Rapid Determination of Total Biomass from a Yeast Fermentation Using Sequential Injection

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An assay for the measurement of the total biomass in an unfiltered fermentation sample has been developed using a simple sequential injection system, with either total transmitted light or light scattered at 90° as the analytical signal. The biomass is determined without pre-treatment or dilution of the original sample. In the past, the use of unfiltered bioprocess samples in flow injection has been avoided due to problems associated with a complex sample matrix, *e.g.*, cell aggregation, clogging of the flow system, cells sticking to tubing walls and cross contamination. The sample range for the assay is 0.2 to at least 80 g l^{-1} biomass with relative standard deviations of <3%. The results from a single assay are available 3 min after assay initiation.

Keywords: *Biomass; yeast fermentation; turbidimetry; flow injection; bioprocess monitoring*

Introduction

A common theme in bioprocess literature is the measurement of biomass.[1–3] Total biomass is an important parameter that is often used as a measure of product formation, substrate consumption, and process disturbances.[4] More precise control of the bioprocess can be obtained by rapid and frequent measurement of total biomass. Few advances have been made recently in the area of biomass monitoring. At present, most measurements are indirect, typically relating *in-situ* measurements of dissolved oxygen uptake or carbon dioxide evolution to the total biomass. The traditional direct determination of biomass by measurement of dry mass is both time and labour intensive and is not suitable for on-line bioprocess control. Other methods such as total wet biomass and turbidimetry are much faster, but are less accurate.[3,5] The obvious trade-off seems to be speed *versus* accuracy. The most accurate and sensitive method is direct counting, either by electronic counting ('Coulter' counter), flow cytometry or microscopic enumeration,[6] yet these laboratory techniques are much less suitable for process-control applications. Other factors that will influence the method used to measure the total biomass include the type and number of different organisms and the medium that is used for the bioprocess. The general working assumption for all methods currently in use is that the medium does not contain any suspended or undissolved matter, *i.e.*, the microbes are the only particles in the process. Assays that quantitate cellular components for correlation to the total biomass, such as total deoxyribonucleic acid (DNA),[5] ribonucleic acid (RNA),[7] adenosine 5′-triphosphate (ATP)[1] or protein[7–10] content, can accurately measure the component of interest, but the correlation to biomass is not always straightforward.[5] Various spectroscopic methods have also been suggested, including fluorescence of intracellular cofactors such as reduced nicotinamide–adenine dinucleotide (NADH) and reduced nicotinamide–adenine dinucleotide phosphate (NADPH).[2,11,12]

Regardless of the advances made in the area of biotechnology, there is still a need for an analytical method that can quickly measure or estimate the total biomass of a system. It should have a wide dynamic range and a high linearity of response. Ideally, the method should sample directly from the bioprocess and eliminate or minimize preparation for analysis, thus more closely approximating real-time unattended continuous monitoring.

As a result, many researchers have focused their efforts on the development of such probes and/or assays.[3,8,11,13,14] Emphasis has been placed on the development of the spectrophotometric probes that are either non-invasive or can be placed *in situ*. The most notable examples include fluorometric measurement of reduced nicotinamide nucleotides[11] and Fourier transform infrared (FTIR) photoacoustic spectroscopy.[8] These non-invasive monitoring probes would be ideal, but have an insufficient dynamic range and are non-linear. *In-situ* sensors must be sufficiently robust to withstand sterilization and cope with the complexity of the environment. They also suffer from instability and have short lifetimes. On the other hand, the use of turbidity as an analytical signal for the measurement of biomass is well documented, but has rarely been exploited. Benthin *et al.*[13] described an on-line flow injection analyser using absorbance as the analytical signal. This analyser suffers from a poor dynamic range (0–4 g l^{-1}). As absorbance measurement can be made in real time and only a small amount of sample is required, it appears to form the basis of a nearly ideal method.

The platform of the biomass assay developed in our laboratory was an aerobic fermentation of *Saccharomyces cerevisiae*. The assay utilizes both turbidimetric (absorbance) and nephelometric measurements at a wavelength that is not absorbed by the liquid medium. In contrast to previously described spectroscopic methods, the optical and mechanical components used in this assay are simple, robust, and inexpensive. The assay uses a sequential injection (SI) system (Fig. 1) to sample a precise volume of biomass obtained from the fermentor and to deliver it to a flow cell (Fig. 2) where it is quickly mixed. The SI technique has features lacking in traditional stopped-flow injection. For instance, the sample size is not defined by a fixed sample loop, but rather by the length of time for which the pump pulls sample into a holding coil. This time can be easily changed *via* the computer keyboard, resulting in no hardware changes being necessary. The multi-position valve allows several samples, standards, and reagents to be available, which is not possible with flow injection. Finally, methods can be easily optimized by changing parameters such as sample size and flow rate without any reconfiguration of the hardware. The theory of flow and sequential injection has been discussed elsewhere.[15,16]

Experimental

Sequential Injection Analyser

A schematic diagram of the entire SI system is shown in Fig. 1. The pump and valve movements are precisely timed and

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controlled by the FIALab program. The peristaltic pump can rotate in both a clockwise and anti-clockwise direction with instant 'no-coast' stop-and-go action. By changing the direction of the pump, the sample is aspirated into the holding coil and then expelled to the flow cell for analysis.

The geometry of the flow cell was designed to accommodate optical fibre bundles facing each other for the absorbance measurements (turbidimetry) or at right angles for the scattered light measurements (nephelometry). A diagram of the flow cell is shown in Fig. 2, where the fibre optic bundles are shown collecting the absorbance signal. A stir bar contained within the flow cell was controlled manually to mix the biomass sample as it entered the dilution flow cell.

The analyser consisted of a peristaltic pump (Model C4V, Alitea USA, Medina, WA, USA) coupled to an electrically actuated 10-port selection valve (Model SD10P, Valco, Houston, TX, USA). The valve came equipped with 1/16″ fittings. All connections and plumbing were made from 0.8 mm Teflon tubing (Upchurch, Oak Harbor, WA, USA). The pump tubing, Viton No. 13 (Cole-Parmer, Chicago, IL, USA), was connected to the Teflon tubing with flow injection

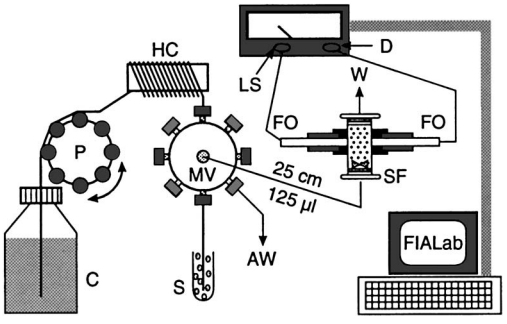

Fig. 1 Schematic diagram of the sequential injection (SI)-biomass manifold. The peristaltic pump (P), 10-position multi-port valve (MV), and data acquisition system are all computer controlled *via* FIALab. LS, Light source; D, detector; W, waste; AW, auxillary waste; S, sample; C, carrier; FO, fibre optic bundles; SF, stirred flow cell; and HC, holding coil. See text for a full description of the components. Tubing diameter, 0.8 mm; flow rate, 2.2 ml min^{-1}; and carrier, de-ionized water with 2 ppm Brij-35.

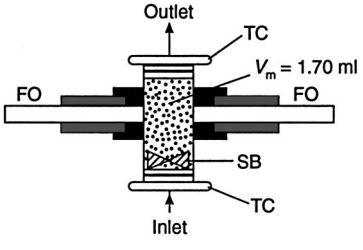

Fig. 2 Stirred flow cell used as both dilution chamber and detection cell. The cell is made of 1.0 mm i.d. glass tubing with Teflon caps (TC) at each end. Tubing used for the inlet and outlet of the flow cell is 0.8 mm i.d. Teflon. The cell is held in position by a cell holder produced in-house, which allows measurement in either 180 ° or 90° mode. SB, Stir bar; and FO, fibre optic bundles. Flow cell internal volume, V_m = 1.70 ml.

Table 1 Fermentation process

Fermentation broth—	
Yeast extract	25 g l^{-1}
$(NH_4)_2SO_4$	10 g l^{-1}
KH_2PO_4	3 g l^{-1}
$MgSO_4$	2 g l^{-1}
Antifoam	1 g l^{-1}
Feeds—	
Glucose	50% w/w
NH_4OH	20% v/v
H_2SO_4	10% v/v
Parameter—	
Airflow	3 l min^{-1}
Temperature	30.0 °C
pH	4.50
Agitation	500 r.p.m.
Glucose feed	0–30 g h^{-1}

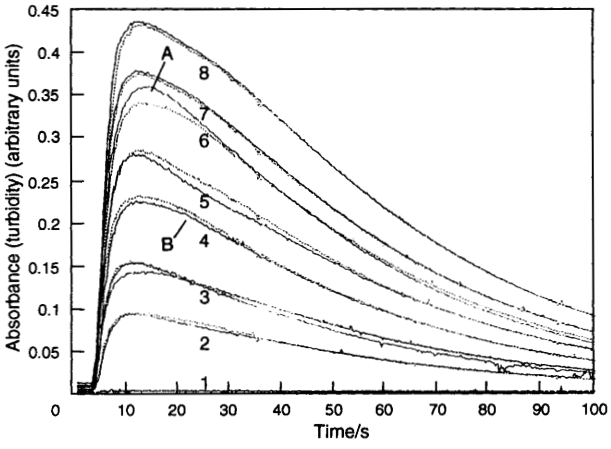

Fig. 3 Typical response profiles (performed in triplicate) observed with various concentrations of raw biomass using the SI biomass assay in the turbidimetric mode. Data acquisition begins when the sample is pushed to the flow cell and continues for 100 s. The peak with a sharp leading edge and long trailing edge is typical of what is observed with a tank with continuous dilution and stirring. Any point along the time axis can be used to determine the biomass. Total biomass (determined by the dry mass method) and fermentation run times, respectively: 1, 0.21 g l^{-1}, 0 h; 2, 13.27 g l^{-1}, 15 h; 3, 23.31 g l^{-1}, 19 h; 4, 34.25 g l^{-1}, 23 h; 5, 44.37 g l^{-1}, 27 h; 6, 53.42 g l^{-1}, 32 h; 7, 60.49 g l^{-1}, 36 h; and 8, 76.07 g l^{-1}, 48 h. A, 32 h sample; and B, 23 h sample.

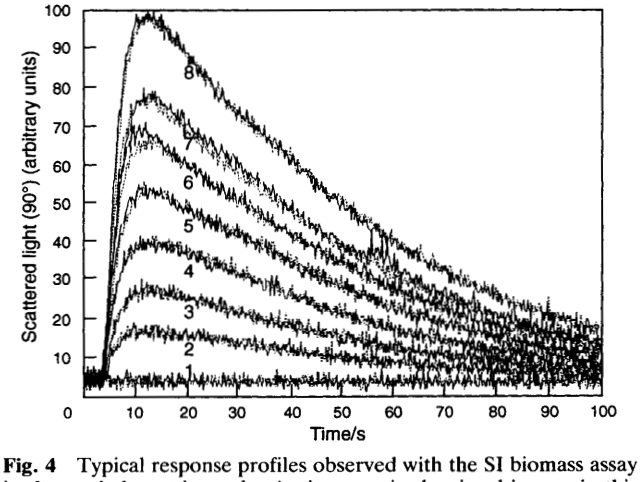

Fig. 4 Typical response profiles observed with the SI biomass assay in the nephelometric mode. An increase in the signal is seen in this mode as the biomass concentration increases. This is as expected, as there will be more light scattering particles to scatter the light at 90° at the highest biomass concentrations. This detection mode is more sensitive at lower biomass concentrations. Numbers on the curves indicate the total biomass (determined by the dry mass method) and the fermentation run time, respectively: 1, 0.21 g l^{-1}, 0 h; 2, 13.27 g l^{-1}, 15 h; 3, 23.31 g l^{-1}, 19 h; 4, 34.25 g l^{-1}; 23 h; 5, 44.37 g l^{-1}, 27 h; 6, 53.42 g l^{-1}, 32 h; 7, 60.49 g l^{-1}, 36 h; and 8, 76.07 g l^{-1}, 48 h.

analysis (FIA) tubing adaptors (Upchurch, Oak Harbor, WA, USA). A flow cell (Fig. 2) with an internal volume (V_m) of 1.70 ml served as both reactor and detector vessel. It was constructed of glass tubing (1.0 cm i.d. × 3.8 cm) and Teflon caps (TC). It contained the cross-shaped magnetic stir bar (9.5 cm × 9.5 cm, No. 77-8640-02, PGC Scientific, Gaitherburg, MD, USA) that kept the cell suspension well-mixed. The stir bar was controlled by a remote-controlled magnetic stirrer (Variomag-USA, South Daytona, FL, USA), which was kept at a fixed distance (3 cm) from the stirred cell. The black delrin cell holder was designed to allow signal collection at 180° and 90° from the incident light and to hold the flow cell and fibre optic bundles in place. The fibre optic bundles were 60.9 cm long with a diameter of 0.23 cm. Both the stirred flow cell and cell holder were designed and built in-house.

A colorimeter (Model PC-701, Brinkman Analytical, Westbury, NY, USA) served as both the light source and detector. Light was delivered to the flow cell and the signal was delivered to the detector by optical fibre bundles (Twarty Technology, Darien, CT, USA).

All hardware was controlled by a data acquisition board (Model ADA-1100, Real Time Devices, State College, PA, USA) from a personal computer (PC) compatible Model 486DX computer (Comtrade, City of Industry, CA, USA). A software package (FIALab, Alitea USA, Medina, WA, USA) was used to control the pump and valve movements as well as collect the data from the colorimeter. Further data manipulation and analyses were performed using MatLab (MathWorks, Natick, MA, USA) and CA-Cricket Graph III (Computer Associates, Islandia, NY, USA).

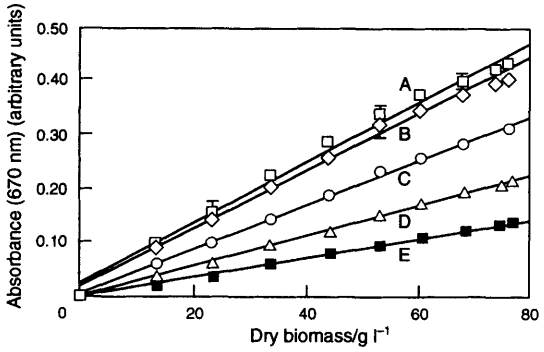

Fig. 5 Turbidimetric (absorbance) calibration plots constructed using the response curves in Fig. 3. The signal at five different time points (peak maximum, 20 s, 40 s, 60 s, and 80 s) is plotted against the biomass concentration (determined independently by the dry mass method). A, Peak maximum, B, 20 s; C, 40 s; D, 60 s; and E, 80 s.

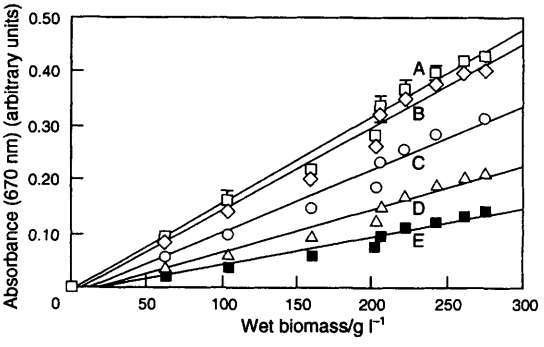

Fig. 6 Turbidimetric calibration plots determined using the response curves in Fig. 3. The signal at each time point (see Fig. 5) is plotted against the biomass concentration (determined independently by the wet mass method). A, Peak maximum; B, 20 s; C, 40 s; D, 60 s; and E, 80 s.

Biomass Assay

The assay is initiated by rinsing the flow cell with carrier for 30 s. Next, the valve moves to the sample port and the pump reverses direction to pull up enough sample to fill the sampling line (3 s). The valve is then switched to the auxillary waste port and the pump rinses any sample that is in the holding coil (20 s). In the next step, the valve moves back to the sample port and a 38 µl sample is pulled into the holding coil (1.0 s). Finally, the sample is pushed to the stirred flow cell and the signal is collected (170 s). The entire assay is completed in under 4 min. Although the assay has not been implemented on-line, we believe that a connection by steam or by a chemically sterilizable barrier[17] is all that is required.

Chemicals

Yeast extract, agar and peptone were obtained from Difco (Detroit, MI, USA). Brij-35 (30% w/v) was obtained from Sigma (St. Louis, MO, USA). All other chemicals were reagent grade or higher. The wash solution was prepared using de-ionized water (Barnstead NANOpure II, Dubuque, IA, USA) with 2 ppm Brij-35 to prevent cells from adhering to the tubing walls. The sample inlet line was rinsed with 0.5 mol l^{-1} acetic acid between each biomass sample to ensure no cross-contamination occurred. Bromothymol Blue (BTB) was obtained from Aldrich (Milwaukee, WI, USA) and was

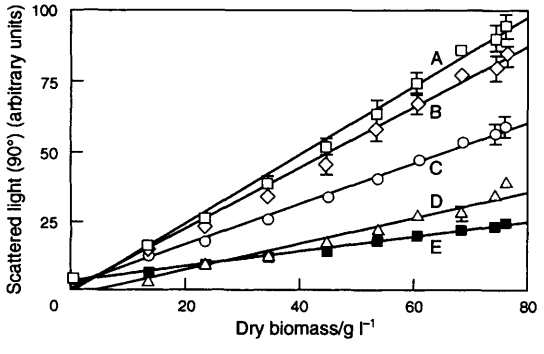

Fig. 7 Nephelometric calibration plots determined using the response curves in Fig. 4. Signal at each time point (see Fig. 5) is plotted against the biomass concentration (determined independently by the dry mass method). A, Peak maximum, B, 20 s; C, 40 s; D, 60 s; and E, 80 s.

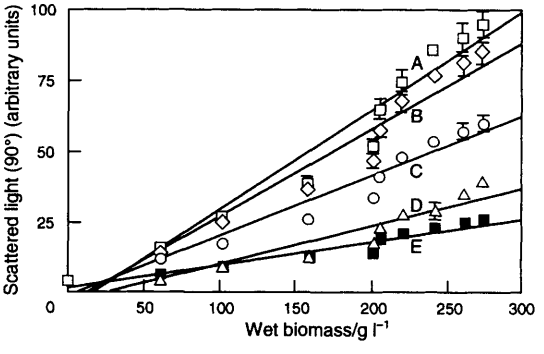

Fig. 8 Nephelometric calibration plots determined using the response curves in Fig. 4. Signal at each time point (see Fig. 5) is plotted against the biomass concentration (determined independently by the wet mass method). A, Peak maximum; B, 20 s; C, 40 s; D, 60 s; and E, 80 s.

prepared as a stock solution of 0.01 mol l^{-1} in 20% aqueous ethanol. Working solutions were diluted from the stock using distilled de-ionized water.

Samples were obtained directly from a feed-batch aerobic fermentation of *Saccharomyces cerevisiae* and were not washed, filtered or otherwise pre-treated prior to the assay.

Fermentation

Fermentation broth (3 l) was prepared using the recipe given in Table 1. The fermentor vessel containing the broth, glucose feed, water for the acid, and base feeds, and all tubing lines were autoclaved for 45 min at 121 °C. On cooling, the appropriate amounts of acid and base were added to their respective flasks. The fermentation vessel was cooled to 30 °C before inoculation. The pH was maintained at 4.5 by the acid and base feeds. The glucose feed was automated *via* a program written in-house for the Bioflo III 5 l fermentation unit (New Brunswick Scientific, Edison, NJ, USA). Acid, base, and glucose feed rates, as well as agitation, temperature, and percentage O_2 were recorded by the program and saved to disk.

Samples were taken automatically at the time of inoculation (time zero) and every 4 h thereafter by a refrigerated autosampler designed in our laboratory, and were delivered into poly(tetrafluoroethylene) (PTFE) bottles. A typical fermentation ran for 60–72 h before one of the feeds was exhausted.

Dry and Wet Mass Measurements

The total biomass was determined by both wet[18] and dry[12,18] weight methods. Five or 10 ml of fermentation sample were accurately delivered to a tared centrifuge tube and the mass was recorded. The sample was centrifuged for 3 min and the broth was discarded. The biomass pellet was spun again to ensure that all the broth was decanted. The pellet was then weighed and recorded. This measurement was used to calculate wet mass. The pellet was resuspended in distilled water and centrifuged. This was repeated two or three times. The final washed pellet was resuspended in water and poured into a tared aluminium weighing pan and dried at 85 °C for 20 h. This was used to calculate the dry mass. Both wet and dry mass samples were prepared in duplicate.

Results and Discussion

Stirred Flow Cell Characteristics

Dilution in the flow cell was examined using the same protocol as for the biomass samples except that a dye solution was used in place of the sample. The reproducibility of the dilution graphs obtained with the flow cell was determined from pentuplicate runs using various concentrations of BTB as sample. The BTB served as a visual tracer to investigate the stirred tank properties of the flow cell and was not expected to stimulate the fermentation samples. However, the yeast cells are about 5 μm in diameter and are expected to mix similarly to the dye solution. The relative standard deviation (RSD) ranged from 0.29% to 6.3%. The higher errors were always associated with the lower signals at later measurement times (*i.e.*, higher dilution due to continuous flow through the flow cell).

Biomass Measurements

The fermentations run during the development and characterization of the biomass assay were designed to have a high biomass yield. The final biomass was typically between 70 to 80 g l^{-1} dry biomass. This was desired in order to test the ability of the flow system to handle the concentrated fermentation broths without becoming clogged. The recipe for the broth that was used, as well as the feeds and physical parameters for the fermentations are listed in Table 1.

Typical response graphs for the biomass assay are shown in Fig. 3 and 4. Fig. 3 shows the signal obtained in the absorbance detection mode. Note that the curves have typical rise-and-fall sections associated with the flow characteristics of a mixed

Table 2 Calibration graphs for the two detection modes and two standardization methods

			Calibration plot		
Detection mode	Standard method	Signal time/s	Slope arbitrary units g^{-1} l	*y* Intercept/ arbitrary units	r^{2*}
Turbidimetric	Dry	Peak maximum	0.0056	0.021	0.991
Turbidimetric	Dry	20	0.0053	0.016	0.992
Turbidimetric	Dry	40	0.0041	0.003	0.999
Turbidimetric	Dry	60	0.0028	−0.0004	1.00
Turbidimetric	Dry	80	0.0018	−0.002	0.999
Turbidimetric	Wet	Peak maximum	0.0016	−0.008	0.984
Turbidimetric	Wet	20	0.0015	−0.011	0.981
Turbidimetric	Wet	40	0.0012	−0.015	0.971
Turbidimetric	Wet	60	0.0008	−0.013	0.968
Turbidimetric	Wet	80	0.0005	−0.01	0.963
Nephelometric	Dry	Peak maximum	1.231	−1.023	0.994
Nephelometric	Dry	20	1.086	0.004	0.996
Nephelometric	Dry	40	0.741	0.927	0.996
Nephelometric	Dry	60	0.462	−1.814	0.950
Nephelometric	Dry	80	0.283	−2.10	0.997
Nephelometric	Wet	Peak maximum	0.348	−6.16	0.944
Nephelometric	Wet	20	0.308	−4.632	0.949
Nephelometric	Wet	40	0.21	−2.25	0.950
Nephelometric	Wet	60	0.129	−3.427	0.877
Nephelometric	Wet	80	0.08	0.866	0.954

* r = Correlation coefficient.

tank. As the fermentation progresses from 0 to 48 h the amount of biomass increases. Above 0.4 absorbance units, the correlation between absorbance and total biomass fails and therefore turbidity values obtained at delay times longer than 10 s have been used. Each biomass sample was run three times. For the most part, the signal was noise-free and the dilution curves for the assays were reproducible. Differences were due to the fact that cell samples were heterogeneous. As yeast cells tend to stick together in small clusters, a clump of cells would cause an aberrant signal, which may be either higher or lower than expected, depending on whether the sampling step or absorbance monitoring had been affected. This is most easily seen with the 32 and 23 h samples labelled A and B, respectively, in Fig. 3. Fig. 4 shows the responses collected in the nephelometric detection mode. The signal was smaller and noisier than with in absorbance mode, yet the dilution curves were still reproducible, particularly for the higher biomasses.

Calibration

The biomass assay was calibrated against both classical wet and dry biomass methods. Because the dilution curves are reproducible, the analytical signal does not necessarily have to be the maximum signal. Any time point along the curve can be chosen as the analytical signal. This is advantageous when the biomass concentration becomes so high that the maximum signal falls outside the linear range of the detector. When this occurs, a signal corresponding to a greater dilution can be used. Arbitrary time points of the peak maximum, as well as 20, 40, 60, and 80 s after the sample was pushed to the dilution flow cell, were used to prepare calibration graphs using both wet and dry biomass methods as the standard. For both detection modes, the exact time points reported are an average of 11 points bracketing the actual time, resulting in the averaging of 2 s of data acquisition, which provides some signal averaging. This was especially important for the nephelometric detection mode, which was noisier than the turbidimetric mode.

The calibration graphs for the absorbance mode are given in Figs. 5 and 6. In Fig. 5, the apparent absorbance at 670 nm is plotted against the total biomass determined using the dry mass method. In Fig. 6, total biomass using the wet mass method was used. Similarly, Figs. 7 and 8 show the calibration graphs for the same time points using the nephelometric detection mode. Table 2 lists the calibration equations determined from the plots shown in Figs. 5–8. It should be noted that the 0,0 data pair was omitted in the fitting of the calibration graphs, because the analytical signal is based on light scattering rather than a strict absorbance reading. An empty flow cell will have no light scattering (and zero absorbance). Instead, the light scatter resulting from a fermentation sample containing broth only was measured following the protocol used for the SI assay and that signal was subtracted from the gross signal from each sample.

The dry mass method produced more linear calibration graphs than the wet mass method. We believe this is due to greater error in the wet mass method (data not shown) rather than in the biomass assay. Errors associated with the wet mass method are primarily due to the difficulty in separating the fermentation broth completely from the yeast pellet without losing any of the pellet. This may in part contribute to the loss of linearity in the calibration graphs around 200 g l^{-1}. It is also possible that the signal is not linear beyond 200 g l^{-1} wet mass.

It was expected that the time dependence of the slopes of the calibration graphs for the two detection methods would be the same. However, this was not the case for the normalized curves. The time dependence of the slopes was linear for the turbidimetric detection mode, but not for the nephelometric mode. This is probably due to the fact that the signal for the nephelometric mode is not only noisier than the turbidimetric mode, but is also half the magnitude (apparent absorbance of the most concentrated sample at the peak maximum in the turbidimetric mode is 0.432, for the nephelometric mode it is 0.191).

The calibration graphs were used to predict the biomass in a subsequent fermentation run. The results are shown in Table 3. If the fermentation samples with a total dry biomass of less than 1 g l^{-1} are disregarded (as the deviations in both methods are high), the RSDs for the biomass predictions using the SI biomass assay and of the same order as for the more precise gravimetric method.

Generally, it appears that the SI analysis method yields a higher estimation of the total biomass than the dry mass method. Although the reason for this is unknown, it is possible that some of the cells break up during the sampling and dilution process, resulting in a larger number of scattering particles without a concomitant increase in cell mass. A *t*-test was performed on the data and the differences between the traditional dry mass measurement and the SI biomass measurements were found not to be significant ($p = 0.01$).

Table 3 Prediction of total biomass using standard graphs from a previous fermentation

Dry biomass/ g l^{-1}	RSD (%)	SI biomass*/ g l^{-1}	RSD (%)
0.13	38.08	0.43	19.87
4.95	0	5.14	2.66
11.29	1.19	12.65	0.39
25.07	0.17	27.4	1.53
47.41	0.33	49.63	2.22
56.75	0.02	56.38	0.96
70.63	0.42	71.63	2.56

* SI = Sequential injection. t Score = 3.268 with 6 degrees of freedom. 99% Confidence limit = 3.707.

Conclusions

There is a wealth of literature that underscores the need for a simple technique that is capable of fast, on-line determinations of total biomass.[2] The sequential injection technique utilizing a stirred flow cell fulfills this need as it is fast and allows the calibration graphs generated from one fermentation to be used to predict or estimate the total biomass in subsequent fermentations. Although other fermentations besides that of *Saccharomyces cerevisiae* have not been tested, we feel that either absorbance or light scattering measurement will work on any fermentation that contains cells as the only source of particulate matter. Fungal and bacterial fermentations remain to be investigated. It is conceivable that many other types of fermentation, such as those containing additional light scattering particles (such as cellulose[7]) or mammalian cells attached to carrier beads, might be monitored by a fluorescence-based variant of this technique. This is the goal of our future research.

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