

Non-Invasive Biomass Monitor with Wide Linear Range

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Abstract

A new on-line biomass instrument (BugEye® 100 sold by BugLab® LLC) has been developed to monitor biomass non-invasively in fermentors having a glass wall or glass viewing port. The instrument consists of an optical sensor that is mounted onto the exterior of the fermentation vessel, and a monitor that processes the sensor signals. By combining reflectance signals measured at multiple source-detector distances, linear response to biomass change is achieved over a wide range of biomass. The performance of the instrument was tested under varying fermentation conditions. Eleven *E. Coli* fermentations and one *S. cerevisiae* fermentation were run in three vessel sizes (5L, 28L, and 250L) in batch and fed-batch modes, up to ODs as high as 130. Averaged over the twelve fermentations, the squared linear correlation coefficient (R^2) between the on-line biomass monitor and off-line OD was 0.99, for ODs greater than 0.1. Real-time prediction of OD was accomplished by using earlier fermentations run under similar conditions for calibration. Averaged over the seven calibrated fermentations, ODs greater than 0.5 were predicted with 15% RMS error.

Introduction

Many methods are available for determining biomass in liquid cultures.^{1,2} The measurement generally considered to be the most direct and accurate, dry cell weight, requires sampling, washing, drying, and weighing, which is laborious and time-consuming. More importantly, the result is not available in real-time to be used as a feedback variable for process control. Wet cell weight can be measured more rapidly, but is also generally less accurate.

A less direct, but more rapid method for estimating biomass is off-line OD measurement. This method has been shown to correlate well with biomass for mono-disperse cultures.² While more convenient than determining dry cell weight, this method has several disadvantages: (1) each time that an aliquot is withdrawn, there is a risk that the culture will be contaminated, (2) the method is not continuous, and (3) the method is cumbersome, labor intensive and prone to systematic error, requiring frequent extraction and precise volumetric dilution of the extracted liquid when high cell concentrations are measured. A number of efforts have been made to automate the process of sample extraction for external assessment in a spectrophotometer.⁵⁻⁸ In order to maintain a linear relationship between OD and biomass, the instruments developed have included means of auto-diluting the sample⁵ or varying the path length through the transmission cell.⁶⁻⁸ However, the complexity and the risk of sample contamination risk presented by such instruments have prevented their wide-spread adoption.

As a result, a number of different instruments, both optical and non-optical, have been developed to estimate biomass directly within a bioreactor. Non-optical methods that have been investigated include nuclear magnetic resonance, acoustic resonance densitometry, and electrical conductivity and capacitance.¹⁻⁴ While correlation with biomass has been demonstrated under some conditions, what these methods generally have in common is high complexity and cost and low sensitivity to biomass.¹⁻⁴

Optical probes that can be immersed directly into liquid cultures have been developed based on techniques including transmission,¹⁰⁻¹⁴ reflectance,^{15,16} combined transmission and reflectance,^{17,18} fluorescence,¹⁹ and image and particle analysis.^{20,21} Unfortunately, such devices are prone to drift, particularly due to growth of cells or microorganisms on the photosensor itself. In addition, the range of biomass that can be measured is generally limited by the path length between source and detector. Many microorganisms, particularly strains of yeast (e.g. *Pichia Pastoris*), are grown to much higher concentrations (e.g. ODs of 100 and higher) than can be measured with devices based on a single source-detector pair. In addition, the non-linear response of such devices to biomass changes can make calibration challenging.

Non-invasive probes for biomass have the advantage of avoiding sensor fouling while eliminating the possibility of cross-contamination. Previously a non-invasive

optical transmission probe was developed that was limited to a very low range of biomass due to the long path length (50 mm) between the source and detector.²² More recently a fiber optic reflectance probe was developed that uses the near infrared reflectance spectrum to predict cell density.²³ Due to the use of a single source-detector geometry, accurate biomass prediction was limited to the range of 1 to 40 g/L.

In the present publication, we describe testing and comparison of a new non-invasive probe based on optical reflectance at multiple source-detector separations. By combining the signals from these multiple source-detector pairs a linear response to biomass is maintained over a very wide range of biomass: 0.05 to 200 g/L. The non-invasive nature of the measurement avoids the problems of sensor fouling and cross-contamination. The need to sterilize the probe is also eliminated, reducing the cost of the materials used in sensor production.

Methods

On-Line Biomass Monitoring

Two commercial on-line optical monitors were tested in these studies: the Model FSC402 Turbidity Analyzer (Mettler-Toledo AG) and the BugEye 100 Biomass Monitor (BugLab LLC). The FSC402 Turbidity Analyzer consists of an immersion probe and a monitor. The immersion probe contains a pair of optical fibers, one to carry laser excitation into the culture and one to collect the light reflected back from the culture. The reflectance is quantified and reported by the monitor.

The BugEye Biomass Monitor used in these experiments consists of a non-invasive optical sensor and a monitor. The sensor is mounted on the exterior of a fermentor having a glass wall or a glass viewing port and held in place with an adjustable strap and buckle. The sensor contains an array of Vertical Cavity Surface Emitting Lasers (VCSELs) emitting at 850nm and detectors arranged to detect the laser light reflected from the organisms in the fermentor at multiple laser-detector distances.²⁴ The detected light is optically and electronically filtered to reduce sensitivity to ambient light.

The monitor drives the lasers, reads the detectors, and combines the signals generated by the individual laser-detector pairs. The result is then reported to the user

through a fluorescent display. In addition, the result can be accessed in digital form through a RS-232 communications port or in analog form through a 4-20 mA output. Real-time display, automatic saving, and calibration of the result into biomass units (OD, dry weight, etc.) is accomplished through a windows-based data acquisition program (BugFree® sold by BugLab).

For the fermentation studies described below the BugEye sensor reading measured shortly before inoculation of the culture was subtracted from all subsequent readings. The BugEye sensor was removed from the vessel between fermentation runs.

Theory of Sensor Operation

In operation, light emitted from the BugEye sensor is passed through the glass fermentor wall or window into the liquid culture. The light is then scattered by the cells or microorganisms, creating a glow ball. The intensity and size of the glow ball are dependent on the biomass within the liquid culture. At early stages of growth, when the biomass is low, the glow ball will be large in size and weak in intensity. As the cells or microorganisms grow and divide, the concentration will increase and the glow ball will reduce in size and increase in intensity. The detectors in the photosensor are sensitive to the intensity of light emanating from the glow balls. Further, the photosensor is arranged such that there are multiple separation distances between the detectors and light sources. Each of the detectors is sensitive to the glow ball generated from the light emitted by each of the light sources. In this way, not just the intensity but the size of the glow ball is measured. By combining the signals from the multiple detectors due to the multiple light sources, the biomass in the liquid culture is determined with linear dynamic range orders of magnitude larger than immersion transmission or reflectance techniques.²⁴

Sensor Comparison Testing

Comparison of transmission and reflectance sensors operating at different source-detector separations was performed by dissolving Baker's yeast (Red Star Active Dry Yeast, Lesaffre Yeast Corporation) into 0.9% saline. Sensitivity to changes in container wall thickness was tested by constructing a box from acrylic with 3 different wall thicknesses: 6.3, 9.5, and 12.7 mm. The box was filled with dissolved Baker's yeast and

continuously agitated while the sensor was re-positioned onto the different walls of the container.

Fermentation Methods

Sensor testing was performed during 11 *E. coli* and one *S. cerevisiae* fermentation runs with 3 different fermentor types: (1) 5 liter all-glass (7.0 mm thick) vessel (Bioflo, New Brunswick Scientific Co.), (2) 28 liter stainless-steel vessel with rectangular glass (12.5 mm thick) viewing port (W.B. Moore, Inc.), and (3) 250 liter stainless-steel vessel with circular glass viewing port (W.B. Moore, Inc.).

Five *E. coli* batch fermentations were run in 5 liter vessels with the temperature fixed at either 30 or 37C, agitation rates between 400 and 600 RPM, and aeration between 4.7 and 5.0 LPM. Both simple and complex (Terrific broth with soy and yeast added) media were tested. The maximum off-line OD measured during these batch fermentations was 22 during a fermentation run lasting 24 hours.

Three *E. coli* fed-batch fermentations were run in 5 liter vessels with the temperature fixed at either 30 or 37C, agitation rate continuously varying between 200 and 1000 RPM, and aeration varying between 4.3 and 5.2 LPM. Reisenburg medium was used with an initial addition (1% V/V) of glycerol followed by a continuous feed (50% V/V) of glycerol. The agitation rate was regulated so that the minimum dissolved oxygen was maintained at 20% of atmospheric oxygen. The total amount of fed glycerol ranged between 830 and 1040 mL for the three cultures while the maximum off-line OD measured was 133 during a fermentation run lasting 65 hours.

One *E. coli* batch fermentation was run in a 28 liter vessel with the temperature fixed at 36C until induction, at which point the temperature was reduced to 24.5C. The fermentation run lasted 13 hrs, during which time the agitation was varied between 167 and 300 RPM while aeration was varied between 2 and 11 LPM.

Two *E. coli* batch fermentations were run in a 250 liter vessel, with maximum off-line OD reaching 4 and 26. One *S. cerevisiae* fermentation was run in a 5 liter vessel, with maximum off-line OD reaching 2.4.

Off-Line Methods

Off-line OD was measured using a Milton Roy Spectronic 601 spectrophotometer. OD was measured at 600 nm in a 1.00 cm cuvette. Samples were diluted to an OD range of 0.05 to 0.2 prior to spectrophotometric analysis. The measured OD was then multiplied by the dilution factor.

Dry weight was determined by sampling 1.00 mL of liquid culture, centrifuging the sample, discarding the supernatant, resuspending the solids in 1.0 mL phosphate buffer (50 mM, pH 7), centrifuging again, discarding the supernatant again, drying the sample for 2-3 days at 60-80C, and then recording the final weight.

Results

Sensor Comparison Testing

In Figure 1a the on-line OD measured by a transmission probe (10 mm path length) immersed in the undiluted liquid culture is compared with the off-line OD measured after dilution. A linear relationship between on-line and off-line OD is maintained only up to an off-line OD of approximately 0.4. At this OD value the on-line OD underestimates the off-line OD by 10%. The under-estimation increases to 20% at an off-line OD of 1.0, and by ODs of 3 to 4 the on-line response is nearly constant with further increases in off-line OD. The useful linear range of this on-line transmission sensor therefore spans approximately 1 order of magnitude. By changing the transmission distance this curve can be shifted to the left (shorter path length) or right (longer path length), but the span over which the sensor is linear will remain approximately 1 order of magnitude.

Reflected intensity measured by a single emitter-detector pair spaced 6 mm apart is shown in Figure 1b. Compared to the transmission sensor in Figure 1a the dynamic range of the reflectance sensor is wider, but the reflectance relative to off-line OD is still non-linear and rolls off at ODs greater than 100. As with the transmission probe, the range of maximum sensitivity of the reflectance probe can be tuned by changing the separation between the source and detector.

The BugEye sensor response measured under the same conditions as the transmission and reflectance probes is shown in Figure 1c. By combining the reflectance

signals measured by pairs of emitters and detectors at multiple separations, the combined response is linearized across a very wide range relative to off-line OD.²⁴ The useful linear range of the BugEye sensor spans approximately 4 orders of magnitude.

The effect of window thickness on the BugEye sensor response is shown in Figure 2. The sensor response through the 6.3 and 9.5 mm thick windows is nearly indistinguishable. The sensor response through the 12.7 mm thick window is consistently lower than for the 2 thinner windows by approximately 15%.

Fermentation Results

In Figure 3, the raw output of the BugEye 100 Biomass Monitor and the FSC402 Turbidity Analyzer measured during a 5L *E. Coli* fermentation are shown plotted versus the off-line OD measured at 600 nm. The relationship between the BugEye output and off-line OD is well-approximated by a linear function ($R^2 = 0.992$) across the full range of biomass encountered during the fermentation run. The FSC402 is well approximated by a linear function at OD values higher than about 8, but in lower OD range there is no clear relationship between the sensor output and the off-line OD. Near the end of the fermentation run the agitation rate was stepped from 600 to 400 rpm. Both on-line instruments showed a small but significant off-set from the linear relationship to off-line OD established at a fixed agitation rate.

Linear relationships between off-line OD and the BugEye Biomass Monitor were also observed during *S. cerevisiae* batch cultures grown over a low OD range (Figure 4) and *E. coli* fed-batch cultures grown to high OD (Figure 5). Table 1 summarizes the results of the 12 fermentations during which the on-line biomass monitor was tested. High correlation coefficients were seen for both simple and complex media. The average squared linear correlation coefficient between off-line OD and the BugEye Biomass Monitor was 0.986, for all OD measurements greater than 0.1.

Dry weight was also measured off-line for some of the fermentations. A highly linear relationship ($R^2 = 0.997$) was observed between dry weight and OD, as seen for a 5L *E. coli* batch fermentation in Figure 6. The above results suggest that a linear function is adequate to calibrate the BugEye Biomass Monitor to either OD or dry weight during *E. Coli* fermentations.

An example of applying a calibration to predict OD on-line with the BugEye Biomass Monitor is shown in Figure 7. The calibration data was collected during a 5L fed-batch *E. Coli* fermentation (fermentation number 4 in Table 1). Over the course of the calibration run, agitation was varied between 200 and 700 rpm, aeration (using room air) was varied between 4.5 and 4.8 LPM, and temperature was fixed at 30C. The data shown in Figure 5 were measured during a subsequent fermentation (fermentation number 6 in Table 1) run under similar conditions. The relative root-mean-squared error (RMSE) between the OD predicted by the calibrated BugEye Biomass Monitor and the off-line OD was 14%, for ODs greater than 0.5. For ODs less than 0.5 the absolute RMSE was 0.1.

In addition to that described above, five other fermentations were run in calibrated mode. The prediction results are summarized in the last two columns in Table 1. Overall, the relative RMSE was 15%, for ODs greater than 0.5. For ODs less than 0.5, the absolute RMSE was 0.2. In general, the on-line OD predictions were most accurate when run under similar conditions to the fermentation that was used for calibration. However, the on-line OD of *S. cerevisiae* (fermentation number 12 in Table 1) was accurately predicted from an *E. Coli* fermentation (number 2 in Table 1) run under similar conditions.

Conclusions

Comparison testing between and transmission and reflectance immersion sensors based on a single emitter-detector pair and the non-invasive BugEye 100 Biomass Monitor demonstrates the advantage of employing reflectance at multiple emitter-detector spacings: greatly increased linear dynamic range. Minimal sensitivity of the BugEye sensor response to window thickness was observed across the range typical of commercial glass fermentor vessels and stainless steel vessels with glass windows.

When tested on *E. coli* and *S. cerevisiae* fermentations run under a variety of conditions, the BugEye 100 Biomass Monitor responded linearly to changes in biomass over a range of 0.1 to 130 OD units. This high linearity was observed for both simple and complex media, run in small (5L) and large (28L and 250L) fermentors. Some

sensitivity to agitation rate was observed. However, when calibrated and then run under similar fermentation conditions, the on-line monitor was able to predict OD with high accuracy.

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Table 1.

Ferment. Number	Org.	Vessel Size	Med.	Max OD	No. of Ref. ODs	R²	% Error (OD>0.5)	Abs. Err. (OD<0.5)
1	<i>E. Coli</i>	5L	Comp	22	18	0.992		
2	<i>E. Coli</i>	5L	Comp	6	8	0.989	15.3	0.06
3	<i>E. Coli</i>	5L	Comp	13	6	0.997	15.6	0.07
4	<i>E. Coli</i>	5L		130	8	0.996		
5	<i>E. Coli</i>	5L		37	8	0.998	10.1	
6	<i>E. Coli</i>	5L		133	7	0.995	14.4	0.10
7	<i>E. Coli</i>	5L	Simp	6	5	0.995		
8	<i>E. Coli</i>	5L	Simp	5.5	6	0.998	24.5	0.34
9	<i>E. Coli</i>	28L		0.8	6	0.990		
10	<i>E. Coli</i>	250L		4.2	8	0.933		
11	<i>E. Coli</i>	250L		26	11	0.974	15.8	0.52
12	<i>S. cerevis.</i>	5L		2.4	8	0.980	12.8	0.08
Average:						0.986	15.5	0.19

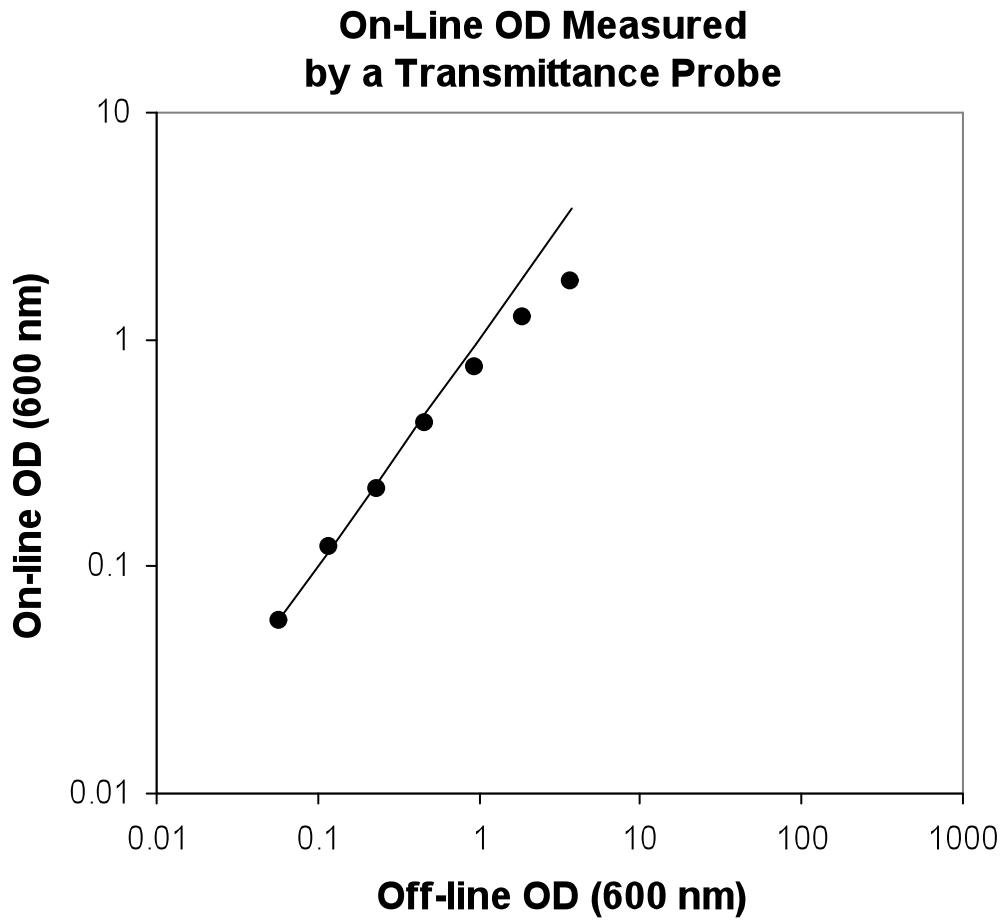


Figure 1a

Reflected Intensity for a Single Emitter-Detector Pair

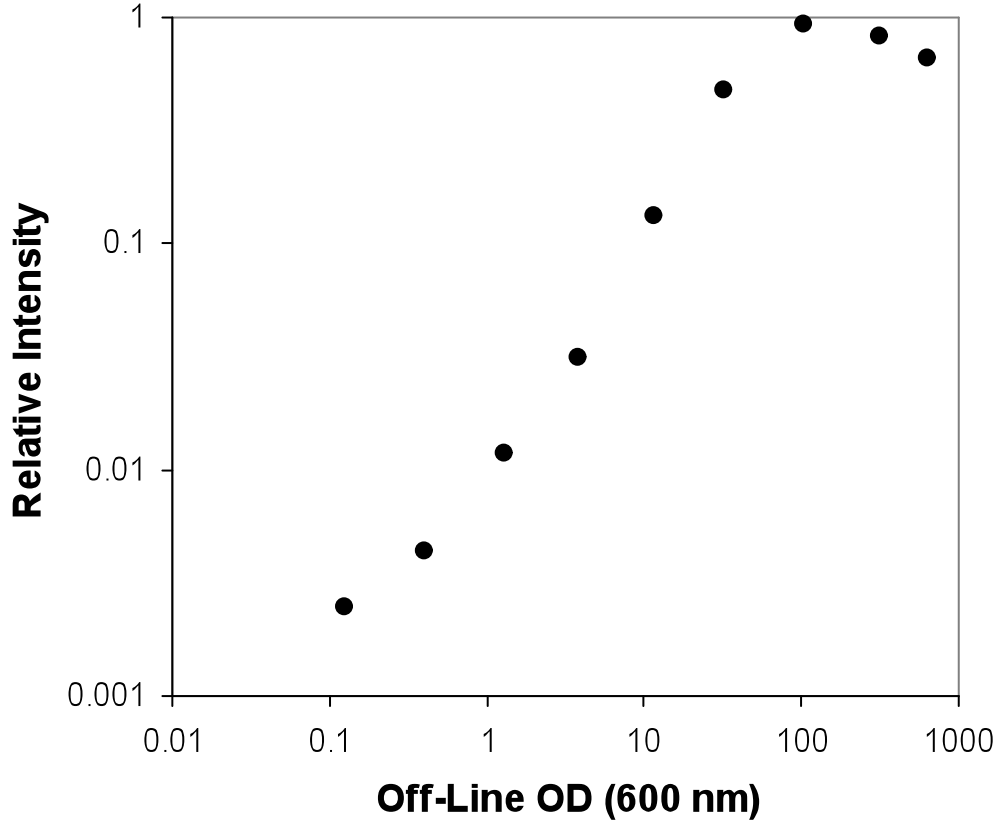


Figure 1b.

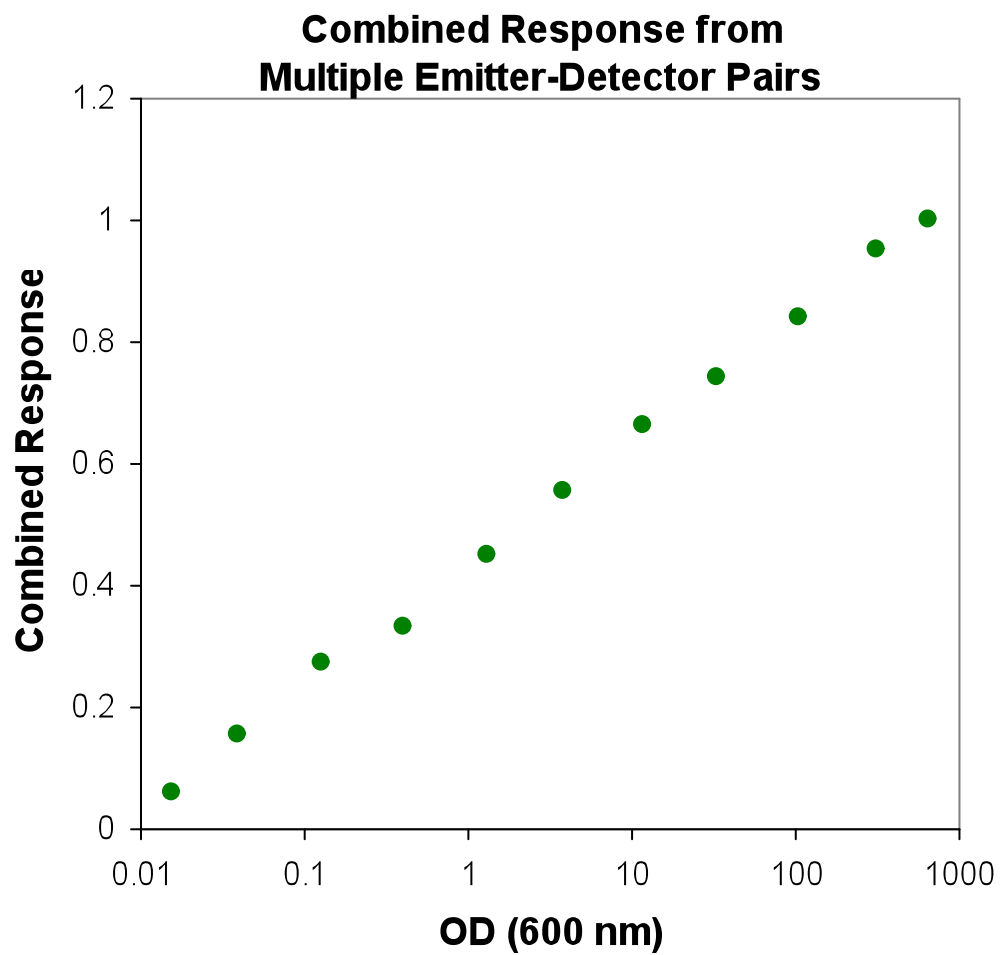


Figure 1c.

Effect of Glass Thickness on Combined Response

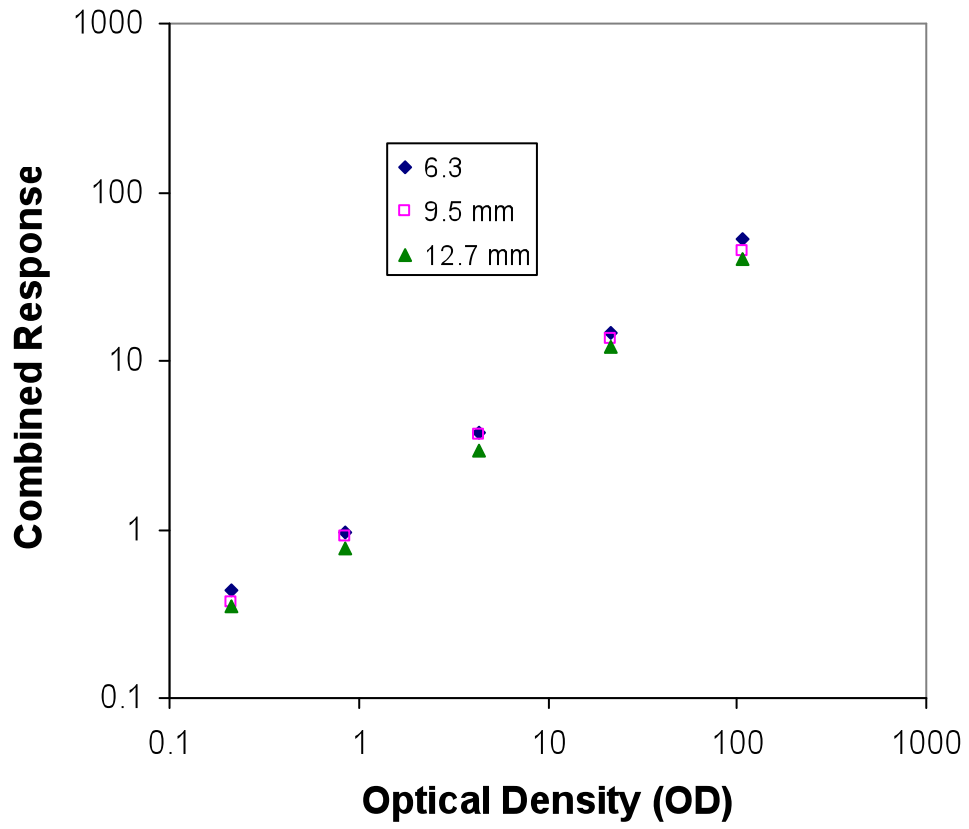


Figure 2.

5L *E. Coli* Batch Culture

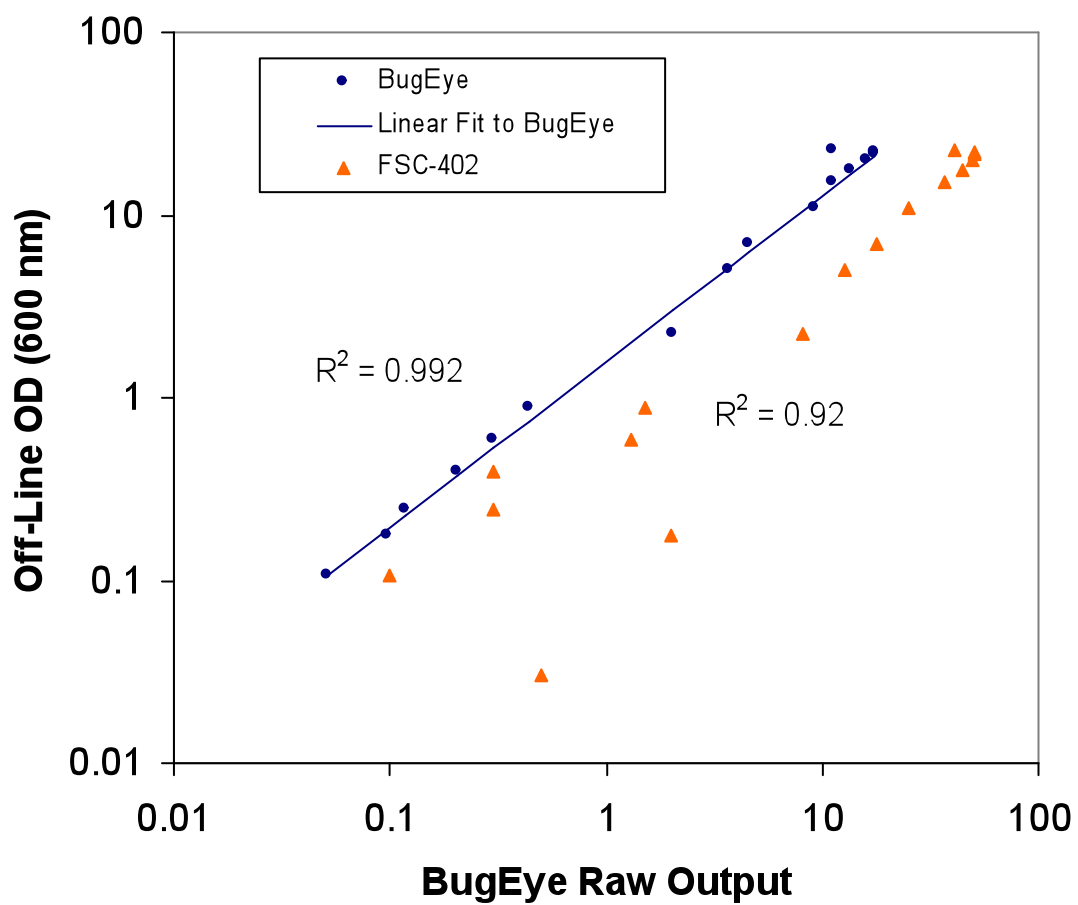


Figure 3.

5L *S. cerevisiae* Batch Culture

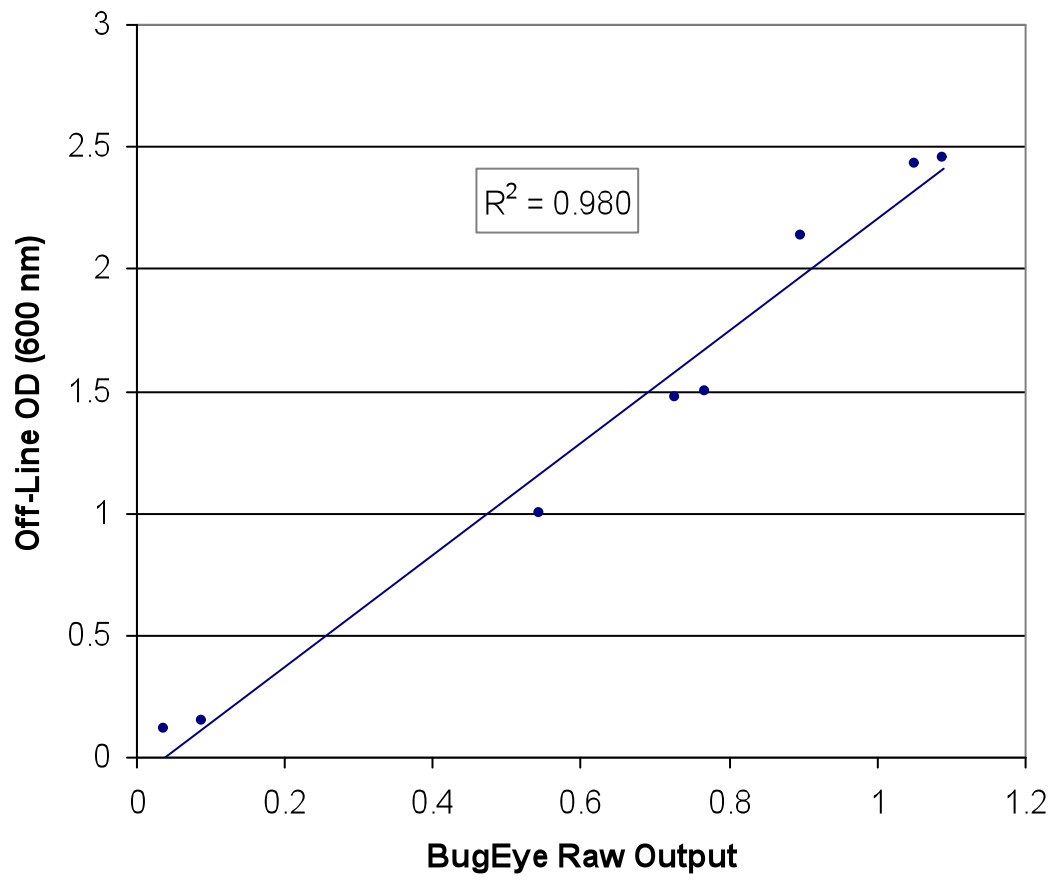


Figure 4.

5L E. Coli Fed-Batch Culture

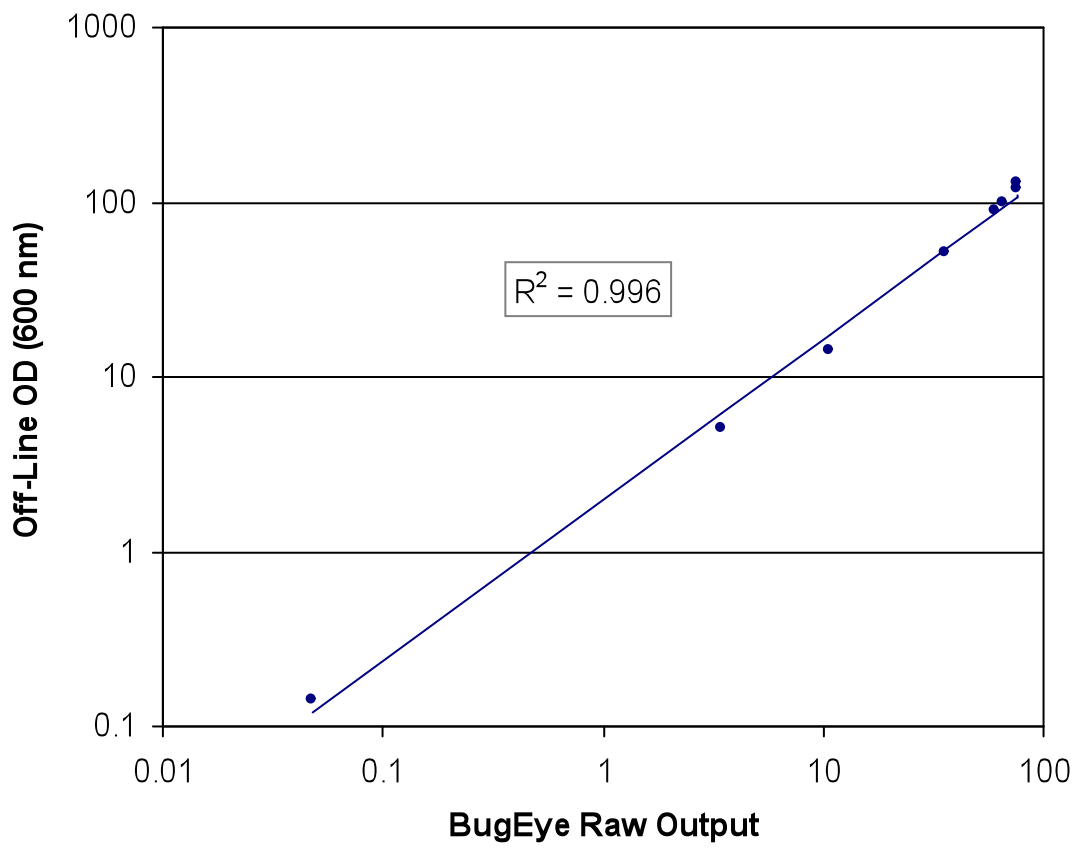


Figure 5.

5L *E. Coli* Batch Culture

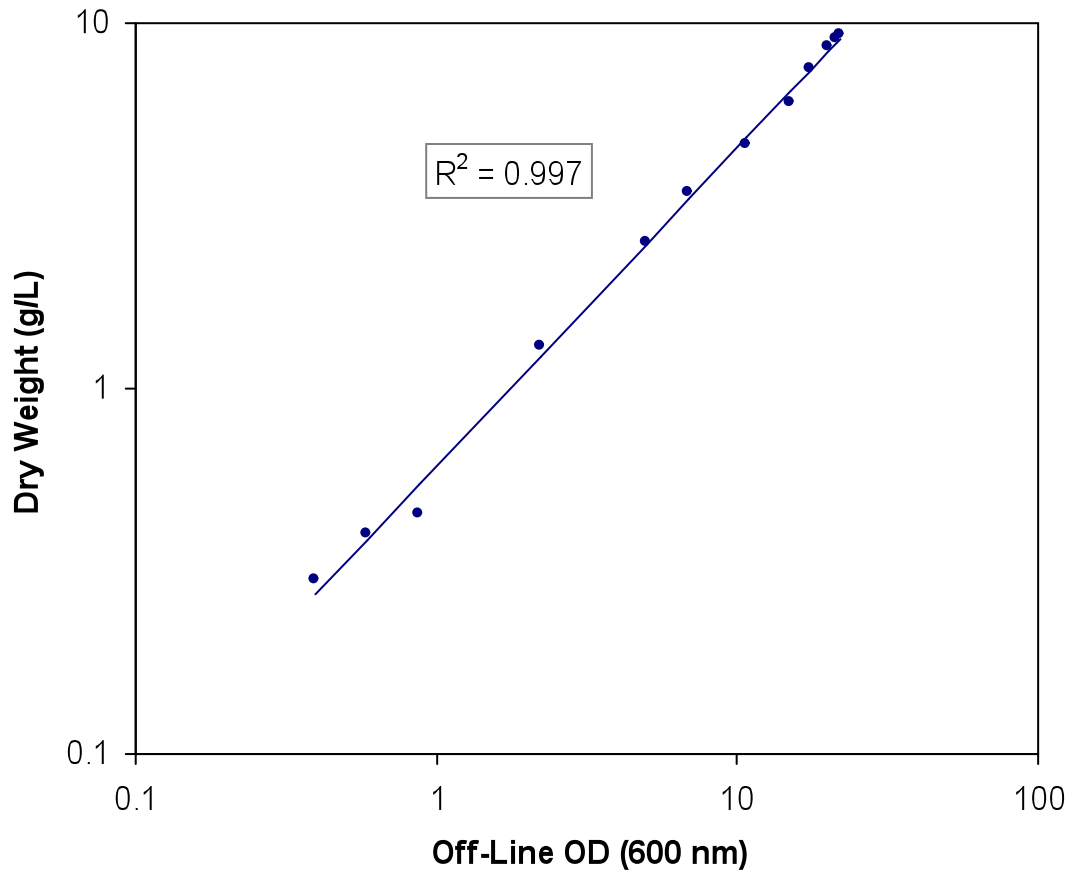


Figure 6.

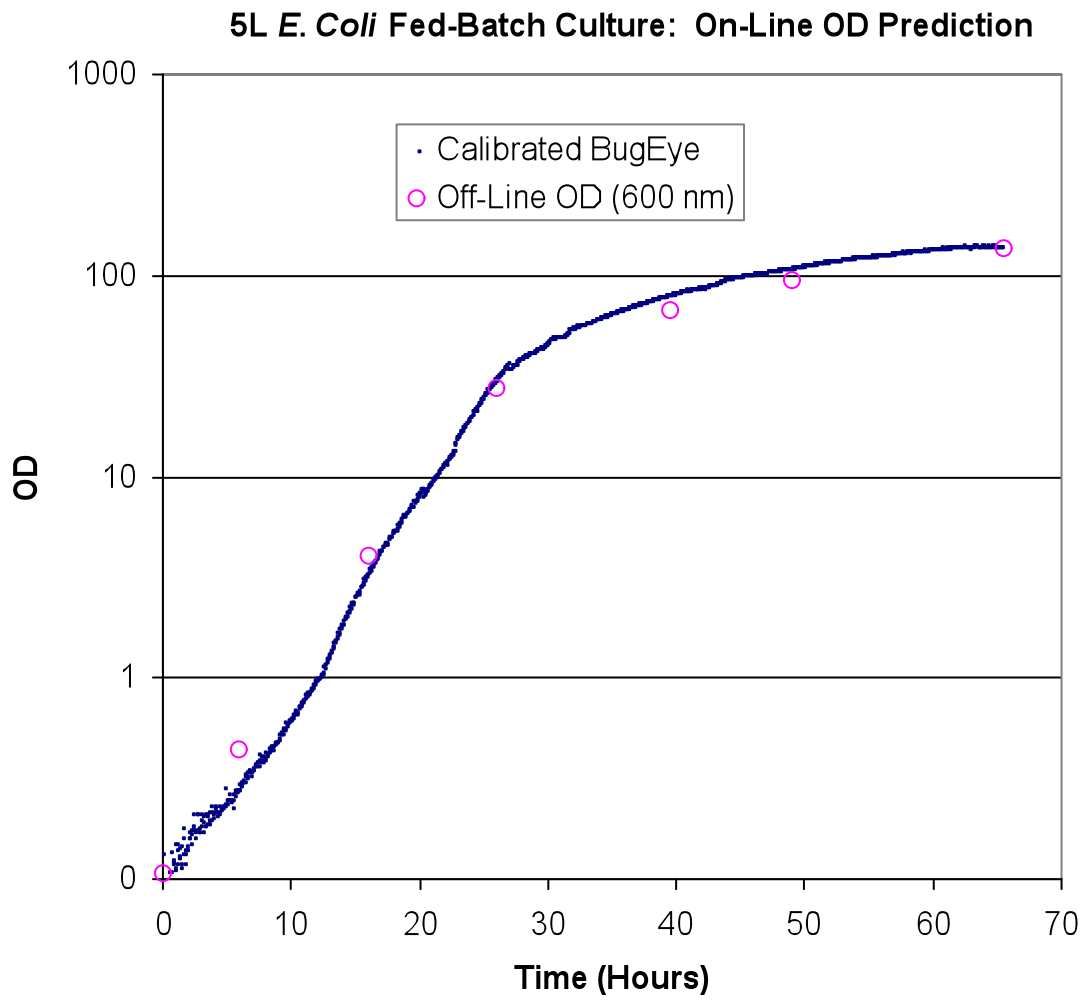


Figure 7.