5-2015

A study on the BE2100 Noninvasive Biomass Sensor as an instrument for measuring optical density during fed-batch fermentation at various stir rates

Hannah Moreland
University of Arkansas, Fayetteville

A Study on the BE2100 Noninvasive Biomass Sensor as an Instrument for Measuring Optical Density During Fed-Batch Fermentation at Various Stir Rates

An Undergraduate Honors College Thesis
in the

Department of Biological Engineering
College of Engineering
University of Arkansas
Fayetteville, AR

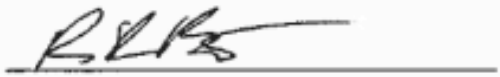
by

Hannah Elizabeth Moreland

April 26th, 2012

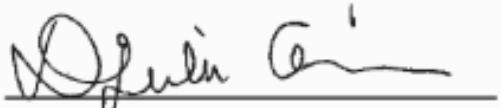
This thesis is approved.

Thesis Advisor:



Dr. Robert Beitle

Thesis Committee:



Dr. Julie Carrier

Dr. Thomas Costello

Table of Contents

Acknowledgements

I would like to thank Dr. Robert R. Beitle, Jr. of the Ralph E. Martin Department of Chemical Engineering for allowing me to work in his lab and for his guidance and assistance on all aspects of this project. I would also like to thank McKinzie Fruchtl, a graduate student in the Ralph E. Martin Department of Chemical Engineering, for her extensive assistance and training in the lab. Finally, I would like to thank Dr. Joshua Sakon of the Department of Chemistry for his assistance and contribution to the overall project and Dr. Thomas Costello and Dr. Julie Carrier for serving on my thesis committee. This project was funded with the help of grants from the University of Arkansas Honors College and the State Undergraduate Research Fellowship program through the Arkansas Department of Higher Education.

Abstract

Fusion proteins, engineered proteins that combine the DNA sequences and therefore properties of two different proteins, can be used in a variety of therapeutic purposes. One example of a therapeutic fusion protein is collagen binding domain-parathyroid hormone glutathione-S-transferase (CBD-PTH-GST), which can combat osteoporosis by binding specifically to collagen in the vertebral column and promoting bone growth through the release of calcium. This fusion protein is being expressed with *Escherichia coli* at the University of Arkansas through fed-batch fermentation, a method that produces large volume of cells through fermentation in a controlled environment in a bioreactor. It is necessary to track cell growth during fed-batch fermentation, and typical methods include measuring optical density (OD) with a spectrophotometer. However, this machine is limited in the range of OD it can measure without dilution and requires removal of samples from the bioreactor. Alternatively a noninvasive OD probe can be used to monitor cell growth during fed-batch fermentation, such as the BE2100 Noninvasive Biomass Sensor, or "BugEye" probe. Unfortunately the relationship between BugEye units and spectrophotometer OD is not well understood. This study was conducted in order to investigate the relationship between the BugEye probe and spectrophotometer OD and whether or not this relationship was affected by the RPM of the stirrer in the bioreactor during fermentation.

Introduction

Fusion proteins are proteins that are genetically engineered to contain sequences from two different genes [1]. These sequences result in the expression of a protein that has new properties, generally combining the characteristics of each of the joined proteins. Fusion proteins are useful in that they allow researchers to observe how proteins interact with each other and how proteins move, appear, and degrade in living cells [2]. Fusion proteins are also employed in purification through the use of affinity chromatography, where the second protein is an affinity tag that does not affect the activity of the target protein [2-3]. Increasingly, however, fusion proteins are being employed for pharmaceutical purposes [4]. Using fusion proteins in therapeutics offers opportunity to increase solubility and stability of the therapeutic protein and provide a method of targeting the protein to a specific location in the body [3].

One method of increasing the targeting or attachment abilities of therapeutic proteins is to employ collagen binding domain (CBD). CBD, through its specificity to collagen, anchors therapeutic proteins to collagen in the human body, without affecting the activity of its fusion partner [5]. For example, CBD fused to epidermal growth factor (EGF) has been shown to promote faster integration of a collagen patch into a perforated rat eardrum. CBD-EGF binds to the collagen patch, maintaining concentration and activity of the growth factor at the eardrum, thus repairing damage more quickly than without the fusion protein supplement [6]. CBD has also been fused to parathyroid hormone (PTH) for applications in treatment for osteoporosis. CBD binds to the discs in the vertebral column of rats, while its fusion partner PTH activates bone growth through the release of calcium [5].

Fusion proteins such as CBD-PTH are produced through recombinant expression in bacteria such as *Escherichia coli* [3-6]. The DNA sequence coding for the fusion protein and a

DNA molecule of *E. coli* (called the vector DNA or plasmid) are digested separately by a restriction enzyme. The two DNA fragments are mixed and joined using DNA ligase, so that the DNA for the fusion protein is part of the plasmid. The plasmid is then inserted into *E. coli* cells where it is replicated as the cells grow and divide [7]. The cells can then be induced with compounds such as isopropyl-β-D-thiogalactopyranoside (IPTG) or lactose, which act as "on-off switches" in the expression of the protein from the plasmid DNA [5]. After a desired amount of product has accumulated, it can be retrieved from the cells through sonication and purification [4-7].

Recombinant expression of fusion proteins with *E. coli* can be performed on a variety of scales, including shake flask, batch, and fed-batch fermentation. At the shake flask level, small (250 mL) solutions of cells in media can be cultured. The flasks are shaken to maintain a productive level of dissolved oxygen in the media. This scale of fermentation is useful in optimizing formulations for the media in which the bacteria will express the largest amount of fusion protein [5-6]. Batch fermentation is a larger scale fermentation performed in a bioreactor that typically lasts no longer than 24 hours. Fed-batch fermentation is on the same scale as batch fermentation, but nutrients are added during the fermentation process. Also, parameters such as pH, dissolved oxygen, and temperature are controlled [4-6].

During fed-batch, cell concentrations are monitored throughout the process, so that the cells can be induced at a time of high concentration (since protein expression can result in metabolic burden and slower cell growth) [5]. Cell concentration can be quantified by using optical density (OD) measuring equipment such as a spectrophotometer. Samples of cell culture solution are taken from the bioreactor and placed in the spectrophotometer to be analyzed. The absorbance measured by the spectrophotometer can then be converted to dry cell weight as

demonstrated by the curve in Appendix 1. However, cell concentration can also be monitored noninvasively with an instrument called the BE2100 Noninvasive Biomass Monitor (BugLab LLC, Concord, CA), or the "BugEye." The BE2100 sensor is strapped to an open viewing port in the heating jacket around the bioreactor. The sensor uses a variety of infrared lasers and detectors, which are each "sensitive to a different range of biomass changes." This information is then communicated to the base unit software, which linearizes the response to change in biomass and records the data in a file on the computer used to run the bioreactor [8]. Figure 1 depicts the BugEye probe and how it attaches to a bioreactor.

a) b)

Figure 1: a) The BE2100 "BugEye" Noninvasive Biomass Sensor and Base Unit; b) The BE2100 "BugEye" Noninvasive Biomass Sensor attached to a glass bioreactor [8]

The BugEye probe is valuable in monitoring cell concentration during fed batch because, unlike any other probe, it is able to measure OD at the high levels reached during fed batch. In order to determine OD at such high levels with a spectrophotometer, the samples must be diluted in order to get an accurate reading, which can introduce error.

The OD data given by the BE2100 OD and the spectrophotometer are not identical. This is because the BE2100 reads OD in the bioreactor as it is being stirred and the spectrophotometer reads OD of still culture media. The relationship between the OD data given by the two instruments is not entirely understood, especially in terms of varying rpm of the stirrer motor [9]. Additionally, the OD readings of the BE2100 may be affected by factors such as dissolved oxygen concentration in the culture media.

Objective

Currently, the expression of the fusion protein CBD-PTH-GST (CBD-PTH with a Glutathione-S-Transferase affinity purification tag) through fed-batch fermentation of *E. coli* is being investigated at the University of Arkansas. Both spectrophotometer and BugEye probe are employed to monitor OD during fermentation. The objective of this project was to determine the relationship between the BE2100 OD data, the spectrophotometer OD data, and the rpm of the stirrer motor and to mathematically model this relationship for future use in monitoring cell concentration during fed-batch fermentation.

Materials and Methods

Materials and Equipment

Cultures of recombinant, ampicillin resistant *E. coli* BL21 DE_3 pCHC305PTHdEco2 were used to produce the fusion protein product, CBD-PTH-GST. *E. coli* is usually grown in Luria-Bertani (LB), but for mass production of a biopharmaceutical, LB is not favored since it cannot be easily validated and is therefore not typically permitted by the Food and Drug Administration. Modified M9 medium was used instead and consists of, per liter of medium:

- 10 g glucose
- 15 g K_2HPO_4
- 7.5 g KH_2PO_4
- 2 g citric acid
- 2.5 g $(NH_4)_2SO_4$
- 2 g $MgSO_4$-$7H_2O$
- 1 ml trace element solution

where trace element solution is, per liter of 1M HCl:

- 2.8 g $FeSO_4$-$7H_2O$
- 2 g $MnCl_2$-$4H_2O$
- 2.8 g $CoCl_2$-$7H_2O$
- 1.5 g $CaCl_2$-$2H_2O$
- 0.2 g $CuCl_2$-$2H_2O$
- 0.3 g $ZnSO_4$-$7H_2O$

The M9 media was supplemented with 5g/L glucose. An antifoam emulsion agent was added once before cultivation and once during fermentation. Cells were induced with lactose approximately 22 hours after innoculation.

The method of production was fed-batch fermentation in a 3 L Applikon Bioreactor (Foster City, CA). Equipment associated with the bioreactor was controlled with BioXpert Advisory software from Applikon. During the fermentation, pH was monitored with an Applisense pH probe and maintained near 6.8 using concentrated NH_4OH. A heating jacket was placed around the bioreactor to maintain media temperature at approximately 37° C. The media was supplemented with a stream of air and then oxygen (about 4.5 hours after inoculation) so that dissolved oxygen concentration was greater than 35%. Agitation was achieved with a stirrer attached to a motor; stir rate was managed using the bioreactor's software. Optical Density was monitored using a BE2100 Noninvasive Biomass Monitor (BugLab LLC, Concord, CA) and a DU800 Beckman Coulter spectrophotometer (Brea, CA). Because this investigation was more concerned with the method of monitoring cell concentration over time and not with achieving the highest product yield, glucose was not fed into the bioreactor during fermentation.

All media and equipment were sterilized before use in an autoclave. Figure 2 shows the bioreactor and monitoring equipment set up.

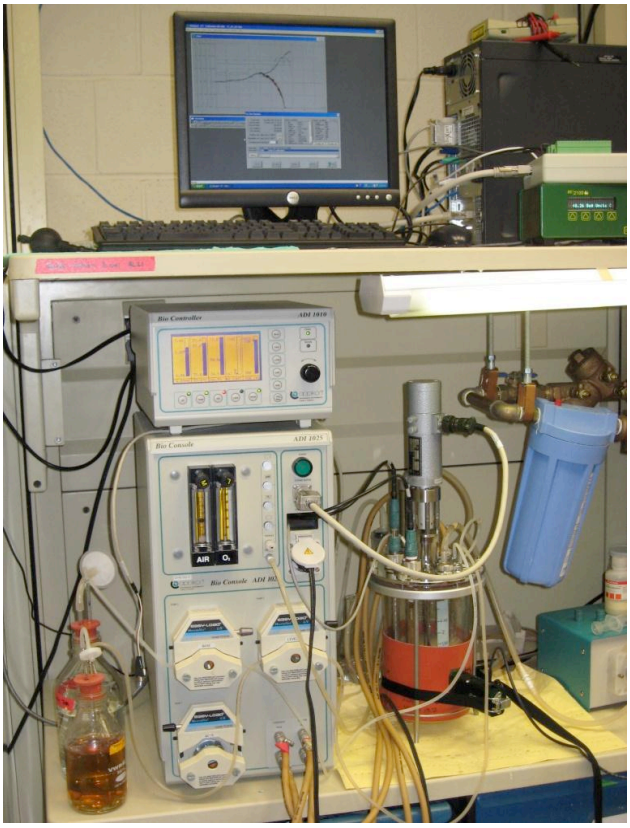


Figure 2: Set up of the bioreactor and monitoring equipment used for fed-batch fermentation of *E. coli* [5]

Day Zero

A 5 mL culture of the recombinant *E. coli* was cultured overnight in M9 medium. The culture was placed in a flask in a shaking incubator at 37°C.

Day One

The Applikon Bioreactor and associated equipment and 1L of M9 medium were washed with soap and water and then sterilized in an autoclave. The cells from the overnight culture were transferred to 15 mL of fresh M9 medium. Ampicillin was added to inhibit the growth of microbes other than the desired *E. coli* in the media. The new culture was once again stored

overnight in the shaking incubator at 37°C. The pH probe for the bioreactor was calibrated. The bioreactor and equipment were then set up. The BugEye was attached and allowed to achieve a baseline with the stirrer spinning at 500 RPM overnight.

Day Two

The media in the bioreactor was inoculated with cells from the overnight culture. An initial OD reading was then taken with the spectrophotometer. A 1 mL sample of the cell culture was taken from the bioreactor using a pipette. The sample was placed in a cuvette, which was then placed in the spectrophotometer. The machine was blanked with a 1 mL cuvette of water. The absorbance at 600 nm was then measured and recorded. The BugEye probe software was set to record the BugEye OD data once each minute for the duration of the fermentation. The BugEye number while the stirrer was at 500 RPM was recorded by hand in a notebook. The stir rate was then increased to 750, 1000, and 1250 RPM and the greatest BugEye number given at each RPM was recorded in the notebook. The stirrer was then placed back at 500 RPM until the next reading was taken an hour later.

Because the BugEye number fluctuated so greatly after each change in RPM, all other BugEye readings were recorded in the notebook after allowing the stirrer to spin for approximately 5 minutes at each RPM. In this way, approximate average BugEye readings were noted for 500, 750, 1000, and 1250 RPM each hour for 11 hours. OD was also measured with the spectrophotometer every hour. All samples for the spectrophotometer were taken when the stirrer was at 500 RPM. The stirrer was kept at 500 RPM between readings.

After the 11 hours of growth, the culture was left to grow overnight in the reactor before it was induced with lactose. A graduate student associated with a separate part of the project then processed the resulting cell mass and protein product.

Results and Discussion

The raw OD data recorded by the computer over the course of the fermentation is plotted below in Figure 3. Each point on the graph represents the BugEye number every 60 s during the fermentation period. The baseline curve at the bottom of the graph represents the increase in BugEye number over time when the stirrer was at 500 RPM. All points plotted above this baseline represent the OD observed by the BugEye probe during minutes when the stirrer RPM was increased, or when the probe was adjusting after an increase or decrease in RPM. Curves representing the BugEye number at 750, 1000, and 1250 can be observed as shown in Figure 3.

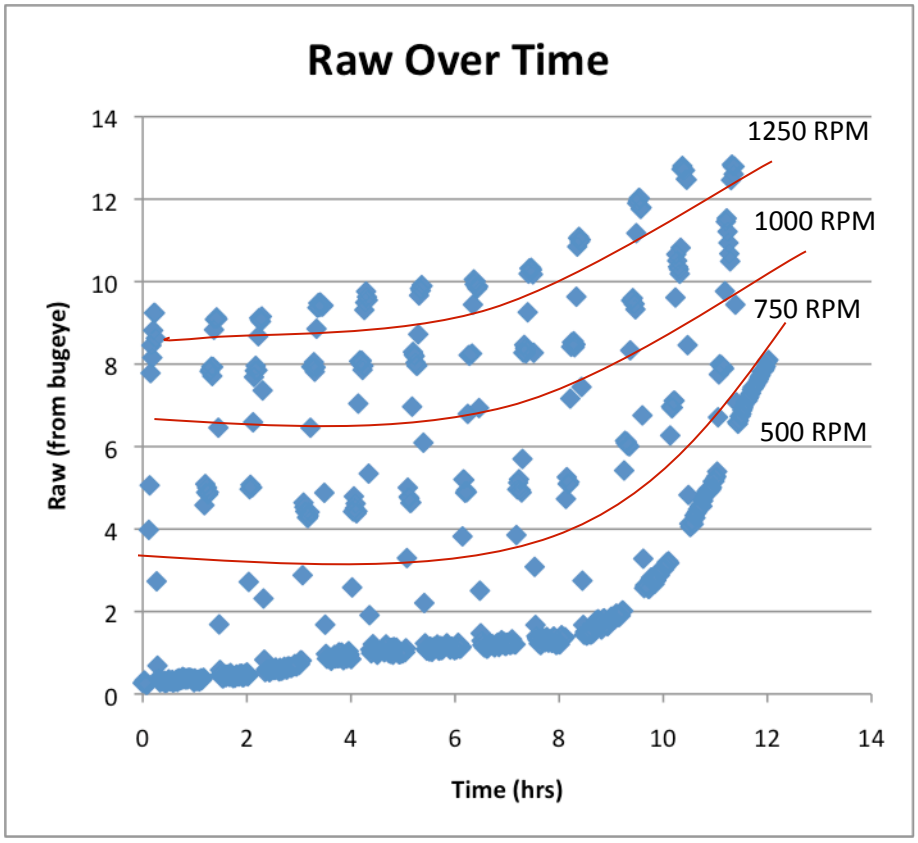


Figure 3: Plot of the raw OD data from the BE2100 "BugEye" OD probe over time during the fed-batch fermentation of *E. coli*; the baseline growth curve represents the bioreactor stirrer at 500 RPM, while the points above the baseline represent an increase in the RPM of the stirrer.

Figure 4 below shows the change in OD over time as measured by the spectrophotometer during the fermentation. The plot mimics a cell growth curve, confirming that as cell concentration increases over time, so does OD. Because of the similarities between Figures 3 and 4, one can see that the OD measured by the BugEye represents cell growth similarly to the OD measured by the spectrophotometer. The numbers, however, given by the BugEye differ from those given by the spectrophotometer and can differ more greatly as RPM is increased.

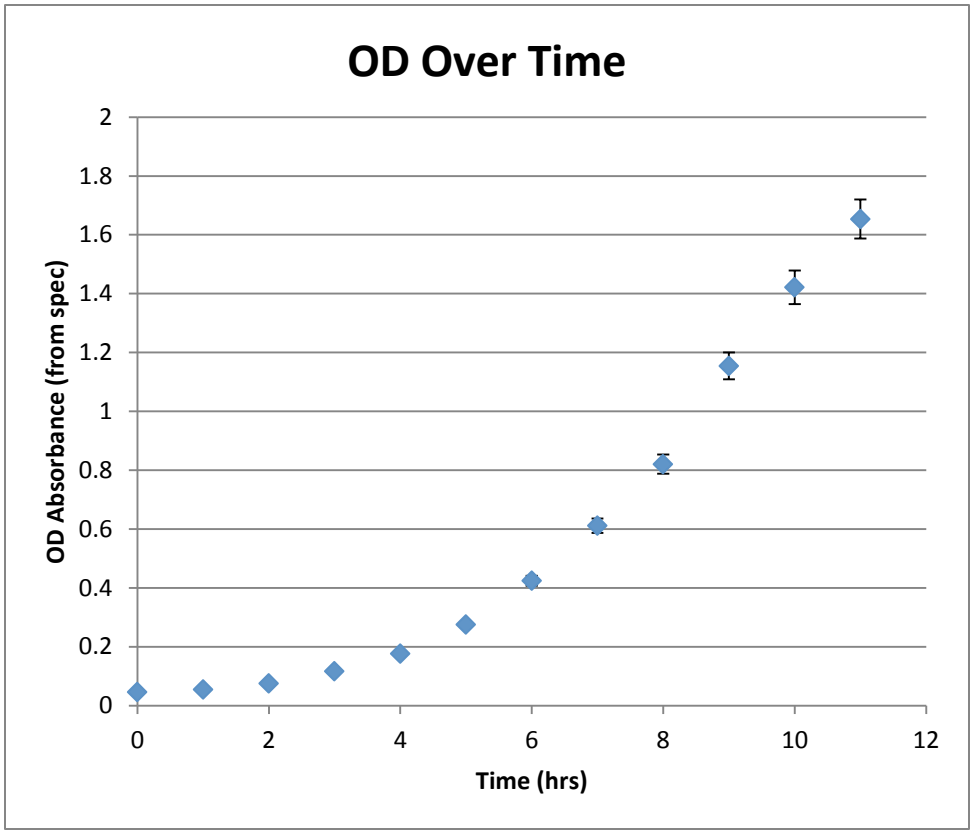


Figure 4: Plot of the OD data from the spectrophotometer over time during the fed-batch fermentation of *E. coli.* Graph shows the typical 4% error.

Figures 5, 6, 7, and 8 show the BugEye data plotted against spectrophotometer OD when the stirrer was set at 500, 750, 1000, and 1250 RPM, respectively. The BugEye data used for this plot was the approximate average BugEye number that was recorded by hand after allowing the probe to "settle" after the RPM had been changed for 5 minutes (except for the data given in Figure 5, as the stirrer was set at 500 RPM for approximately 40 minutes in between readings).

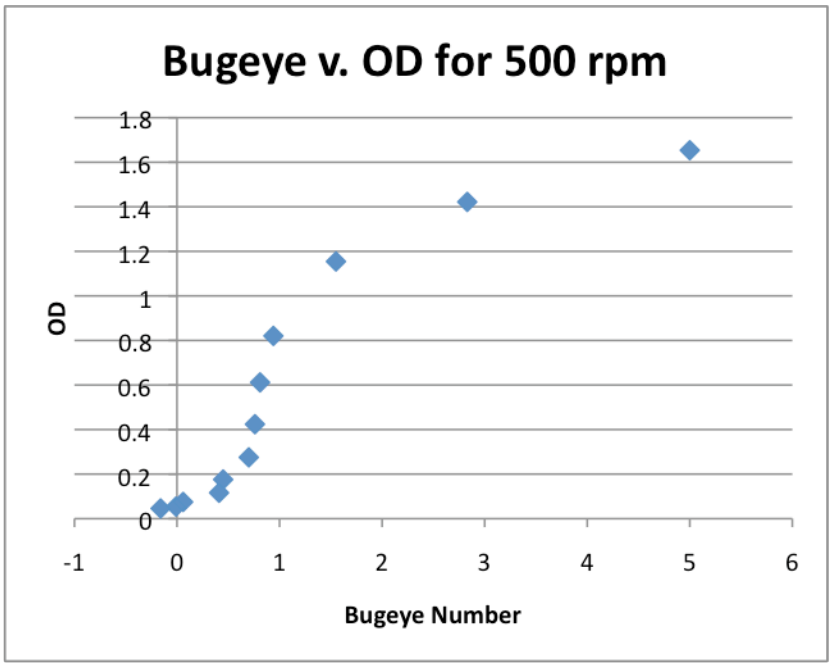


Figure 5: Approximate average BugEye OD Number vs. OD given by the spectrophotometer over the course of fermentation when the bioreactor stirrer was set at 500 RPM

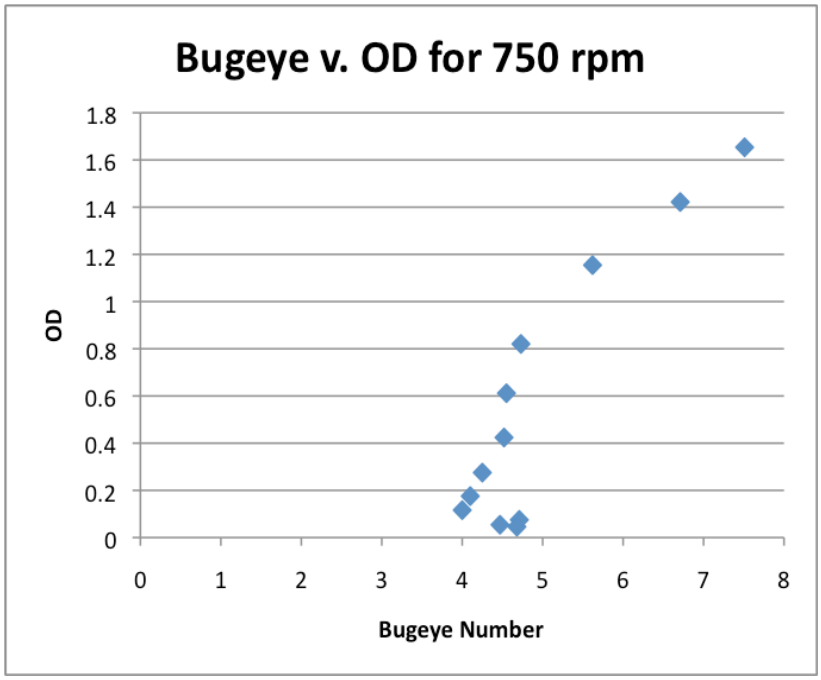


Figure 6: Approximate average BugEye OD Number vs. OD given by the spectrophotometer over the course of fermentation when the bioreactor stirrer was set at 750 RPM

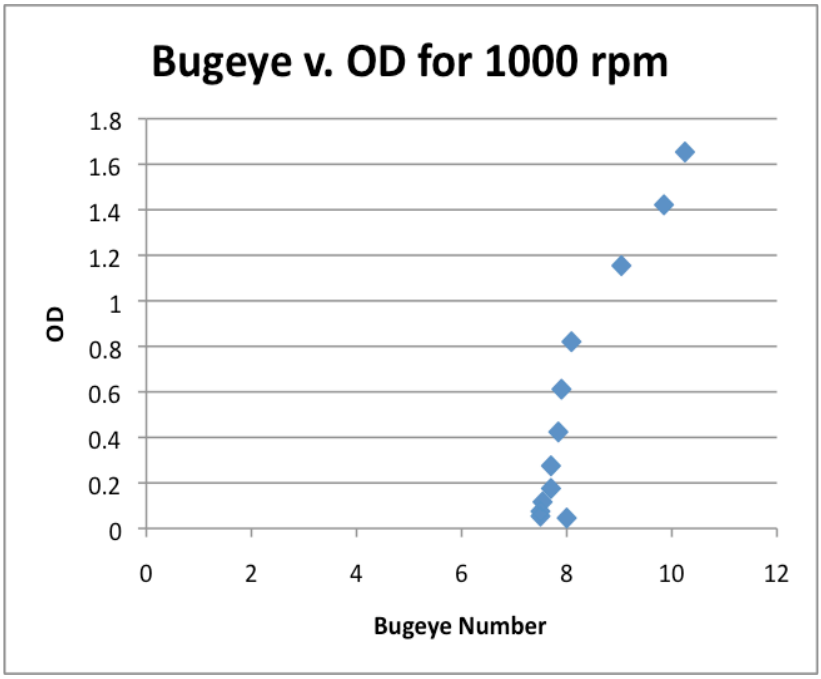


Figure 7: Approximate average BugEye OD Number vs. OD given by the spectrophotometer over the course of fermentation when the bioreactor stirrer was set at 1000 RPM

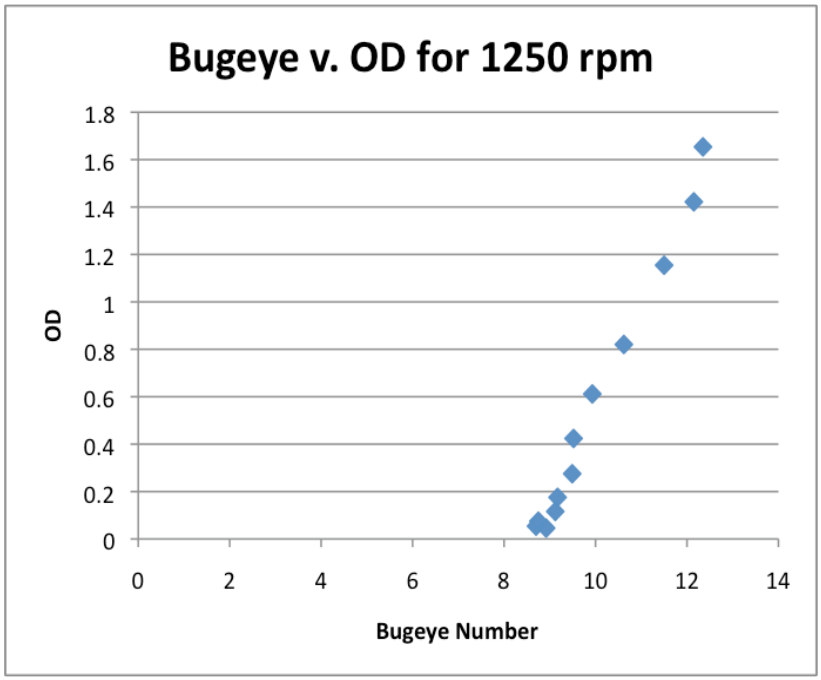


Figure 8: Approximate average BugEye OD Number vs. OD given by the spectrophotometer over the course of fermentation when the bioreactor stirrer was set at 1250 RPM

Each of the above figures displays that as the BugEye number increases, so does the OD given by the spectrophotometer. However, the shapes of each plot differ depending on RPM. For example, the shape of the data plotted in Figure 5, when the stirrer was at 500 RPM, resembles an S-shaped curve, while the shape of the data plotted in Figure 8, when the stirrer was at 1250 RPM, displays a more linear relationship between BugEye Number and spectrophotometer OD. Figure 9 below compares the data given in Figures 5-8.

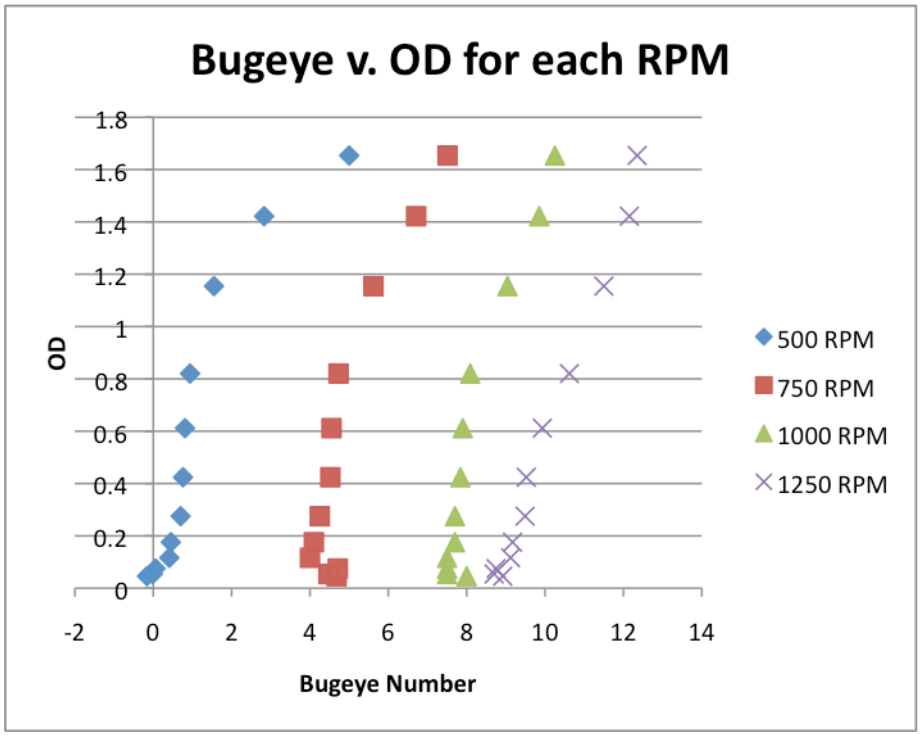


Figure 9: Approximate average BugEye OD Number vs. OD given by the spectrophotometer over the course of fermentation when the bioreactor stirrer was set at varying RPM

Because the shapes of the plots are different depending on RPM, no single correlation can be observed between BugEye Number, spectrophotometer OD, and stirrer RPM. However, when looking more closely at how the shapes of the data plots compare as RPM is increased, one can see that BugEye Number and spectrophotometer OD approach a linear relationship as RPM

of the stirrer is increased. Regardless of the difficulty of achieving a single mathematical model for BugEye Number and OD as RPM changes, the plots still show that the BugEye number given, and therefore the relationship between BugEye number and OD, depends on the stir rate in the reactor.

Even though one mathematical model could not be achieved, individual models could be determined to relate BugEye number and OD for each stirrer RPM. The overall "flattening" or linearization of the data with increasing RPM of the stirrer suggests that OD at a lower RPM is more difficult to represent with the instrument.

Additionally, for each OD value measured, the BugEye number was plotted against the RPM of the stirrer. This data shows a similar linear relationship between RPM and BugEye number for each OD. These plots are given in the appendix to this study.

Conclusions

Overall, the results of this investigation show that the BugEye BE2100 Noninvasive Biomass Sensor is useful in monitoring cell concentration during fed-batch fermentation. The relationship between the OD given by the BugEye probe and the OD given by a spectrophotometer is dependent on the RPM of the stirrer in the bioreactor. However, a single mathematical model to relate BugEye number and spectrophotometer OD across all stir rates could not be achieved, because the relationship between the two parameters changes as RPM increases. The data suggests that the relationship between BugEye number and spectrophotometer OD becomes more linear at higher RPMs. Therefore, it seems the probe is more capable of representing accurate OD ratings at higher stirrer RPM.

The information gathered in this study will be used to create calibration charts to relate BugEye number to spectrophotometer OD for specific stir rates. Higher stir rates, near 1250 RPM, will most likely be used in the future, for easier and more accurate relating of BugEye number to OD. Continuation of this study will include investigating the relationship of BugEye number to spectrophotometer OD as dissolved oxygen concentration in the bioreactor changes. After both studies are concluded, the data expressed by the BugEye BE2100 Noninvasive Biomass Sensor will be more easily understood and used in future fed-batch production of the therapeutic fusion protein CBD-PTH-GST.

References

[1] Cammack R, Atwood T, Campbell P, Parish H, Smith A, Vella F, Stirling J. Fusion Protein. Oxford Dictionary of Biochemistry and Molecular Biology 2008. Available at: www.oxfordreference.com.library.uark.edu/views/ENTRY.html?subview=Main&entry=t219.e7526

[2] Ewalt KL, Schimmel P. Protein Engineering. AccessScience, McGraw-Hill 2008. Available at: http://www.accessscience.com.

[3] Pyne ME, Sukhija KS, Chou CP. Strain improvement of *Escherichia coli* to enhance recombinant protein production. Manual of Industrial Microbiology and Biotechnology 2010; 3:273-286.

[4] Shiloach J, Rinas U. Bacterial cultivation for production of proteins and other biological products. Manual of Industrial Microbiology and Biotechnology 2010; 3:132-144.

[5] Fruchtl M, Moreland H, Beitle RR, Sakon J. Collagen binding domain fusion proteins for therapeutics: expression and isolation. AIChE Annual Meeting Proceedings 2011.

[6] Beitle R, Sakon J. Novel Wound Dressing Strategy to Promote Vascularization and Integration with Host Vasculature to Treat Perforated Tympanic Membrane. *NIH*. Grant Proposal 2010.

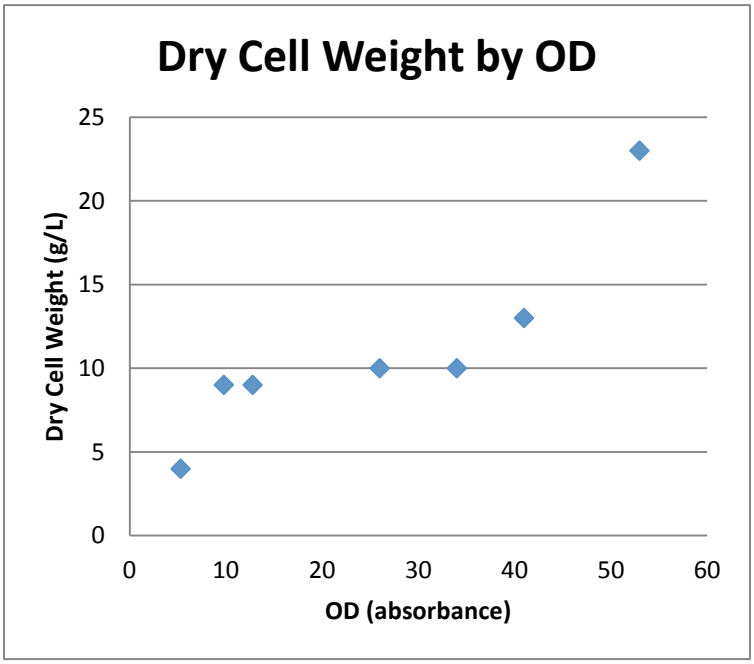
[7] Guilfoile, PG. Recombinant DNA. Genetics, Macmillian Reference USA 2003; 4:5-7. Available at: http://go.galegroup.com/ps/i.do?id=GALE%7CCX3406500242&v=2.1&u=faye28748&it=r&p=GVRL&sw=w

[8] BugLab, LLC. Product website 2012. Available at: http://buglab.com/index.html.

[9] Beitle R. Interpersonal Communication 2012.

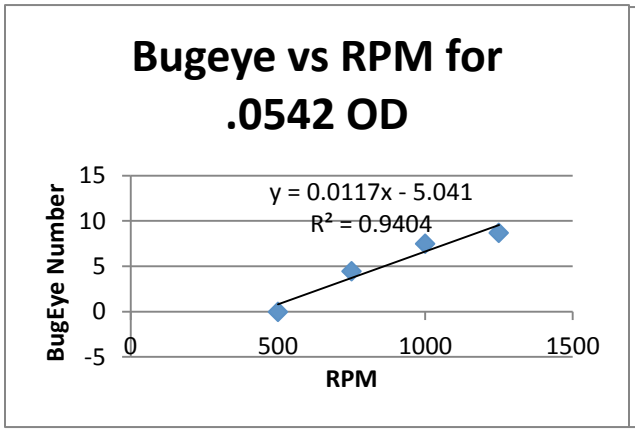
Appendix 1

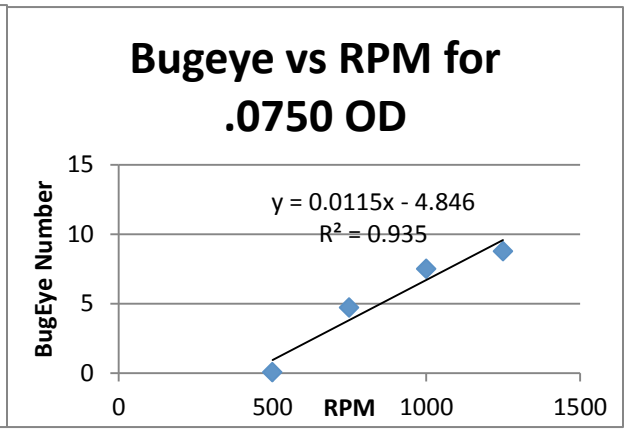
Below is the relationship between OD (given by the spectrophotometer) and dry cell weight for *E. coli* grown in fed-batch fermentation in M9 media.

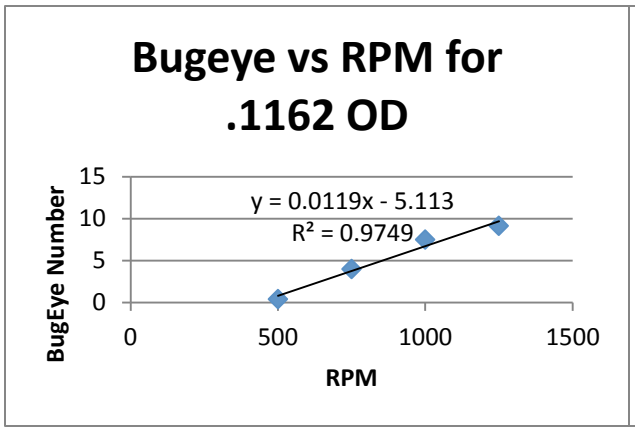


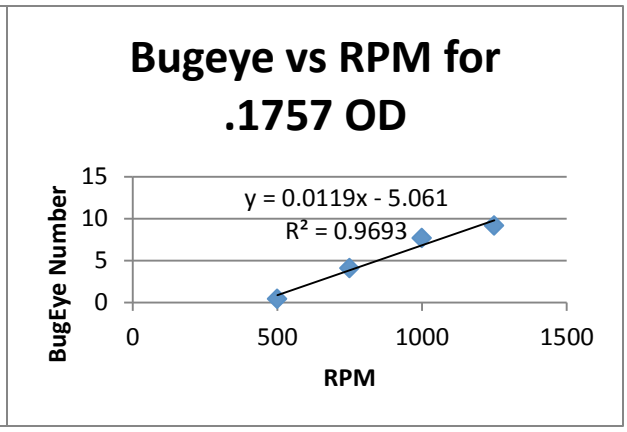
Appendix 2

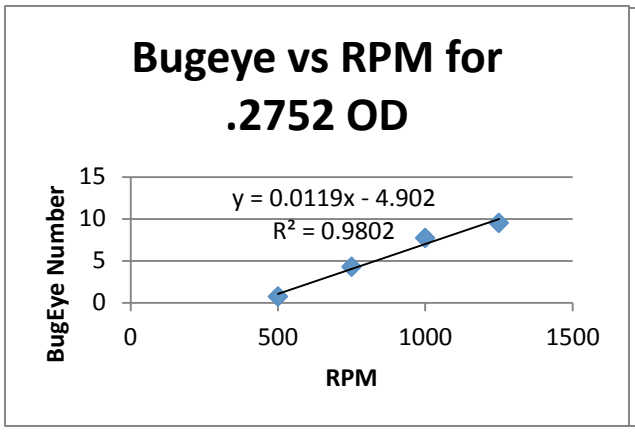
Below is the data for the BugEye number plotted against RPM for each OD. Each shows a linear relationship with similar trendline equations.

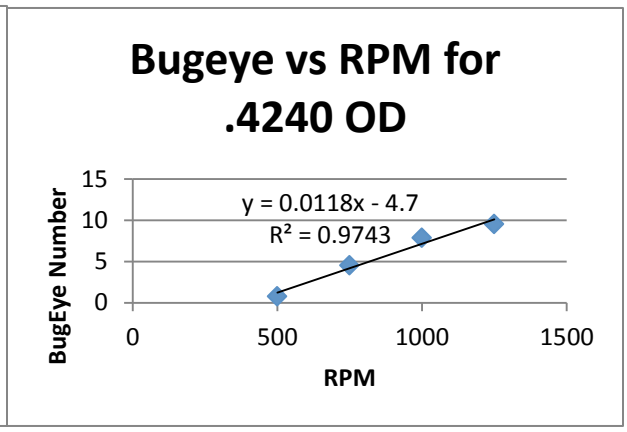


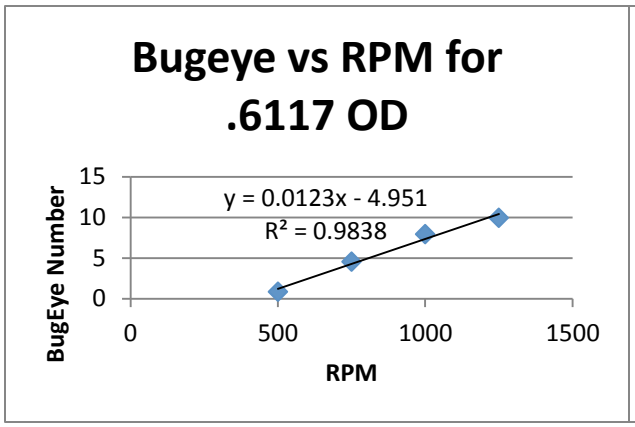


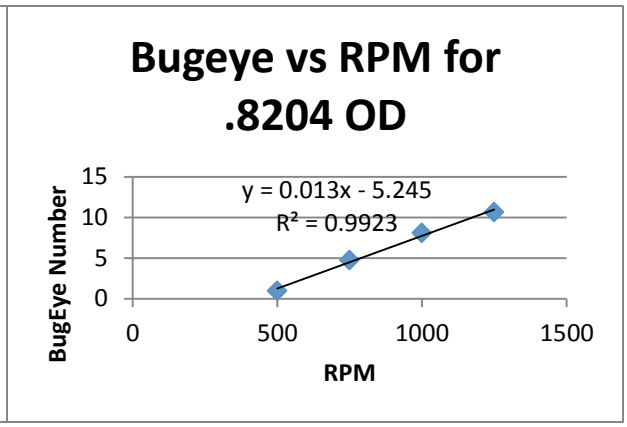


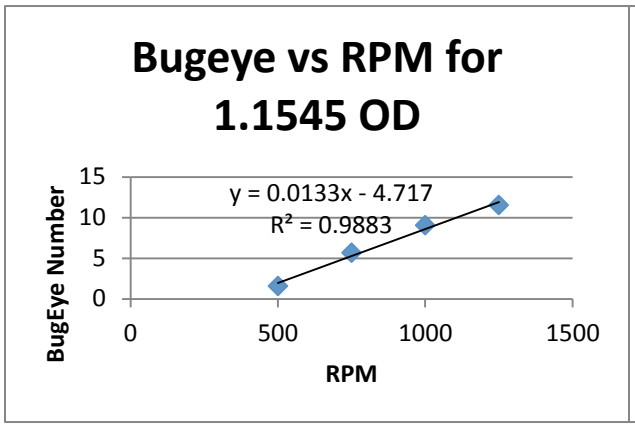


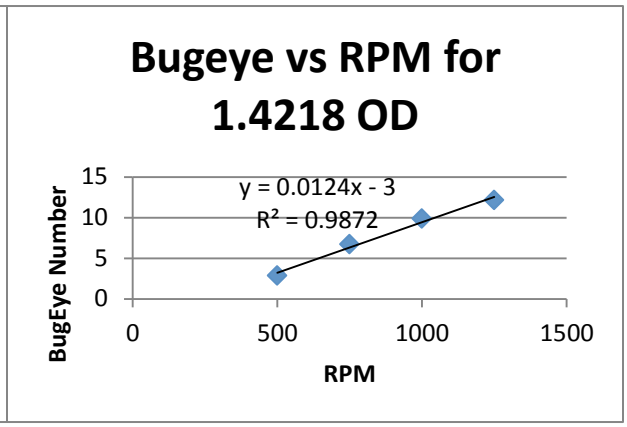


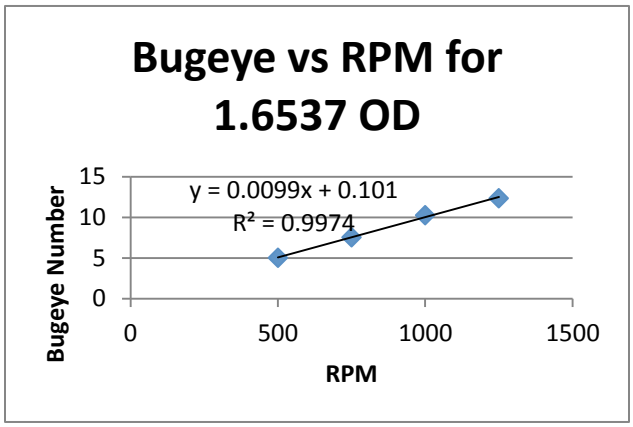



Bugeye vs RPM for .6117 OD
y = 0.0123x - 4.951
R² = 0.9838
BugEye Number
RPM
0
5
10
15
0
500
1000
1500


Bugeye vs RPM for .8204 OD
y = 0.013x - 5.245
R² = 0.9923
BugEye Number
RPM
0
5
10
15
0
500
1000
1500


Bugeye vs RPM for 1.1545 OD
y = 0.0133x - 4.717
R² = 0.9883
BugEye Number
RPM
0
5
10
15
0
500
1000
1500


Bugeye vs RPM for 1.4218 OD
y = 0.0124x - 3
R² = 0.9872
BugEye Number
RPM
0
5
10
15
0
500
1000
1500


Bugeye vs RPM for 1.6537 OD
y = 0.0099x + 0.101
R² = 0.9974
Bugeye Number
RPM
0
5
10
15
0
500
1000
1500