of the induced culture samples.

Figure 4: Effect of Glucose on HA2_185 Expression



Lane	Glucose	Fraction	Induced	Lane	Glucose	Fraction	Induced
1	2 g/L	soluble	yes	6	20 g/L	TCP	yes
2	20 g/L	soluble	yes	7	2 g/L	TCP	no
3	2 g/L	insoluble	yes	8	20 g/L	TCP	no
4	20 g/L	insoluble	yes	9	2 g/L	TCP	0 hr
5	2 g/L	TCP	yes				

4.3 Discussion

The SGR was again found to be independent of the glucose concentration. Also, the average SGR calculated in these experiments, 0.35 hr^{-1}, was similar to the SGR of

0.39 hr^{-1} found in the experiment previously discussed. Induction, however, had a very significant effect on the growth rate. Two hours after induction, the OD_{600} of the uninduced cultures had increased by approximately three times the magnitude of the increase in the OD_{600} of the induced cultures. This could have been a result of product inhibition, toxicity of the IPTG, or the diversion of cellular resources to the production of HA2_185.

The SDS-PAGE analysis shown in Figure 4 indicated that the Rosetta/pET expression system had the ability to express HA2_185 at high enough concentrations to satisfy the mass and purification requirements of the project. Lanes 1 through 6 of Figure 4 had 23 kDa bands that were missing in the uninduced fraction. HA2_185 has a weight close to 23 kDa. The similarity of the intensity of the HA2_185 bands in lanes 5 and 6 indicated that the difference in glucose concentration between the two cultures had not resulted in a variation in expression levels.

Lanes 1 through 4 shown in Figure 4 compared the insoluble and soluble fractions of the TCP samples ran in lanes 5 and 6. The insoluble fractions contained the dense inclusion bodies formed by the HA2_185, while the soluble fractions contained the HA2_185 that had not aggregated into inclusion bodies during expression and lysis. This HA2_185 fraction was assumed to primarily consist of membrane bound and detergent solubilized HA2_185 in its native conformation. For the purposes of this thesis, the fraction of expressed protein that did not form inclusion bodies was designated "soluble HA2_185". The heavy bands in lanes 3 and 4 suggested that the induction conditions of 37 °C and 1 mM IPTG utilized in this experiment resulted in the formation of inclusion bodies of HA2_185. In addition, the bands were more intense than those displayed in

lanes 1 and 2. This suggested that less protein remained in the soluble than in the insoluble form after two hours of induction at these conditions. The visual appearance of the gel suggested that a higher portion of the HA2_185 was found in the insoluble fraction of the culture sample at 20 g/L glucose than in the 2 g/L sample. The total amount of all the proteins in the 20 g/L insoluble fraction was also greater, however. Correspondingly, the 20 g/L soluble fraction had a smaller total amount of protein. It was likely that the separation of the soluble fraction from the insoluble fraction during preparation of the 20 g/L sample was incomplete. This would account for the appearance of the higher mass of inclusion bodies in the 20 g/L culture sample.

An inducer concentration of 1 mM IPTG is recommended for maximum expression levels in the pET vector (Novagen, 2002). The T7 promoter used in the pET expression system is very strong. Upon full induction, the pET vector is designed to set all of the anabolic activity of the cell towards production of the recombinant protein. As shown in Figure 3, this causes a significant reduction in cell growth. In addition, the high induction activity causes large levels of inclusion body formation. This suggests that the induction conditions used in this experiment should be modified if increases in the overall yield of the soluble fraction versus the insoluble fraction are desired.

5. Effect of Temperature and IPTG Concentration on Induction

5.1 Background

As discussed in section 4, the Rosetta/pET expression system tends to form inclusion bodies at high induction strength. In recombinant protein expression, it is often desirable to have control of the yield of soluble protein. A reduction in inclusion body formation can be accomplished by decreasing the induction levels during expression (Laage and Langosch, 2001; Novagen, 2002). Lower induction levels act to reduce the rate of protein formation and lower the concentration of unbound protein in the cytoplasm. This reduced protein concentration decreases the interaction between the hydrophobic regions of the induced proteins and lowers the probability of aggregation.

Induction levels in Rosetta can be reduced by lowering the IPTG concentration. Wild type E. coli utilize lac permease for active uptake of lactose analogs (Garrett and Grisham, 1999). Rosetta, however, has a mutation that inactivates the lacYI gene that encodes for this transport protein (Novagen, 2002). Therefore, IPTG enters the cell via passive diffusion that is dependent on the concentration of IPTG in the media. Since the activity of the lac promoter is dependent on the local IPTG concentration, the induction level can be controlled by varying the amount of IPTG added to the fermentation broth.

Induction levels are also dependent on temperature because protein is formed by enzymatically catalyzed reactions. Enzymatic activity decreases with decreasing temperature. Therefore, if the temperature in the bioreactor is reduced, the rate of protein formation is also reduced.

An experiment was conducted to determine the effect of temperature and IPTG concentrations on cell growth, protein expression, and inclusion body formation. A single test tube culture of Rosetta was used to inoculate 300 mL of M9 media containing 4 g/L of glucose and the required antibiotics as described in Appendix A. The culture was incubated in a 1 L Erlenmeyer flask until an OD_{600} of 1.0 was reached at 10 hours. It was then split into six 50 mL fractions in 250 mL shake flasks. IPTG was added to the flasks to produce duplicate cultures with inducer concentrations of 0.5, 0.05, or 0 mM. Half of the duplicate shake flask cultures were then transferred back to the 37 °C incubator. The other three flasks were kept at room temperature (~25 °C). Samples were collected over five hours and analyzed by SDS-PAGE.

5.2 Results

Figure 5 shows the effect of the IPTG concentration and the temperature on the cell densities of the cultures after induction.

Figure 5: Effect of Temperature and IPTG Concentration on Growth

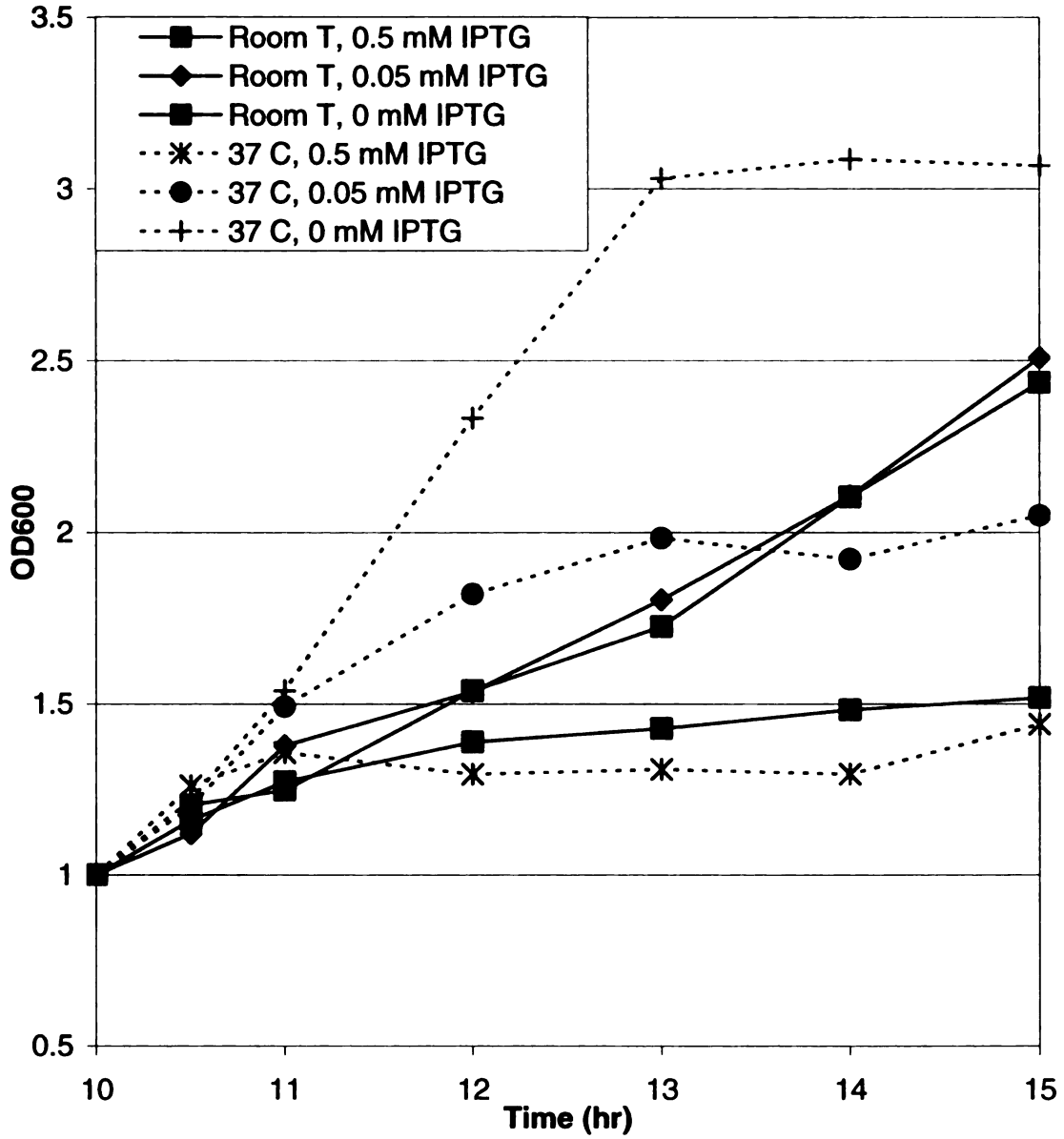
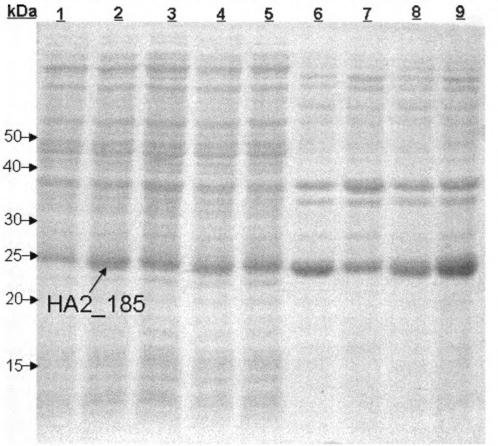


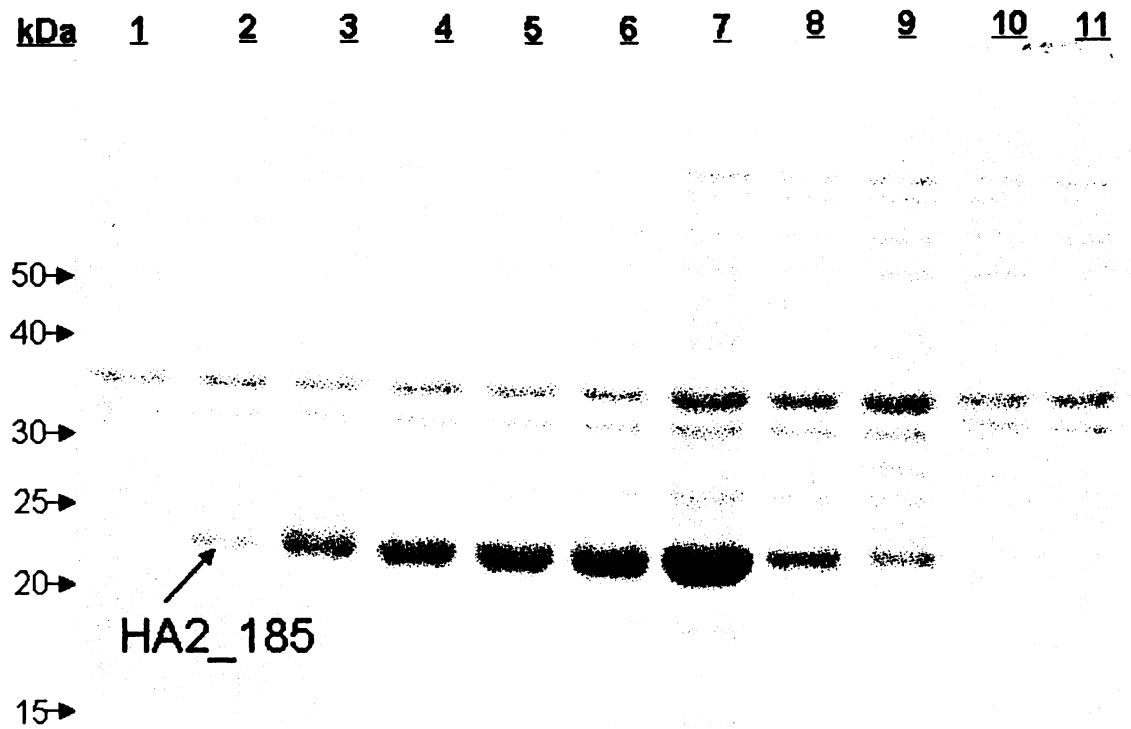
Figure 6 shows the results from the SDS-PAGE analysis of the soluble and insoluble fractions of the induced cell culture 3 hours after induction. Figure 7 shows the insoluble fractions from the induced cultures grown at room temperature.

Figure 6: Effect of Temperature and IPTG on HA2_185 Expression after 3 hrs



Lane	IPTG	Fraction	Temp.	Lane	IPTG	Fraction	Temp.
1	0 mM	soluble	room	6	0.05 mM	insoluble	room
2	0.05 mM	soluble	room	7	0.5 mM	insoluble	room
3	0.5 mM	soluble	room	8	0.05 mM	insoluble	37 °C
4	0.05 mM	soluble	37 °C	9	0.5 mM	insoluble	37 °C
5	0.5 mM	soluble	37 °C				

Figure 7: Effect of IPTG on Inclusion Body Formation



Lane	Time	IPTG	Lane	Time	IPTG	Lane	Time	IPTG
1	0 hr	0.5 mM	5	3 hr	0.5 mM	9	2 hr	0.05 mM
2	0.5 hr	0.5 mM	6	5 hr	0.5 mM	10	1 hr	0.05 mM
3	1 hr	0.5 mM	7	5 hr	0.05 mM	11	0.5 hr	0.05 mM
4	2 hr	0.5 mM	8	3 hr	0.05 mM			

5.3 Discussion

As was expected, the uninduced culture grew faster at 37 °C than at room temperature (~25 °C) (Shuler and Kargi, 2002). Induction also had a very significant effect on the growth rate and the extent of the growth inhibition during induction was dependent on the temperature. The OD_{600} of the cultures induced with an IPTG concentration of 0.5 mM IPTG increased very little after induction. The culture at 37 °C

and 0.5 mM IPTG entered a stationary phase within 1 hour of induction while the cell density of the culture at room temperature continued to increase slightly. At 37 °C, the growth rate of the culture with 0.05 mM IPTG was substantially inhibited in comparison to the uninduced culture. Up until 3 hrs after induction, both of the cultures at 37 °C increased cell mass at a greater rate than the cultures at room temperature. After 3 hrs of induction, the cultures at 37 °C entered into a stationary phase that probably resulted from nutrient depletion. The cultures at room temperature continued to grow until the end of the 5 hour induction period. Also, induction at 0.05 mM IPTG did not appear to have inhibitory affects on the cellular growth rate at room temperature.

The intensity of the HA2_185 band was not noticeably different in lanes 2 through 5 as shown in Figure 6. All of the cultures had produced about the same amount of soluble HA2_185 after 3 hrs of induction. In fact, a comparison of the soluble fractions from all of the time points showed that the quantity of soluble HA2_185 displayed in Figure 6 was the maximum attainable quantity in the culture in these experiments (results not shown). Within 3 hours of induction, all of the cultures had reached a maximum limit on the amount of soluble HA2_185 that they could produce and this maximum quantity was the same irrespective of induction conditions. The cultures with higher induction strengths reached their soluble HA2_185 limits within an hour, while the cultures with lower inducer concentrations took longer. A difference, though, could be found in the total mass of inclusion bodies formed. As can be seen in lane 11 of Figure 6, the culture with the highest induction strength, 37 °C and 0.5 mM IPTG, produced significantly more insoluble HA2_185 than the others. The samples ran in lanes 8 though 10 and all exhibited similar HA2_185 band intensities. The culture

induced at room temperature and 0.5 mM IPTG exhibited slightly less insoluble protein, but this was attributed to its lower cell density relative to the other two samples.

The gel in Figure 7 was run in order to determine the time rate of change of inclusion body formation. Lanes 1 through 6 showed that inclusion bodies continued to increase with time in the culture at 0.5 mM IPTG and room temperature. A significant mass of insoluble protein was present within half an hour of induction. In comparison, inclusion bodies did not appear in the culture subjected to a lower concentration of inducer until 2 hours after induction. Its overall production of HA2_185 after five hours of induction, however, had far exceeded that created by the culture at 0.5 mM IPTG. This is a result of the higher cell density obtained in the culture at 0.05 mM IPTG.

The induction process has a toxic effect on cell growth that is relevant to the optimization of overall protein yield. If recombinant protein expression causes a large enough decrease in cell growth or cellular metabolism, then the overall protein yield may be very low. This is exemplified by a comparison of the final HA2_185 mass obtained in the cultures displayed in Figure 7. While the culture at 0.5 mM IPTG initially had a greater rate of HA2_185 production, the culture at 0.05 mM IPTG ultimately produced a higher mass of HA2_185 because it did not enter into a stationary phase as did the other. Optimally, inducer activity should be high enough to direct the majority of the cellular anabolic activity towards recombinant protein production. The induction level, however, should not be so high that it causes a stress response in the cells and forces the culture into a stationary phase. In some cases, the stress response resulting from recombinant protein expression has been shown to become more pronounced at higher temperatures (Hunke and Betton, 2003). This effect was seen in these experiments and suggests that

both the temperature and inducer strength should be lowered to in order to maintain cell growth.

The total amount of soluble HA2_185 that is obtained per mass of culture is independent of the induction strategy. This suggests that the amount of HA2_185 that can remain in a soluble state in a Rosetta cell is limited by the membrane surface area with which the protein can interact. During expression, the hydrophobic fusion peptide of HA2_185 must become incorporated into the hydrophobic portion of a lipid bilayer in order for it to remain soluble. Otherwise, interactions between the peptides causes them to aggregate and create inclusion bodies. A saturation limit is reached after a specific quantity of the peptide has become embedded in the membrane. When this solubility constraint is attained, additional expressed fusion protein must necessarily form inclusion bodies.

Under the above hypothesis, the yield of soluble protein can be maximized by developing an induction strategy that results in the formation of the maximum amount of allowable soluble protein while minimizing inclusion body formation. For the cultures induced at lower temperatures in this experiment, inclusion bodies only began to form after the saturation limit for the soluble protein was reached. Kim et al. (1996) found the maximum incorporation level of the fusion protein to be 50% of the total protein in the membrane. In the experiment, the culture subjected to the lower inducer concentration did not form significant quantities of inclusion bodies until much later in the induction phase. It also did not experience the growth inhibition displayed by the cultures at higher induction levels. Therefore, recommended conditions for production of soluble HA2_185 in E. coli include lower temperatures and reduced IPTG concentrations.

6. Expression of HA2_185 in a Fed-Batch Bioreactor

6.1 Background

Based on the results of the batch growth and expression experiments, an induction scheme was devised to produce isotopically labeled HA2_185 in a fed-batch bioreactor. Optimal results were based on four criteria:

1) High overall yield of active HA2_185 per mass of cell protein
2) Maximum incorporation of the labeled amino acid
3) Minimal isotopic dilution
4) Minimal cost

HA2_185 needs to be produced in high overall concentrations due to the requirements of the purification process. The immobilized metal affinity chromatography (IMAC) operation used to purify HA2_185 preferentially binds the heterologous protein relative to the native constituents of the cell. If the majority of the affinity sites are occupied by HA2_185 after the cell lysate and column resin are equilibrated, then the other proteins can be easily washed from the chromatography column and the HA2_185 can be eluted at a high purity. If, however, the HA2_185 is at a low overall concentration compared to the contaminant proteins, equilibrium conditions favor that at least some contaminant protein binds to the column. This reduces the effectiveness of the chromatography purification.

The purified protein must also be in its native, solubilized state or the NMR results are not indicative of the active structure of the protein. An attempt was made to solubilize the HA2_185 inclusion bodies using the denaturant urea. The unfolded protein

was then allowed to renature by removing the urea from the buffer solution in the presence of detergent. Unfortunately, the solubilized protein was only partially active in producing fusion events at low pH (results not shown). This suggested that a portion of the protein remained in a denatured state after solubilization. Due to time constraints, more complex renaturation procedures were not attempted.

Therefore, only the soluble fractions of HA2_185 are suitable for use in NMR experiments. The shake flask experiments suggested that the maximum overall concentration of soluble HA2_185 is dependent on the saturation limitations of the lipid bilayer of the cell. After the maximum amount of soluble protein is produced, the rest enters into inclusion bodies. Since inclusion bodies cannot be used for NMR, there production is a waste of cellular capacity and should be minimized. This is especially true considering the cost of the labeled amino acid used to form them.

The issue of isotopic dilution can be handled in multiple ways (Muchmore et al, 1989). The most effective solution is to utilize an auxotrophic host strain that can neither break down nor produce specific amino acids. The expression system for this project had already been chosen, however. Another common method of reducing isotopic dilution is to use a defined media that includes a full complement of all of the amino acids required by the cell. If the culture does not need to produce amino acids, then the transamination reactions required for amino acid production are not active and isotopic dilution is unlikely. This type of defined media is very expensive, however, so this approach conflicted with the desire to minimize cost. Therefore, M9 medium was used, and the labeled amino acid was added at induction. This labeling procedure has worked successfully for other researchers (Verdemato et al., 2000)

Practical steps can be taken to decrease the likelihood that the labeled amino acid are metabolized by the cell. First, as glucose is the preferred substrate, it can be kept at concentrations above 1 g/L for the duration of the fermentation, which would exceed the uptake saturation level for the sugar (Levisauskas, 2003). Secondly, very high induction levels can cause a stress response in the cell that leads to the production of proteolytic enzymes (Medina et al., 2002; Schweder et al., 2002). This may cause unnecessary degradation of HA2_185 and increase the possibility of isotopic dilution. Therefore, lower levels of expression are preferred.

Based on the rationalization given above, a strategy for HA2_185 expression in fed-batch fermentations was developed. The set-up and operation of the BioFlo IIC were as described in the FED-BATCH section of Appendix A. M9 medium with an initial concentration of 4 g/L glucose, 20 mg/L chlorimphenicol and 50 mg/L kanamycin was employed as the growth media. The bioreactor was inoculated with 45 mL of Rosetta at an OD_{600} of 1.8. Glucose was fed manually throughout the fermentation at a rate required to keep the substrate from becoming depleted. A total of 18.5 g glucose was added by the end of the fermentation. The BioFlo IIc was set to control temperature at 37 °C, and air flow was maintained at 0.1 L/min throughout the exponential growth phase of the culture. Agitation was controlled to maintain a dissolved oxygen concentration of 30%. Ammonium hydroxide was added as needed to maintain the pH at 7.0. When the culture reached an approximate OD_{600} of 7 at 12.75 hrs after inoculation, the temperature in the bioreactor was lowered to 26 °C. The culture and BioFlo IIc controller were given 15 minutes to adjust to the change in temperature. Then, 200 mg of leucine and 0.2 mmols of IPTG were added to the 1 L of fermentation broth. The induction phase was

allowed to proceed for two hours during which culture samples were taken every half hour. The samples were fractionated into soluble and insoluble fractions and analyzed by SDS-PAGE. At the end of the induction period, the culture was harvested so that the HA2_185 could be purified and analyzed by NMR.

6.2 Results

Figure 8 shows the OD_{600} of the fermentation culture in the BioFlo IIc prior to induction. The calculated SGR for the culture was 0.40 hr^{-1}. The OD_{600} of the culture at the conclusion of the induction phase was 8.6. Figure 9 is the SDS-PAGE analysis of the soluble and insoluble fractions over the two hour induction period.

Figure 8: Growth in the BioFlo IIc

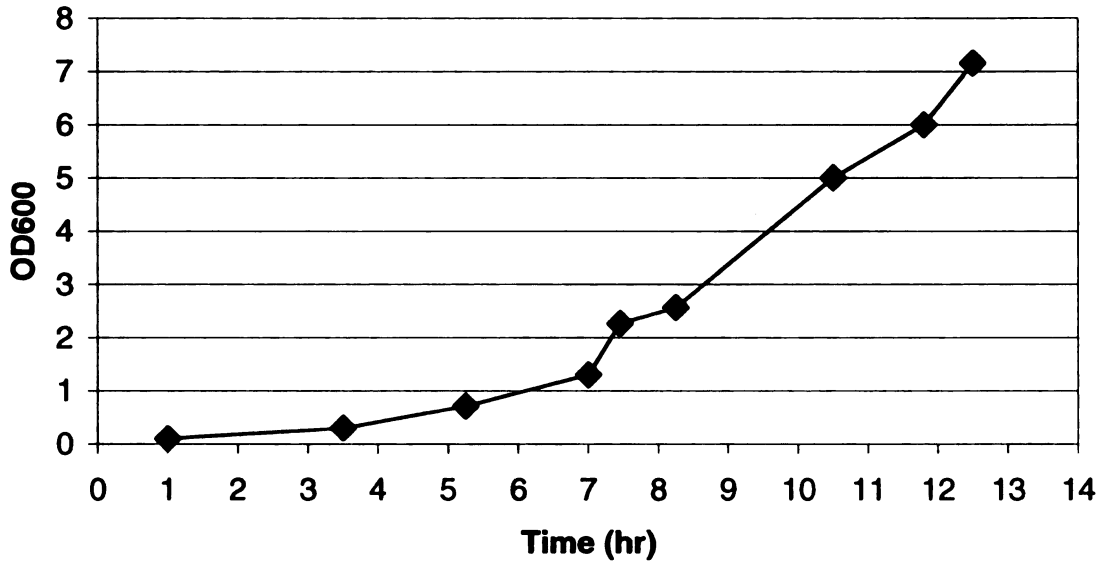
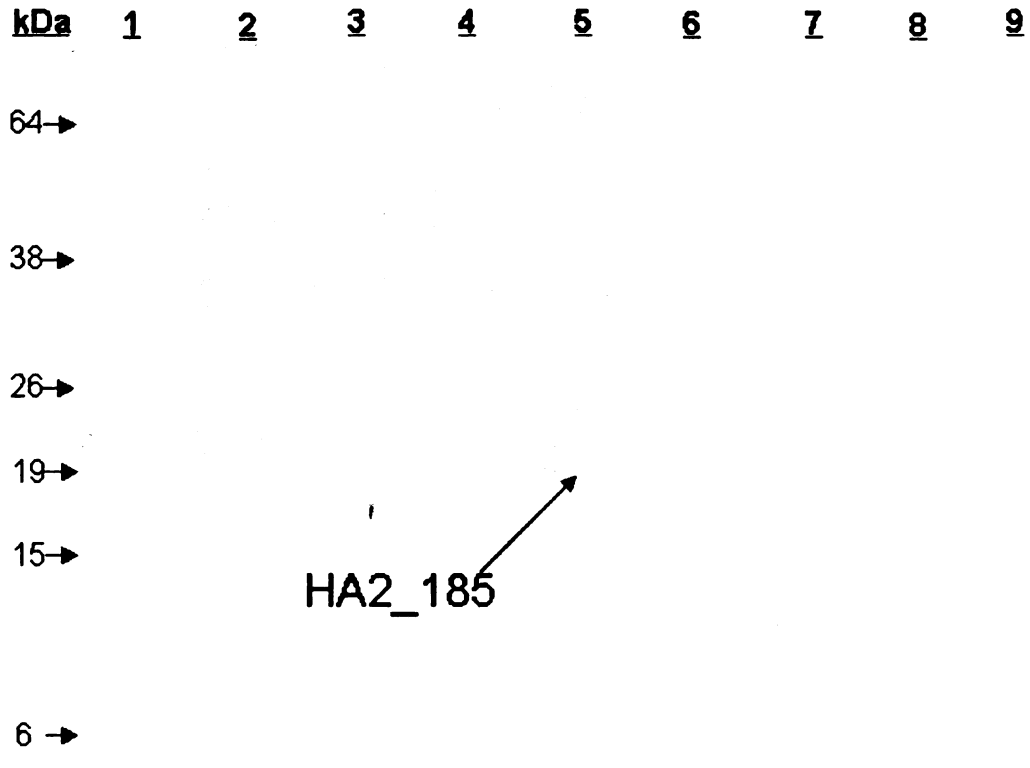


Figure 9: Expression of HA2_185 in the BioFlo IIc



Lane	Time	Fraction	Lane	Time	Fraction
1	0 hr	soluble	6	0.5 hr	insoluble
2	0.5 hr	soluble	7	1 hr	insoluble
3	1 hr	soluble	8	1.5 hr	insoluble
4	1.5 hr	soluble	9	2 hr	insoluble
5	2 hr	soluble			

6.3 Discussion

Growth and expression in the bioreactor closely mimicked the results found in the shake flask experiments. The SGR was the same in both the fed-batch fermentations and the batch fermentations. Nutrient depletion or excessive fermentation byproduct creation also did not appear to be an issue at the cell densities reached in this fermentation; the growth rate was nearly constant prior to the time of induction.

The IPTG concentration of 0.2 mM and the expression temperature of 26 °C were specifically chosen to reduce the likelihood of a stress response in the culture. The lowered temperature decreased the overall growth rate, but the cells continued to grow and reached a final OD_{600} over 1 unit higher than at the time of induction. In addition, the oxygen uptake rate of the culture continued to increase as indicated by the continuing ramp of the agitation rate (results not shown). In order to maintain a constant dissolved oxygen concentration, the agitation rate had to increase in order to balance the rate of oxygen transfer with the increased rate of oxygen uptake required for cell growth and protein expression.

As shown in Figure 9, very little inclusion body mass was found in the insoluble fractions of the culture samples. The overall quantity of insoluble protein found in the SDS-PAGE analysis was also very low, though, as indicated by the faint bands in lanes 6 through 9. Therefore, it was difficult to draw conclusions about the extent of inclusion body formation in relation to the overall cell mass. Because the insoluble HA2_185 band never exceeded the intensity of the band at ~38 kDa, it was assumed that inclusion body formation was not excessive. HA2_185 levels in the soluble fraction noticeably increased over the course of the induction phase. Comparing the relative intensities of the HA2_185 bands in Figure 4 and Figure 6 implied that more soluble protein could have been produced before the solubility limit was reached. Even so, the mass of HA2_185 in the harvested cell culture was high enough relative to contaminating proteins that satisfactory purity was obtained through the purification procedure. When Rosetta was allowed to express for six hours at the same conditions in a separate experiment, the

HA2_185 concentration far exceeded the previously observed soluble fraction limit within three hours (results not shown).

Incorporation of the isotopically labeled leucine into the protein for purposes of NMR study was also successful. Based on the NMR signal, the ratio of labeled leucine to unlabeled was estimated at 50%, a high enough incorporation rate for analysis by REDOR (results not shown). Isotopic dilution was not significant enough to affect the results of the NMR studies.

These experiments illustrated the use of the protocols described in Appendix A to optimize protein production in recombinant expression systems. Results from batch experiments were used to develop a successful expression strategy for fed-batch fermentations. In addition, simple tests such as OD_{600} and SDS-PAGE were used to determine the SGR of a host strain, inhibitory effects of the induction system, the rate of inclusion body formation in recombinant E. coli, and media selection.

Significant limitations to this work and the supplied protocols exist with regards to fed-batch fermentations. Relatively small quantities of protein were required for the REDOR analysis, so the growth of high cell density cultures in the BioFlo IIc was not mandatory for the success of this project. The maximum dry weight of the culture never exceeded 5 g/L. Attempts were made to grow this host to high cell densities in conditions similar to those used in this experiment, but growth inhibition was found to varying degrees when the culture reached dry weights above 5 g/L (results not shown). Others have obtained dry weights of E. coli greater than 150 g/L (Lee, 1996). At these cell concentrations, inhibition by toxic byproducts and oxygen transfer limitations become increasingly problematic. The ability to optimize the control scheme to

overcome these limitations was addressed in the development of Presto Control, however.

7. Presto Control

7.1 Background

Presto Control is the name given to the controller software designed for conducting fed-batch fermentations in the BioFlo IIc. The reasons for its creation have already been discussed in the introduction in section 1. A description of Presto Control's features and an introduction to fermentor control can be found in Appendix B.

Riesenberg (1999) and Rani (1999) review the vast number of control schemes and concepts developed to produce high cell density cultures. Other than the stress response caused by anoxic conditions in a bioreactor, poor control of substrate concentration is the principal concern in high cell density culture growth and recombinant protein expression (Konz et al., 1998; Notley and Ferenci, 1996). For example, starvation for more than a brief period of time can irreparably reduce both growth rate and maximum cell density if it is allowed. Generally, though, feed depletion is not harmful if it can be corrected within a few minutes (Suzuki et al., 2000).

The Standard Fed Batch control scheme available in Presto Control utilizes a specific method for detecting and correcting substrate starvation. This scheme is described in detail in Appendix B. In brief, the control unit detects a spike in the dissolved oxygen (DO) caused by a starvation induced drop on the cellular respiration rate of the cells. It then reacts by adding a specified amount of feed. This is a commonly utilized feed strategy (Nor et al., 2001; Knop, 2002; Suzuki et al., 2000; Oh et al., 1998).

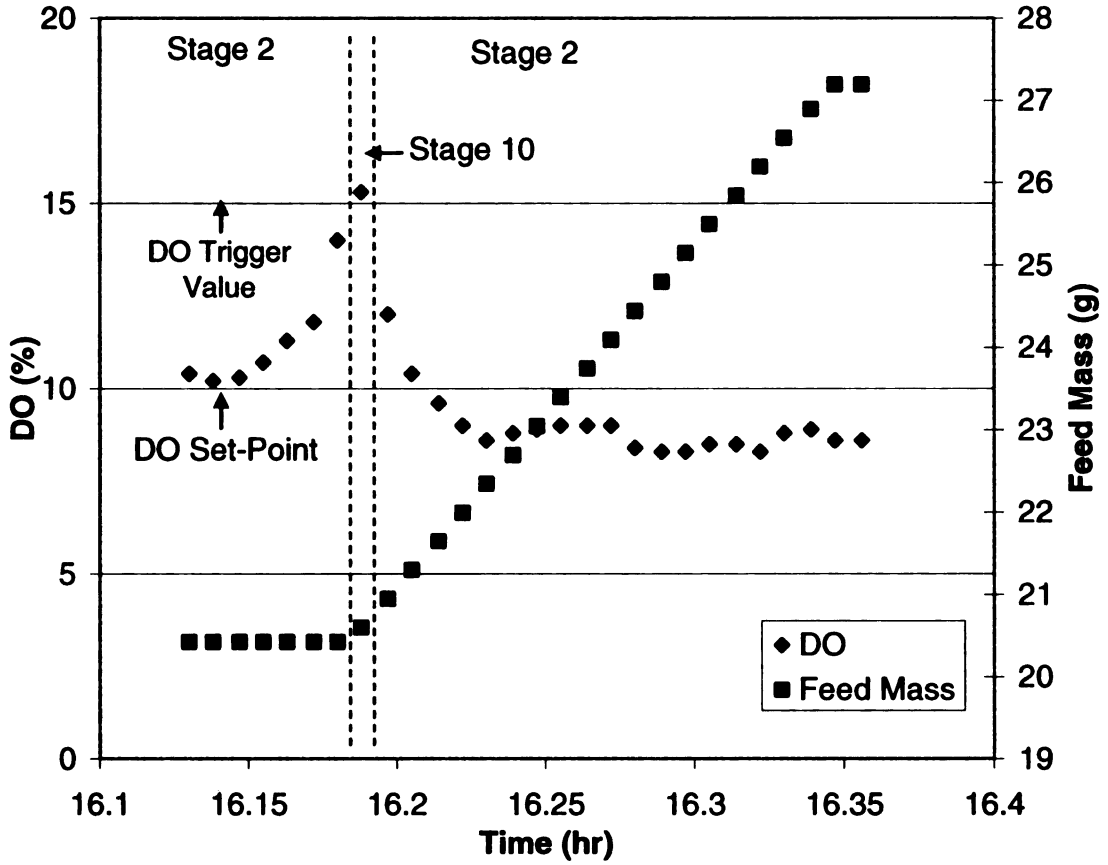
To test this controller response, the BioFlo IIc was prepared and inoculated with Rosetta following the protocols in Appendix A. The Standard Fed Batch control recipe

was utilized as described in the protocols, and an initial 10 g charge of feed was added in the first stage. Eight hours after inoculation, substrate depletion was detected by the controller, and the results showing controller compensation are presented below. The response of the controller was governed by stage 2 and stage 10 of the Standard Fed Batch control scheme.

7.2 Results

Figure 10 shows the data points taken by the fermentation logging function of Presto Control at the time of the feed depletion event. The time points of the DO and the total mass of glucose added were recorded every thirty seconds. The stage logging feature was referenced to give the exact times of the stage switches. The DO set-point and trigger values are indicated on the graph. Also, the period of each stage is designated with the dotted lines. At the time of feed depletion, the control scheme was in stage 2. At a recorded time of 16.1843 hrs into the fermentation, the controller detected that the DO in the BioFlo IIc had exceeded 15%. Since Trigger A of stage 2 is set to call stage 10 when the DO exceeds 15 %, stage 10 was initiated. Immediately, the feed pump began adding feed as specified in stage 10. At 16.1912 hrs (25 seconds later), the controller detected that the DO had dropped below 15% again. This triggered stage 2 and the final 10 grams of feed specified in stage 10 were added.

Figure 10: Presto Control Response to Substrate Depletion



7.3 Discussion

The Standard Fed Batch control scheme responded as expected to the feed depletion event. Unfortunately, due to the 30 second scan rate for variable logging, only a broad understanding of the probe and controller responses can be obtained from the data shown in Figure 10. The following description should provide greater insight into the events.

Before feed depletion, the DO began to slowly rise over a two minute period as the glucose concentration dropped to inhibitory levels. When the glucose was completely consumed, the DO rose from 12% to 15% within 5 seconds. In response, the feed pump

immediately began to add glucose at a rate which brought the glucose to a concentration above 0.05 g/L within 5 seconds. Uptake of the glucose and reestablishment of the original oxygen uptake rate was nearly instantaneous. The DO probe, however, exhibited an approximate 5 second time lag, which delayed the drop in measured oxygen. This response delay allowed the DO to spike to over 20% before the DO began to decrease. Initially, the DO decreased very quickly but. as it got closer to its steady state value, the rate of decrease declined. At 25 seconds, the DO finally dropped below 14% and the rest of the 10 grams was added.

If the DO had not dropped below 14% within 30 seconds, feed would have been cut off by the triggering of stage 11. This action is designed to prevent excessive amounts of feed from being added due to rises in the DO that are not caused by feed depletion. It is possible that the DO response to feed might be slow enough that a control scheme might discontinue feeding even if the detected DO spike was due to glucose depletion. At the tubing size and glucose concentration recommended in Appendix A, 0.35 g of glucose would have been added to the fermentation media before the thirty second cut-off point. This is well above the saturation limit of glucose for E. coli (Levisauskas et al., 2003). Therefore, even if the control scheme did halt feeding early by triggering stage 11, the DO would quickly drop back down to steady state and the controller would be prepared to detect the next DO spike.

The response of Presto Control to a feed depletion event is a very basic example of the capabilities of the program. Appendix B explains its features and its use in controlling a fed-batch fermentation. More importantly, Presto Control is flexible

enough that an operator can tune the control parameters and design control schemes according to the needs of each protein expression system.

APPENDICES

APPENDIX A

PROTOCOLS FOR PROTEIN EXPRESSION

APPENDIX A: TABLE OF CONTENTS

INTRODUCTION

BACKGROUND

This is intended as a general protocol for producing protein in microbial cultures using the equipment available in room 3262 of the Engineering Building at Michigan State University. The included material is particularly directed towards optimizing intracellular protein production in recombinant E. coli. It is split into three sections. The first section describes batch growth and production experiments in shake flasks and test tubes. The second, much longer section, contains protocols for completing fed batch protein production experiments using the BioFlo IIc. The third section contains descriptions of tools and techniques that are relevant to both batch and fed-batch projects. It includes a protocol for SDS-PAGE, cell density measurements, autoclaving, centrifugation of the culture, and various media recipes.

This manual assumes that the operator has already created or obtained the host strain and induction procedure. It does not include information on the creation or transformation of host cells and vectors for recombinant engineering. Molecular Coning: A Laboratory Manual (Sambrook and Russell, 2001) can be referenced for these procedures. In addition, the "pET System Manual" (Novagen, 1992) has a good overview of common recombinant E. coli strains and induction systems.

These protocols assume that the operator has some knowledge of aseptic culturing techniques and cellular biochemistry and growth kinetics. Every operator has their own personalized style and beliefs on cell culturing and protein production, so view this manual as suggested practice and not as scientific law.

A choice needs to be made regarding the growth media. The best media for use in fermentations varies depending on the cell type and the final product desired. Different media compositions and their applications can be found in MEDIA on pg. 85.

The goal in recombinant engineering is to overexpress a heterologous protein in a cell culture. Two common methods for achieving this goal exist. The first is to express the protein as a batch culture in a shake flask or test tube. The second is to grow the culture in a stirred tank bioreactor using probes and media feeds to control the environmental conditions. Other methods such as continuous growth reactors and immobilized cell biocatalysis exist, but they require special equipment and are not covered in this protocol. Batch suspension cultures and fed batch fermentations in bioreactors each have specific advantages over the other.

The major advantage of batch fermentations in shake flasks or test tubes is that they do not require specialized bioreactor equipment. In addition, the preparation time for the experiment is much lower. All that is required is an enclosed vessel and a shaker to keep the culture suspended. The disadvantage is that the environmental conditions of a suspension cultures are difficult to control. Feed concentration, pH levels, and oxygen

concentration in the media can be impossible to maintain. Therefore, the cell density in a shake flask culture is limited by the buffering capacity of the media and the minimal oxygen transport allowed by the shaker and the flask. Generally, the maximum dry weight of cells per liter of media that can be obtained in a batch culture is 2.5.

In contrast, fed batch fermentations in a bioreactor can produce cell densities as high as 50 times those in a shake flask. Substrate can be fed on demand to keep the culture from starving without toxic overfeeding. Acid or base can be added to maintain physiological pH. Agitation and aeration can be ramped up to maintain the desired dissolved oxygen concentration for growth. All of these processes, however, require specialized equipment and probes. In addition, the culture volume is limited by the size of the bioreactor. The bioreactor described in this protocol, the BioFlo IIc, has a working volume of 1 L. Each experiment requires a relatively large quantity of media and, therefore, a large expenditure of time and money. Furthermore, if only one bioreactor is available, fermentations cannot be run simultaneously and the results compared.

For these reasons, the choice to use a suspension culture or a stirred tank bioreactor is dependant on the goals of the fermentation. Batch fermentations work well as preliminary steps in optimizing cell growth and protein production. Small batch experiments with differing media or induction conditions can be run simultaneously and the results used to determine the optimum expression strategy. The expression strategy can then be employed in high cell density, fed batch fermentations in order to produce large amounts of the desired protein. In some cases, however, the expression strategy is dependant on the cell density, so the BioFlo IIc is required for the final optimization steps. Conversely, the protein being produced might only be needed in small quantities and expression in a shake flask might be ideal.

GENERAL SAFETY GUIDELINES

- If any problem occurs, immediately contact the safety representative for the lab. The name and number of the contact person is posted on the door to the lab.
- The pressure in the air lines and the bioreactor needs to be kept under 15 psig.
- Filters and drains should be monitored for flow resistance or plugging.
- Eye, arm, leg, and feet protection should be worn while working in the lab.
- Gloves should be worn when working with materials that have been exposed to the culture.
- Rubber gloves and splash goggles must be worn when working with acid and base tubing and containers.
- Acid and base containers should only be opened in the fume hood.
- All vessels, including the bioreactor, must be vented during autoclaving.
- Thick autoclave gloves must be worn when handling recently autoclaved vessels.
- All centrifuges need to be properly balanced and the tops to centrifuge tubes and containers must be tightly sealed.
- All surfaces and materials that come into contact with the culture must be sterilized.
- Pipette tips and syringes used to handle the culture should be placed in a biohazard bag after use.

BATCH FERMENTATIONS

These protocols assume that the purpose of the project is to test multiple conditions in simultaneous experiments in order to determine the optimum expression strategy. They are broken into two categories: test tubes and shake flasks. Ultimately, the optimized expression strategy can be used to produce large amounts of protein in large volume shake flasks or in the BioFlo IIc.

When working with a new host or induction system, certain environmental parameters should be checked for their influence on protein expression. They are:

- Temperature
- Bulk media composition
- Initial substrate concentration
- Inducer concentration
- Induction time

Knowledge of the expression host or recombinant protein can supply information on which variables might be important. Other environmental conditions such as cell density, pH, and dissolved oxygen concentration can only be analyzed in the BioFlo IIc.

As with all fermentations, the most reliable method of beginning a project is to use an inoculum culture grown up in a series of increasing volume transfers. A full discussion of inoculum cultures can be found in INOCULUM CULTURES on page 95. In addition, thought needs to be given to the way in which simultaneous batch culture experiments are to be analyzed. If batch cultures are to be compared, they must all come from the same inoculum culture. Furthermore, the inoculum culture should only be separated at the time that the variance in the tested environmental condition is introduced. The growth and expression characteristics of separated cultures can drift apart and cause non-specific variability in the compared results.

The following is an example culture transfer scheme for simultaneous comparison of IPTG inducer concentrations and induction time. The listed steps include inoculum culture transfers.

- Streak glycerol freeze sample onto agar plate.
- Grow to until visible colonies are formed.
- Transfer single colony from plate to 5 mL of media in a 25 mL test tube.
- Grow to OD_{600} of 1.5.
- Transfer 5 mL test tube culture to 200 mL media in 1 L shake flask.
- Grow to OD_{600} of 1.0.
- Separate 200 mL culture into four 50 mL cultures in 250 mL shake flasks.
- Add either 0.1 mM, 0.2 mM, 0.4 mM. or 0.8 mM IPTG to each flask.
- Grow and take samples at 0, 1, 2, 4, and 6 hour time points.

TEST TUBE CULTURES

Due to the small volumes available in test tubes, it is difficult to take more than one culture sample without significantly changing the culture conditions. Therefore, test tubes are primarily used to determine the final results of an expression strategy. They are also useful for testing a large range of varying media compositions. Each test tube contains a specific media type or carbon source and the final expression levels are tested to determine if a specific media type acts to inhibit protein production.

Supplies

Media
Inoculum
Sterilized flask
1 or more sterilized test tubes with tops
Sterilized pipette tips
Sterilized wooden colony applicator or metal loop
Sterilized graduated cylinder (optional)
Sterilized pipette tips
Pipetter
Inducer
Marking tape
Permanent marker

Time: 9 hrs to 18 hrs

Procedure

1) Prepare an inoculum culture on an agar plate or in a test tube according to INOCULUM CULTURES on pg. 95.
2) Prepare the media to be tested according to MEDIA on pg. 85.
3) In the laminar flow hood, transfer the sterilized media to the desired number of sterilized test tubes with caps.
   - Suggested: 5 mL of media in a 25 mL test tube.
4) Move the inoculum plate or test tube culture from the incubator to the laminar flow hood.
   - The culture should not have been refrigerated before transfer. It should be in mid-exponential growth phase at an $OD_{600} \approx 1.0$.
5) Inoculate the test tube with a single colony from the plate or a small volume from the test tube.
   - Remove the cap to the test tube and sterilize the lip of the glass by passing it through a flame from the Bunsen burner. For an agar plate, remove the cover and touch a sterilized wooden applicator to a visible colony then carefully swirl it in the media in the test tube. For inoculum from a test tube, use the pipette to add 1% to 10% culture volume to fresh media.
6) Label the tubes with your name, date, and the components of the culture.
7) Incubate the tubes in the desired growth conditions.
   - Suggested: 37 °C, 250 RPM

8) When the culture reaches the desired cell density for induction, transfer the test tube back to the laminar flow hood.
   - Suggested: When the broth becomes opaque (OD_{600} of approximately 1.0).
9) Add the required amount of inducer to bring the inducer concentration to the desired induction strength.
   - Suggested: Dilute 0.1 mL of 1 M IPTG to 1 mL with sterilized RO water. Add 50 µL of 0.1 M IPTG to the 5 mL test tube culture for an induction strength of 1mM.
10) Return the culture to an incubator which has been set it to the desired temperature.
   - Maximum total expression in E. coli usually occurs at 37 °C.
   - Do not allow the culture to remain unagitated for extended periods of time at high cell concentrations or the culture can become oxygen starved.
11) At the end of the induction period, take a 1 mL culture sample according to CULTURE SAMPLES on pg. 101 and a cell density measurement according to CELL DENSITY – OD600 on pg. 99.
   - Suggested: 4 – 6 hr induction period.
12) Sterilize the remaining culture in the test tubes and inoculum vessel.
   - Method 1: Place in a test tube rack and autoclave the culture according to AUTOCLAVING on pg. 92.
   - Method 2: Move the test tubes to the walk-in hood in 3269 or a fume hood. Fill each test tube with 10% bleach solution and let sit for 10 minutes.
13) Clean the test tubes.
   - Dump the sterilized culture down the drain and wash the test tubes with soap and water. Then rinse them with RO water and allow them to dry.
14) Analyze the culture samples according to SDS-PAGE on pg. 107.

SHAKE FLASK CULTURES

Shake flasks have a larger volume then test tubes and can supply multiple culture samples from a single vessel. Therefore, the operator can obtain a time course analysis of the growth rate or expression levels of the culture. Any experiment that can be done in a test tube can be expanded to a shake flask experiment. Test tube cultures, however, require less time and material.

Large volume shake flask cultures can also be used to produce protein for experimentation purposes. Many biochemistry labs that do not have stirred tank bioreactors use shake flasks for all of their recombinant protein work. If a shake flask is to be used to produce protein, the operator must remember that the maximum cell density in a batch experiment is inhibited by the buffering capacity of the media. In general, the maximum OD_{600} that can be reached before the physiological pH is lost is between 3 and 4. M9 media results in acidic conditions while LB media tends towards basic conditions. Also, for E. coli, 1 g of glucose provides enough substrate for a little over a half unit increase in the OD_{600}. Therefore, 4 grams of glucose can potentially give a final cell culture density indicated by an OD_{600} of approximately 2.5. The quantity of substrate available in the normal recipe for LB media that does not include glucose provides similar results. For these reasons, it is common practice to induce protein expression in a

batch culture when the OD_{600} has reached 1.0. This allows the culture to continue to grow and produce protein before the limitations of the media are reached.

Supplies
Media
Inoculum
Multiple sterilized Erlenmeyer flasks
Sterilized pipette tips
Pipetter
Inducer
Sterilized graduated cylinder (optional)
Marking tape
Permanent marker

Time: 9 hrs to 18 hrs

Procedure
1) Prepare an inoculum culture in a test tube or shake flask according to INOCULUM CULTURES on pg. 95.
2) Prepare the media according to MEDIA on pg. 85.
3) Transfer the sterilized media to one or more sterilized shake flasks.
   - For maximum oxygen transport, the media should not be greater than 20% of the total flask volume.
4) Transfer the inoculum culture from the incubator to the laminar flow hood.
   - Suggested: OD_{600} of 1.0 for inoculum.
   - Do not allow the culture to sit too long without agitation or the cells can suffer from the stringent response caused by oxygen starvation.
5) Inoculate the shake flasks with the inoculum culture.
   - Remove the covering from both culture vessels and flame the opening to sterilize. Use the pipette to add 1% to 10% culture volume to the fresh media in the shake flasks. Otherwise, just the flame the lip to the inoculum vessel and pour the culture directly into the flask.
6) Label the flasks with your name, date, and the components of the culture.
7) Incubate the shake flasks.
   - Suggested: 37 °C and 250 RPM in the enclosed incubator in the teaching lab.
8) When the culture the reaches the desired cell density for induction, transfer the shake flasks back to the laminar flow hood.
   - Suggested: OD_{600} of 1.0.
9) Remove the top to the shake flask and flame the opening.
10) Take a 1 mL culture sample according to CULTURE SAMPLES on pg. 101 and a cell density measurement according to CELL DENSITY – OD600 on pg. 99. This will be the non-induced 0 hr time point for the expression.
11) If multiple environmental conditions or media component concentrations are to be tested for their impact on induction, split the large shake flask culture among smaller shake flasks.

- Remove the coverings to the sterilized shake flasks and flame the openings. Use either large volume sterilized pipette tips or a sterilized graduated cylinder to split the culture equally among the given containers. Maintain a culture volume of approximately 20 % of the total shake flask capacity.

12) Add any additional media components to the separated cultures.
    - A change in carbon source for the culture source cause a lag phase in growth or change in the metabolism of the culture. This makes determination of the effect of substrate on the rate of protein expression difficult.
13) Add the required amount of inducer to bring the inducer concentration to the desired induction strength.
    - Suggested: 1 mM IPTG for full induction of Lac promoters.
14) Incubate the cultures in the desired environmental conditions.
    - Do not allow the culture to remain unagitated for extended periods of time when at high cell concentrations or the culture can become oxygen starved.
15) Take additional culture samples and OD_{600} readings during the course of induction.
    - Suggested: 0.5, 1, 2, 3, 4, 6 hrs.
16) At the end of the induction period, determine the cell density and take a final 1 mL culture sample.
    - Suggested: 4 – 6 hr induction period.
17) If the culture is to be processed and the protein purified, harvest the cells according to CULTURE HARVESTING on pg. 105.
18) Sterilize the remaining culture in the shake flasks and inoculum vessels.
    - Method 1: Partially fill the flasks with water and autoclave the culture according to AUTOCLAVING on pg. 92.
    - Method 2: Move the shake flasks to the walk-in hood in 3269 or a fume hood. Fill each test tube with bleach to produce a 10% final concentration and let sit for 10 minutes.
19) Clean the glassware.
    - Dump the sterilized culture down the drain and wash the flasks with soap and water. Then rinse them with RO water and allow them to dry.
20) Analyze the culture samples according to SDS-PAGE on pg. 107.

FED BATCH FERMENTATIONS

BIOFLO IIC INTRODUCTION

The BioFlo IIc is a 1 L working volume, bioreactor vessel and control console designed for growing microbiological cultures to high cell densities. This section is intended as an introduction and step by step procedure to operating the BioFlo IIc bioreactors in room 3262 of the biochemical engineering teaching lab. The following protocols include tips and suggested operating parameters for first time users of the equipment. Many of the suggestions primarily target projects involving induced, intracellular protein production by recombinant E. coli, so other microbiological systems require modifications to this protocol.

Opto 22 is a set of software programs and processing units designed for industrial automation projects. Due to the limited control capabilities built into the BioFlo IIc, Opto 22 has been used to add functionality to the bioreactor system. "Presto Control" is the name given to the controller and display programs designed using Opto 22 that allow staged controller recipes to regulate the process variables of the BioFlo IIc. While an understanding of the software driving the controller of the BioFlo IIc is not required to run a project, it is helpful. It is possible for the culture or the equipment to function outside of the parameters expected by the controller and quick changes might need to be made to the controller recipe to keep the project from failing. The Presto Control Manual, describes the features of the program and how individualized recipes can be created.

For background on fed batch bioreactors, New Brunswick has published some introductions to fermentation and equipment in "Fundamentals of Fermentation: Techniques For Benchtop Fermentors" (R & D Lab, date unknown) and "An Introduction to Fermentation: Fermentation Basics" (Pumphrey and Julien, 1996). Useful information on bioreactors can be also be found in reviews by Lee (1996), Liden (2002), and Riesenberg (1999).

BIOREACTOR PREPARATION CHECK LIST

Prepare and sterilize media components

Prepare and autoclave feed vessels, shake flasks, and test tubes

Prepare and pour plates

Inoculate plates

Inoculate test tube cultures

Inoculate shake flask cultures

Calibrate pH probe

Clean and assemble bioreactor

Autoclave bioreactor

Add media components to bioreactor and feed vessels

Hook up bioreactor to BioFlo IIc Console and feed vessels

Ionize DO probe

Calibrate DO probe

Setup Presto Control and BioFlo IIc Console for bioreactor control

Inoculate bioreactor

BIOREACTOR PREPARATION

The bioreactor needs to be sealed and sterilized before introduction of the culture. The following procedure is a list of all of the basic tasks that must be accomplished before the bioreactor is ready to be used. Due to the requirements of the DO probe calibration procedure, the equipment must be autoclaved at least six hours before the culture is introduced. Do not wait until the culture is nearly ready for transfer before starting this part of the protocol.

All of the BioFlo IIc control parameters are set using the "Selector" and "Mode" knobs on the front of the BioFlo IIc console. The "Increase/Decrease" switch changes the values in each mode.

Mode Options:

- CONTROL: Shows the actual value of the selected monitored variable. This does not have to be selected for the BioFlo IIc to control the variable; the control is a continuous process that cannot be turned off.
- SET POINT: Shows the set point value for the selected environmental variable. When in DO control mode, the displayed set point value for agitation is the maximum RPM value allowed.
- ZERO: Shows the current value of the selected variable and used for calibrating the pH and DO probes. When this mode is selected and the Increase/Decrease button is pressed, the current value of the monitored variable is set to the new displayed value.
- SPAN: Shows the current value of the selected variable and used for calibrating the pH and DO probes. When this mode is selected and the Increase/Decrease button is pressed, the current value of the monitored variable is set to the new displayed value. In conjunction with the value set in the "ZERO" mode, the span value creates a calibration curve for the entire range of values.

Selector Options:

- RPM: Controls the motor that spins the impeller. In DO control mode, this option is used to set the minimum and maximum allowed RPM values.
- NUTRIENT: Disabled. This function has been bypassed so that it can be controlled by Presto Control.
- pH: Monitors the pH of the media and controls both the acid and the base pumps.
- DO: When the DO control mode is selected, the set point for this mode will control the agitation between the maximum and minimum set points for RPM. Otherwise, this mode just monitors the DO and allows calibration of the DO probe.
- ANTIFOAM: The probes are not available for the use of this controller.
- TEMPERATURE: Controls the pump, heater, and valves that are used to supply cooling or heating water to the water jacket.

pH Probe Calibration

The pH probe calibration should be performed prior to autoclaving the bioreactor. Otherwise, the probe and the bioreactor can become contaminated when the probe is removed and reinstalled during the calibration process. Autoclaving the probe in a liquid environment does not affect its functionality and the BioFlo IIc maintains calibration set points even when it is turned off. The probe should be calibrated in buffers heated to the operating temperature of the bioreactor but this can be difficult to accomplish.

Supplies

Bioreactor pH probe
BioFlo IIc pH probe cable
RO water bottle
Rinse container
pH 7.0 buffer
pH 4.0 buffer

Time: 10 min

Procedure

1) Remove the probe from its storage solution and rinse it with RO water.
2) Attach the pH cable to the probe and to the BioFlo IIc.
3) On the BioFlo IIc control unit, turn the Selector knob to "pH" and the Mode knob to "Zero".
4) Insert the pH probe into pH 7.0 buffer and wait for the reading to stabilize.
   - The nonrefillable pH probes respond slowly and may take a couple minutes to fully stabilize.
5) Use the Increase/Decrease button to adjust it to 7.0.
6) Remove the probe from the pH 7.0 buffer and rinse it with RO water.
7) Place the probe in pH 4.0 buffer and switch the Mode knob to "Span".
8) After the reading has stabilized, use the Increase/Decrease button to adjust it to 4.0.
9) Detach the pH probe and rinse it with RO water. The probe is now calibrated and ready to be autoclaved with the bioreactor.
10) Return the probe to its storage buffer until the bioreactor is ready for the head plate to be installed.

Bioreactor Assembly

The exact requirements for assembling the bioreactor depend on the parts available and the type of ports on the head plate. Some engineering ingenuity might be required to get everything installed and functioning properly.

Supplies

Media
Bioreactor vessel
Head plate
Feed port
Culture/media/antifoam port

3 head plate screws
Inoculation port cap
Stoppers or caps for unused ports
Thermowell port
Sample port and assembly
Sparging port and assembly
Impeller shaft and assembly
2 impellers
Condenser port
Two 0.2 μm air filters
Baffles
Scissors
Multiple ID tubing
pH probe
DO probe
Hex head wrenches

Time: 20 minutes

Procedure

1) Check that the bioreactor vessel, baffles, head plate and attachments are all clean.
   - It is difficult to remove all of the detergent from the vessel when it is cleaned. Rinse the vessel thoroughly before use or excessive foaming can occur during agitation.
   - If feed needs to be controlled precisely, a piece of tubing attached to a pipette or syringe tip should be attached to the outlet of the feed port on the underside of the head plate. The outlets of the standard metal ports can cause erratic feeding due to large drop sizes or feed solution clinging to the underside of the head plate.
2) Check that the impeller drive shaft does not easily slide up and down and that it turns with only moderate effort and without the bearings squeaking or grinding.
   - If the shaft slides up and down, a new underside seal assembly will need to be ordered and installed.
   - If the shaft is grinding, a new ball bearing assembly will need to be ordered and installed.
3) Install the impeller blades in the desired positions on the impeller shaft.
   - Suggested: Use two of the six spoke, flat bladed impellers with a diameter of 2 1/8". The lower impeller should be positioned at the end of the impeller shaft just above the sparger. The second impeller should be just below the surface of the liquid (about 2 ½" above the lower impeller).
4) Place the baffles in the bioreactor
   - In order to allow all of the port connections to be made, the gap in the baffles needs to face the cooling water ports on the bioreactor base.
5) If they are present, seal the addition ports in the sides of the glass vessel.
6) Add media to the vessel according to MEDIA on pg. 85.
7) Place the head plate on the glass vessel.

- Make sure that the black rubber O-ring is in place in the groove on the underside of the head plate and that it forms a seal with the lip of the glass vessel.
- Line up the pH and DO probe ports with the gap in the baffles and look through the ports to ensure there is adequate space for safe probe insertion.

8) Remove the pre-calibrated pH probe from its storage buffer, rinse it with RO water and slide it into the head plate.
   - Most likely, the pH probe being used does not screw into the head plate and the seal is maintained with just an O-ring.
   - If adjustments need to be made to the baffles or the head plate, do not use the pH probe as a lever to force movement because it may break.
9) Check the membrane of the DO probe for damage. Insert the probe into the head plate and screw in the adapter.
   - If the probe appears damaged, the probe was responding slowly or erratically in previous fermentations, or it has been over six months since changing the electrolyte, check the probe's instruction manual for the protocol on how to service it.
   - The DO probe shaft may be too long for the vessel. A black O-ring can be slid partially up the body of the DO probe and used to form a seal between the adapter, the DO probe, and the head plate.
10) Raise the stainless steel ring encircling the glass vessel and secure the head plate with the thumbscrews.
   - Do not fully tighten the thumb screws before autoclaving or the glass lip may crack.
11) Recheck that the impeller can spin freely and that all of the probes and ports line up correctly
12) Attach an autoclavable 0.2 μm air filter to the air inlet hose on the head plate. Attach a second filter to the air outlet hose on top of the exhaust gas condenser.
   - Twist the filter onto the end of the tubing so that the tubing reaches at least to the second ridge of the filter. Filters and the filter tubing can be secured with zip ties.
   - The filters can be reused from previous experiments if they have not become plugged and significantly impede airflow. If the removal of either the inlet or outlet filter from the airflow path causes a large airflow spike by the controller, the filter should be replaced.
   - If plugging is a concern, T-adapters can be utilized to allow two filters to be used in parallel on the inlet or outlet line. If one filter becomes plugged, the other will still function.

Bioreactor Sterilization

Certain principles should be kept in mind when preparing the bioreactor for autoclaving.

- Do not over tighten any of the screws or caps. They can become overly tight or cause cracking of the glass vessel during autoclaving.
- Any tubing or ports that stick down into the media within the bioreactor must be closed off before autoclaving to prevent media loss. These include the sample port and the air inlet.

- Air must be allowed to move in and out of the vessel during autoclaving in order to allow equilibration of the pressure. Media can be forced out of the vessel during depressurization of the autoclave if equilibration is not maintained.
- Moving metal parts and filter openings should be covered with aluminum foil to prevent damage.
- Certain components of the media should not be autoclaved and must be filter sterilized. These solutions should be added after the bulk media has cooled.

Supplies
Aluminum foil
Clamps

Time: 1 hr

Procedure
1) Clamp off the inlet air hose.
   - Do not clamp the exhaust gas line. The exhaust gas line allows airflow for pressure equilibration during autoclaving.
2) Seal off the acid, base, and glucose addition ports with small sections of clamped tubing.
   - If antibiotics and mineral salts are going to be added after the bioreactor has been autoclaved and hooked up to the BioFlo IIc console, a small 0.2 μm syringe filter can be attached to the tubing of one of the inlet ports before autoclaving and used to aseptically transfer the media components.
3) Clamp the tubing attached to the sampling port and close the sampling port valve.
4) Cover all inlets, outlets, probe connections, and moving metal pieces with aluminum foil.
   - These parts include the impeller shaft, the outlets of the air filters, all clamps, the tops of the pH and DO probes, and any ports that may have been left open.
   - The aluminum foil increases heat transfer and reduces the chances of corrosion due to condensation on the metal parts.
5) Unscrew the cap to the inoculation port so that it is just resting on the port.
   - This keeps pressure from building up within the vessel and keeps the threaded seal from becoming too tight during heating and cooling in the autoclave.
6) Recheck to be sure that all of the ports with openings into the media are sealed.
7) Place the vessel upright in the autoclave.
8) Flip the Power and Control switches to the "On" position on the autoclave.
9) Open the cooling water and steam valves located behind the autoclave.
10) Close the autoclave door and lock it in place by turning the handle all the way to the right.
11) Set the Steam Sterilize Time to 35 minutes and the Steam Dry Time to 00 minutes.
12) Press the Reset button.
13) Press the "Liquid Cycle" button to start the autoclave process.
14) Wait for the autoclave to finish.

- The autoclave will run for 35 minutes once it reaches 132 °C. It will then spend several minutes exhausting the steam. The buzzer will sound when the exhaust cycle is complete. The buzzer can be stopped by pressing the reset button.

15) Close the steam valve behind the autoclave.
16) Open the door, remove the vessel and carry it to the BioFlo IIc stand.
    - If possible, allow the autoclave to continue to cool for at least 15 minutes before opening the door. This prevents excessive volume reduction of the media due to boil off when the door is opened and the remaining pressure is released.
    - Do not leave liquid in a closed autoclave with the steam valve open for more than a couple of hours or a large portion of the media may evaporate.
17) Close the cooling water valve to the autoclave.

DO Probe Ionization

The DO probe functions by measurement of the current produced by a continuous voltage between an anode and a cathode. Oxygen diffuses across the probe membrane that separates the potassium chloride electrolyte solution from the bioreactor media and is reduced at the cathode. The reduction is balanced by the oxidation of silver at the anode. The current produced is directly proportional to the oxygen concentration if the voltage is constant. Therefore, the electrodes of the DO probe must be fully polarized before calibration. Ionization of the DO probe is accomplished by having it hooked to the BioFlo IIc console with the consoles power turned on for a minimum of 6 hours. Deionization is not instantaneous, so the probe can be unhooked for a short period of time during the ionization process if need be. The first steps of the procedure are concerned with connecting the BioFlo IIc to its console.

Time: 6 hrs

Procedure

1) Make sure that the main water supply to the BioFlo IIc is turned on.
2) Check that the water line into the back of the BioFlo IIc is open.
3) Turn on the power to the BioFlo IIc.
4) Remove the foil from the probe connections to allow any trapped water to evaporate quickly.
5) Remove the clamp on the air inlet.
    - During autoclaving, the air hose may become permanently compressed. Open the clamp slightly and rotate it 90 degrees so that it forces the compressed portion of the tubing to remain open.
6) Lightly tighten any of the loosened screws or port caps.
7) After the vessel is cool enough to touch, attach the DO probe cable to the probe and the BioFlo IIc.
    - The bioreactor can be quickly cooled down by using the cooling jacket as described in *Temperature Control* on pg. 67.

FEED VESSELS

The optimum feed source for culture growth is dependant on the host strain and the expression system. In all cases, the feed should consist of a highly concentrated carbon source. In order to reduce the volume increase caused by the addition of feed, the carbon source should be near to its solubility limit while not being too viscous to pump. A nitrogen source is also be needed if the base being used to maintain the pH is not ammonium hydroxide or ammonia.

- **Glucose:** E. coli grow preferentially on glucose but certain protein expressions systems are catabolically inhibited by glucose. In some cases this is a desired effect because it inhibits basal level (leaky) expression that can inhibit the culture during the growth phase. Glucose can also lead to excessive levels of acetic acid formation by certain strains of bacteria.
- **Glycerol:** Glycerol does not lead to catabolite inhibition, but its uptake is slower than that of glucose. Glycerol has been shown to stop acetate production by some strains of E. coli (Lee, 1996).
- **Lactose/Galactose**: Galactose induces expression in systems utilizing a Lac promoter. Some host strains are incapable of active uptake of the catabolite.
- **LB**: LB supplies complex forms of all the needed nutrients including nitrogen, but continuous feeding of this source can lead to the buildup of slowly metabolized, toxic components.
- **Other sources**: Fructose, maltose, lactate, acetate, ethanol, complex oils.

The following procedure explains how to set-up a feed vessel. Besides the feed source, these vessels are also used to pump in acid, base, non-autoclavable components and the culture.

Supplies

1 ft of medium diameter tubing
1 ft of small diameter tubing
500 mL screw top glass jar
Circular metal plate with inlets and outlets
Cylindrical screw top for sealing the metal plate to the glass jar
Tubing clamp
Small air filter
Tin foil
Plastic tubing adapter

Procedure

1) Thoroughly wash and rinse the glass jar and its top.
2) Rinse water through the tubing to make sure that it is not plugged or dirty.
3) Cut a length of medium diameter tubing that is slightly longer than the length of the glass jar. Attach it to the outlet on the underside of the metal plate.
4) Attach a foot long piece of medium diameter tubing to the outlet on the top of the plate that is in-line with the tubing that was already connected.

5) Cut a short length of medium diameter tubing. Attach an air filter to one end. Attach the other end to the opening on the top of the plate that is not in line with the piece of tubing that was already connected.
6) Add the feed solution if it is to be autoclaved in the vessel rather than being supplied from a stock solution after autoclaving.
7) Thread the tubing and filter through the screw top and lightly screw the assembly to the top of the bottle.
8) Use a tubing adapter to attach the foot long section of smaller diameter tubing to the medium diameter tubing attached to the head plate.
    - For best control of the rate of feed addition, this tubing should have a bore size of 1.0 mm. This smaller tubing allows more precise control of the feed. If precise control is not needed or the vessel will be used to add culture media, larger diameter tubing can be used. The mixed assemblage of larger and smaller diameter tubing is required because it can be very difficult to get the smaller diameter tubing attached to the metal ports of the feed vessel.
9) Clamp the outlet to the tubing.
10) Wrap the clamp, the outlet of the tubing, and the filter in aluminum foil.
11) Place the vessel(s) in an autoclave tray.
    - Wrap the end of the tubing around the top of the jar so that outlet is not lying in condensed steam at the end of the autoclave cycle.
12) Autoclave for at least 20 minutes as described in AUTOCLAVING on pg. 92.
13) Remove from the autoclave and tighten the screw top.

LOADING PRESTO CONTROL

The following protocol demonstrates a quick procedure for starting Presto Control. The controller is needed for attachment of the feed tubing and calibration of the DO probe. Three components of the system have to be operating correctly for everything to be working:

1) The snap control unit and I/O modules must be operating and being sent signals from the BioFlo IIc and its probes.
2) The control unit must have the correct OptoControl strategy downloaded and in "Run" mode and it must be communicating with the computer.
3) The computer must have the correct OptoDisplay Project loaded for communicating with the OptoControl program downloaded to the control unit.

The Quick Start procedure starts Presto Control if the correct strategy and display program have already been loaded. If the controller or display programs are incorrect or they are not communicating properly, use the Full Load procedure.

Time: 2 min

Quick Start Procedure

1) Open the "Shortcut to OptoDisR" icon on the desktop. The Presto Control display screen should open.
2) Event Log Viewer should list:
    - ◊ Comm Port: snap=>Open Primary
    - ◊ Controller: snap=>Attaching to Scanner
3) Close the Event Log Viewer.
    - If the program opens to a screen displaying a green stop button at the top, a recipe is already started. Click on "Stop" to stop the recipe and exit to the set-up screen.

Full Load Procedure

1) Click on the "OptoControl" icon on the desktop to start OptoControl.
2) Click on "File", "Open Strategy", and select the strategy located at D:\PrestoControl\ControllerProgram\PrestoController.
3) Select "Download Strategy" from the "Controller" menu. Wait for the control program to download.
4) Select "Debug" from the "Mode" menu.
5) Select "Play" from the "Debug" Menu
6) Exit OptoControl.
7) Click on the "Shortcut to OptoDisC" to start OptoConfigurator.
8) Click on "File", "Open Strategy", and select the open the project located at D:\PrestoControl\DisplayProgram\PrestoDisplay.
9) Click on "File", and "Save Project and Load Runtime".
10) Event Log Viewer should list:
    - ◊ Comm Port: snap=>Open Primary
    - ◊ Controller: snap=>Attaching to Scanner
11) Close the Event Log Viewer.

CONTROL PREPARATION

The following sections describe how to set up the BioFlo IIc for control operations. The procedures can be done at any time before inoculation. In general however, there is no need to have agitation, pH, or temperature control of the bioreactor while ionization of the DO probe is occurring.

Mixing

Agitation is controlled directly by the BioFlo IIc. The Presto Control program can only monitor the RPM. Two modes of control exist: manual RPM set-point, and PID control by DO set point. When the temperature or pH control is active, the agitation should be set to at least a minimum value in order to maintain mixing in the bioreactor. This section describes setting the system for manual set-point control of agitation for mixing purposes. The agitation can be set between 25 and 1000 RPM.

Supplies

Motor
Hex head wrenches

Time: 1 min

Procedure

1) Place the servo motor on top of the drive shaft and make sure that the multi-jaw coupling is linking the drive shaft and the motor.
   - Over time, the socket on the top of the drive shaft may drop too low. If the coupling is no longer connecting with the drive shaft, use a hex head wrench to raise the socket.
2) Connect the motor's control cable to the BioFlo IIc.
3) Turn the Selector knob to "RPM" and the Mode knob to "Set Point".
4) Using the Increase/Decrease switch to adjust the set point to 50 rpm.
5) Turn on the motor using the "Motor" switch on the front of the BioFlo IIc console.

Temperature Control

The temperature of the bioreactor is monitored by a resistance temperature detector inserted into the thermowell in the head plate and controlled by a heating/cooling jacket cradling the bottom of the bioreactor. The default operation of the temperature controller is to recirculate the water through the jacket of the bioreactor. When the temperature is too low, a heater is turned on to warm the water circulating through the jacket. When the temperature is too high, the BioFlo IIc stops recirculating the jacket water and allows fresh cooling water to enter from the water lines. The process is controlled by a PID controller with a set point that can range between 20 °C and 60 °C.

Supplies

Resistance temperature detector
Glycerin or water

Time: 1 min

Procedure

1) Insert the temperature probe into the thermowell on the head plate and connect the cable to the BioFlo IIc.
   - Heat transfer to the temperature probe can be improved by partially filling the thermowell with water or glycerin.
2) Turn the Selector knob to "Temperature" and turn the Mode knob to "Set Point".
3) Use the Increase/Decrease switch to adjust the set point.
   - Suggested: 37 °C is the standard growth temperature for E. coli.
4) Turn the Mode knob to "Control".
5) Connect the upper water lines to the exhaust gas condenser.
   - The top line goes to the top port for each set of connections. The top lines are always connected first.
6) Attach the lower water lines to the metal bioreactor heating jacket.
   - Do not attach the water lines unless the temperature probe is connected and operating properly. The heating can become uncontrolled and the water jacket can become very hot.
7) Prime the recirculation loop for 30 seconds by holding down the Prime switch on the right side of the BioFlo IIc.

pH Control

The pH is controlled directly by the BioFlo IIc. When the pH is outside of the dead band established by the set point, either the base or acid pump is set to operate until the pH is returned to the set point. With large diameter tubing and/or highly concentrated acids or bases, the control parameters of the BioFlo IIc can lead to overshoot and a cycle of alternating acid and base addition. This leads to a buildup of the salt concentration in the media and can cause culture death. To prevent this, only put one pump in control of maintaining the pH. A minimal media with ammonium as the source of nitrogen leads to the production of hydrogen ions due to cell metabolism. The pH of this type of media can be maintained with just the base pump placed on "AUTO". The acid pump can be turned off. In addition, if the base being used is concentrated ammonium hydroxide or ammonia, the culture has a continuously replenishing nitrogen source. Complex media tends toward basic conditions and can usually be controlled with just the acid pump. 6 N hydrochloric acid is commonly used. The following procedure should be done while wearing rubber gloves, full coverings for the arms and legs, and chemical splash goggles.

Supplies

Protective clothing
Acid vessel
Base vessel
Acid
Base
Labeling tape
Permanent marker

Time: 10 min

Procedure

1) Add the acid and base to the sterilized feed vessels described in FEED VESSELS on pg. 64.
   - Do this in a fume hood. Do not do it in the laminar flow hood. Wear rubber gloves with gauntlets, a long sleeved lab coat, and chemical splash goggles. Under no circumstances open an ammonium hydroxide or sulfuric acid container outside of a fume hood.
   - Acid and base vessels can be reused in subsequent bioreactor projects. The solutions do not need to be replaced between each use.
   - Always keep the tubing outlets above the liquid level of the acid and base containers and firmly clamped.
   - Inspect any plastic tubing adapters to make sure that they are not damaged by previous acid or base contact.
2) Label the vessels with contents, name, and date.
3) Connect the acid and base lines to their ports on the head plate.
   - The acid and base ports are the small, bent, ridged tubes that are permanently attached to the head plate. Make sure that the ports and tubing outlets are above the liquid level of the acid and base containers. Quickly remove the tubing used to seal the ports during autoclaving and push the acid and base tubing into place. If possible, attach the tubing before removing the clamps.
   - Airflow through the bioreactor keeps positive pressure in the vessel and limits the chance of contamination.
   - If the tubing is being reused from a previous experiment or the clamp is close to the outlet, attach a second clamp slightly farther up the tubing. Then, scissors can be used to cut off the outlet clamp and a sterile portion of the tubing exposed for attachment to the head plate. Be very careful to protect against any splashing that may occur due to acid or base caught between the two clamps.
4) Thread the acid, base and feed lines into their respective pumps.
   - Determine the rotation direction of the pump head and the amount of tubing needed to reach the bioreactor from the pump. The tubing with the smallest inner diameter (1 mm bore size) should be wound through the pump head. Switch the pumps to "ON" and carefully thread the tubing into the moving pump. If the tubing gets caught on one of the metal guide pegs, it can be torn. Switch the pumps to "OFF" when completed.
5) Prime the tubing.
   - Prime the tubing by switching the pumps to "ON" until drops can be seen coming from the outlet of the ports.
   - If the pumps do not seem to be able to start the fluid flowing, priming can be accomplished by either lifting the outlet of the media container above the inlet to the pump or by pulling the fluid through the tubing with a sterilized syringe before attaching it to the port.
6) Attach the pH probe cable to the probe.

- If the pH probe does not screw into the head plate, then the sterile seal is maintained with O-rings only. Because of this, excessive movement of the probe can introduce contamination into the vessel. To avoid this, detach the cable from the BioFlo IIc and screw the cable onto the probe by turning the cable, not the probe. The other end of the cable can then be connected to the BioFlo IIc.

7) Turn the Selector knob to "pH" and the Mode knob to "Set Point".
8) Use the Increase/Decrease switch to adjust the set point.
   - A pH of 7.0 is standard physiological pH.
9) Return the Mode knob to Control.
10) Flip the Acid and Base switches on the side of the BioFlo IIc to "AUTO".
    - When the pH controller has added the necessary amounts of acid and base to reach its set point, switch "OFF" the pump that is not needed for maintaining the pH during culture growth.

Feed Pump Calibration

This procedure can be used to determine the exact operating parameters of the nutrient pump for the BioFlo IIc. The pump speeds do not drift so, if the speed is already known or can be read from the table in Feed Control on pg. 72, this section can be skipped. Feed control on the BioFlo IIc is complicated by the fact that the pumps only have one speed when they are turned. Therefore, the digital feed pump must use an on/off cycle to mimic an analog rate. Presto Control has been designed to allow the user to determine the "Pulse Length" and "Pump Calibration" needed for tight control of feed by the digital mimic. The following procedure is not required if the Presto Control recipe being used is a batch fermentation or does not utilize open loop or PID control of the feed.

This protocol directly determines two parameters.

1) The maximum feed rate of the pump when it is turned on
2) The minimum length of time needed for the pump to form one full feed droplet.

This 2nd parameter is important because effective feed control requires that at least one feed droplet is added to the bioreactor every time the feed pump is turned on. Otherwise, the analog rate might specify the current feed rate to be a certain value even though the actual feed rate during the current on/off cycle is zero. This can be especially problematic at very low feed rates when the on/off cycle length is very long. This same consideration needs to be taken into account when deciding on a length of time for the "PID On/Off Cycle Length". If a full droplet takes 2 seconds to form and the "PID On/Off Cycle Length" is set to 4 seconds, an output of the PID controller of less than 50% of maximum might allow the pump to cycle on and off without any feed actually being added. If the "PID Scan Rate" is also set to 4 seconds, the controller would update its output based on its previous specified feed rate even though no feed had actually been added. This would contribute to poor controller performance. Therefore, the feed PID scan rate and cycle length should be scaled according to the maximum feed rate of the tubing and the feed requirements of the culture. Alternatively, a better fix for this control limitation is to attach a small pipette or syringe tip to the outlet of the feed line. This reduces the amount of feed required to form a full droplet. If the added liquid volume is not a consideration, the feed concentration can also be reduced.

Supplies
10 mL graduated cylinder
1 ft feed tubing
Outlet of feed line
Small Erlenmeyer flask
Water or feed solution

Time: 15 min

Procedure

Pump Calibration

1) Thread a 1 ft length of feed tubing into the nutrient pump head.
   - This tubing needs to be the same as the tubing used to pump the feed into the bioreactor. The outlet should also to be the same as the outlet of the feed port into the bioreactor. Depending on the preparation of the bioreactor, this may be a metal tube, another piece of plastic tubing, or a pipette or syringe tip.
   - Switch the pump to "AUTO". In the "Feed" section of Presto Control, set the "Pump Time" to 30 seconds or more. Set the feed to "Full On" and carefully thread the tubing into the moving pump. If the tubing gets caught on one of the metal guide pegs, it can be torn. Leave an equal amount of tubing exiting and entering the pump head.
2) Fill the Erlenmeyer flask with at least 10 mL of feed solution or water.
   - If the feed solution is exceptionally viscous, the substitution of water may not be appropriate.
3) Place the inlet of the tubing into the solution in the Erlenmeyer flask and the outlet of the tubing into the mouth of a 10 mL graduated cylinder.
4) Prime the tubing.
   - Set the "Pump Time" to 2 or 3 minutes. Prime the tubing by setting the feed to "Full On" until drops can be seen coming from the outlet of the tubing. Set the feed to "Full Off".
   - If the pump does not seem to be able to start the fluid flowing, priming can be accomplished by either lifting the liquid level of the Erlenmeyer flask above the inlet to the pump or by pulling the fluid through the tubing with a syringe.
5) Determine the maximum feed rate of the pump.
   - Set the "Pump Time" to 120 or more seconds. Set the feed to "Full On". When the feed timer has stopped, calculate the maximum feed rate of the Nutrient pump based on the "Pump Time" and the volume of feed added to the graduated cylinder.
6) Click on the orange "Control" button.
7) Click on "Pump Calibration" and enter the calculated maximum feed rate of the nutrient pump.

Pulse Length

1) Maintain the same preparation as used for calculating the "Pump Calibration".
2) Click on "Pulse Length" in the FEED CONTROL section of the main window and enter 200 ms.

3) In the same window, click on "Fraction of Time On" and enter 0.5 or an alternate desired value.
4) Click on feed time and enter 60 seconds or more.
5) Click on "Feed Pulse Control".
   - The nutrient pump will turn the pump on for the time specified in "Pulse Length" and turn it off for a length of time specified by "Fraction of Time On". For example, the pump would be on for 0.2 s and off for 0.6 s when the "Pulse Length" equals 200 ms and "Fraction of Time On" equals 0.25.
6) Increase the "Pulse Length" in increments until at least one full liquid droplet is produced from the end of the tubing outlet.
   - For a very small pipette syringe tip, this may be as little as 200 ms. Small bore size tubing may produce one droplet every 1 second. Large metal tubing may require 2 seconds.
7) Click on the orange "Control" button.
8) Enter the new "Pulse Length" in the Feed Control section of the pop-up window.
9) If desired, enter a corresponding "PID On/Off Cycle Length" and "PID DO Scan Rate".
   - Assuming that the PID feed control output will demand a feed rate of at least 20% of the maximum rate, the "PID On/Off Cycle Length" should have a value of 5 times the "Pulse Length". This will allow at least one drop to be formed each time the pump switches on during PID feed control.

Feed Control

The feed should be consistent with the carbon source chosen from MEDIA on pg. 85. Do not add more feed solution to the feed vessel than is needed for culture growth or can be added to the bioreactor without fear of overflowing. A controller problem can lead to uncontrolled overfeeding.

Supplies

Feed
Feed vessel
Labeling tape
Permanent marker

Time: 10 min

Procedure

1) On the main screen of the Presto Control program, click on the orange "Control" button. Make sure that the current parameters are set to the desired values.
   - If tight control of feed is desired, and the minimum "Pulse Length" and exact "Pump Calibration" have not been determined, check Feed Pump Calibration on pg. 70 for the procedure.
   - Suggested "Feed" control parameters:
     ◊ Pulse Length: 1000 ms
     ◊ Feed Concentration: 0.50 g/mL
     ◊ Pump Calibration: 1.41 mL/min

- ◊ PID On/Off Cycle Length: 5000 ms
- ◊ PID DO Scan Rate: 5.0 s
- ◊ Maximum Volume: 200 mL
- ◊ Proportional: -1.00
- ◊ Integral: 1.00
- ◊ Derivative: 0.00

2) If applicable, transfer the feed from the stock feed bottle to the feed vessel.
   - Take the bottle of sterilized stock feed solution to the laminar flow hood and open it. Use the Bunsen burner to flame the top of the bottle. Unscrew the cap of the feed vessel and flame its top. Pour the desired amount of feed from the feed stock bottle to the feed vessel while attempting to keep the feed from dripping down the sides of the bottle. Reflame the lips of the containers and replace the tops.
3) Label the vessel with contents, name, and date.
4) Connect the feed line to its port on the head plate.
   - Use the same technique described for attaching the pH tubing.
5) Thread the feed line into the nutrient pump head.
   - Determine the direction of rotation of the pump head and the amount of tubing needed to reach the bioreactor from the pump. The tubing with the smallest inner diameter should be wound through the pump head. Switch the pump to "AUTO". In the "Feed" section of Presto Control, set the "Pump Time" to 30 seconds or more. Set the feed to "Full On" and carefully thread the tubing into the moving pump. If the tubing gets caught on one of the metal guide pegs, it can be torn.
6) Prime the tubing.
   - Set the "Pump Time" to 2 or 3 minutes. Prime the tubing by setting the feed to "Full On" until drops can be seen coming from the outlet of the port. Set the feed to "Full Off".
   - If the pumps do not seem to be able to start the fluid flowing, priming can be accomplished by either lifting the outlet of the media container above the inlet to the pump or by pulling the fluid through the tubing with a sterilized syringe before attaching it to the port.

Air Control

Two gas sources exist for sparging into the bioreactor and the desired source is selected by a three way valve attached to the top of the BioFlo IIc. The first source comes from a nitrogen cylinder that is controlled manually. The second source is pressurized environmental air controlled by an airflow regulator in conjunction with Presto Control. Both have to pass through a manual regulator on the front of the BioFlo IIc. This valve must always be opened far enough so that the added flow resistance of the valve does not prevent the controller from reaching the maximum desired airflow output.

Time: 2 min

Procedure

1) Click on the orange "Control" button. Make sure that the current parameters are set to the desired values.
   - Suggested "Air" control parameters:

- ◊ PID DO Scan Rate: 5.0 s
- ◊ Minimum Air Flow Rate: 0.05 L/min
- ◊ Maximum Air Flow Rate: 1.05 L/min
- ◊ Proportional: 1.00
- ◊ Integral: 5.00
- ◊ Derivative: 0.00

2) Uncover the air inlet and exhaust filters and attach their respective lines.
   - Make sure that the tubing is not kinked on either of the air lines.
3) Twist the air flow source valve attached to the top of the BioFlo IIc to the right so that it selects for "air".
4) Make sure that the air flow regulator on the front of the BioFlo IIc is open.
5) Check to make sure that the airflow regulator attached to the air line on the south wall has pressure and that the valves are open.
6) Test various "Air Flow Rates" in the feed section of Presto Control to make sure that the controller can reach and maintain the chosen air flow.

DO Probe Calibration

The electrolyte solution in the DO probe must be ionized before it can be calibrated. Therefore, the probe must be connected to the BioFlo IIc with its power turned on for at least 6 hours before inoculation. The output of the DO probe depends heavily on the temperature of the media, so the probe should be calibrated at the operating temperature. The zero point oxygen concentration is set using nitrogen saturated media. The 100% DO concentration value is set using air saturated media at the maximum mixing conditions.

Time: 15 min

Procedure

1) Turn the Selector knob to "Temperature" and the Mode knob to "Set Point".
2) Use the Increase/Decrease switch to change the set point to the operating temperature.
3) Switch the Selector knob to "RPM" and the Mode knob to "Set Point".
4) Increase the agitation speed to the maximum process value.
   - The maximum suggested agitation rate is 750 RPM.
5) Select nitrogen as the inlet gas by turning the 3-way valve on top of the BioFlo IIc so that it points left.
6) Open the main valve on the nitrogen cylinder.
7) Open the smaller, pressure control valve by twisting it clockwise until the line has approximately 15 lb*ft of pressure
8) Set the flow rate of nitrogen entering the vessel to 1 L/min using the manual flow regulator on the front of the BioFlo IIc.
9) Wait for the DO reading on the BioFlo IIc to stabilize – it should be near to 0%.
   - Stabilization takes a few minutes.
   - If nitrogen is not available or the DO probe needs to be quickly recalibrated without nitrogen sparging, the zero point can be established by unplugging the DO probe from the BioFlo IIc.
10) Turn the Selector knob to "DO" and the Mode knob to "Zero".
11) Push the Increase/Decrease switch once to adjust the display reading to 0%.

12) Turn off the main valve on the N_2 cylinder.
13) When the gauges on the regulator drop to 0 psi, shut the secondary valve.
14) Turn the 3-way valve to point to the right so that it selects for environmental air.
15) Click on "Airflow Set Point" in Presto Control to set the maximum air sparge rate that will be used in the control recipe.
    - The maximum suggested air flow rate is 1 L/min.
    - Open up the manual air flow regulator on the front of the BioFlo IIc by giving it an extra counter-clock wise turn. This reduces the restriction on the airflow and allows the controller to reach its maximum air flow.
16) Wait for the DO reading on the BioFlo IIc to stabilize – it should be near 100%.
17) Turn the mode knob to "Span".
18) Use the Increase/Decrease switch to adjust it to 100%.
19) Click on "Airflow Set Point" in Presto Control and enter "0" into the pop-up window to stop the air flow.
20) Decrease the agitation to 50 rpm by moving the selector knobs to "RPM" and "Set Point" and press the decrease button until desired value is reached.

Agitation Control

When in DO control mode, the BioFlo IIc sets the agitation rate based on the DO set point entered into the BioFlo IIc console. Presto Control cannot control or monitor the DO set point used for control of agitation. Therefore, the independent control processes of the BioFlo IIc can interfere with some of the staged controller recipes of Presto Control. Unless the DO set point control is manually switched off on the BioFlo IIc, the BioFlo IIc continues to correct for errors in its DO set point by changing the agitation. This can conflict with PID and open loop control of the airflow or feed by Presto Control. The rate of change of agitation through the BioFlo IIc is so slow, however, that it rarely interferes with the normally faster response of the PID controller used in Presto Control or conflicts with the detection of a DO spike monitored in some controller schemes. A large problem can result, though, if the DO set point of the BioFlo IIc is accidentally set to a different value than the DO set point in Presto Control.

A few methods can be used to make sure that the lack of interaction between Presto Control and the agitation controller does not become a problem. The first method is already a part of the control program. The RPM value reported to Presto Control is greater than the actual reading taken by the BioFlo IIc. Therefore, if a control recipe calls for a maximum agitation rate of 750 RPM before switching to the next stage, and the maximum agitation rate set on the BioFlo IIc is 750 RPM, it is still possible for a stage switch to occur. If this signal offset had not been implemented, signal drift could cause the RPM value reported to Presto Control to become smaller than the actual reading taken by the BioFlo IIc. Therefore, 750 RPM would never be detected and the stage switch could never occur. A second method of reducing controller conflict is implemented by just switching off the DO control mode on the BioFlo IIc console when the RPM has reached its maximum value. The third method is to set the DO set point on Presto Control slightly lower than the set point on the BioFlo IIc. Therefore, when the agitation has reached its maximum value, the switch to PID control of airflow or feed maintains

the DO below the DO set point for agitation control and forces the agitation to remain at its maximum value.

If the Presto Control recipe being used does not rely on ramping of the agitation rate, this next part can be skipped. Instead, just set the desired, constant agitation rate as described in Mixing on pg. 67.

Time: 1 min

Procedure

1) Turn the Selector knob to "DO" and the Mode knob to "Set Point".
2) Use the Increase/Decrease switch to adjust the set point to the desired value.
   - Use the same or slightly higher value used in Presto Control. The minimum set point to ensure aerobic growth is 10%.
3) Turn the Selector knob to "Agitation" and the Mode knob to "Set Point".
4) Use the Increase/Decrease switch to adjust RPM to the desired minimum value.
   - Suggested: 50 rpm.
5) Press the DO Control Active button. The DO Control Active light will come on.
   - Once this control has been set, do not press the button again while agitation is ramping up or it will force the RPM to immediately go to its maximum value.
6) Use the Increase/Decrease switch to adjust the set point to its maximum value.
   - 750 RPM is the maximum suggested agitation rate.
   - The agitation will ramp up slightly while the maximum value is being set, but it will slowly settle back down to its minimum value if the DO is above its set point.

MEDIA ADDITIONS

The salts, antibiotics, and trace minerals that can not be autoclaved with the buffered media in the bioreactor need to be added individually. The technique for these additions varies depending on the circumstances. Three methods are listed here.

Method 1

If the tubing and probes have not been connected to the BioFlo IIc and none of the salts, amino acids, or antibiotics degrade quickly, the media components can be added in the hood after the bioreactor has cooled down and before the DO probe has been hooked up for ionization.

Time: 5 min

Supplies

Media components
Sterilized pipette tips
Pipetter
Sterilized graduated cylinders (optional)

Procedure

1) Move the bioreactor into the laminar flow hood.
2) Unscrew the inoculation part, one of the smaller ports, or remove the tubing covering the feed ports.
3) Use sterilized pipettes or sterilized graduated cylinders to add each of the media components.
4) Replace the port covers or tubing and move the bioreactor back to the BioFlo IIc stand.

Method 2

If the media components are in small volumes, they can be added through a syringe filter into one of the ports.

Time: 5 min

Supplies

Media components
Rinse water
Sterilized pipette tips
Pipetter
Sterile 0.2 μm syringe filter
Syringe

Procedure

1) Unclamp the piece of tubing covering one of the open ports.

2) Quickly attach a small 0.2 μm syringe filter onto the end of the sterile tubing attached to the port.
   - The syringe filter can be attached before autoclaving in order to reduce the chance of contamination.
3) Screw the end of a 3 or 5 mL syringe onto the filter.
4) Remove the plunger.
5) Pipette the desired quantity of one of the components into the syringe body.
6) Replace the plunger and force the media component into the bioreactor.
   - If the volume being added is very small, water can be used to flush the solute through the filter.
   - Antifoam cannot be passed through a filter.
7) Repeat steps 4 through 6 for each component.

Method 3

If a large volume needs to be added and none of the components might come out of solution due to interactions at higher concentrations, they can all be pumped into the bioreactor together.

Time: 10 min

Supplies

Media components
Sterilized pipette tips
Pipetter
Sterilized graduated cylinders (optional)
Sterilized feed vessel

Procedure

1) Move all of the media components and a sterilized feed vessel into the laminar flow hood.
2) Unscrew the top of the feed vessel and flame the glass lip.
3) Add each of the media components to the feed vessel.
   - Either aseptically pipette each component from a stock feed solution or dissolve the solutes and sterile filter them into the vessel using a syringe and filter.
4) Flame the lip of the feed vessel and replace its top.
5) Move feed vessel to the BioFlo IIc stand.
6) Connect the feed tubing to an open port on the head plate.
   - Use the same technique described for attaching the pH tubing.
7) Thread the feed tubing into the antifoam pump head.
   - Use the same technique described for the pH tubing.
8) Set the antifoam pump to "ON" and wait for all of the media to be added to the bioreactor.
9) Switch the antifoam pump to "OFF".

INOCULATION

The inoculation process can cause a stress reaction in the culture or lead to contamination of the bioreactor. The following method describes the best way to keep the bioreactor from becoming contaminated. Unfortunately, it also allows the culture to become starved of oxygen as it is being pumped. The best way to prevent oxygen starvation is to take the inoculum culture directly from the incubator, unscrew the large inoculum port of the bioreactor, and dump the culture directly into the bioreactor. It is also the best way to cause contamination.

It is important to keep the cell density of the culture within the optimum bounds of the exponential growth phase. Allowing the culture to stagnate too long at too high of a cell density can lead to a long lag phase, or poor growth and production at the beginning of the run. In contrast, a low inoculating cell density can lead to a lengthy lag phase.

If the initial feed source for the culture is added by the first stage of the Presto Control control scheme, the control scheme should be started while the inoculum is being pumped into the bioreactor.

Supplies

Inoculum
Sterilized feed vessel

Time: 10 min

Procedure

1) In the laminar flow hood, withdraw up to 1 mL of culture and pipette it into a cuvette.
10) Check the OD_{600} of the sample according to CELL DENSITY – OD600 on pg. 99.
    - The culture should have an OD_{600} between 0.8 and 2.0.
2) If the OD_{600} is within the proper range, transfer the culture to a sterile feed bottle described in FEED VESSELS on pg. 64.
3) Connect the inoculum tubing to an open port on the head plate.
    - Use the same technique described for attaching the pH tubing.
4) Thread the feed tubing into the antifoam pump.
    - Use the same technique described for the pH tubing.
5) Set the antifoam pump to "ON" and wait for all of the inoculum to be added to the bioreactor.

BIOREACTOR GROWTH

The best control scheme for a project depends on the specific requirements of the culture for growth and production. The control scheme recipes available for download are described in the Presto Control Manual along with a full description of program operations. Individualized recipes can also be designed and saved using Presto Control.

Ideally, once the control scheme is started, the operator should no longer need to make changes to the system except during induction or antifoam additions. Culture conditions, however, should be monitored at least every 6 hours. Check to make sure that the DO is being maintained at the desired set-point. Also, verify that the agitation, airflow, and feed profiles represent what would be expected from the control recipe and exponential growth. Recognize that the OD_{600} of an E. coli culture can double in less than an hour. Therefore, if the oxygen transfer capability of the bioreactor is at 50 % of maximum, it can become maxed out within an hour. If one of the goals of the project is to keep the culture in an exponential growth phase, the culture might need to be harvested or induced close to this time point. The bioreactor should be monitored closely near to the end of the run.

The length of the growth phase in the bioreactor can vary drastically depending on the culture and the media and the exact timing cannot be standardized. The cell density should be checked regularly as described in CELL DENSITY – OD600 on pg. 99. If the culture needs to be tested for product formation, samples can be taken as described in CULTURE SAMPLES on pg. 101. Periodically, antifoam needs to be added as described in ANTIFOAM ADDITION on pg. 82.

Time: 12 hrs – 48 hrs

Procedure

1) Verify that all of the control parameters are set correctly in the "Control" window.
2) Check to make sure that the feed pump is set to "AUTO".
3) Check that all of the set points and control values are at their desired values.
4) If applicable, check to make sure that the BioFlo IIc DO control light indicates that the RPM ramp is active.
5) Click on the desired control strategy recipe to download (both parts if applicable).
   - For induced protein expression in recombinant E. coli, use Standard Fed Batch Control.
   - The screen will freeze for about 1 minute while downloading.
6) Press the "Start" button to begin the recipe.
   - A dialog box will appear listing the name of the logging file. Record this name.
7) Make any online changes that are needed for the recipe.
8) Monitor culture growth.
   - Check the OD_{600} and the environmental conditions at least every 6 hours. Monitor the culture more regularly as the cell density increases.
   - Take culture samples if necessary.

9) When the culture has achieved the desired cell density, induce according to INDUCTION on pg. 103.

ANTIFOAM ADDITION

As the culture grows, foam is sometimes produced on top of the liquid. If this foam enters the exhaust filter it can block the air flow and lead to a pressure build up in the vessel. This changes the oxygen transfer characteristics of the system and it can negatively impact culture growth. Also, if the foam leaks past the seals of the bioreactor, it can allow culture to escape or lead to contamination of the bioreactor. The addition of antifoam dissipates this buildup and prevents these problems. Antifoam is an oil that reduces the surface tension of the media. It is standard practice to add a few drops of antifoam every six hours or whenever the cell density is measured. If the culture produces high levels of foam it might need treatment more often.

Supplies

Sterilized antifoam agent
Feed vessel
Labeling tape
Permanent marker

Time: 5 min

Procedure

1) If needed, transfer sterilized antifoam to a sterile feeding bottle with the appropriate tubing and connections.
   - The antifoam vessel can be reused in subsequent bioreactor projects. The antifoam does not need to be replaced between each use.
2) Label the antifoam bottle with name, date, and contents.
3) Connect the antifoam tubing to an open port on the head plate.
   - Use the same technique described for attaching the pH tubing.
4) Thread the tubing into the antifoam pump.
   - Use the same technique described for the pH tubing.
5) Prime the tubing by setting the antifoam pump to "ON" and wait for drops to form at the port outlet.
6) Let a few drops fall into the media.
   - A single drop should noticeably reduce foaming.
   - Addition of antifoam affects the surface tension of the media and will immediately cause a large decrease in dissolved oxygen. An increase of 100 rpm or 0.1 L/min of airflow may be required to maintain a constant DO concentration after antifoam addition. If the current stage in the controller recipe is set to detect a sudden change in dissolved oxygen or any of the process control parameters, the stage may need to be put on manual hold and the expected response monitored closely until the PID controllers have adjusted to the change.
7) Flip the antifoam switch back to the "OFF" position.
8) If the foam level does not drop within a few seconds, repeat the procedure.
9) Repeat whenever a time point is taken or foam forms.

BIOREACTOR SHUTDOWN

If the culture needs to be harvested, shutdown of the bioreactor and harvesting of the cells should be performed as quickly as possible. Without feed, airflow, or agitation, the culture might exhibit a stringent response that can negatively affect the product. This entire procedure should be done wearing hand and eye protection. Furthermore, disconnection of the acid and base vessels should be done with splash goggles, rubber gloves, and covering for the arms and legs.

Everything that has come into contact with the culture must be sterilized. This can be accomplished by either soaking it in 10 % bleach or autoclaving it. Preferentially, large liquid volumes should be autoclaved, but the bioreactor and unclean glass vessels should not be autoclaved without liquid in them.

Supplies

Large and small flasks and beakers
10 % bleach solution
RO water bottle
Clamps
Soap and brushes

Time: 1 hr

Procedure

1) Click the "Stop" button in Presto Control. This will stop the control recipe and stop data recording.
   - The log of bioreactor data can be accessed in the folder D:\ Presto Control\Controller Program\Fermentation Logs. It will be named according to the date it was recorded.
2) Turn off the Power switch to the BioFlo IIc.
3) Shut off the water supply to the unit.
4) Close the air/nitrogen valve by turning it so that it points away from the front of the BioFlo IIc.
5) Use clamps to close off the tubing to the acid, base, antifoam, and glucose feeds.
6) Disconnect the tubing from the bioreactor.
   - Use the proper protection for handling the acid and base lines.
   - Keep the outlets of all of the tubes above the liquid levels of the vessels.
7) Remove the motor and place it on its stand on top of the BioFlo IIc console.
8) Disconnect the pH and DO cables and remove the temperature probe from the thermowell.
9) Remove the pH and DO probes. Rinse them with RO water. It may be necessary to lightly scrub them to remove culture and wipe them with 70% ethanol solution to remove culture. Return them to their stands.
   - The pH probe should be stored with it tip covered in a KCl solution or a buffer solution with a pH between 4.0 and 7.0.

10) Disconnect the water lines from the heat exchanger and the exhaust condenser.
    - Remove the bottom connector first, followed by the top.
11) Disconnect the air inlet line.
12) Carry the bioreactor to a nearby sink or to the wash basin. Set it in the basin and unscrew the head plate.
13) Remove the head plate and set it in the sink. Rinse it with a 10% bleach solution to kill the culture on it.
14) Remove the air inlet and exhaust filters. Dispose of them in a biohazard bag or save them for future use.
15) Carefully pour the culture into a large beaker.
16) If the cell culture is to be processed for product, see the HARVESTING CELLS protocol on pg. 105.
17) If the culture is not going to be harvested, it needs to be sterilized. This can be done using one of two methods. The first method is the best option.
    - Method 1: Cover the beaker containing the culture with aluminum foil. Autoclave the culture according to AUTOCLAVING on pg. 92. Any extra liquid cultures, flasks, or test tubes can also be sterilized in this manner.
    - Method 2: Pour the culture into a bucket and place it in the walk-in hood in 3269 or in a fume hood. Make sure the hood fan is on. Add ~500 mL of bleach and let the culture stand for at least 30 minutes. Disposing of the culture in this way produces poisonous chlorine gas, so it must be done in the hood.
18) Return the bioreactor to the basin. Rinse it with a 10% bleach solution to kill any remaining cells. Let the bleach contact it for 10 minutes.
19) Wash the head plate and bioreactor and any feed vessels thoroughly with detergent and tap water. Be sure to scrub the impeller shaft, impellers and baffles. Rinse all ports (i.e. air inlet, sparger, acid, base, antifoam, glucose, condenser and sampling port) and tubing by running water through them.
20) Rinse the head plate and bioreactor several times to remove any remaining detergent.
21) Give all items two final rinses with RO water.
22) Reinstall the head plate and return the bioreactor to the BioFlo IIc console or to the designated storage area.
23) If applicable, remove any autoclaved culture containers from the autoclave after the autoclave has cooled down. Pour the liquids into the drain and wash and rinse the containers.

SUPPLEMENTARY MATERIAL

MEDIA

The following is a list of commonly used culture media types and stock solutions. See the review by Zhang and Greasham (1999) for a discussion of other types of media and the nutrient needs of microorganisms. The preparation procedures group the media components that can be mixed together before autoclaving without fear of degradation or reaction. The components listed after the autoclaving step should be added after the bulk media has cooled. The given amounts are the quantities that would be required to prepare the 1 liter of media used in the BioFlo IIc. The recipes should be scaled down as appropriate for producing agar plates and inoculum media, but do not mix and autoclave incompatible components.

M9

E. coli are facultative microorganisms and can produce nearly every component of their cellular structure from a nitrogen, oxygen, and carbon source. M9 (minimal media) supplies these components in their simplest form and is the cheapest of the growth media. The carbon source is glucose. Nitrogen is supplied via ammonium chloride and its uptake by the cells causes hydrogen ion formation. Therefore, potassium phosphate and sodium phosphate are used as buffering agents. Sodium chloride increases the ionic strength of the media and magnesium sulfate provides the trace mineral magnesium. Not all strains of E. coli are able to grow on basic minimal media. In such cases, other trace minerals and nutrients can be added.

Composition

1 g/L NH_4Cl
6 g/L Na_2HPO_4
3 g/L KH_2PO_4
0.5 g/L NaCl
4 g/L glucose
2 mM $MgSO_4$

Preparation (1 L)

- 200 ml 5X M9 salts
- 790 ml deionized H20
- 20 g agar if creating plates
- Autoclave in bioreactor or flask
- Cool to 60°C
- Sterilized 8 ml 50% glucose
- Sterilized 2 ml 1 M MgSO4
- Antibiotics
- Immediately pour plates if applicable

Reference: pET System Manual (2002)

Modified M9
This is an example of a modified minimal media. The following additions to M9 are recommended when culturing JM109.

Composition
M9 media
0.1 mM $CaCl_2$
1 mM thiamine-HCl

Preparation (1 L)
- Standard M9 preparation
- Sterilized 0.1 mL 1M $CaCl_2$
- Sterilized 1 mL 1 M Thiamine-HCl
- Immediately pour plates if applicable

Reference: Yanisch-Perron et al. (1985)

LB
LB is the most common complex media used for cell culturing. It relies on processed cell material and protein supplements to supply nutrients, and extra buffering components and salts are not needed. Cultures grow better and faster on LB than on minimal media. Unfortunately, the media is more expensive and does not allow tight control of amino acid, protein, or sugar concentration for purposes of labeling and protein expression.

Composition
10 g/L Bacto tryptone
5 g/L Yeast extract
10 g/L NaCl

Preparation (1L)
- 200 mL 5X LB
- 800 mL deionized H20
- 20 g agar if creating plates
- Autoclave in bioreactor or flask
- Cool to 60°C
- Antibiotics
- Immediately pour plates if applicable

Reference: pET System Manual (2002)

TB
Terrific broth is a variation of LB that is designed to improve growth in certain cultures by adding more carbon and nitrogen sources.

Composition
12 g/L Bacto tryptone
24 g/L Yeast extract
4 ml glycerol
2.31 g/L KH_2PO_4
12.54 g/L KH_2PO_4

Preparation (1 L)
- 900 ml deionized water
- 12 g Bacto tryptone
- 24 g Yeast extract
- 20 g agar if creating plates
- Autoclave in bioreacotor or flask
- Cool to 60°C
- Sterilized 5 ml 80% glycerol
- Sterilized 100 ml sterile K phosphate
- Antibiotics
- Immediately pour plates if applicable

Reference: Sambrook and Russel (2001)

Defined Media
This media was designed to allow isotopic labeling of specific amino acids in proteins expressed by E. coli. In order to suppress amino transferase and proteinase activity of the culture, the media supplies excess concentrations of most of the amino acids and metabolites needed by E. coli for growth and protein expression. For strains exhibiting poor growth on M9, this media is also a good reference for possible additions to minimal media that might improve metabolism.

Composition
0.50 g/L alanine
0.40 g/L arginine
0.40 g/L aspartic acid
0.05 g/L cystine
0.40 g/L glutamine
0.65 g/L glutamic acid,
0.55 g/L glycine
0.10 g/L histidine
0.23 g/L isoleucine
0.23 g/L leucine
0.42 g/L lysine hydrochloride
0.25 g/L methionine
0.13 g/L phenylalanine
0.10 g/L praline
2.10 g/L serine

0.23 g/L thrionine
0.17 g/L tyrosine
0.23 g/L valine
0.50 g/L adenine
0.65 g/L guanosine
0.20 g/L thymine
0.50 g/L uracil
0.20 g/L cytosine
1.50 g/L sodium acetate
1.50 g/L succinic acid
0.50 g/L NH_4Cl
0.85 g/L K_2PO_4
2 g/L glucose
4 mM $MgSO_4$
0.01 mM $FeCl_3$
2 mg/L $CaCl_2*2H_2O$
2 mg/L $ZnSO_4*7H_2O$
2 mg/L $MnSO_4*H_2O$
50 mg/L L-tryptophan
50 mg/L thiamine
50 mg/L niacin
1 mg/L biotin

Preparation

- 960 mL of water
- 0.50 g alanine
- 0.40 g arginine
- 0.40 g aspartic acid
- 0.05 g cystine
- 0.40 g glutamine
- 0.65 g glutamic acid,
- 0.55 g glycine
- 0.10 g histidine
- 0.23 g isoleucine
- 0.23 g leucine
- 0.42 g lysine hydrochloride
- 0.25 g methionine
- 0.13 g phenylalanine
- 0.10 g praline
- 2.10 g serine
- 0.23 g thrionine
- 0.17 g tyrosine
- 0.23 g valine
- 0.50 g adenine
- 0.65 g guanosine

- 0.20 g thymine
- 0.50 g uracil
- 0.20 g cytosine
- 1.50 g sodium acetate
- 1.50 g succinic acid
- 0.50 g NH_4Cl
- 0.85 g K_2PO_4
- Autoclave in bioreactor or flask
- Cool to 60°C
- Sterilized 40 mL 50% glucose
- Sterilized 4 mL MgSO4
- Sterilized 1 mL 0.01 M FeCl3
- 10 mL trace minerals
- Antibiotics

Reference: Muchmore et al. (1989)

Stock Solutions

80% Glycerol (100 mL)
- 20 mL of RO water in 100 mL graduated cylinder
- Add glycerol to 100 mL by volume displacement

50% glucose (1 L)
- Heat and stir 600 mL of RO water
- Slowly add 500 g of glucose (dextrose)
- Add water to 1L
- Autoclave

K phosphate (100 mL)
- Stir 80 mL of RO water
- Add 2.31 g KH2PO4
- Add 12.54 g KH2PO4
- Add water to 100 mL
- Autoclave

5X M9 salts (1 L)
- Stir 900 mL of RO water
- Add 5 g of NH_4Cl
- Add 30 g Na2HPO4
- Add 15 g KH2PO4
- Add 2.5 g NaCl
- Adjust pH to 7 with 1N NaOH
- Add water to 1 L

- Autoclave

5X LB (1 L)
- Stir 800 mL of RO water
- Add 50 g Bacto tryptone
- Add 25 g Yeast extract
- Add 50 g NaCl
- Adjust pH to 7 with 1N NaOH
- Add water to 1L
- Autoclave

1M $MgSO_4$ (100 mL)
- Stir 90 mL of RO water
- Add 12 g $MgSO_4$
- Add water to 100 mL
- Autoclave

1 M IPTG (isopropyl β-D,thiogalactopyranoside) (10 mL)
- Open 1 g IPTG container.
- Dissolve contents in total 4.2 mL of water
- Filter sterilize
- Store at –20°C.

0.01 M $FeCl_3$ (100 mL)
- Stir 90 mL of RO water
- Add 0.16 g FeCl3
- Add water to 100 mL
- Autoclave

Trace Minerals and Vitamins (100 mL)
- Stir 90 mL of RO water
- Add 20 mg $CaCl_2*2H_2O$
- Add 20 mg $ZnSO_4*7H_2O$
- Add 20 mg $MnSO_4*H_2O$
- Add 500 mg L-tryptophan
- Add 500 mg thiamine
- Add 500 mg niacin
- Add 10 mg biotin
- Add water to 100 mL
- Filter sterilize

Antibiotics

The use of antibiotics in the media serves two purposes. One is to prevent contamination by undesired microorganisms. The second purpose is to select for host strains that contain the plasmids that express the protein. Each plasmid encodes for both a

component of the protein expression system, and a protein that makes the cell immune to a specific antibiotic. Therefore, only the cells containing the expression plasmid(s) can survive in the growth media. The following is the preparation procedures for certain antibiotics used in the pET expression system (Novagen, 2002). The needed antibiotics should be added to the desired concentration at each phase of the culture transfer. Antibiotics should never be autoclaved; use a 0.2 μm syringe filter and syringe for sterilization. They should be stored at -20°C once they are in solution.

Carbenicillin (disodium salt) (5 mL)
- 250 mg in 5 mL deionized water.
- Filter sterilize
- Store at –20°C
- Use at 50 μg/ml (1:1000)

Ampicillin (sodium salt) (5 mL)
- 125 mg in 5 mL deionized water.
- Filter sterilize
- Store at –20°C.
- Use at 50 μg/ml (1:1000)

Chloramphenicol (5 mL)
- 170 mg in 5 mL ethanol.
- Filter sterilize
- Store at –20°C
- Use at 34 μg/ml (1:1000)

Kanamycin (sulfate) (5 mL)
- 150 mg in 5 mL deionized water.
- Filter sterilize
- Store at –20°C.
- Use at 30 μg/ml for cells containing kanR plasmids (1:1000)
- Use 15 μg/ml for cells with a chromosomal kanR gene (1:500)

Tetracycline (5 mL)
- 62.5 mg in 5 ml ethanol.
- Filter sterilize
- Store at –20°C.
- Use at 12.5 μg/ml (1:1000)

Rifampicin (5 mL)
- 5 mg in 5 ml 67% methanol, 0.17 N NaOH.
- Filter sterilize
- Use at 200 μg/ml within 5 days (1:50)
- Protect from light.

AUTOCLAVING

All liquids added to the bioreactor should be sterilized. The autoclave can be used for sterilization if the media components are not temperature sensitive and do not react at high temperatures. Media that degrades in the autoclave or small volumes of solutions can be sterilized by passing them through a 0.2 μm filter. The autoclave is also used to kill cell cultures so that they can be poured down the sink or thrown out with the trash. Some points:

- Glucose has a tendency to turn media brown when autoclaved with other media components.
- Certain salts (i.e. $MgSO_4$) react with other salts at high temperatures or high concentrations.
- Antibiotics are degraded by heat.
- If a trace mineral is to be added to the media but its reactivity is unknown, it is safer to autoclave it separately from the bulk media and add it after the liquids have cooled.
- DO and pH probes should only be autoclaved if their tips are submersed in media.
- Always use autoclave gloves when removing objects from the autoclave.
- Do not overfill containers or they might boil over.
- The "Liquid Cycle" slowly releases the pressure as the autoclave cools. This prevents liquid from violently boiling out of its container. The "Dry Cycle" immediately vents the steam at the end of the autoclave cycle and can be used to sterilize empty vessels.
- If agar plates or plastic biohazards are being destroyed, they should be placed in an orange biohazard bag and set in an autoclave tray with water covering the bottom. The bag should be autoclaved for 45 minutes on the "Liquid Cycle". It can be thrown out with the rest of the trash if it is sealed up in a secondary black garbage bag.

Supplies

Bottles, flasks, or containers
Aluminum foil
Labeling tape
Permanent marker

Procedure

1) Prepare the media or desired stock solutions.
   - If the autoclave is not immediately available, store in the refrigerator to prevent contamination.
2) Pour the solution into a bottle or Erlenmeyer flask.
   - To prevent boiling over, do not fill the container more than 2/3rds full.
3) Cover the top of the container.
   - If the bottle has a screw top, loosely screw on the cover.

- If the container is a flask, seal the opening by crushing a piece of aluminum foil around the outside edges of the mouth.
- The containers cannot be sealed airtight or pressure equilibration cannot occur.

4) Use labeling tape and a permanent marker to label the flask with name, contents, and date.
5) Set the container(s) in an autoclave tray and place the tray in the autoclave.
6) Flip the Power and Control switches to the On position on the autoclave.
7) Open the cooling water and steam valves, located behind the autoclave.
8) Close the autoclave door and lock it in place by turning the handle all the way to the right.
9) Set the Steam Sterilize Time to at least 20 minutes and the Steam Dry Time to 00 minutes. Larger media volumes will require more time to fully sterilize.
10) Press the Reset button.
11) Press the "Liquid Cycle" button to start the autoclave process.
12) Wait for the autoclave to finish.
    - The autoclave will run for the set length of time once it reaches 132 °C. It will then spend several minutes exhausting the steam. The buzzer will sound when the exhaust cycle is complete. The buzzer can be stopped by pressing the reset button.
13) Close the steam valve behind the autoclave.
14) Shut off the valve to the steam supply.
    - If possible, allow the autoclave to continue to cool until the internal temperature is below 100 °C before opening the door. This prevents excessive volume reduction of the media due to boil off when the remaining pressure is released by the door being opened.
    - Do not leave liquid in a closed autoclave with the steam open for more than a couple of hours or a large portion of the media will evaporate.
15) Open the autoclave door.
    - Some steam will be released when the door is opened. Do not place your head directly over the top edge of the door while the pressure is being released.
16) Remove the autoclave tray using a pair of heavy autoclave gloves.
    - Check to make sure that the aluminum foil has not been blown off the top of the containers. If it has, it can be quickly replaced without a strong risk of contamination.
17) Shut off the cooling water supply.

AGAR PLATES

Preparing agar plates is tricky when media components need to be added to the bulk media after it has cooled. A subjective measurement of the correct time to finish mixing the media and pour the plates is when the flask containing the solution is no longer uncomfortably hot to hold. Condensation might form on the top of the plate during cooling and storage, so they should be stored upside down. Try not to allow this to drip down onto the agar and cause mixing of the culture colonies during inoculation and incubation. The following protocol is taken from the Experiment Manual for the Biochemical Engineering Laboratory (Worden et al., 2002).

Supplies

Autoclaved media with 2 % agar
Extra components and antibiotics
Sterile Petri dishes
Parafilm or bag
Permanent Marker

Procedure

1) Prepare and autoclave the media in a flask with 2% agar added to the solution.
   - After autoclaving, the solutions may be cooled by partially submerging the flasks in cold water. The agar solution must not be cooled below 50°C, however, and it must also be swirled continuously during cooling. Otherwise, the agar will solidify on the inner wall of the flask. Its freezing point is 42°. Once the agar has solidified, it must be reheated to 100°C to be liquefied again.
2) Remove the aluminum from the mouth of the flask and flame the lip until it is hot.
3) If applicable, add the other media components, such as antibiotics, by sterile pipette.
   - In order to make sure that the addition of the other media components does not cause solidification of the media, swirl the media while adding the solutions.
4) Lift the lid of a plate, and pour enough agar solution into the plate to just cover the bottom (about 15-20 mL). Replace the lid.
   - Any bubbles that may have formed during pouring may be broken by gently swirling the plate. They may also be broken after the gel has solidified by passing a Bunsen burner flame across the top of the gel.
   - Stack the next plate to be poured on top of the one just poured to insulate the plate and minimize condensation inside the lid.
5) Repeat the previous step until all of the plates are poured.
6) Immediately after pouring the plates, rinse the remaining agar down the drain with plenty of hot water. Once the agar has solidified, it can be difficult to remove from the flask.
7) Wait for the plates to cool completely.
8) If not being used immediately, wrap Parafilm around the outside edge of each plate or place all of the plates into a sealed bag.
9) Label the plates with name, contents, and date.
10) Store the plates upside down at 4°C.

INOCULUM CULTURES

In order to easily monitor cell growth and reduce the lag in growth caused by a massive dilution in cell concentration, inoculum cultures are first grown up in a series of increasing volumes of broth. The dilution ratio for each volume transfer can be as high as 1:50 without a significant risk of a long lag phase. For 1 L fermentations, a common series of culture transfers is: glycerol freeze → agar plate → 5 mL media in a test tube → 50 mL media in a shake flask → 1 L media in a bioreactor. A culture should be transferred to a larger volume of media during mid exponential growth. Therefore, it is best to transfer the culture when it has reached an OD_{600} of between 0.8 and 2.0. Most shake flasks and test tubes cannot support exponential growth at a cell concentration indicated by an OD600 above 2.0 due to oxygen transfer or buffer limitations. On the opposite extreme, an OD_{600} below 0.8 might lead to a lag phase in growth at any dilution ratio greater than 1:10. The procedure for the OD_{600} measurement can be found in CELL DENSITY – OD600 on pg. 99.

A single cell line should be used as the basis for an inoculum culture. Individual colonies of a single cell line can be obtained by streaking a culture sample onto an agar plate. Then, after the cells have multiplied to form visible, separated colonies, a colony is removed from the plate and transferred to growth media in order to start a suspension culture from a single cell line. Ideally, the cell culture sample for an inoculum plate should come from the original glycerol freeze created directly after the culture was transformed. Cultures that have been grown to high cell densities and transferred to fresh media multiple times might grow and express proteins poorly due to a loss of plasmid(s) or natural selection for slower growing or more stringent cell lines. If a glycerol freeze is not available, however, the culture used to start the inoculum plate can come from a suspension culture or a different plate. If it comes from a plate, the plate should not have been incubated at growth temperatures (37 °C) for more than 24 hours and should not have been stored in the refrigerator (8 °C) for more than a week before transfer. Similarly, the cell density in a shake flask should not have grown above an OD_{600} of 2.0 and should not have been allowed to sit without agitation for extended periods of time.

If possible, the culture should be grown on the same carbon source from one media transfer to the next. Switching its primary substrate causes a lag in growth as the culture adapts its metabolism to the new substrate. In general, E. coli grows quicker on LB broth than on minimal media and glucose. Also, if the product is expressed by genes on plasmids, all of the growth media should contain the antibiotics that select for those plasmids.

Agar Plates

Supplies

1 or more agar plates
Glycerol freeze or other culture source
Wand or wire loop

Parafilm
Permanent marker

Time: LB – 12 hrs; M9 - 24 hours

Procedure

1) Warm the plates to incubation temperature and place in the laminar flow hood.
   - If the culture to be used was stored on a plate in the refrigerator, warm the culture back up to growth temperature for an hour in the incubator.
2) Inoculate the plate with cells from the culture sample
   - Flame the wand or wire loop and allow it to cool. Push the tip of the wire loop into the glycerol freeze or the culture source and remove. Starting in one corner of the plate brush the loop back and forth across one side of the plate. Reflame the loop and allow it to cool. Then, starting on the edge of the section of the plate that was previously streaked, brush the loop across a new corner of the plate while make sure to cross over into the previous section to pick up cells. Repeat this dilution procedure until every section of the plate has been streaked.
   - Do not allow the glycerol freeze to warm up to room temperature. Return it to the -80 °C freezer immediately after use.
3) Place the cover on top of the plate and wrap the outer edge with parafilm.
4) Label the plate with your name, date, and the components of the culture.
5) Incubate the plate upside down in the incubator.
   - Suggested: 37 °C
   - Rich media plates (i.e. LB) are usually incubated overnight for 8 to 12 hr. Minimal media plates (i.e. M9) may take 24 hr before visible colonies are formed.
6) If the cultures are not going to be used immediately after incubation, they should be stored upside down at 4 °C.

Test Tube Cultures

Supplies

Media
Inoculum
Sterilized flask
1 or more sterilized test tubes with tops
Sterilized pipette tips
Sterilized wooden colony applicator or metal loop
Sterilized graduated cylinder (optional)
Marking tape
Permanent marker

Time: LB – 6 hrs; M9 - 12 hrs

Procedure

1) Prepare the media according to MEDIA on pg. 85.
   - Prepare enough media for at least two test tube cultures and two shake flask cultures (100 mL total).

2) In the laminar flow hood, transfer the sterilized media to one or more sterilized test tubes with caps.
   - Suggested: 5 mL of media in a 50 mL test tube.
3) Move the inoculum plate from the incubator to the laminar flow hood.
   - If the culture to be used was stored on a plate in the refrigerator, warm the culture back up to growth temperature for an hour in the incubator.
4) Inoculate the test tube with a single colony from the plate.
   - Using a sterilized wooden applicator or 200 µL pipette tip, touch it to the visible colony on the plate and then carefully drop it or swirl it in the media in the test tube.
   - Prepare a second culture at the same time in case the first culture has too long or too short of a lag phase for the time schedule.
5) Label the tubes with your name, date, and the components of the culture.
6) Incubate the tubes.
   - Suggested: 37 °C and 250 RPM in the enclosed incubator in the teaching lab.
   - Transfer the culture when it begins to become opaque (OD_{600} of approximately 1.0).
   - LB cultures usually need to incubate for 6 hours. Minimal media cultures may require 12 hours or more. Allow more time if the culture is being transferred to a media with a different culture source.

Shake Flask Cultures

Supplies

Media
Inoculum
Sterilized 250 mL Erlenmeyer flask
Sterilized pipette tips
Pipetter
Sterilized graduated cylinder (optional)
Marking tape
Permanent marker

Time: LB – 3 hrs; M9 - 6 hrs

Procedure

1) Prepare the media according to MEDIA on pg. 85.
2) Transfer the sterilized media to one or more sterilized shake flasks.
   - Suggested: 5 mL culture into 45 mL of fresh media in a 250 mL Erlenmeyer flask.
   - For maximum oxygen transport, the media should not be greater than 20% of the total flask volume.
3) Transfer the inoculum test tube culture from the incubator to the laminar flow hood.
   - Do not allow the culture to sit too long without agitation or the cells can suffer from the stringent response caused by oxygen starvation.
4) Inoculate the shake flasks with test tube culture.
   - Flame the edges of the test tube and pour the culture directly into the media in the flask.

5) Label the flask with your name, date, and the components of the culture.
6) Incubate the shake flasks.
   - Suggested: 37 C and 250 RPM in the enclosed incubator in the teaching lab.
   - Transfer the culture when it begins to become opaque (OD_{600} of approximately 1.0).

CELL DENSITY – OD600

Below an optical density (OD) of 1.0, a cultures absorbance at a wavelength of 600 nm is directly proportion to its cell density. Therefore,

$$A = CX \tag{1}$$

where A is the OD_{600}, C is the proportionality constant, and X is the cell mass concentration. The OD_{600} can be used to determine the specific growth rate, μ, of the culture. The specific growth rate of the culture is defined as:

$$\mu \equiv \frac{1}{X}\frac{dX}{dt} \tag{2}$$

where t is the time. Assuming that the specific growth rate is constant, Equation 2 can be integrated to obtain an expression for X.

$$X = X_0 e^{\mu t} \tag{3}$$

where X_0 is the cell mass concentration at time equals 0. As can be seen, the cell mass is an exponential function of the specific growth rate. Rearranging equation 3 and substituting the OD_{600}, A, for the cell concentration, Equation 4 is obtained.

$$\text{Ln}\ \frac{A}{A_0} = \mu t \tag{4}$$

Where A_0 is the OD_{600} at time equals zero. A semi log plot of the ratio of A to A_0 versus the time produces a line with a slope equal to the specific growth rate. Note that these results are only applicable when the culture is in exponential growth phase and the measured OD_{600} is below 1.0.

This procedure relates to the use of the Turner SP-890 UV/VIS Spectrophotometer located in room 3262 of the Engineering building. The instrument has multiple modes depending on whether it is to be run as a stand-alone unit or connected to a computer. See the manual for general instructions on its use and how to change modes. The protocol listed here for determining cell density applies to most host types used in recombinant engineering. The optimal wavelength used for the measurement is different for some species, and optical measurements are not suitable for determining the density of some cultures such as fungi. If the accuracy of these methods is in doubt, serial dilution and weighing experiments should be used to check the assumption of linear dependence.

Supplies

1 or 2 cuvette tubes
Dilution media
Test tubes for dilutions
Pipette tips
10% bleach and flask

Procedure

1) Turn on the power switch in the back.

2) Wait for the system to initialize and the System Main Menu screen to be displayed.
   - Do not select a mode.
   - If the system is already in a specific mode, press "ESC" to return to the main menu.
3) Press the "λ" button. LAMBDA should be displayed on the top line of the screen.
4) Enter "600.0" in the "λ" section of the screen. Then press "ENTER" and wait for the spectrophotometer to adjust to the new wavelength. The new wavelength will be displayed in the upper right corner, along with the absorbance.
5) Insert a cuvette containing your blank solution into the back sample holder.
   - Minimal media and water do not significantly absorb light at 600 nM. Water can be used as the blanking medium with little loss in accuracy.
6) Close the lid and press "AUTO ZERO". Remove the cuvette and discard the blank.
7) Pipette your culture sample into the cuvette and return it to the back sample holder.
8) Close the lid and record the absorbance reading displayed in the "DATA" field of the screen.
9) Use serial dilutions to dilute the culture until the OD_{600} is below 1.0.
10) Calculate and record the absorbance based on the dilutions made.
11) Rinse the cuvettes out into a labeled flask containing 10% bleach to kill the culture cells.

Calculations

Cells/mL= OD_{600} x $8x10^8$ Cells / dilution ratio
Cell Dry Weight = OD_{600} x 0.43 g/L /dilution ratio
Cell Wet Weight ≈ 3 x Cell Dry Weight

CULTURE SAMPLES

In order to monitor protein expression or cellular responses to environmental variables, culture samples are collected and stored for later analysis by SDS-PAGE or enzyme assay. Culture can be collected in 1.5 mL microcentrifuge tubes and spun down to obtain cell samples for analysis. At cell densities indicated by an OD_{600} below 5, 1 mL samples generally produce the required protein concentrations for analysis by SDS-PAGE. At high cell densities, a 1 mL sample volume requires large volumes of Laemmli buffer for solubilizing the cell mass. Therefore, the sample volume should be reduced in relation to the increase in cell density as the OD_{600} rises above 5. When checking protein expression after induction, a sample should be taken before induction, at 0.5 hours after induction, at 1 hour after induction, and every hour after that until the culture is harvested.

Supplies

Syringe
1.5 mL microcentrifuge tubes
Test tubes
Pipette tips
Pipetter
Permanent marker
Small flask
10% bleach solution

Time: 5 min

Procedure

1) Connect a 5 mL syringe to the end of the tubing attached to the sampling port.
2) Open the sampling valve.
3) Flush the sampling port.
   - Withdraw ~5 mL of culture with the syringe. Close the sampling valve to prevent drainage of the culture out of the bioreactor and remove the syringe. Discard the first 5 mL into a flask containing 10% bleach solution to kill the cells.
4) Take a culture sample.
   - Reattach the syringe and open valve as before. Withdraw a second 5 mL of culture. Close the clamp and valve and remove the syringe.
5) Transfer the culture sample to a test tube.
6) Use a portion of the culture sample to determine the OD_{600}. Record this for reference to the 1 mL pellet sample.
7) Pipette 1 mL of the culture sample into one or more 1.5 mL microcentrifuge tubes.
   - At high cell concentrations, smaller volumes of culture sample may be easier to prepare for analysis by SDS-PAGE. For reference, a 1 mL culture sample harvested at an OD_{600} of 10 produces the desired cell mass for viewing by SDS-PAGE using 1 mL of Laemmli buffer. Higher cell concentrations will require more work to digest with Laemmli buffer due to the limited volume of the microcentrifuge tube.

- If only one sample is taken, fill a second microcentrifuge tube with 1 mL of water. This tube will serve to balance the microcentrifuge.

8) Place the tubes across from on another in whatever microcentrifuge is available. The hinge to the lid of each tube should face the outside of the rotor.
9) Centrifuge at full speed for 30 seconds.
10) Remove the tubes.
    - If available, save the counter-balance tube for later use.
11) If the product being checked is intracellular, discard the culture supernatant by pouring it into a flask containing 10% bleach to kill any remaining cells. If the product is extracellular, pour the supernatant into a new microcentrifuge tube.
    - A twisted piece of KimiWipe can be used to mop up any liquid left in the pellet sample tube. Make sure not to disturb the pellet.
12) Label the sample tube or a box containing all of the sample tubes with your name, contents, and date.
13) Store the sample in whatever freezer is available.
    - A -40 °C or -80 °C freezer is ideal.

INDUCTION

In recombinant engineering, the desired protein product is usually encoded on a plasmid that has been introduced into the host cell. Transcription of the DNA on this plasmid is designed to only occur when induced by a change in the media or environmental conditions by the operator. This allows the host culture to grow to a high cell density without the heterologous protein interfering with the metabolism of the cells. Induction strategies include changes in temperature, changes in pH, and the introduction of a new chemical. The following protocol describes a protocol for induction by the addition of isopropyl β –D,thiogalactopyranoside (IPTG). IPTG is a non-metabolizable analog of lactose that can induce transcription of genes controlled by Lac promoters. This form of induction is utilized by the pET expression system and it is the most common inducer used in this lab. In fed batch fermentations in the BioFlo IIc, the temperature, pH, or DO can also be changed to decrease degradation of the protein being expressed.

Supplies

Sterilized 1 M IPTG or other inducer
Sterilized pipette tips
Pipetter

Time: 1hr - 6 hrs

Procedure for Fed Batch Fermentations in the BioFlo IIc

1) Make any changes to the control recipe needed for induction.
2) If applicable, change the set point of any of the controlled environmental conditions such as pH or temperature that need to be changed. Allow the bioreactor to reach its new set point.
   - A drop in temperature or change in pH will slow down the metabolism of the culture and cause an increase in the DO. If the current stage in the controller recipe is set to detect a sudden change in dissolved oxygen or any of the process control parameters, the stage may need to be put on manual hold and the expected response monitored closely until the PID controllers have adjusted to the change.
3) Unscrew and remove the cap to the inoculation port.
   - This exposes the bioreactor to the environment and a chance of contamination. This late in the fermentation and at this high of a cell density, contamination is no longer a concern as long as the length of the induction phase is less than several hours.
4) Add the IPTG or other inducer to the broth.
   - 1 mL of 1 M IPTG will give the standard induction concentration of 1 mM IPTG.
   - IPTG is toxic to the cells and may cause a rise in the DO as the oxygen uptake rate decreases. As with a change in the pH or temperature described above, the stage may need to be put on manual hold and until the PID controllers have adjusted to the change. If the recipe is currently employing PID control of the DO by feed rate, the drop in the metabolism rate will likely cause overfeeding. The controller increases the feed rate in an attempt to reduce the rising DO

concentration that is a result of the maximum oxygen uptake rate decreasing below oxygen transfer rate of the bioreactor. In other words, the DO concentration is forced above the set-point and the controller tries to respond by dumping feed into the bioreactor even though the culture's rate of oxygen consumption is no longer being limited by a lack of substrate. The correct controller response is to return the recipe to DO control by airflow with an open loop feed scheme to keep the culture from becoming substrate limited.

5) Return the cap to the inoculation port.
6) Periodically monitor and take samples from the bioreactor during the induction phase.
7) When the induction phase is complete, stop the recipe and harvest the culture.
   - 4 to 6 hours is the standard length of most IPTG induction phases.

Procedure for Batch Fermentations

8) If the temperature is to be reduced to improve expression, set-up a water bath shaker for use.
   - Water bath shakers are located on the back wall of near to the door that connects the two rooms of the Biochemical Engineering Teaching Lab. Use a bucket to add water to the basin until the flask clamps are covered. Turn of the power and the heater and set the temperature knob to the desired set-point. Monitor the temperature to make sure that the heating coils of the shaker are producing the desired bath temperature.
9) Remove the culture from the incubator and place it in the laminar flow hood.
   - Do not allow the culture to sit without agitation for extended periods of time. The DO in the flask can become low enough to trigger a stress response in the cells that will affect protein expression.
10) Remove the foil or cap from the batch culture container and flame the opening.
11) If desired, take a cell density measurement and culture sample for analysis as the zero time point of the induction phase.
12) Add the IPTG or other inducer to the broth.
   - Suggested: 1 M IPTG diluted 1:1000 by the batch fermentation broth will give the standard induction concentration of 1 mM IPTG.
13) Reapply the tin foil or cap to the opening of the batch culture container.
14) Return the culture to the incubator or to the water bath shaker.
15) If desired, periodically take cell density measurements and culture samples from the shake flask in order to determine the time course of the induction phase.
16) When the induction phase is complete, stop the recipe and harvest the culture.
   - 4 to 6 hours is the standard length of most IPTG induction phases.

CULTURE HARVESTING

At the end of the project, the entire culture needs to be harvested from the bioreactor. If the product is an intracellular protein created by induction, the cell mass must be separated from the broth and the product purified. If the product is extracellular, only the liquid needs to be saved. Do not allow the culture to sit at room temperature for extended lengths of time or product degradation can occur. This is why the centrifuge is cooled to 4 °C. Also, it is imperative that the centrifuge is balanced properly.

Supplies

Several 500 mL centrifuge bottles with lids
Spatula
Plastic storage container with lid
10% bleach
Soap and brushes
Labeling tape
Permanent marker

Time: 20 min

Procedure

1) Turn on the Sorvall RC-5B centrifuge in Room 3269.
2) Press the "Door" button while lifting the lid to access the rotor.
3) Install the correct rotor for the by gently placing it on the spindle in the center of the centrifuge.
   - The KA-10 composite rotor should be used for 500 mL bottles.
   - The Sorvall SA-600 rotor should be used for 30 mL samples
4) Close the door and adjust the centrifuge temperature to 4 °C
   - The controls are located below the temperature gauge on the left side of the unit. The blue line represents the low temperature limit and the red line represents the upper limit. The red line should be placed at 5 °C and the blue line just above 0 °C. Allow some time for the centrifuge to cool down.
5) Carefully pour the culture into the centrifuge bottles.
   - The bottles should be filled no higher than 80% full. Overfilling them can cause them to leak while being centrifuged.
6) Take the samples to the double pan balance in 3269 and zero the balance using the sliding weights.
7) Balance the bottles.
   - Inspect the rotor to determine the number of sample slots that must be used to provide counter balancing. If only one sample bottle is being used, a water-filled counter balance will be required. Two bottles containing cells can be placed on the balance and culture transferred from one to the other until they are balanced. While all the bottles placed in the rotor should contain approximately the same volume, only those placed across from each other need to be carefully balanced.

- Make sure that the lids of the 500 mL bottles have o-ring seals and are fully sealed to prevent leakage.

1) Check that the centrifuge has reached 4 °C and open the door and unscrew the rotor lid.
   - Even if the centrifuge has not completely reached 4 °C, it is better to centrifuge and freeze the culture immediately rather than allow degradation of the product due to the stress response of the cells.
2) Place the sample bottles in the centrifuge and make sure that the placement acts to counterbalance the rotor.
3) Replace the rotor lid and screw it counter clockwise into place. Tighten the second screw to lock the lid in place.
4) Close the door.
5) Set the Angular Velocity and the Time.
   - Suggested: 5,000 rpm; 10 minutes
6) Press the Start button.
   - The centrifuge must be balanced or the moorings of the spindle may snap. If a high-pitched whine or a vibration begins to occur, immediately press the "Stop" button and allow the rotor to slow down. When the "Door" button is lit, you can press it to open the door. Remove the centrifuge tubes and double check that they are balanced. Clean and disinfect any culture sample that may have spilled into the centrifuge. If leakage or poor counterbalancing did not cause the problem, contact the lab safety representative because there may be some other problem.
7) When the rotor has stopped and the "Door" light is lit, open the lid and unscrew the rotor lid clockwise. Remove the rotor.
8) Remove the tubes from the rotor.
   - Check for spills inside the rotor or centrifuge. If culture has leaked into the centrifuge, clean and sterilize the area.
9) If the supernatant is to be discarded, autoclave it or add bleach to it as described for culture sterilization in BIOREACTOR SHUTDOWN on pg. 83.
10) If the product being checked is intracellular, discard the culture supernatant by pouring it into a flask and either add 10% bleach or autoclave it as described for culture sterilization in BIOREACTOR SHUTDOWN on pg. 83. If the product is extracellular, pour the supernatant into a new sample bottle.
11) Determine the weight of a plastic container that will be used to store the cells.
12) Use a spatula to spoon the cells from the centrifuge tubes into to the sample container.
13) Measure the weight of the plastic container with the added cells.
14) Calculate the weight of the cell pellets and label the culture container with your name, contents, date, and cell mass. Store it in a -40 °C f or -80 °C freezer.
15) Rinse the 500 mL centrifuge bottles and any other containers with 10% bleach to kill the remaining cell culture. Wash and rinse the centrifuge bottles and other containers.
16) Store it in an available freezer.
   - A -40 °C or -80 °C freezer is ideal.

SDS-PAGE

Sodium Dodecyl Sulfate Poly-Acrylimide Gel Electrophoresis (SDS-PAGE) is a ubiquitous and non-specific method of determining protein composition in biological samples. SDS-PAGE separates proteins along a linear sample lane on a gel sheet according to MW. If the size of a protein is distinct from the rest of the proteins in the sample, it is viewed as a sharp band in the sample lane. Multiple samples can be run side by side and the intensity of the bands compared to each other to determine the relative concentrations of each protein in the sample.

SDS-PAGE functions by unfolding the protein and attaching negative charges along its length. The protein is then loaded onto a gel matrix and subjected to a voltage potential. The voltage draws the protein through the gel and the gel acts as a selective inhibitor to the protein's migration. The larger the size of the protein, the more resistance it experiences and the slower it migrates through the gel. The smaller proteins encounter less resistance and therefore travel through the gel faster than the larger proteins. When the proteins have been fully separated across the length of the gel, they are dyed. The intensity of the bands that are created by the dyed protein is directly proportional to the mass of protein in that band.

SDS-PAGE generally serves two purposes as a tool for optimizing recombinant protein expression. First, it allows the researcher to get a general idea of the purity of a protein sample that has been purified. Be separating the proteins by weight, any bands that are visible besides the one for the desired protein indicate that the product sample has not been fully purified. The second use of SDS-PAGE is to determine the expression level of an induced protein in a host organism. The strength of the product band relative to the other bands of the sample indicates the extent to which the protein was overexpressed.

The following protocol is intended for use specifically with the FB-VE10-1 10 mL Fischer vertical electrophoresis stand. All SDS-PAGE equipment function similarly, though. Wear gloves and safety goggles at all times during these procedures. Many of the components such as acrylimide, and 2-mercaptoethanol are toxic. Others chemicals can stain the skin and clothing. In addition, proteins from exposed flesh on the hands can contaminate the samples.

Gel Casting

The electrophoresis stand allows two gels to be run at once. This can save a great deal of time and it allows more samples to be compared to each other. As long as the gel and sample compositions are similar, the procedure and results for running two gels as compared to one gel should be the same. In order to make sure that the two gels do not vary, the gels should be poured at the same time using a single set of solutions. The gel caster has the capability to hold two sets of plates at one time. The metal plates must face each other on the inside so that the clear glass plates allow the operator to view the gels that are being poured.

The gels are poured in two layers having differing acrylimide concentrations and differing pH's. The top layer, in which the protein is loaded, is called the stacking gel. The low polymer concentration in stacking gel allows all of the proteins to migrate at the same velocity and forces them to stack up at the interface between the stacking gel and separating gel. The separating gel is the lower layer that is used to separate the proteins. The desired composition of the separating gel is dependant on the size of the protein that is to be separated and viewed. Gels are defined by their concentration of polymerizable component. The stock monomer solution used in forming the gel is a mix of 30% acrylimide and 0.8% bisacrylimide for cross linking. A 12 % gel contains 12% by weight of acrylimede. Free radical polymerization of the gel is initiated by ammonium persulfate. N,N,N',N' tetramethylethylenediamine (TEMED) is added to protect the free radicals from quenching.

Each acrylimide concentration has only a certain range of protein weights that it can separate. Gels with a very high polymer concentration resist the movement of the larger proteins to a greater extent than the smaller proteins. Therefore, the smaller proteins reach the bottom of the gel before the larger proteins begin to visibly separate near the top of the gel. Lower polymer concentrations can be used to separate larger proteins. The larger proteins are allowed to travel through the gel, but the smaller proteins all crowd together at the bottom of the gel.

The following tables contain the recommended concentrations of each component for the stacking and separating gel solutions. The solution that is prepared can be used to create two gels. The ammonium persulfate and TEMED should only be added right before the gel is poured.

Table 2: Separating Gel Solutions (15 mL)

Polymer Concentration	6 %	8 %	10 %	12 %
Target Protein Weights	110 kDa	70 kDa	40 kDa	25 kDa
Water	8.1 mL	7.1 mL	6.1 mL	5.1 mL
Separating Buffer	3.8 mL	3.8 mL	3.8 mL	3.8 mL
30:0.8 Acrylimide/Bis	3.0 mL	4.0 mL	5.0 mL	6.0 mL
10 % Ammonium Persulfate	150 µL	150 µL	150 µL	150 µL
TEMED	12 µL	9 µL	6 µL	6 µL

Table 3: Stacking Gel Solution (10 mL)

Polymer Concentration	3 %
Water	6.5 mL
Stacking Buffer	2.5 mL
30:0.8 Acrylimide/Bis	1 mL
10 % Ammonium Persulfate	60 µL
TEMED	10 µL

Supplies

DI water
Separating buffer

30:0/8 acrylimide/bis solution
10% ammonium persulfate
Stacking buffer
Pipette tips
Gel caster stand
Glass gel plate
Notched metal plate
White Teflon plate spacers
White Teflon sample comb
Gel plate plastic pouch
Gel caster pressure plate
Butanol
Two small beakers or 25 mL tubes

Time: 45 minutes

Procedure

1) Clean the gel caster stand and the plastic front pressure plate with soap and water. Wipe dry.
2) Clean the glass plate and the white metal plate with the tabs at the upper corners. Rinse with ethanol or acetone and allow it to dry.
   - The gel that is formed will not stick to wet surfaces, so the plates must be completely dry before they are placed in the pouch. This is why the plates are rinsed with a volatile liquid such as acetone or ethanol. The acetone or ethanol can be quickly removed from the plates by holding the plate with tongs and passing it over the flame from a Bunsen burner. They can also be wiped dry with a KimiWipe.
3) Clean the plastic pouch and allow it to dry.
   - Check that the pouch does not leak.
   - The pouch can be dried quickly by rinsing it with ethanol or acetone forcing air to circulate inside it.
4) Place the glass plate, the notched metal plate and two white teflon spacers into the pouch.
   - The white spacers are placed between the edges of the two plates to form a space in which to pour the gel.
   - Make sure that spacers have the same thickness as the comb that is to be used to create the sample reservoirs.
5) Adjust the thumb screws on the front of the caster to create room for the pouch between the front pressure plate and the rear wall of the caster.
6) Place the pouch into the caster so that it rests against the rear wall. Lightly tighten the thumb screws to hold the pouch upright.
7) Straighten the spacers near the edges of the two gel casting plates.
   - This is easily done by sliding a third spacer between the two plates and using it to press out against the edges the other two spacers.
8) Finish tightening the thumbscrews until the spacers and the bag have formed a firm seal with the plates.

- The plates can crack if too much pressure is used. Stop tightening when the screws require extra effort to turn them.

9) Level the gel caster using the leveling screws at the four corners of the gel caster.
   - Adjust the front two leveling screws until the air pocket is centered in the plastic bubble in the middle of the gel. Most likely, the caster will be balancing on only three of the four leveling screws. Lower the fourth leveling screw until it also begins to support a portion of the caster's weight.

10) Mark the desired height of the separating gel.
   - Slide the sample well comb into the slot in the top of the gel plates until the tabs are completely below the notch in the metal plate. Measure one centimeter down from the bottom of the wells and mark the spot.

11) Prepare the separating gel according to the Table given above.
   - Wear gloves.

12) Add the required amount of ammonium persulfate and TEMED to the separating gel solution.
   - These components are used to initiate the polymerization. Only add them when the gel is ready to be poured.

13) Thoroughly mix the separating gel solution
   - Cap the resolving solution container and gently mix the polymerization initiators by flipping the container upside down and back again. Do not shake the container to mix it. The SDS will cause a foam to be created that cannot be easily poured. The solution can also be mixed by gently stirring with a pipette tip or spatula.

14) Pour the separating gel solution into the space between the plates.
   - This can be tricky. The easiest method is to use a pipette to add separating gel solution until the top of the liquid reaches the marked line. Otherwise, if the container has a small diameter opening, the solution can be slowly poured into the space between the plates. When done, check to make sure that solution is not leaking around the edges of the spacers and into the sides of the pouch. If the level drops, use the thumb screws to add more pressure to the plates and add more solution if needed.
   - Do not wait to pour the gels. Polymerization begins immediately and the solution will become too viscous to pour.

15) Immediately cover the polymerizing separating gel with water-saturated butanol. Ethanol will also work if butanol is not available.
   - The butanol must be saturated with water or it will dehydrate the acrylimide solution and shrink and concentrate the gel. The alcohol layer serves three purposes. One, it removes any bubbles that formed while pouring the gel. Air pockets in the gel will stop the migration of the protein and ruin the results. Two, the butanol protects the solution from the air while it is polymerizing. Oxygen that is present in the solution will interfere with the free radical polymerization used to link the acrylimide. Three, the pressure on the separating solution helps to keep it level.

16) Allow the gel to sit until polymerized.
   - The expected time requirement is 15 minutes. If the gel has not hardened within 20 minutes, a mistake has probably occurred in the preparation of the gelling solution.

- It can be difficult to determine if the gel has polymerized due to the liquid layer on top. A plug of solution can be kept in the pipette tip or sealed in the acrylimide mixing container. When this has hardened, so has the gel in the stand.

17) Pour off the butanol solution from the top of the gel.
18) Gently wash the top of the gel with water to remove any remaining alcohol or unpolymerized separating gel solution.
19) Pour off the water from the top of the gel.
    - The surface tension of the liquid can make it difficult to pour all of it out from between the plates. The edge of a KimiWipe held against the lip of the plates while they are turned upside down will draw the rest of the liquid out.
20) Prepare the stacking gel according to the table given above.
21) Add the required amount of ammonium persulfate and TEMED to the stacking gel solution.
22) Pour the stacking gel solution onto the separating gel until the solution just reaches the opening created by the notch in the metal plate.
23) Insert the sample reservoir comb into the resolving gel solution in the space between the plates.
    - The friction of the plates will hold the comb in place.
    - Do not allow air bubbles to be trapped between the comb and the separating gel. They will block the movement of the sample through the gel or allow adjoining sample reservoirs to mix. Bubbles can sometimes be removed by slightly tilitng the gel stand to one side and tapping on the glass. If all else fails, remove the comb before the gel has polymerized, add more solution if any leaked out, and reinsert the comb.
24) Allow the plates to sit for 15 min or until the gel has polymerized.
25) Unscrew the thumbscrews and carefully remove the gel pouch.
26) Gently slide the gel plates out of the pouch.
    - First, check if gel leaked out during the polymerization and bonded the pouch to the outside of the plates. If a seal has formed, gently separate the plate from the pouch in order to keep the plates from pulling apart as they are being removed.
27) Scrape away any gel that has stuck to the outside walls or edges of the plates.
28) Inspect the gels.
    - The gel should not have any air bubbles and the spacers and comb should be flush with the edges of the plates. It is best if the bottom of the wells are approximately 1 cm above the interface of the stacking and the separating gel layers. If a visible bubble exists in the gel, the well directly above the air pocket should not be used to separate a protein sample. Instead, fill it with 1x Laemmli buffer during sample loading.
29) If the gels are not going to be used immediately, place them back into the pouch or some other bag or container. Seal the pouch shut and store it in a refrigerator.
    - Do not leave the gel open to the environment for long periods of time or it can become dried out.

Cell Fractionation

The following protocol only pertains to experiments in which the operator wishes to determine if inclusion body formation during protein expression is an issue. If the

protein is known to be soluble or the operator only wants to determine the overall extent to which the protein is being expressed, then this section can be skipped.

A common problem with expression of proteins in recombinant E. coli is the production of insoluble protein aggregates called inclusion bodies. Inclusion bodies are usually formed by heterologous proteins that have a number of hydrophobic regions that make them difficult to maintain in solution. During expression, the protein might come into contact with another protein with a similar hydrophobic region and the two proteins might denature and fold together to shelter the hydrophobic region from the aqueous cytoplasm of the cell. This is the seed for an inclusion body and it continues to grow as more hydrophobic proteins are drawn into it. Due to the denaturing that occurs when the proteins form aggregates, proteins that are recovered from inclusion bodies must be refolded to be active. This can be a very complicated procedure.

Inclusion body formation is especially a problem for eukaryotic, integral membrane proteins that are extremely hydrophobic and are only stable in the hydrophobic regions of a cellular membrane. In their native environments, these types of proteins generally require chaperone proteins to protect them from denaturing conditions as they are transported to the cell membrane. When expressed in prokaryotic hosts such as E. coli, the proteins generally rely on random diffusion for transport. At the high expression levels and protein concentrations that characterize recombinant protein expression strategies, it is very likely that the proteins collide and form inclusion bodies before they reach the cell membrane. Furthermore, the cell membrane might become saturated with the expressed protein and either kill the cell or increase the rate of inclusion body formation. Therefore, the most common first step in reducing insoluble protein levels in the cell is to reduce the rate of formation of the expressed protein. This is usually accomplished by reducing the temperature and the concentration of the inducer.

SDS-PAGE can be used to determine the extent of inclusion body formation during expression in recombinant hosts. The inclusion bodies are very dense compared to the soluble components of the cell. Therefore, normal centrifugation can be used to separate the inclusion bodies and the rest of the insoluble fraction from the soluble fraction of the cell. By comparing the electrophoresis bands of the expressed protein from both fractions, the operator can determine whether a change needs to be made in the expression strategy in order to produce more of the soluble protein.

The following protocol is a simple method of fractionating a 1 mL cell pellet sample that does not require any specialized equipment. The freeze/thaw method lyses the cell by quickly freezing the cell samples to create ice crystals that fracture the cell membrane. The procedure is more effective the quicker the sample freezes, so liquid nitrogen should be used in place of the dry ice/ethanol combination specified in this protocol if it is available. A tip sonicator can also be used.

Supplies
1 mL cell culture samples
Sturdy plastic bowl

Styrofoam ice box
1 cup of dry ice
Ethanol
Metal tongs or tweezers
Microcentrifuge tubes
Marker
Beaker with water

Time: 30 minutes

Procedure

1) Thaw the 1 mL cell culture sample pellets.
2) Spoon the dry ice into the plastic bowl and place the bowl in the Styrofoam box.
   - Use enough ice to cover most of the bottom of the bowl while leaving some open space between the nuggets.
3) If applicable, return the dry ice to the freezer from which it was obtained.
4) Add ethanol to the bowl until the liquid level is just above the level of the dry ice.
   - If available, liquid nitrogen works much better for quickly freezing the culture samples.
5) Make sure that the culture samples are well marked.
   - The ethanol will tend to strip permanent marker from the outside of the centrifuge tubes. It is best to mark both the top and the side of the tubes in case the tube becomes submerged. An even better strategy is to create a harness using a microcentrifuge tube rack that can be dipped in and out of the ethanol bath.
6) Vortex the samples to resuspend the cell material.
   - Suggested: 30 seconds
7) Use the tongs, to dip the centrifuge tubes in the dry ice/ethanol bath until they are completely frozen.
   - Suggested: 2 minutes
   - Try to position the microcentrifuge tubes in the dry ice so that the top is not submerged.
8) After the cell samples in the tubes have frozen, remove them from the dry ice bath and place them in the water bath.
9) When the samples have thawed, remove them from the water bath and wipe them dry.
   - Do not allow the samples to reach room temperature. It reduces the speed with which the samples can be frozen and increases the rate of protein degradation in the sample.
   - If proteolytic activity is causing the appearance of bands of degraded protein, a protease inhibitor can be added to the buffer.
10) Vortex the samples again to resuspend the cells.
11) Repeat the freeze/thaw procedure multiple times.
   - Suggested: 5 freeze/thaw cycles
12) After the final thaw, place the samples in a microcentrifuge and pellet the insoluble fraction;
   - Suggested: 15,000 RPM; 20 minutes

- Use a microcentrifuge that is kept in refrigerated conditions if one is available. It reduces protein degradation. One can be found in the Protein Expression Laboratory.
- Remember to balance the centrifuge.

13) Label empty microcentrifuge tubes in a way that indicates that they will contain the soluble fractions of the samples that have just been fractionated.
14) When the centrifugation is complete, remove the sample tubes.
    - Be careful not to unsettle the pellet by shaking or dropping the tubes.
    - A small pellet should be visible on the bottom of each tube. If one cannot be seen or the insoluble material appears to have formed a viscous liquid layer instead of a hard pellet, the sample may need to be centrifuged for a longer time period.
15) Pipette the supernatant from the sample tube to the newly marked microcentrifuge tube.
    - Be careful not to suck up a portion of the pellet. It is unlikely that the pellet is fully compacted against the bottom of the centrifuge tube. Use a small volume pipette whose intake rate can be easily controller. Keep the pipette tip as far away from the pellet as possible while removing the supernatant. When only a small volume is left in the tube, it can be rotated to horizontal to open up more area of the supernatant to the pipette. Remove as much of the supernatant as possible.
16) Add the same amount of 1X PBS buffer to the pellet as was added to the cell sample initially.
17) Vortex the pellet to suspend the insoluble material
    - Suggested: 30 seconds
18) If the samples are not going to be analyzed immediately, place them in the freezer.
    - Suggested: -80 °C

Sample Preparation

As a first step in determining expression levels in recombinant E. coli, it is often easiest to solubilize an entire 1 mL culture sample from the fermentation and examine the total cell protein (TCP) by SDS-PAGE. Comparing the band of the recombinant protein to the bands from the rest of the proteins gives the operator a good idea of how well the desired product is being overexpressed. If the product band cannot be seen when comparing the uninduced sample to the induced sample, then the expression levels are either poor or nonexistent and a new expression host might be required.

The following sample preparation procedure is based on the assumption that the sample is a cell pellet taken directly from a fermentation culture. The sample can, in fact, be a solution of purified protein, lysate from a cell fractionation, or the insoluble portion of a cell fractionation. A method for fractionating and analyzing a cell sample based on its insoluble and soluble components is given in Cell Fractionation on pg. 111.

The primary consideration for sample preparation is having the correct amount of total protein per SDS PAGE Sample. Too much protein causes the gel lane to be a large blue streak that expands outward against the other lanes. Too little protein and the operator might be unable to view the desired protein even if it had a reasonable expression level. Here are some common principles:

- At least 1 μg of protein is required to be visible by SDS-PAGE.
- 4 - 10 μg is the optimum quantity of protein for viewing of the band.
- The total amount of protein per well should not exceed 100 μg.
- The sample well can hold between 10 – 20 uL of prepared sample.

A first estimate of the volume of buffer required for solubilizing a full cell pellet for SDS-PAGE can be obtained from the optical density of cell sample. For every unit of OD_{600}, a 1 mL E. coli pellet should be solubilized in 200 μL of SDS-PAGE buffer. For example, if the OD_{600} of the culture was 2.00 at the time that the 1 mL culture sample was pelleted, the final SDS-PAGE sample should have a volume of 400 μL: 200 μL of PBS and 200 μL of 2X Laemmli sample buffer. The sample buffer is named after the researcher who developed the SDS-PAGE technique (Laemmli, 1970).

Laemmli sample buffer contains:

- A buffering reagent: Tris-HCl
- A charged ionic component for bonding to the protein: SDS
- A reagent for increasing density so that the sample will sink into the wells: glycerol
- A chemical that cleaves disulfide bonds between proteins: 2-mercaptoethanol
- A low MW dye that signifies the protein front during diffusion: bromophenol blue
- A denaturing reagent for untangling insoluble components: urea

SDS is an anionic surfactant with very strong ability to denature and solubilize proteins. The amount of SDS that becomes attached to the protein is, in most cases, directly proportional to the weight of the protein. SDS is the component of the buffer that causes the protein to migrate towards the cathode of the SDS-PAGE stand. Standard Laemmli buffer does not require urea. It has been added to this protocol to help solubilize inclusion bodies and other normally insoluble components that can be found in an unpurified cell sample. Urea is often not included in the Laemmli buffer if the sample is culture lysate or purified protein. All of the chemicals that make up the buffer can be premixed and stored frozen except for the 2-mercaptoethanol. 2-mercaptoethanol should be added directly before use.

Supplies

2X Laemmli sample buffer
PBS
TEMED
Pelleted culture samples
Heated water bath
Microcentrifuge
Vortex

Time: 20 minutes

Procedure

1) Remove the 1 mL culture sample pellets or protein samples from the freezer and allow them to thaw.
2) Remove a 9 mL aliquot of 2X Laemmli sample buffer from the freezer and allow it to thaw.
   - The urea in the buffer is near its saturation limit. Therefore, it is like that crystals will from when the buffer is thawed. This does not cause problems.
3) Add 1 mL of 2-mercaptoethanol to the 9 mL aliquot of sample buffer.
   - Wear gloves.
   - The Laemmli buffer can still be used for a week after the 2-mercaptoethanol is added without noticeable degradation in the results. It can be stored at room temperature.
4) Begin heating a water bath for boiling the samples.
5) Add PBS to all of the sample pellets if the culture samples have not yet been resuspended in buffer or purified.
   - Suggested: 0.1 mL per unit OD_{600} of the culture sample.
6) Add equal volumes of 2x Laemmli buffer to the volumes of buffer already present in the sample tubes.
7) Vortex the microcentrifuge tubes to suspend the samples.
   - Suggested: 30 seconds
8) Check to see if the water bath has begun to boil.
   - The temperature can range between 85 to 100 °C.
9) Boil the samples for five minutes.
   - Do not heat the samples for too long or significant cleavage of peptide bonds can occur.
10) Centrifuge the samples to pellet any remaining insoluble cellular components.
   - Suggested: 14,000 RPM; 5 minutes.
   - Insoluble material that is added with the sample will cause streaking in the gel.
11) If the sample is not going to be immediately run, label it with name and date and store it in the refrigerator. Heat it back up to 37 ° C and centrifuge it again before use.

Electrophoresis Procedure

In accordance with the instruction manual for the Fischer vertical electrophoresis stand, the upper buffer chamber (UBC) is the vertical stand to which the gel plates, power chords, and cooling water lines are attached, and the lower buffer chamber (LBC) is the electrophoresis buffer pool in which the UBC sits. With the gel plates firmly sealed against the sides of the UBC, the top of the gel is only open to the buffer that is in contact with the anode and bottom of the gel is only open to the buffer in contact with the cathode. This allows a voltage source to be used to create a potential across the length of the gel as long as a leak is not present between the buffers in the LBC and UBC.

The sample experiences more random diffusion the longer it remains in the gel. Therefore, the quicker and more efficiently that the samples can be loaded and run, the better the results will be. The migration speed of the protein being analyzed is dependant on the voltage. The optimum voltage is empirically determined and it is bound by two considerations. If the voltage is too high, a large amount of current is forced through the

gel and the gel heats up. Heat dissipation in the electrophoresis stand is aided by the use of the cooling water and the use of metal plates to increase transport. If the gel becomes too hot, however, the gel and the samples deform and the resulting dye front looks like its smiling. If this occurs, lower the voltage. In contrast, if the voltage is too low, the length of time required for the proteins to travel through the gel is too long and undirected diffusion allows the bands to spread. When running a gel for the first time, it is good practice to monitor the gel regularly to determine whether the dye front and samples are distorting.

The following protocol is an adaptation of the instructions supplied with the gel stand.

Supplies

Upper buffer chamber (UBC)
Lower buffer chamber (LBC)
Water
5X electrophoresis buffer
500 mL or larger volumetric flask or beaker
Prepped samples
Protein ladder
Small pipette and tips or Hamilton syringe
Two prepped gel plates or one gel plate and a plastic plate
Voltage regulator
Power chords
3/8" cooling water tubing

Time: 2 hrs

Procedure

1) Inspect the white rubber gaskets that line the outer walls of the UBC.
   - These rubber gaskets seal the plates against the UBC and separate the electrophoresis buffer in the UBC from the buffer in the LBC. If a gasket is damaged or improperly seated in the UBC, buffer can leak around the gel plates and cause drainage of the UBC buffer reservoir or allow electric current to pass directly from the UBC to the LBC electrodes without passing through the gel.
2) Unscrew the black knobs on the sides of the UBC to loosen the black clamps that seal the gel plates against the UBC.
3) Seal the plate or plates against the sides of the UBC.
   - Slide the plate down between the black clamps and the UBC. The metallic plate with the notch in the top should face the inside. Make sure that the plates are centered and both of the lower edges are settled into the well cut into the lower section of the UBC. Position the clamps so that the notch between the thinner section of the clamp and the base of the clamp cradles the edge of the plates. Tighten the knobs until the white gasket between the plate and the UBC wall begins to compress and form a seal. It is very easy to break the clamps or crack the plates with too much pressure. Do not over tighten.

- In order to function, both sides of the UBC must be sealed. If two gels are not going to be run, one of the sides can be sealed with the thick glass plate that is supplied with the electrophoresis stand.

4) Place the UBC into the LBC so that openings in the slots of the LBC are facing the pegs extruding from the lower sides of the UBC. Slide the UBC pegs into the slots on the LBC.
5) Mix 100 mL of 5X electrophoresis buffer with 400 mL of DI water to make 1X electrophoresis buffer.
6) Add 1X electrophoresis buffer to the UBC.
   - Suggested: 200 mL
   - The buffer should reach to about 5 mm from the top of the glass slide while completely covering the opening to the gel created by the notch in the metal slide. If the comb used to create the wells has not yet been removed after pouring the plates, this opening in the gel will still be covered.
   - Inspect the seal between the UBC and gel plates to make sure that buffer is not leaking around the gasket and pooling in the LBC. If a leak exists, pour off the buffer, remove the gel plates and start over.
   - Foaming of the electrophoresis buffer can impede sample loading. If any bubbles have formed in the buffer, use a pipette to suck them off.
7) Add 1X electrophoresis buffer to the LBC to approximately 5 mm
   - Suggested: 200 mL
   - The buffer should reach to about 5 mm above the opening in the bottom of the gel plates.
   - If the electrophoresis buffer level drops during a run and contact is lost with the gel, more buffer can be added and the voltage potential reestablished
8) Remove the comb from the gel.
   - It can be difficult to view the sample well positions after the comb is removed. A marker can be used to outline the positions of the wells on the glass plate.
   - The comb should be removed slowly and carefully. A vacuum tends to form between the notches in the comb and the gel as they are separated from each other. If the comb is gently twisted and bent back and forth while being removed, buffer from the UBC is allowed to drain between the plates and the comb and fill the space that is created. After the comb has been removed, use a thin pipette tip to reposition any of the sample well walls that have been bent or displaced. If the gel of the wall cracks or tears, the two wells that it separates cannot be used for running samples.
9) Attach one end of a piece of 3/8" ID clear flexible lab tubing to the nozzle marked as "in" on the UBC. Attach the other end to the spigot on a cold water tap.
10) Attach another piece of tubing to the nozzle marked as "out" on the UBC and allow the outlet of the tubing to drain into a sink.
11) Open the water tap and allow cooling water to flow through the UBC.
   - The pressure and flow rate of the water does not need to be high. Water flow should be below 2 L/min
12) Load samples onto the gel.
   - Suggested: 10 to 20 μL per sample. Only 10 μL of the protein ladder is required.
   - Remember to include a protein ladder for comparison to known MW.

- Use a Hamilton syringe and rinse the syringe with buffer between each sample. Otherwise, use a 200 μL pipette and replace the pipette tip between each sample.
- Place the tip of the syringe or pipette against the back wall of the glass slide and above the opening to the well. Slowly add the sample so that it sinks through the buffer and into the well.
- Voltage must be applied immediately after the samples are added to keep the protein from diffusing out of the well.
- Load empty unused wells with 1X sample buffer to prevent the spreading of neighboring samples.

13) Set the safety lid onto the unit on so that power chords connect with the electrodes.
- Make sure that the colors of the electrodes and power chords align. If the voltage is reversed, the samples will be lost.

14) Plug the power chords into the corresponding electrodes on the voltage supply.
15) Turn on the voltage supply and set the desired voltage.
- Suggested: 170 V – 200 V
- When voltage is initially applied to the gel, the current transfer can be quite high. A common practice is to use a lower voltage than 170 V to pull the sample through the stacking gel. An 80V stacking gel voltage loads the protein into a single band at the top of the resolving gel and takes about 15 min. The current will drop and the voltage can be increased to its full running value.
- If the electrophoresis buffer level drops during a run and contact is lost with the gel, more buffer can be added and the voltage potential reestablished.
- The samples can be saved if a leak develops between the gasket and the gel plate after the samples have already entered the separating gel. Quickly pour off the buffers, dismantle the apparatus and reset the seals. Then, add the buffers back and reestablish the voltage

16) Maintain the voltage until the dye front reaches the bottom of the gel.
- Suggested: 90 minutes – 120 minutes

17) Turn off the power to the voltage supply.
- The gels need to be processed immediately after the voltage is removed or the protein bands will spread due to diffusion.

18) Unhook the voltage supply, remove the cover to the UBC, and dump the buffer.
19) Remove the plates or plates from the UBC.
- Immediately begin the staining procedure or the separated protein bands will become diffuse and difficult to analyze.

Gel Staining and Analysis

After the proteins are separated in the gel, they must be stained to be made visible. The dye that is often used is Coomassie Brilliant Blue which bonds specifically to certain amino acids in the protein. Other dyes can be used that are either much more specific to certain proteins or are more sensitive to lower concentrations. These stains are either more expensive or toxic, however.

The mixture of acid and methanol in the staining solution acts to shrink the gel and precipitate the protein. This keeps the protein from diffusing out of the gel and maintains the sharpness of the bands. The specific staining and destaining solution compositions

given in this protocol are not essential to the success of the protocol. In addition, a large number of different staining and destaining procedures can be used. The only firm rule is that the operator must not allow the gel to sit in pure water for extended periods of time. Pure water causes the gel to swell and release the protein.

Analysis of the gel is a subjective measurement. The visible intensity of the protein bands is proportional to the mass of the protein in the band. Certain results can make analysis difficult, however. If too much protein is included in the sample, the bands widen and run into each other. If the sample contains too little protein, it can be difficult to see the protein. In addition, large amounts of DNA or RNA in the sample or an especially large concentration of one specific protein size can distort or cause streaking in the gel lane. Multiple gels and sample concentrations might need to be run to get a good sense of the relative protein concentrations. If the expressed protein band is difficult to view due to the extraneous bands created by the other proteins in the sample, increasing the sample size does not help. It just causes the bands to become oversaturated. Purification procedures might be required to remove some of the extraneous protein and cellular components.

Supplies

Coomassie staining solution
Destaining solution
Razor
Glass staining container with cover
Water bottle
Plastic sheets or clear overhead slides

Time: 13 hours

Procedure

19) Separate the gel from between the plates.
    - Use gloves.
    - It is very easy to tear the gel, especially at low acrylimide concentrations. The gel will be more tightly bonded to the metal plate than to the glass. First, use a razor to separate the outer edges of the gel from the surface of the glass plate. Use the razor as a lever to very slowly pry the glass plate away from the gel. Then, gently separate the bottom of the gel from the bottom surface of the metal plate. Lay the bottom of the plate against the floor of the glass staining container so that the bottom of the gel can flop against the container. Slowly lift up on the plate so that gravity and surface tension will cause the gel to slide into the container.
20) Rinse the gel with a small quantity of water and pour off the water.
21) Add enough coomassie stain to the glass staining container to just cover the surface of the gel.
    - Suggested: 100 mL
    - Use gloves. The stain cannot be washed off.
22) Cover the container and place it on a rocker to allow the stain to set.
    - Suggested: 1 hr

- Staining times are not especially important. As long as the staining and solutions contains a large percentage of methanol, the protein bands will become fixed as the gel becomes dehydrated and shrinks. If the gel is allowed to sit in a high concentration of methanol for too long of a time, the gel might shrink too much.

23) Pour out the stain and rinse the gel with a small amount of RO water.
- Save the stain. It can be reused multiple times.

24) Add destaining solution to coat the gel and place it back on the rocker.
- Suggested: 100 mL; 12 hours
- The destaining solution has the same general composition as the staining solution. The low concentration of coomassie blue in the destaining solution acts to draw out the dye from the gel that has not bonded to the protein.
- The destaining process can be sped up by replacing the destaining solution at regular intervals.
- A folded paper towel placed in the destaining bath will soak up excess stain and allow the re-use of the destaining solution.
- The methanol concentration in the destaining solution can be reduced to 20% if the destaining process is causing the gel to shrink too much.

25) Dump out the destaining solution and wash with RO water.
- If the destaining solution does not have a high concentration of coomassie blue, it can be used as an initial destaining wash for another gel. Save it.

26) Cut two pieces of thin plastic laminating sheet or obtain two overhead slides.

27) Add a couple of drops of water to the fronts of both sheets.

28) Trap the gel between the two sheets.
- Make sure that air bubbles are not caught between the sheets and the gel. Air bubbles distort the images.
- If desired, the two sheets can be taped closed.

29) Pictures of the gel can be captured by a scanner and analyzed using a computer.

Stock Solutions

80% Glycerol (100 mL)
- 20 mL of RO water in 100 mL graduated cylinder
- Add glycerol to 100 mL by volume displacement

0.2 % Bromophenol Blue (100 mL)
- Dissolve 0.2 g of bromophenol blue in 5 mL of EtOH
- Add water to 100 mL

20% SDS (Sodium Dodecyl Sulfate) (50 mL)
- Stir 30 mL of water
- Add 20 g of SDS
- Add water to 50 mL

0.5 M Tris (pH= 6.8) (100 mL)
- Stir 60 mL of water
- Add 6.1 g of Tris base

- Adjust pH to 6.8 with 5M or concentrated HCl
- Add water to a final volume of 100 mL

Stacking Buffer (100 mL)
- 98 mL of 0.5 M Tris (pH 6.8)
- Add 2 mL of 20% SDS

Separating Buffer (100 mL)
- 98 mL of 1.5 M Tris (pH 8.8)
- Add 2 mL of 20% SDS

1.5 M Tris (pH=8.8)
- Stir 60 mL of water
- Add 18.2 g of tris base
- Adjust pH to 8.8 with 5M or concentrated HCl
- Add water to a final volume of 100 mL

2x Laemmli Sample Buffer (90 mL)
- Stir 20 mL 0.5 M Tris pH6.8
- Add 25 mL 80% Glycerol
- Add 48 g Urea
- Add 2 g SDS
- Add 1 mL 0.2% Bromophenol Blue
- Add water to 90 mL
- Separate into 9 mL aliquots and store at -20 °C

5x electrophoresis buffer (100 mL)
- Stir 80 mL of water
- Add 1.5 g Tris base
- Add 7.2 g glycine
- Add 0.5 g SDS
- Add water to 100 mL
- Confirm that pH is near 8.3 but do not adjust
- Store in a glass container

10% Ammonium Persulfate (10 mL)
- 10 mL of water
- Add 1 g of ammonium persulfate
- Store in at 4 °C

Butanol
- Mix water and butanol
- Allow to separate

Destaining Solution (100 mL)

- Add 40 mL methanol
- Add 10 mL acetic acid
- Add 50 mL water

Coomassie Blue Stain (100 mL)
- Stir 100 mL Destaining Solution
- Add 0.1 g Coomasie Brilliant Blue R250
- Filter the solution to remove undissolved dye
- The stain can be saved and reused

Phosphate Buffer Saline (PBS) Solution (100 mL)
- Stir 80 mL of water
- Add 0.26 g sodium phosphate monobasic
- Add 1.28 g sodium phosphate dibasic heptahydrate
- Add 0.7 g NaCl
- Confirm that pH is near 7.2.

APPENDIX B

PRESTO CONTROL MANUAL

APPENDIX B: TABLE OF CONTENTS

Introduction

Control System Overview

"Presto Control" is the name given to the controller and display programs designed using Opto 22 that allow knowledge based (KB) control of the BioFlo IIc. Opto 22 is a set of software programs and processing units designed for industrial automation applications. Due to the limited control capabilities built into the BioFlo, Opto 22 has been used to add functionality to the bioreactor system. Presto Control utilizes a number of interconnected components to monitor the bioreactor environment and implement control schemes. These components are the OptoDisplay program, the OptoControl program, the control unit, the recipe data file, the individual I/O modules, and the BioFlo unit.

OptoDisplay

OptoDisplay is the visual interface program that allows the operator to interact with the control unit. It displays the monitored fermentation variables and transmits the values that the operator enters into the computer. The computer and OptoDisplay, however, do not actually make any process calculations or controller decisions. In fact, the control unit can control the bioreactor and implement a control scheme without being connected to the computer. The majority of this manual is dedicated to describing the graphical interface that is governed by the OptoDisplay program.

OptoControl

OptoControl is a computer program that is used to design control strategies. It uses a graphical programming interface to represent multiple command loops running in parallel. The program also includes built in control processes such as PID calculations and timers. After an OptoControl strategy is created and compiled, it must be downloaded into the control unit before it can be run.

Control Unit

The control unit is a stand alone processor that implements the control strategy created using OptoControl. It calculates all of the process values and sends and receives signals from the computer and the I/O modules.

Recipe

All of the parameters that define the stages of a control scheme in Presto Control are represented as tables of values within the OptoControl strategy. For example, a table titled "Stage_Time_Trigger_t" holds a list of 1's and 0's that signifies for the controller whether the time trigger is active for the corresponding stage. A feature of Opto22 is its ability to use text files called recipes to store data for table variables in control strategies. Presto Control utilizes these recipes to save a control scheme so that it can be used in future fermentations by the operator.

I/O Modules

The output from the probes that monitor the bioreactor conditions are analog signals. The I/O modules convert the analog signals to digital signals that the control unit can

read. The I/O modules also convert the digital signals that the control unit sends to the BioFlo to analog. The calibration of the signals is a function of how the variables are defined in the OptoControl strategy.

BioFlo Unit

A description of the BioFlo IIc can be found in the Protocols for Protein Expression. The BioFlo controls the agitation, temperature, and pH in the bioreactor and transmits data on those parameters to the control unit. The control unit handles air flow and feed rate using its own processes and individual control modules.

Bioreactor Control Overview

The goal of fermentation control is to establish environmental conditions within the bioreactor that optimize cell growth and/or product accumulation. The optimum environment can vary greatly from one project to the next and it depends on factors such as the host organism, the type of product being produced, and the media and expression system being employed. Also, the amount of knowledge that an operator has of the bioreactor environment is limited by the probes available for monitoring it. Less information results in fewer variables available for modeling and control of the cellular state. Therefore, a large number of possible control schemes exist. Advanced methods include the use of artificial neural networks (ANN), physiological state (PS) variables, and modeling of the enzymatic pathway fluxes. Presto Control does not utilize any direct mathematical modeling. Instead, it relies on the operator's expectations of how a culture should react to changes in the control parameters. These expectations are used to set up a knowledge based (KB) control scheme.

Advanced modeling of a bioreactor requires a wide variety of probes. For example, a flow through spectrophotometer can be used to calculate cell growth, a glucose analyzer can monitor substrate consumption, and off gas analysis of CO_2 production can indicate the respiration rate. In order to reduce the cost and complexity of the system, Presto Control employs a relatively small number of probes to measure environmental conditions. The monitored variables are temperature, pH, and dissolved oxygen concentration (DO).

Most fermentations are run at a constant temperature and constant pH. The BioFlo controls the temperature through the use of a water jacket and the pH through the use of acid and base pumps. They are neither controlled nor monitored by Presto Control. If a pH or temperature change needs to be made to increase protein production, the adjustments must be manually implemented by the operator.

The primary environmental condition monitored by Presto Control is the DO. Even without other information such as substrate concentration, the DO can be used to determine growth rate, substrate consumption and depletion, and cell concentration. The fermentation parameters that Presto Control can use to control the DO are the agitation rate, the air flow rate, and the feed rate. Most control schemes are designed to keep the oxygen concentration constant throughout the growth of the culture because anoxic conditions can irreversibly harm the growth of certain host organisms such as E. coli.

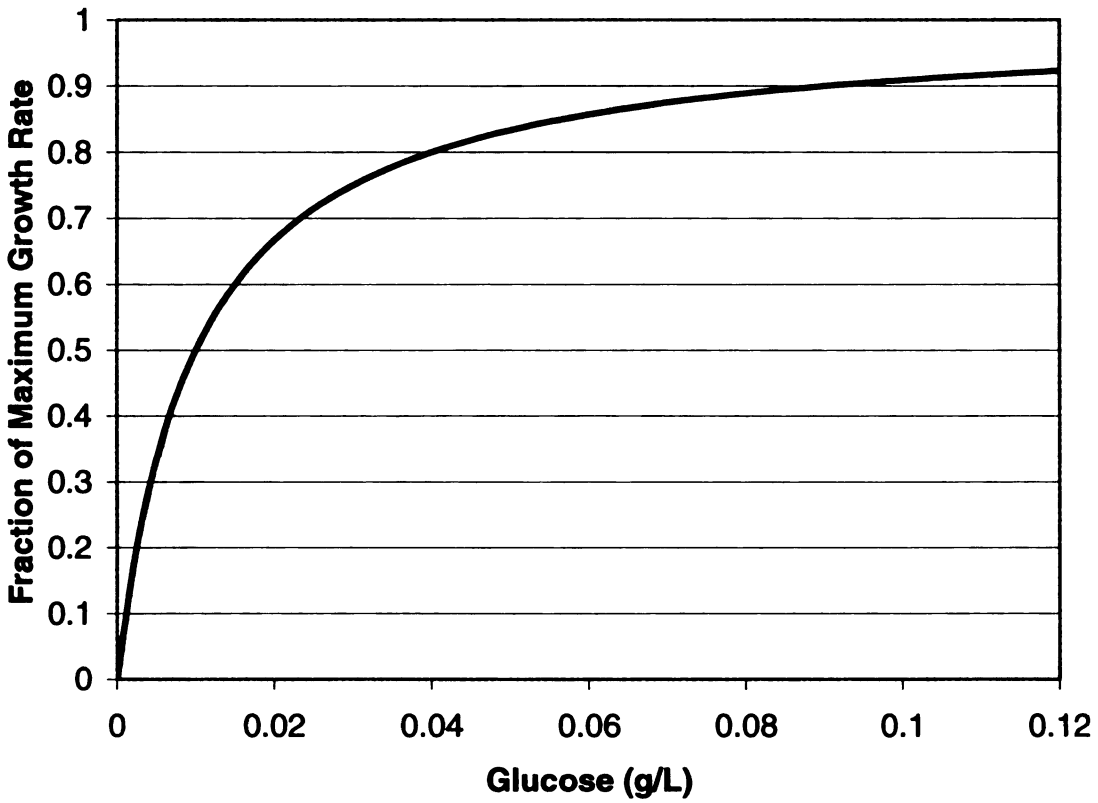
During exponential growth, the rate of increase in cell mass can be defined by the Monod Equation:

$$\frac{dX}{dt} = \mu_{\max} \times \frac{S}{K_S + S} \times X \qquad (1)$$

Where X is the cell concentration, μ_{max} is the maximum specific growth rate, S is the substrate concentration, K_S is the kinetic constant for the substrate, and t is the time. The value of μ_{max} is heavily dependent on temperature, pH, media composition, and the host

strain. Note that the model does not take into account volume increases due to feed addition during fed batch growth. Also, the growth rate can be inhibited by low oxygen concentrations, but the relationship is much more complex than the standard Monod model can accurately predict. A measured DO of 10% in a well mixed lab-scale bioreactor can assure that no portion of the cell culture is starved of oxygen. A graphical representation of how the growth rate varies with the substrate concentration when K_s equals 0.001 g/L (kinetic constant for E. coli growing on glucose) can be seen in Figure 11.

Figure 11: Glucose Limited Growth



Except in specialized cases, neither the oxygen nor the substrate concentrations should be growth rate limiting. The cellular uptake mechanisms for both of the molecules should be saturated to produce optimum growth kinetics.

Oxygen is consumed by the cell culture as a result of cellular growth, cellular maintenance, and protein production. The accumulation of oxygen in the bioreactor is equal to the difference between the rate of oxygen transport and the consumption of oxygen by the culture. It can be mathematically represented by:

$$\frac{dO}{dt} = k_L a(O^* - O) - q_O \qquad (2)$$

Where $k_L a$ is the mass transfer coefficient of the sparged air, O^* is the concentration of oxygen in the media in equilibrium with the sparged air, O is the concentration of oxygen in the vessel, and q_O is the rate of consumption of oxygen by the cells per unit volume.

Equation 2 assumes that the system is well mixed and the concentration of oxygen in the sparged gas does not decrease as the bubble travels through the bioreactor. This assumption is acceptable for lab-scale fermentations.

A simple representation of q_O is:

$$q_O = \frac{1}{Y_{X/O}} \frac{dX}{dt} + m_O X \tag{3}$$

Where $Y_{X/O}$ is the cell/oxygen yield coefficient for growth, and m_O is the rate of oxygen consumption by the cells for maintenance. Combining Equations 1 and 3 produces:

$$q_O = \left(\frac{1}{Y_{X/O}} \mu_{max} \times \frac{S}{K_S + S} + m_O \right) X \tag{4}$$

Equation 4 demonstrates that the rate of oxygen consumption is proportional to the cell concentration during exponential growth. Therefore, in order to maintain a constant oxygen concentration within the bioreactor, the rate of oxygen transport, k_La (O^*-O) will have to increase in relation to the cell concentration. The oxygen concentration gradient, (O^*-O), can be increased by increasing the oxygen concentration in the sparged gas. Presto Control cannot control the composition of the gas. Therefore, the area available for oxygen mass transfer must be increased, and Presto Control has two methods for influencing the value of k_La. One is to increase the volume of air entering the bioreactor by increasing the air flow rate. The other method is to increase the agitation rate, which acts to break up the sparged air into smaller bubbles and increase the overall surface area. Increasing the agitation rate also increases the rate of mixing which helps to maintain a consistent oxygen concentration throughout the bioreactor. A common practice in fermentation control is to first increase the agitation to a maximum value and then ramp up the air flow rate.

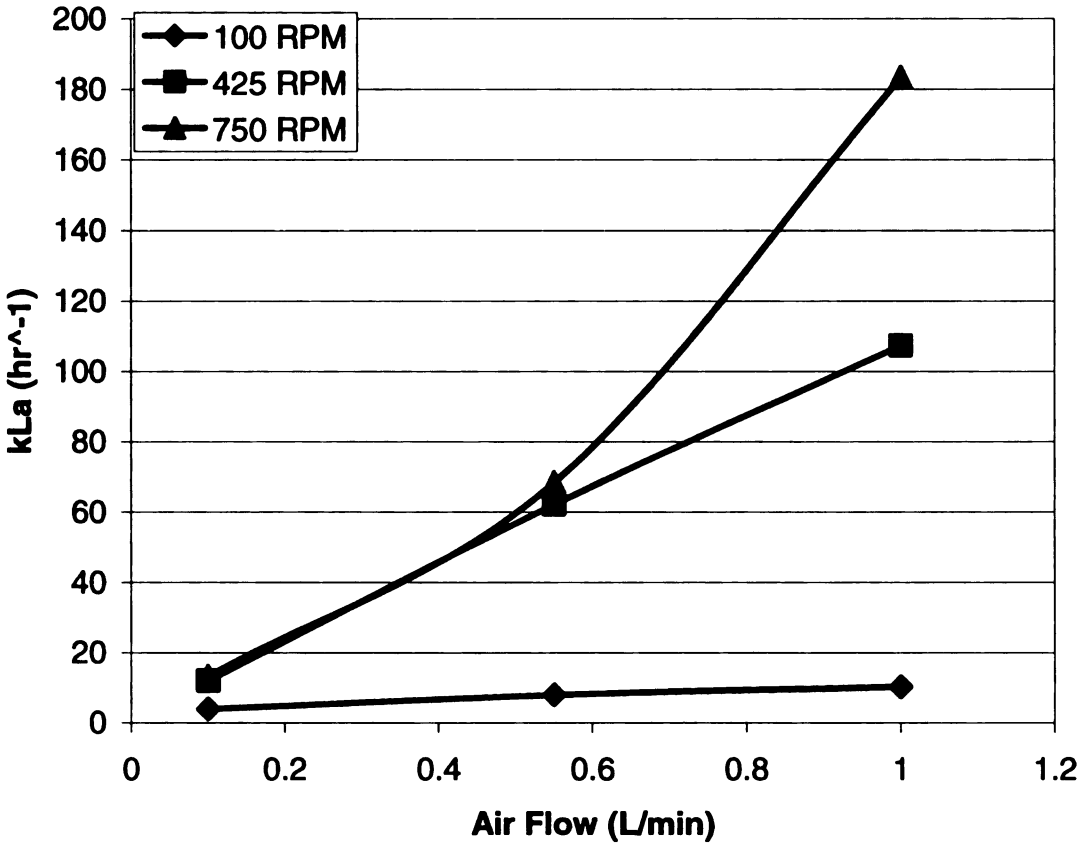
Figure 12 is a graph of k_La for the BioFlo for various air flow and agitation rates. The values were determined based on experimental data and the experimental protocol outlined in the Experiment Manual for the Biochemical Engineering Laboratory (Worden et al., 2002). Equation 2 can be integrated to obtain:

$$\ln\left(\frac{O^* - O}{O^* - O_0} \right) = k_L a \times t \tag{5}$$

Where O_0 is the measured oxygen concentration at time equals 0. Equation 5 is obtained by assuming no oxygen consumption and constant O^*. The latter assumption is valid when the rate of oxygen transfer is small compared to the response of the DO probe and the composition of the air bubbles in the bioreactor is uniform. Mass transfer coefficients were obtained by forcing step changes in the composition of the sparged gas and using least squares analysis to fit the resulting changes in the measured oxygen concentration to Equation 5. In order to allow the assumption of a constant O^* to be valid, only the data obtained as the concentration approached steady state were used. O^* was then assumed to be the equilibrium solubility of oxygen in contact with the inlet gas stream. Bioreactor conditions were as described in the BioFlo Protocol. The bioreactor contained 1 L of LB media with 0.05 mL of added antifoam agent. Agitation was created by two six spoke, flat bladed impellers with a diameter of 2 1/8". The lower impeller was placed just above

the sparging ring at the end of the impeller shaft. The second impeller was completely submerged just below the surface of the liquid. The measured k_La values were very much dependent on these conditions. Increasing the media volume or changing the positions of the impeller could increase the maximum k_La rates.

Figure 12: kLa for the BioFlo IIc



The maximum agitation and air flow rates are limited by the foaming and frothing they create. If either is too great, the broth can be forced out the seals in the head plate or into the air filters. This may cause pressure to build up or contamination to occur. In addition, shear forces from bubble coalescence and agitation can kill certain cell types. Therefore, the respiration rate of the culture is limited by the oxygen transfer restrictions as indicated by the maximum k_La of the bioreactor. When the airflow and agitation have reached their maximum rates, the rate of oxygen consumption by the cells needs to be limited so that the cells can continue to grow in an aerobic state. This is done by reducing the growth rate of the cells. According to Equation 1, this can be accomplished by limiting the substrate concentration in the bioreactor until the cells are essentially starved of food and can no longer grow at their maximum specific growth rate. In most microbes, the stress response to oxygen starvation is much more harmful than the response to substrate depletion.

Combining Equations 2 and 4 produces:

$$\frac{dO}{dt} = k_L a(O^* - O) - \left(\frac{1}{Y_{X/O}} \mu_{max} \times \frac{S}{K_S + S} + m_O\right) X \quad (6)$$

During substrate limited growth, the DO can be maintained by control of the feed rate. Substrate accumulates in the bioreactor at a rate that is equal to the difference between its feed rate and it consumption. Its representative Equation is:

$$\frac{dS}{dt} = D_S - q_S \quad (7)$$

Where D_S is the feed rate of the substrate per unit volume of the culture, and q_S is the rate of substrate consumption by the cells per unit volume of the culture. If the controller needs to maintain a steady, growth limiting concentration of substrate, then the feed rate must equal the rate of feed consumption. When the dissolved oxygen increases above its desired value, the feed rate is increased in order to increase the substrate concentration and allow a higher rate of cell growth. When the DO drops below its set-point, the feed rate is decreased in order to limit growth.

A simple representation of q_S is:

$$q_S = \frac{1}{Y_{X/S}} \frac{dX}{dt} + m_S X \quad (8)$$

Where $Y_{X/S}$ is the cell/substrate yield coefficient, and m_S is the rate of substrate consumption by the cells for maintenance. As with oxygen consumption, combining Equations 1 and 8 produces:

$$q_S = \left(\frac{1}{Y_{X/S}} \mu_{max} \times \frac{S}{K_S + S} + m_S\right) X \quad (9)$$

As with the respiration rate, the overall rate of substrate consumption is proportional to the cell concentration.

It is often assumed that the rate of oxygen consumption is proportional to the rate of substrate consumption during exponential growth. Oxygen and substrate consumption are linked by their yield coefficients and the molecular composition of the cell. Overall yield coefficients, $Y'_{X/S}$ and $Y'_{X/O}$, can be obtained when the individual yield coefficients and maintenance requirements of the cell are constant during exponential growth.

$$\frac{1}{Y'_{X/S}} = \left(\frac{1}{Y_{X/S}} \mu_{max} \times \frac{S}{K_S + S} + m_S\right) \quad (10)$$

$$\frac{1}{Y'_{X/O}} = \left(\frac{1}{Y_{X/O}} \mu_{max} \times \frac{S}{K_S + S} + m_O\right) \quad (11)$$

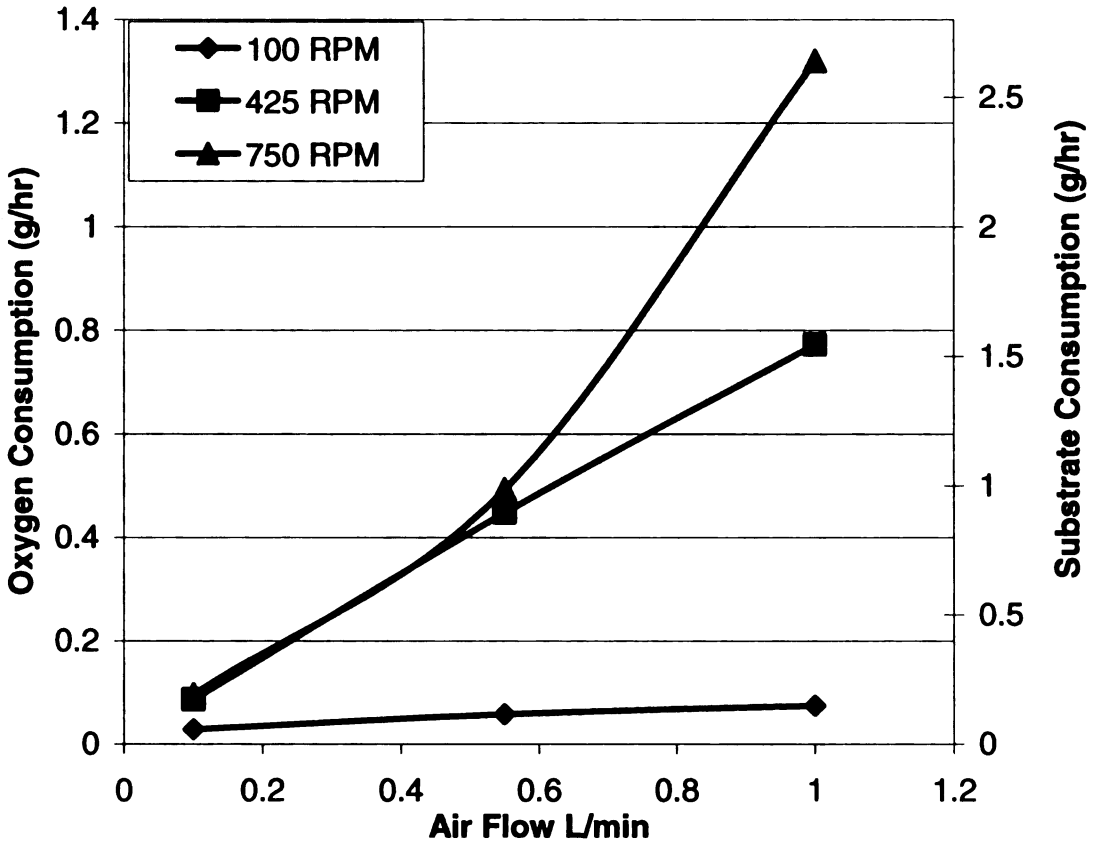
Substituting Equation 10 into Equation 9 and Equation 11 into Equation 4 and dividing the rate of substrate consumption by the rate of oxygen consumption, Equation 12 is obtained.

$$\frac{q_S}{q_O} = \frac{Y'_{X/O}}{Y'_{X/O}} = Y'_{S/O} \quad (12)$$

Where $Y'_{S/O}$ is the mass of substrate consumed per mass of oxygen consumed by the culture. Assuming a value of 0.5 g/g for $Y'_{X/S}$ and a value of 1 g/g for $Y'_{O/S}$ and a maintained DO of 10% of saturated air in the bioreactor, the rate of glucose and air

consumption for various air flow and agitation rates can be obtained by comparison to the graph of k_La values. Figure 13 shows a graph of the expected consumption rates. This knowledge can be useful in designing control schemes for bioreactor control.

Figure 13: Mass Transfer and Consumption in the BioFlo IIc



The above description is a simplified model of cell growth and metabolism. The exponential growth patterns described by Monod kinetics cannot account for lag phases, steady state growth, or inhibition by products or media components. Also, the yield coefficients and maintenance needs vary depending on the metabolic state of the culture. The main points to remember are that, in the absence of inhibitor, oxygen transfer in the bioreactor is the limiting factor in high cell density culture growth, and the rate of oxygen consumption is dependent on the substrate concentration during substrate limited growth.

In recombinant engineering, product expression in the cell is often designed to be induced by a change in the environmental conditions in the bioreactor. For example, induction could be triggered by the addition of a media component such as IPTG, a change in temperature, or a change in pH. Rather than maximizing cell growth, the goal of the bioreactor controller during induction is to maximize product expression. For example, the culture may need to be kept in oxygen starved conditions if the product is sensitive DO levels. Therefore, the control scheme will need to consist of two stages. One stage will be active during culture growth while the other stage will be initiated at the time of induction.

Recipes

Presto Control is fundamentally a simplified programming tool built on the framework of the Opto 22 software. Whereas the OptoControl software offers a generic method of programming a controller, Presto Control limits the variables available for making controller decisions to just those parameters that characterize a fermentation. These variables are the dissolved oxygen, the agitation rate, the air flow rate, the feed rate, the age of the fermentation, and the amount of feed added to the bioreactor. When a specific value of one of these variables is reached, a controller action is triggered. This action is the result of a new stage being called into operation that is defined by different control parameters and trigger values. Therefore, the control scheme must be broken up into specific stages in order to program it into Presto Control. Every controller action requires its own stage. For example, the control scheme may call for PID control of DO to be transferred from agitation to the air flow rate when the agitation reaches 750 RPM. In Presto Control, this is accomplished by first defining a stage in which DO is controlled by agitation and the stage's trigger is an RPM greater than 750. The first stage's trigger calls a second stage in which DO is controlled by air flow rate. Since two sets of triggers exist, the controller has a choice of two paths leading out of a stage. Each of these paths can also branch out, thereby creating a virtually limitless number of choices and control schemes.

Seven example recipes can be directly loaded from the *Recipe Loader Menu* of the *Fermentation Setup Window*. These recipes were developed in order to allow the operator to test the ability of different feed control schemes to deal with each expression system's individual requirements. Due to their complexity and unreliability, some of these schemes have more of a conceptual rather than practical value. The recipe parameters may also need to be customized depending on the growth characteristics or induction strategy of the strain.

Each of the predefined recipes is an adaptation of a control strategy that has been used by other researchers to stop the accumulation of inhibitory concentrations of fermentation byproducts. Other than the toxic effects of induction and recombinant protein expression, the most commonly reported inhibitor of high cell density culture growth in bioreactors are the cellular byproducts lactic and acetic acid that are formed via anaerobic metabolic pathways. The culture creates these byproducts when it consumes more substrate than it can fully oxygenate in the respiratory cycle. Various models for this phenomenon have been proposed, but the environmental indicators are a relatively high cell growth rate, and saturated substrate and oxygen uptake rates.

Culture growth can also be inhibited by the stringent response of the cells due to prolonged starvation. A very brief substrate depletion event, as indicated by a quick spike in the DO due to a reduction in the respiration rate of the cells, is not overly harmful, however. The goal of many control schemes is to keep the feed below the uptake saturation concentration while also keeping the culture from experiencing starvation for long periods of time.

These control schemes all have some characteristics in common. All assume a working volume of 1 L. Also, the required initial feed mass is supplied in the first stage of the control scheme so substrate does not need to be added to the media during bioreactor preparation. In all but the "Maximum DO Control" recipe, this first stage also waits for the oxygen uptake rate of the culture to meet the minimum oxygen transfer rate of the bioreactor. Then, once the dissolved oxygen has dropped to its set-point value, the agitation rate is allowed to ramp up to its maximum value before the air flow ramp is initiated. If both the agitation and the air flow reach their maximum values, the dissolved oxygen is maintained by PID control of feed with checks to determine that excessive feeding does not occur.

Feed depletion in the media is assumed to have occurred when the DO increases a specified amount above its current value or set-point. In most recipes, the feed depletion event is confirmed if the DO drops in response to the feeding of substrate into the bioreactor. If feed does not cause a response, a preset, open loop feed control scheme is used to make sure that the culture does not suffer from starvation before the dissolved oxygen returns to its set-point.

Each recipe description includes the reference from which the control scheme was devised. In addition, a table lists the major features of the recipe and some subjective judgments of its reliability and robustness:

- Dissolved Oxygen Set-Point: The DO set-point can be changed in the first stage and it will carry through the entire recipe for the PID control, but the trigger values in each stage for the detection of feed depletion will also need to be adjusted
- Air Flow Fate Range: Describes the minimum and maximum air flows spanned before DO control is transferred to PID control by feed.
- Agitation Range: The values that need to be input into the BioFlo.
- Initial Feed Concentration: The amount of feed added in the first stage. It can be adjusted with little affect on the performance of the control recipe.
- Momentary Feed Depletion: The expected number of DO spikes caused by feed starvation that will be allowed in the recipe per hour.
- Chance of Excessive Feed: The likelihood that the scheme will fail and dump in more feed than intended by the design of the recipe.
- Chance of Prolonged Starvation: The likelihood that the control scheme will fail and allow the culture to starve for extended periods of time without adding feed.
- Growth Rate Limitations by Feed: The lower the substrate concentration is below its saturation level, the slower the culture will grow.
- Average Feed Concentration: A comparison of how strict the recipe is in maintaining low substrate concentrations relative to the other recipes.
- Reliability of Feed Scheme: Subjective measurement of how well the scheme will function without adaptation or constant monitoring by the operator.

Standard Fed Batch Control

Reference: Knop, 2002

This is an adaptation of a control strategy created by David Knop for growing high cell density cultures in the BioFlo. This type of staged control scheme is common for controlling laboratory scale batch fermentations. A large feed mass is added initially with the intention of sustaining culture growth until both the agitation and the airflow have ramped to their maximum values and DO control by feed rate is initiated. If feed depletion occurs before this point, a measured quantity of feed is added to support growth to the desired density without overshooting. This scheme allows the least chance of feed depletion or starvation during the exponential growth phase.

Table 4: Standard Fed Batch Control

Dissolved Oxygen Set-Point (%):	10
Air Flow Fate Range (L/min):	0.1 – 1.0
Agitation Range (RPM):	50- 750
Initial Feed Concentration (g/L):	10
Momentary Feed Depletion (events/hr):	0
Chance of Excessive Feed:	very low
Chance of Prolonged Starvation:	low
Growth Rate Limitations by Feed:	none
Average Feed Concentration:	very high
Reliability of Feed Scheme:	very high

The following is a walk through of the recipe that defines the *Standard Fed Batch* control scheme. This discussion and the included recipe maps give a better understanding of some of the features of recipe creation in Presto Control.

Stage 1: DO Drop

Control: This stage sets up the initial environmental conditions of the bioreactor. The set-point for the DO is specified as 10% and this value is carried over throughout all of the rest of the stages. In addition, the air flow rate is set to 0.1 L/min and held constant until PID control is specified in stage 5. Stage 1 also adds the initial feed charge of 10 g with the feed pump set to its maximum rate. This mass can be modified without seriously affecting the control scheme.

Trigger A (Stage 2): When the bioreactor is first inoculated, the culture usually cannot consume enough oxygen to cause the dissolved oxygen concentration to drop below its set-point of 10%. At the minimum air flow and agitation rates of the bioreactor, the mass transfer rate of oxygen at its set-point concentration is greater than the culture's oxygen uptake rate. Subsequent stages in the control scheme detect a feed depletion event by the DO rising above 15%. Therefore, trigger A does not call the next stage until the DO has dropped below the set-point. An additional constraint of trigger A is that the feed rate must drop below 0.001 g/hr. This indicates that the initial 30 g of feed has been added and the feed pump has shut off. Trigger A is also set to wait for a fermentation time of 5 hours before activating. The stage called by the trigger checks for both feed depletion and a maximum agitation rate, neither of which is likely to occur within five hours of inoculation. The *Fermentation Time Trigger* keeps the controller from responding to a false feed depletion event, but it can be turned off without affecting the basic operation of the control scheme. When all of these trigger values are met, trigger A calls stage 2.

Trigger B (Stage 14): Trigger B calls stage 14 after 24 hours of fermentation time. This trigger can only be met if the DO does not drop below 10% within a day of inoculation. The only likely reasons for this to happen are culture death or a malfunctioning of the DO probe. In case of a DO probe malfunction, the called stage adds feed at a preset rate in order to keep the culture from starving. The time value can be reduced depending on the operator's expectations of culture growth.

Stage 2: RPM Ramp

Control: This stage is intended to allow the agitation to ramp up to its maximum value of 750 RPM before the air flow ramp is enacted. The air flow rate is again set to 0.1 L/min because stage 4 calls this stage if the air flow drops too low during *Air PID Control.* Feed control is set to *Full On* even though no mass of feed is directly specified. When this stage is called by stage 1, the feed control immediately sets itself to *Full Off* since the feed mass is 0. Stage 10, however, also calls stage 2, and the control scheme has been designed to allow stage 2 to finish feeding the feed mass specified by stage 10.
Trigger A (Stage 3): When the BioFlo has ramped up the agitation rate past 750 RPM, stage 3 is called so that PID control by the air flow rate can be initiated.
Trigger B (Stage 10): Feed depletion is indicated by a spike in the DO. The culture stops growing and consuming oxygen when the available substrate is completely consumed. The result is such a quick accumulation of oxygen in the broth that the controller cannot decrease the agitation rate quick enough to keep the DO from increasing at least 5% above its set-point. Trigger B is used to detect this feed depletion event. When the DO increases above 15%, stage 10 is called to add more feed to the bioreactor.

Stage 3: RPM Check

Control: A malfunction in the impeller motor might cause the agitation to accidentally spike above 750 RPM and activate the triggering of this stage by stage 2. Stage 3 attempts to detect this type of problem before PID control by air flow is initiated in the subsequent stages.
Trigger A (Stage 4): After 30 seconds with an agitation rate greater 750 RPM, it is assumed that the agitation has ramped up to its maximum value due to oxygen consumption by the culture. Trigger A calls stage 4, which initiates the air flow ramp.
Trigger B (Stage 2): If, within the 30 seconds allowed by Trigger A, the agitation drops back below 750 RPM, Trigger B sends the control scheme back to stage 2.

Stage 4: Air Ramp

Control: Stage 4 is designed to allow the PID controller to ramp up the air flow rate to its maximum value. As in stage 2, the feed control is set to *Full On.*
Trigger A (Stage 5): As in stage 2, an increase in the DO above 15% signals that all of the feed has been consumed. This event triggers stage 5.
Trigger B (Stage 2): In case the oxygen uptake rate of the culture substantially decreases during stage 3, trigger B stops the PID controller from forcing the air flow to drop below its minimum value. The trigger sends control back to stage 2 where the air flow rate is reset to 0.1 L/min. This trigger assumes that control of agitation by DO set-point is still

active on the BioFlo. If desired, Trigger B can be inactivated without harming the control scheme.

Stage 5: Feed Depletion

Control: This stage begins to feed substrate into the bioreactor in response to the detection of a feed depletion event by stage 4. It also detects whether the air flow has reached its maximum rate and determines whether PID control by feed should be attempted. This check of the air flow rate is only done after the detection of a feed depletion event because PID control by feed is ineffective if the culture is not substrate limited.
Trigger A (Stage 6): After 5 s with the air flow rate being maintained above 1 L/min, stage 6 is called to transfer PID control by the DO from air flow to feed rate.
Trigger B (Stage 15): If the air flow rate is below 1 L/min, a series of stages is called that determines how much feed should be added to allow the culture to grow until the maximum air flow rate is reached. The first stage of this series is stage 14.

Stage 6: PID check

Control: If PID control by feed rate is attempted too soon in a fermentation, a glucose dump might occur. Stage 6 determines whether PID control by feed has been initiated before the culture has become substrate limited. The stage drops the air flow rate down to 1 L/min and begins feeding 1 g of substrate at an initial rate of 8 g/hr by PID control.
Trigger A (Stage 29): This trigger is met if PID control by feed is not effective in bringing the DO back down to a value below its set point. This would occur if the DO spike in stage 4 was not caused by feed depletion and the bioreactor has not yet reached its maximum oxygen transfer capacity. A gram of feed is allowed to be added before stage 29 is called. The triggering event is a drop in the feed rate below 0.01 g/hr due to the feed control switching to *Full Off*.
Trigger B (Stage 30): If PID control by feed causes the DO to drop below its set-point, the culture is substrate limited. Stage 30 is called to establish the required PID controlled feed rate to support cell growth.

Stage 10: Start Feed

Control: This stage begins feeding substrate at the maximum rate in response to the feed depletion event detected in stage 2. The stage specifies that 10 g should be added to supplement growth. This mass can be changed by the operator depending on the needs of the culture and the goal of the fermentation.
Trigger A (Stage 2): If the addition of the feed mass causes the DO to drop back below 14% within 30 seconds of the triggering of the stage, the assumption built into the control scheme is that the DO spike was a result of a feed depletion event. Therefore, stage 2 is called and the rest of the feed mass is added.
Trigger B (Stage 11): The DO might rise above 15 % in stage 2 due to failure of the DO probe or an accidental spike in the air flow or agitation. Therefore, if the feed pulse is ineffective in reducing the DO, trigger B calls stage 11 in order to halt the addition of feed. Stage 11 is also called if more than 41 g of feed has been added to the fermentation. Significant amounts of feed should not be required to support the culture during the RPM ramp portion of the control scheme. The addition of over 41 g indicates

that the DO might be fluctuating due to controller oscillations or malfunctioning equipment. The specified trigger mass can be changed according to the needs of the operator.

Stage 11: Wait Response

Control: After it is determined that the DO has not spiked above 15% due to feed depletion, stage 11 stops the feed and removes the excess mass specified in stage 10. This keeps stage 2 from adding the rest of the feed when it is ultimately called. Stage 12 also calls stage 11 to have its *Maximum Feed Mass* reset. Stage 11 waits for the DO to return to normal as the bioreactor adapts to whatever caused the DO spike.
Trigger A (Stage 2): When the DO drops below 14%, this trigger calls stage 2 so that normal control operations can be reinstated.
Trigger B (Stage 12): If, after 5 minutes, the DO still has not decreased, it is assumed that the control system has failed and stage 12 is called in order to implement a safety procedure.

Stage 12: Safety

Control: This stage is intended to keep the culture from starving until the control system is corrected by the operator. Feed is added at a constant rate of 4 g/hr. The air flow rate is specified to be 0.1 L/min in order to return the air flow to this minimum value when stage 12 is called by stage 22.
Trigger A (Stage 11): Control is returned to stage 11 and then stage 2 when the DO returns to the expected value.
Trigger B (No Stage): Manual hold.

Stage 15: 0.8 – 1 Flow

Control: Stages 15 through 19 are used to find the current air flow rate and determine the correct quantity of feed to add to support growth until PID control by feed is initiated. Each air flow rate range (i.e. 0.8 to 1.0 L/min) allows a different amount of feed to be added to the bioreactor. The specified mass can be changed by the operator depending on the expectations of the cultures growth rate and oxygen uptake rate. If the correct air flow rate range is represented by the stage, a time delay is allowed before the next stage is automatically called in order to determine if the DO responds to the addition of substrate. Stage 15 allows 1 g of feed to be added.
Trigger A (Stage 20): Trigger A is the same for stages 15 through 19. It gives the DO 30 seconds to drop below 14.9% in response to the addition of the feed. Stage 20 is then called to determine how the culture responded to the feed pulse.
Trigger B (Stage 16): If the air flow rate is not between 0.8 to 1.0 L/min, stage 16 is called in order to find the correct amount of feed to add.

Stage 16: 0.6 – 0.8 Flow

Control: The function is the same as stage 15 except that stage 16 adds an additional 2 g of feed over the previous stage.
Trigger A (Stage 20): Same as stage 15.
Trigger B (Stage 17): This trigger calls stage 17 to check for air flow rates below 0.6 L/min.

Stage 17: 0.4 – 0.6 Flow

Control: The function is the same as stage 15 except that stage 16 adds an additional 2 g of feed over the previous stage.
Trigger A (Stage 20): Same as stage 15.
Trigger B (Stage 18): This trigger calls stage 18 to check for air flow rates below 0.4 L/min.

Stage 18: 0.2 – 0.4 Flow

Control: The function is the same as stage 15 except that stage 16 adds an additional 2 g of feed over the previous stage.
Trigger A (Stage 20): Same as stage 15.
Trigger B (Stage 19): This trigger calls stage 19 if the air flow rate is below 0.2 L/min.

Stage 19: 0.1 – 0.2 L/min

Control: Stage 19 is the same as the rest of the stages in the series except that it allows PID control by air flow. Also, Trigger B is modified.
Trigger A (Stage 20): Same as stage 16.
Trigger B (Stage 10): Since 0.1 L/min is the lowest allowable air flow rate, this trigger sends the control scheme back to PID control by agitation if the air flow rate drops below 0.1 L/min. Stage 10 is called initially in order to allow the control scheme to respond to the DO spike.

Stage 20: Response Check

Control: Stage 20 is called by all of the DO spike response stages to determine how the DO has responded to the addition of feed. The stage checks to see whether the DO is dropping as quickly as would be expected if the feed had become depleted. If the DO is oscillating with a slow period in response to poor PID tuning, this stage keeps excessive feeding from occurring. A check of the total feed added to the bioreactor is also made.
Trigger A (Stage 21): Trigger A gives the DO 10 seconds to drop below 14% before calling stage 21, which cuts off feed. Stage 21 is also automatically called if 60 grams of feed has already been added during the fermentation. The reasoning for this controller response is that the respiration rate of the culture should have exceeded the maximum oxygen transfer rate of the bioreactor by the time 60 grams of feed has been consumed. This mass value can be changed according to the needs of the fermentation.
Trigger B (Stage 4): If the DO drops an additional 0.9% from the calling stage, trigger B calls stage 4 and allows the rest of the feed mass to be added during normal ramping of the air flow rate.

Stage 21: Wait Response

Control: This stage has the same function as stage 11 of the agitation ramp portion of the control scheme. It waits for the DO to approach it set-point value without adding additional feed.
Trigger A (Stage 22): If it takes over five minutes for the DO to return to normal, stage 22 is triggered in order to implement the safety response. This safety response is also called if the DO increases by 5%, indicating that another DO spike has occurred due to

either feed depletion or continuing failure of the control scheme. Another trigger for the safety stage is a drop in the air flow to value below its minimum rate of 0.1 L/min.
Trigger B (Stage 4): When the DO drops back to its set-point, this trigger calls stage 4 to return to normal ramping of the air flow rate.

Stage 22: Safety

Control: Stage 22 has the same purpose as stage 12. It feeds substrate at 8 g/hr to keep the culture from starving. If required, it also calls stage 12 in order to keep the PID controller from starving the cells of air.
Trigger A (Stage 29): When the DO has regained its expected value, stage 29 is called to reset the control scheme.
Trigger B (Stage 12): Stage 12 is called if the high DO that led to the triggering of stage 22 causes the PID controller to drop the air flow rate below its minimum value of 0.1 L/min. Stage 12 reestablishes an air flow rate of 0.1 L/min while waiting for the DO to return to its set point.

Stage 29: Reset

Control: This stage is called for the sole purpose of removing the *Maximum Feed* values specified by stages 22, 29, and 30.
Trigger A (Stage 4): The stage is given 1 s to complete its task before stage 4 is called. The time lag specified by the trigger is not actually required for all of the stage parameters to be loaded.
Trigger B (No Stage): Manual hold.

Stage 30: PID SS

Control: The maximum oxygen uptake rate of the culture must be significantly higher than the maximum oxygen transfer capacity of the bioreactor before PID control by the air flow rate is discontinued. Otherwise, the DO might rise above its set-point irrespective of the feed rate and a glucose dump can occur. Stage 30 is meant to make sure that PID control by feed rate is necessary for keeping the culture from consuming too much oxygen. After the DO drops below 10% in stage 6, PID control by feed is maintained in stage 30 until a gram of feed has been added. At the end of this period, it is expected that the controller has dampened the feed rate oscillations caused by the initiation of PID control. Control of the fermentation is then sent to one of the Feed PID control schemes. If the control scheme detects that a glucose dump is occurring because oxygen transfer is greater than its uptake, control is instead returned to PID control by air flow.
Trigger A (Stage 29): Trigger A attempts to detect whether a glucose dump is occurring. A glucose dump is a result of the PID controller ramping up the feed rate in response to the DO not dropping down to its set-point. Therefore, if the feed rate exceeds 30 g/hr, stage 29 is called in order to return to PID control by air flow. A rise in the DO above 14% also causes the triggering of this response. Both of these indicators of a glucose dump are heavily dependant on the PID control parameters and the maximum oxygen transfer rate of the bioreactor. Therefore, the PID and/or trigger values might need to be tuned.

Trigger B (Stage 31): If a gram of feed has been successfully added without a glucose dump being detected, the first stage (stage 31) of the Feed PID control scheme is triggered.

Reduced Fed Batch Control

Reference: Shiloach, 1996

This scheme is the same as the Standard Fed Batch Control except that the initial feed concentration is smaller. In addition, only a small amount of feed is added when a feed depletion event is detected. This scheme is intended to reduce acetate production due to high glucose concentration in the media, but it allows a high rate of feed depletion events.

Table 5: Reduced Fed Batch Control

Dissolved Oxygen Set-Point (%):	30
Air Flow Fate Range (L/min):	0.1 – 1.0
Agitation Range (RPM):	50- 750
Initial Feed Concentration (g/L):	2
Momentary Feed Depletion (events/hr):	up to 60
Chance of Excessive Feed:	low
Chance of Prolonged Starvation:	low
Growth Rate Limitations by Feed:	low
Average Feed Concentration:	moderate
Reliability of Feed Scheme:	high

Maximum DO Control

Reference: Jun Sun, unpublished

This scheme is similar to a strategy described by Jun for controlling the BioFlo 3000 in the PEL. Unlike the other recipes, this scheme does not employ a ramp of agitation and airflow to maintain the DO at a set-point. Instead, the oxygen transfer rate is maximized by setting the airflow and agitation at their maximum rates and the DO is allowed to ramp down as cell growth increases. When the DO spikes due to feed depletion, a small pulse of feed is added to maintain a low average feed concentration. This is the least complicated recipe.

Table 6: Maximum DO Control

Dissolved Oxygen Set-Point (%):	maximum
Air Flow Fate Range (L/min):	1
Agitation Range (RPM):	750
Initial Feed Concentration (g/L):	10
Momentary Feed Depletion (events/hr):	30
Chance of Excessive Feed:	very low
Chance of Prolonged Starvation:	low
Growth Rate Limitations by Feed:	low
Average Feed Concentration:	moderate
Reliability of Feed Scheme:	high

Exponential Corrected Feed Forward Control

Reference: Nor, 2001

This is a relatively complicated recipe intended to keep the substrate concentration close to the uptake saturation limit by maintaining the feed rate at the maximum substrate uptake rate (MSUR). When the initial feed mass is depleted, a specified quantity of feed is added to the bioreactor. The time required for this feed to be consumed and a DO spike to occur is used to estimate the MSUR. This calculated MSUR is used as the basis for an open loop, exponentially increasing feed rate. After 30 minutes, feed is stopped and the exponential feed ramp rate is either increased or decreased depending on the time required for feed depletion to occur. Then, the MSUR is recalculated as before and the process is repeated.

Table 7: Exponential Corrected Feed Forward Control

Dissolved Oxygen Set-Point (%):	10
Air Flow Fate Range (L/min):	0.1 – 1.0
Agitation Range (RPM):	50- 750
Initial Feed Concentration (g/L):	2
Momentary Feed Depletion (events/hr):	4
Chance of Excessive Feed:	low
Chance of Prolonged Starvation:	moderate
Growth Rate Limitations by Feed:	low
Average Feed Concentration:	moderate
Reliability of Feed Scheme:	moderate

Minimum Feed Rate Control

Reference: Suzuki et al., 2001

This recipe adds feed at a constant rate while attempting to detect relatively small but significant increases in the DO as a signal that the current feed rate is too low. The feed rate is then increased and the process is repeated. This recipe can lead to excessive feed rate limitations on growth if it is not tuned properly for detection of the DO increase.

Table 8: Minimum Feed Rate Control

Dissolved Oxygen Set-Point (%):	50
Air Flow Fate Range (L/min):	0.1 – 1.0
Agitation Range (RPM):	50- 750
Initial Feed Concentration (g/L):	5
Momentary Feed Depletion (events/hr):	minimal
Chance of Excessive Feed:	moderate
Chance of Prolonged Starvation:	very low
Growth Rate Limitations by Feed:	high
Average Feed Concentration:	low
Reliability of Feed Scheme:	low

Maximum Feed Rate Control

Reference: Suzuki et al., 2001

This recipe includes all of the same features as the Minimum Feed Rate Control recipe. In addition, after a gram of feed is added without the DO rising to signify the need for an increase in the feed rate, the feed rate is doubled for a short period of time. The extent of the decrease in the DO as a result of this step increase in feed is used to determine whether the feed should be increased, decreased, or maintained at the current rate. The result is a more robust control scheme that can detect if the feed rate is too high or if the DO rises are not being detected.

Table 9: Maximum Feed Rate Control

Dissolved Oxygen Set-Point (%):	50
Air Flow Fate Range (L/min):	0.1 – 1.0
Agitation Range (RPM):	50- 750
Initial Feed Concentration (g/L):	5
Momentary Feed Depletion (events/hr):	minimal
Chance of Excessive Feed:	moderate
Chance of Prolonged Starvation:	very low
Growth Rate Limitations by Feed:	moderate
Average Feed Concentration:	low
Reliability of Feed Scheme:	low

Saturation Pulse Test Control

Reference: Akesson et al., 1999

This control scheme is based on the use of step changes in the feed rate to determine whether the substrate and oxygen uptake mechanisms of the host are saturated. The recipe attempts to maintain the feed concentration below the uptake saturation level in order to reduce fermentation byproducts. If a step increase in the feed rate causes a drop in the DO, then neither the oxygen nor the substrate cellular uptake mechanisms are saturated and the nominal feed rate is increased. If a step decrease in the feed rate does not induce an increase in the DO, then the substrate uptake mechanism is saturated and the nominal feed rate is decreased. The step rate changes occur on a four minute cycle. This allows 30 seconds for a feed rate step change and 90 seconds to recover from the response. This recipe attempts to detect transient changes in the bioreactor environment due to transient changes in a very complex system, and it can therefore be considered relatively unreliable. Furthermore, at high substrate uptake rates, a step change in the feed rate has a disproportionate effect on the rate of change in the substrate concentration as compared to low substrate uptake rates. The parameters may need to be retuned for different substrate uptake rates in order to be effective.

Table 10: Saturation Pulse Test Control

Dissolved Oxygen Set-Point (%):	30
Air Flow Fate Range (L/min):	0.1 – 1.0
Agitation Range (RPM):	50- 750
Initial Feed Concentration (g/L):	5
Momentary Feed Depletion (events/hr):	minimal
Chance of Excessive Feed:	low
Chance of Prolonged Starvation:	moderate
Growth Rate Limitations by Feed:	high
Average Feed Concentration:	very low
Reliability of Feed Scheme:	very low

Feed PID 1 & 2

The Feed PID recipes are supplementary control schemes for maintaining the dissolved oxygen at its set point by PID control of the feed rate. They are intended to be loaded in conjunction with one of the primary control schemes listed above. All of the primary control schemes trigger the first stage (stage 31) of the Feed PID control scheme when the maximum air flow and agitation rates are reached. If a Feed PID recipe has not yet been downloaded, the controller will become trapped in an undefined stage.

The Feed PID control schemes are designed to be universal; they maintain the DO set-point, air flow rate, and initial feed rate of the primary control scheme. The initial stages of the Feed PID recipe are designed to wait for the PID controller to reach a near steady state feed rate. They also make sure that the DO does not drop far below 10%. When a steady feed rate is attained, the control scheme monitors the feed rate to make sure that the feed concentration is the growth limiting factor. If the feed concentration is not growth rate limiting, the respiration rate of the cells cannot be controlled by the feed rate. Therefore, the DO might rise above its set-point irrespective of the feed concentration, and the controller will respond by ramping up the feed rate in an attempt to reduce the DO. This response is called a "glucose dump" and it can lead to toxic feed concentrations and overfilling of the bioreactor.

If a glucose dump is detected, Feed PID 1 and Feed PID 2 respond in different ways. Feed PID 1 responds by dropping the air flow rate in order to reduce oxygen transfer and bring culture growth back to feed limiting conditions. PID control by feed is maintained. Feed PID 2 responds by switching to DO control by air flow rate. The controller then waits for a DO spike to indicate feed depletion and adds a short pulse of feed. This scheme is similar to that used in the later stages of Reduced Fed Batch Control.

The recipes for both Feed PID 1 and Feed PID 2 contain stages that assist in DO control during induction. Induction almost always causes a reduction in the respiration rate of the cells. This leads to a glucose dump if PID control of the feed rate is active. To stop this from happening, the controller sets the feed rate to be 20% greater than the average PID feed rate and transfers DO control to the air flow rate. This is accomplished if the operator manually triggers stage 50 at the time of induction. The operator should only do

this if PID control of feed is active and at a near steady state rate. If the optimum induction strategy does requires low substrate concentrations, an alternate control scheme should be developed.

Table 11: Feed PID 1 & 2

Dissolved Oxygen Set-Point (%):	undefined
Air Flow Fate Range (L/min):	undefined
Agitation Range (RPM):	undefined
Initial Feed Concentration (g/L):	0
Momentary Feed Depletion (events/hr):	minimal
Chance of Excessive Feed:	high
Chance of Prolonged Starvation:	very low
Growth Rate Limitations by Feed:	very high
Average Feed Concentration:	very low
Reliability of Feed Scheme:	moderate

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