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Sequential Injection Analysis: A Versatile Technique for Bioprocess Monitoring

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With the recent advances made in biotechnology, there is a need for analytical methods that rapidly and systematically provide quantitative measurements of various system parameters. These determinations should provide a "status report" of the bioprocess with a nearly real-time response and have a wide dynamic range. Additionally, the methods should sample directly from the bioprocess to eliminate or minimize sample preparation, thus more closely approximating real-time unattended continuous monitoring.[1]

Over the past several years, our group has developed methods using both flow injection analysis (FIA) and sequential injection analysis (SIA) to bridge the gap between standard off-line monitoring methods and on-line assays. The primary contribution has been proving that FIA and SIA are viable methods for on-line monitoring and implementing these assays on small-scale (5 L) yeast fermentation. More in-depth reviews on bioprocess monitoring are available.[2−6]

The focus of this Account is on the development of methods for bioprocess monitoring using SIA to simulate the manual methods that have traditionally been used. Simulating existing manual methods serves three purposes: (a) by using existing chemistry, there is no need to "reinvent the wheel", (b) the dilemma of deciding on a method for comparison to prove the reliability of the method is avoided, and (c) the sample preparation step is eliminated from the assay. Therefore, the chemistries used in the following assays are well established, with the innovation being in the simpler, faster, and more reproducible applications.

Sequential Injection Analysis Principles

SIA evolved from FIA in order to accommodate more complex chemistries requiring several reagents[7,8] and more flexibility without the need for hardware reconfiguration. It is based on the same principles as FIA (precise sample injection, controlled dispersion, and reproducible timing) and, hence, provides the same sample handling capabilities. The most basic system is comprised of a single bidirectional pump, a holding coil, a multiposition selector valve, a reaction coil (or chamber), and a detector (Figure 1). The purpose of the holding coil is to hold the stacked sample and reagent zones without allowing them to contact the pump. A typical analysis involves aspirating the sample from its assigned port into the holding coil with

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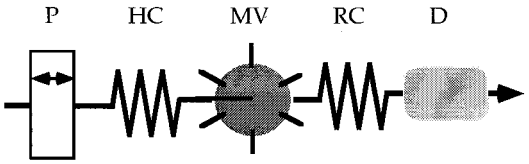

Figure 1. A simple single line sequential injection system. The bidirectional pump (P) aspirates and propels solutions through the system. The sample is introduced into the carrier stream through the multiposition valve (MV) and transported from the holding coil (HC) through the reaction coil (RC) and to the detector (D) which collects the transient SIAgram.

a precisely timed reverse movement. The pump stops, and the valve moves to the reagent position on the valve. Once again, the pump aspirates a zone of reagent into the holding coil. Finally, the valve moves to the detector position, and forward movement of the pump pushes the stacked zones through a reaction coil (or chamber) and through the detector. In a fashion similar to FIA, the sample and reagent become dispersed to form the product to be monitored between the two zones. SIA is more versatile than its predecessor, because rather than relying on a sample loop to define the sample zone, it uses precise timing to stack the required sample and reagent volumes into the holding coil. It also allows more complex chemistries to be used because the number of reagents is no longer limited by pump channels and/or confluence tees. However, SIA has some important requirements that FIA can operate without: SIA requires a computer with sophisticated software and a precise pump. A high-precision pump is necessary for SIA because all volumes are defined by pump timing rather than by a physical loop as in FIA. This allows the operator to optimize the analysis from a computer keyboard. The timing can be precise (on the order of milliseconds), and the small volumes aspirated help minimize wasting expensive reagents.

Sampling for bioprocess monitoring is of great concern.[9] The techniques described above can be used in bioprocess monitoring to aspirate a well-defined aliquot of sample from the process. We now have an analytical technique that can sample the fermentor and perform wet chemical assays that have traditionally been performed manually. This will increase the breadth of knowledge with respect to the fermentation process and allow more precise control. A fermentation that is not running as predicted or that has become contaminated can be immediately repaired or shut down before any more time or feed is wasted.

The primary benefit of using SIA for bioprocess monitoring is in the ability to directly sample the bioreactor for an on-line assay without pretreatment of the sample. The analyte may be measured in the presence of biomass[10,11] or after filtration[12,13] with a tangential-flow filter. When larger inner diameter Teflon tubing is used (0.8 mm), blockages are not a problem, even at high biomass concentrations. If blockages are a problem, a simple rinse step can be added to the assay protocol. This requires a hardware setup that is robust enough to handle samples with biomass concentrations as high as 100 g kg^{-1} without clogging.

Total Biomass

A common theme in bioprocess literature is the measurement of biomass.[14−16] Total biomass is an important parameter that is often used as a measure of product formation, substrate consumption, and process disturbances.[2,17,18] Knowledge of the total biomass provides information on the growth rate of the yeast, which provides insight into how the yeast are using the nutrient feed. Factors that will influence the method used to measure the total biomass include the type and number of different organisms and the medium which is used for the bioprocess. The working assumption for all methods presently 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. This assumption is valid for most fermentations that are fed a soluble carbon source, such as glucose.

Total biomass is also a key variable in the measurement of product synthesis, yield coefficients, and the calculation of specific rates and mass balance in the fermentation.[19] More precise control of the bioprocess can be obtained by rapid and frequent measurement of the total biomass. Classical methods for the determination of total biomass are based on either cell weight or cell enumeration. These methods put the cells in an environment that is very different from the bioprocess environment, thus adding an additional variable that may cause the result to not reflect the true cell count in the bioprocess.[20,21]

There still remains 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. Numerous direct and indirect methods have been described for biomass measurement, and some of them are reviewed below.

Direct Methods of Biomass Measurement

There are both direct and indirect methods for estimating the total and viable biomass in a fermentation. Since the SIA method used a direct method for estimation of total biomass, indirect methods such as relating in situ measurements of dissolved oxygen uptake or carbon dioxide evolution to the total biomass will not be discussed as they fall outside the scope of this mini-review. Direct measurement of biomass

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generally involves measurement of physical properties although chemical properties are utilized in estimating viability.

Cell Mass. The traditional direct determination of biomass is by measurement of dry weight. The biomass is separated from the fermentation medium by either centrifugation or filtration. The cells are then washed with water, dried overnight, and weighed. Drying also causes decomposition of biological material, and some may be lost to volatilization.[22] Of course, this method cannot be used if the medium contains particulate matter. Finally, this method is both time and labor intensive and is not suitable for on-line bioprocess control. However, regardless of the problems associated with the method, dry weight is the accepted method of total biomass measurement.

Enumeration. The most accurate and sensitive method is direct counting, by electronic counting ("Coulter" counter), flow cytometry, or microscopic enumeration.[23] The Coulter counter works by passing a suspension of cells through a small orifice that has two electrodes suspended on either side.[24] The pulse generated by each cell is recorded, giving a count of the number of cells that passed through the orifice. A clump of cells will be registered as either a single cell or an aberrant measurement, both resulting in an inaccurate count. The cell concentration must be kept low to obtain accurate counts; therefore, sample dilution is often necessary. Unfortunately, the instrumentation is expensive, and these laboratory techniques are better suited for clinical rather than process control applications.

Turbidity. Turbidity measurements for estimation of total cell biomass are widely used. Typically, turbidity is measured with a spectrophotometer quantitating the light lost from the beam by scattering. A calibration plot is made using cell count or mass as the comparison method. The turbidity of the sample is dependent on the size as well as the number of cells. Several researchers have built turbidimeters for monitoring fermentations.[25,26] These turbidimeters do not dilute the sample prior to measurement and, thus, limit their linear range. The fiber optic instrument built by Hancher et al. had a linear range to 200 Klett units, or about 20 g of wet cells/L (1 g of dry cells/8 g of wet cells).

Many other groups have developed methods to use turbidimetry for the measurement of cell biomass. Because turbidimetric measurements are linear only to about 0.4 AU, it is obvious that sample dilution is the only way to give the method a greater dynamic range. Thatipamala and co-workers developed flow cells which enabled them to effectively dilute biomass samples by decreasing the path length.[27] Benthin reported an on-line flow injection analyzer which also used optical density as the analytical signal.[28] This analyzer suffered from a poor dynamic range (0−4 g L^{-1}), just as the previous one.

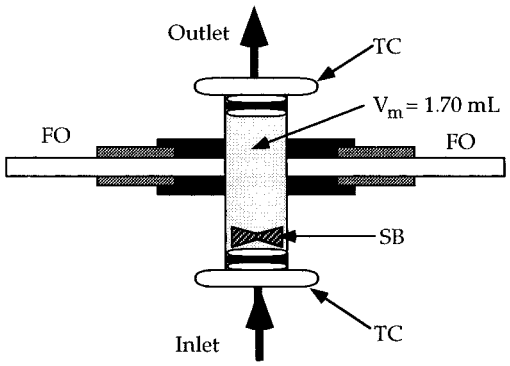

Figure 2. The stirred flow cell is used as both a dilution chamber and a detection cell. The cell is made of 1.0 mm i.d. glass tubing with Teflon caps (TC) on each end. The cell is held in position by a cell holder produced in-house and allows measurement either in a 180° or 90° mode. SB = stir bar, FO = fiber optic bundles, and V_m = 1.7 mL. Reprinted with permission from ref 10. Copyright 1994 The Royal Society of Chemistry.

The SIA assay described here uses turbidity with sample dilution to estimate total biomass. Since optical density needs only a small sample, it appears to be an ideal method. Coupling this with sequential injection analysis should make the assay a simple method for total biomass estimation on a yeast fermentation.

SIA Total Biomass Assay

The SIA total biomass assay[10] uses a simple SIA system (a single peristaltic pump and a multiport selection valve) with a specially designed stirred flow cell. The geometry of the flow cell was designed to accommodate optical fiber bundles facing each other for the optical density measurements (turbidimetry) or at right angles for the scattered light measurements (nephelometry). This type of light scattering measurement is best suited for those organisms that are more spherical, such as *Saccharomyces*. It has not been tested on filamentous organisms and may or may not be applicable. A diagram of the flow cell is shown in Figure 2. A stir bar contained within the flow cell was manually controlled to mix the biomass sample as it entered the dilution flow cell. A colorimeter served as both the light source and detector. The method of light scattering was validated in the turbidimetric mode only using three sizes of micrometer-sized polystyrene (latex) beads approximately the same size as yeast.

Yeast samples were obtained directly from a fed-batch aerobic fermentation of *Saccharomyces cerevisiae* and were not washed, filtered, or otherwise pretreated prior to the assay. The assay is initiated by rinsing the flow cell with carrier (water) followed by aspiration of a fermentation sample directly from the fermentation unit. The valve is then switched to the auxiliary waste port, and the pump rinses any sample that is in the holding coil. This step eliminates sample that may have been retained in the sampling line from the previous assay. Next, the valve moves back to the sample port, and a small sample is pulled into the holding coil. Finally, the sample is pushed to the flow cell and measured. The sampling rate is 18 h^{-1}.

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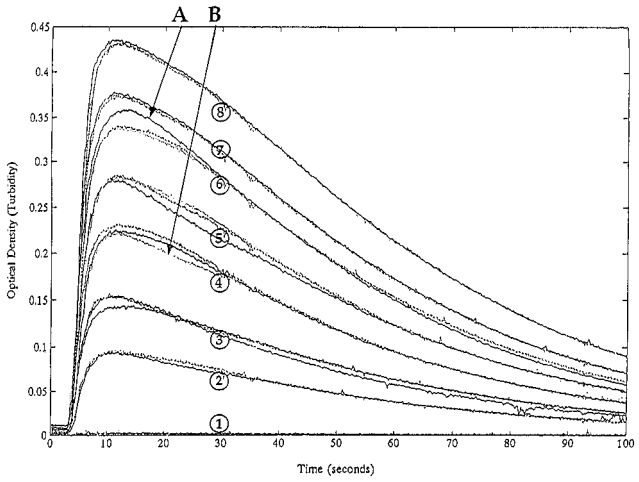

Figure 3. Typical response profiles (performed in triplicate) observed with various concentrations of raw biomass using the SI−biomass assay in the turbidimetric mode. Data acquisition begins when the sample is pushed to the flow cell and continues for 100 s. Any point along the time axis can be used to determine the biomass. Numbers on the curves indicate the total biomass as determined by the dry weight 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, (8) 76.07 g L^{-1}, 48 h. Reprinted with permission from ref 10. Copyright 1994 The Royal Society of Chemistry.

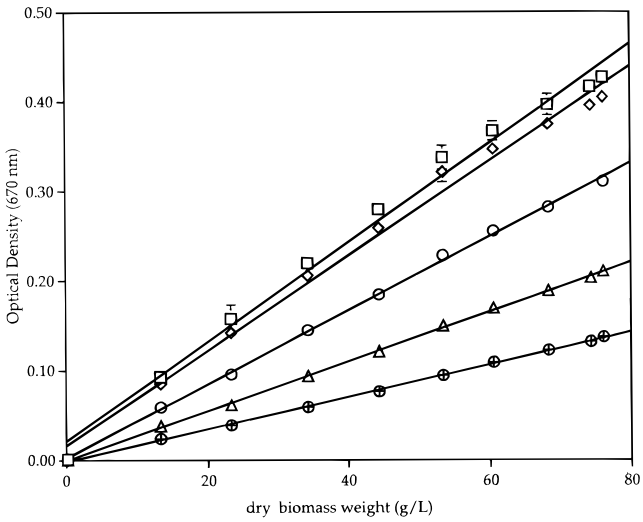

Figure 4. Turbidimetric calibration plots determined using the response curves in Figure 3. The signal at the peak maximum, 20 s, 40 s, 60 s, and 80 s is plotted against the biomass concentration as determined independently by the dry weight method. Error bars are ±1 standard deviation. Legend: peak maximum (□), 20 s (◇), 40 s (○), 60 s (△), 80 s (⊕). Reprinted with permission from ref 10. Copyright 1994 The Royal Society of Chemistry.

The SIA method was calibrated using the traditional gravimetric[23,29] methods. Samples for the manual determination of total biomass concentrations were collected automatically into 60 mL poly(tetrafluoroethylene) (PTFE) bottles at the time of inoculation (time zero) and every 2 h thereafter by a refrigerated autosampler.

Typical response curves for the biomass assay are shown in Figure 3. Note that the curves have typical rise and fall sections associated with the flow characteristics of a mixed tank. Above 0.4 AU, the correlation between optical density and total biomass fails, and therefore turbidity values obtained at delay times longer than 10 s have been used. The signal was noise-free, and the dilution curves for the assays were reproducible. Differences were due to heterogeneity of the cell samples. Cell clumping would cause an aberrant signal which may be higher or lower than expected depending on whether the sampling step or optical density monitoring had been affected to a larger extent. This is important later in the fermentation as yeast cells begin to stick together in small clusters (flocculate). This is most easily seen with the 23 and 32 h samples labeled A and B, respectively. The calibration curves for the optical density mode are given in Figure 4. The apparent absorbance at 670 nm is plotted against the total biomass. Development of a growth film on the inside surface of the flow cell does not affect the analytical measurement since a "blank" measurement is made before the sample is introduced to the cell and this blank is subtracted from the analytical signal.

The calibration curves shown above were used to predict the biomass concentration in a subsequent fermentation run. The results are shown in Table 1. 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 relative standard deviations for the biomass predictions using the SI−biomass assay are on the same order as those of the more precise gravimetric method. A *t*-test was performed on the data and the differences between the traditional dry weight measurement and the SI−biomass measurement were not significant ($P = 0.01$).

Table 1. Prediction of the Total Biomass Using Standard Curves from a Previous Fermentation[a]

dry biomass weight (g L^{-1})	% RSD	SI−biomass[b] weight (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

[a] Reprinted with permission from ref 10. Copyright 1994 The Royal Society of Chemistry. *t* score = 3.268 with 6 DoF (99% confidence limit, 3.707). [b] SI = sequential injection.

Glucose

Another important parameter is the concentration of the carbon source in the fermentation medium. Glucose is one of the most common nutrient carbon sources used and another common variable that is measured in bioprocesses.[30,31] The concentration of glucose in the fermentation medium is extremely important, especially for facultatively fermentative, Crabtree positive yeast such as *S. cerevisiae*. This is because of the effect of glucose concentration on the mode of growth for these yeast.[32] When the glucose concentration is too high, especially at the beginning of the fermentation, the yeast carry out anaerobic

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metabolism,[1] producing ethanol from the feed, even in the presence of oxygen. These effects threaten the stability of the fermentation.

Glucose is traditionally monitored in filtered fermentation samples using an enzymatic/colorimetric reagent such as Trinder reagent. This assay requires 20 min for sample preparation and color development and uses a single wavelength for detection. An alternate method uses a biosensor with glucose oxidase embedded in a membrane. Both manual methods used involve at least one enzymatic reaction and rely upon formation of hydrogen peroxide which is then directly related to the concentration of glucose in the filtered medium.

Colorimetric Methods of Glucose Concentration Measurement

This method of detection is one of the most common manual methods for glucose concentration measurement. The traditional method uses the Trinder reagent (Sigma Diagnostics) and single wavelength detection at the endpoint of the reaction. There have been several methods for glucose determination that use FIA presented in the literature.[12,33−35] Most methods rely on the same enzymes used in the Trinder reaction, those being glucose oxidase (GOx) and peroxidase (POx). One method used in addition mutarotase to convert α-D-glucose to β-D-glucose so that all of the glucose present was oxidized to gluconic acid and hydrogen peroxide.[36]

A similar alternative is chemiluminescence detection, where luminol is substituted for the colorimetric reagent.[28,37,38] The advantage of chemiluminescence is two-fold: sensitivity and low noise. The detection is inherently low noise because the signal is measured as an increase of signal versus the background or blank, in contrast to colorimetric measurements, which measure a decrease in light with respect to the blank signal. Therefore, the blank is obtained when no light reaches the detector. This also gives the method its high sensitivity. The major disadvantage of chemiluminescence detection is its reliance on enzymatic reactors (which will have a limited life span and can become fouled with constant use) and a catalyst.

The multivariate assay that was developed using SIA attempted to create an assay that could simultaneously determine two analytes from the same sample: therefore, this assay determines total biomass in a manner very similar to that of the univariate assay discussed above as well as glucose.

SIA Glucose−Total Biomass Assay

The SIA glucose−biomass assay[11] used the same analyzer as was described in the previous assay.[10] The only difference was that the detector was a diode array spectrophotometer. The assay is initiated by rinsing the flow with the Trinder reagent (carrier solution). Next, the valve rotates to the sample position and pulls enough sample to fill the sample line. At this point, a blank signal is collected from the spectrophotometer. The valve then rotates to the auxiliary waste port to rinse any sample that may be in the holding coil. The system is now ready to accept the sample from the fermentation unit, and the valve rotates to the sample port again and draws 50 μL of a fermentation sample into the holding coil. Next, the sample is propelled to the dilution flow cell, and the flow and stirring are stopped for 3.5 min (the incubation period for the Trinder reaction). At the end of the incubation period, the flow and stirring resume for 20 s in order to dilute the sample sufficiently such that the signal will not be too high for the detector. The flow and stirring are stopped again, and four spectra are collected over the range of 402−800 nm (with 2 nm resolution) and averaged. Finally, the flow and stirring are restarted, and the flow cell is rinsed with carrier to prepare it for the next sample. The entire assay for both total biomass and glucose is complete in less than 8 min. This measurement frequency is slower than that of typical FIA methods due to the incubation period for the Trinder assay. The assay was performed every 30 min for the first 32 h of the fermentation and every hour thereafter on-line for the duration of the fermentation run. This frequency provided adequate feedback on the status of the fermentation.

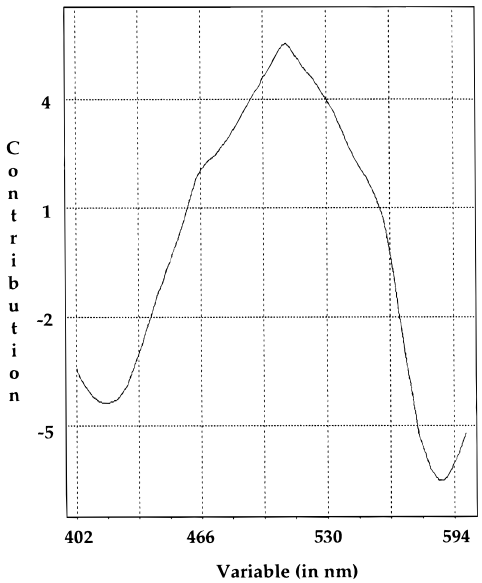

Figure 5. A plot of the glucose model used in this work. The contribution (*y*-axis) is the value each variable (wavelength) contributes to the model. Reprinted with permission from ref 11. Copyright 1995 Ars Polona.

For each fermentation there was a calibration set and an unknown set of samples. Those in the calibration set had the total biomass determined independently by the dry weight method and the glucose concentration (with biomass removed) with the manual Trinder assay. This calibration set was used to calculate the partial least squares (PLS) calibration models for a comparison of the methods. Principle component analysis (PCA) was used to identify outliers.

The final model was used to predict unknown samples, and glucose concentration was determined from fermentation III. A minimum value of the standard error of prediction (SEVP) was obtained using the first three PCs, and it is this model that was

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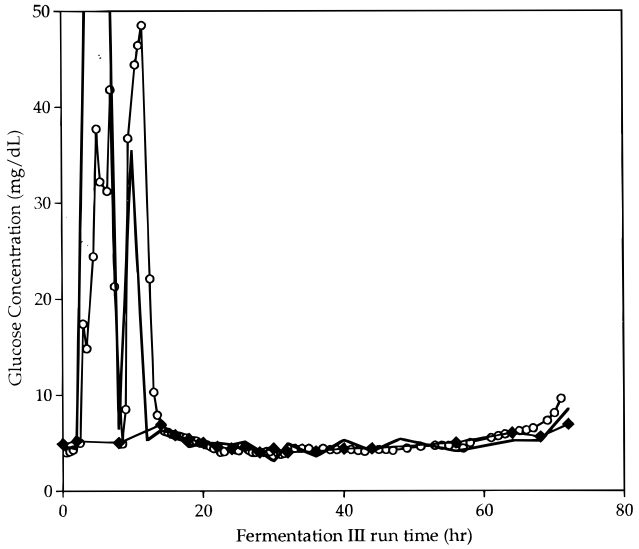

Figure 6. The glucose model is used to predict unknown samples: (—) manual Trinder method, (◆) training set for the PLS model, (○) unknown set predicted by the model. Reprinted with permission from ref 11. Copyright 1995 Ars Polona.

used for prediction. The first two PCs represent the biomass and the glucose while the third PC probably represents the turbidity effect on the glucose assay. The regression model is shown in Figure 5. This is probably the most convincing evidence that the model is actually measuring glucose within the high background signal of total biomass. It has a shape which is very similar to that of the visible spectrum of the Trinder reagent after reaction with glucose with a maximum contribution at 504 nm, the wavelength maximum for the assay. The model was not as quantitative as the biomass model; however, it allowed predictions when the glucose concentrations were below 10 mg dL^{-1}, and it is such a lower concentration range that is of interest to routine industrial fermentations. The contribution value on the *y*-axis indicates the influence or weight each *x*-axis variable has on the final model. Therefore, a large contribution value (either positive or negative) has a high (positive or negative) contribution to the final model. With the aid of this model it was also possible to detect the presence of concentration spikes of glucose in the medium during fermentation, although it was unable to accurately predict the magnitude of the spike. Since the model only included samples in the range of 1−10 mg dL^{-1}, it should not be expected to accurately predict glucose concentrations outside its range of calibration.

The model described above was then applied to the unknown sample set from the same fermentation (III). Figure 6 shows the prediction of the unknown samples from fermentation III along with the calibration set and the manual glucose measurement. When the glucose concentration is within the calibration range of the model, the predictive ability of the model is good. It confirms that the fermentation medium has experienced a spike in the glucose concentration but does not yield a precise value of the spike maximum.

Inorganic Phosphate

Cell growth during fermentation continues as long as all the necessary nutrients are present. When the

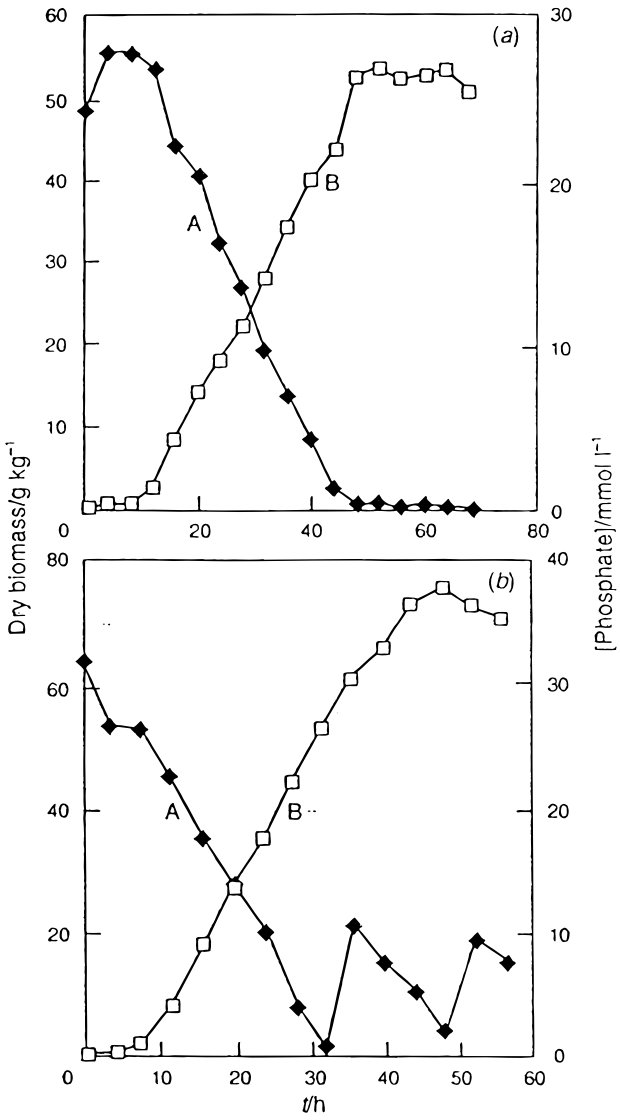

Figure 7. Variation of, A, phosphate concentration ($mmol^{-1}$) and, B, biomass (g kg^{-1}) as a function of fermentation time: (a) phosphate added only at the start of the fermentation and (b) phosphate added at the start and before its depletion from the medium. Reprinted with permission from ref 13. Copyright 1995 The Royal Society of Chemistry.

fermentation is fed a carbon source, such as glucose, an increased period of growth is observed, but this growth ceases even with continuous feed of carbon source. This indicates that some other essential nutrient has been depleted.

Phosphorus is involved in all phases of cellular metabolism, and limitation of phosphorus has been used in the production of alkaloids in plant cell cultures[39] and proteins in yeast and bacterial fermentations.[40,41] A common source of phosphorus is inorganic phosphate. Judicious control over the levels of available phosphorus during fermentation can suppress the production of secondary metabolites.[42,43] The

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best product yields are often achieved when the phosphate level is kept low. Therefore, the ability to carefully monitor the concentration of phosphate on-line in real time during a fermentation can have a direct effect on the production yields.

The standard technique for measuring inorganic phosphate is by the molybdophosphate ("molybdenum blue") method. This method had been adapted to a flow injection method;[10] however, the manifold is composed of many flow channels and requires frequent maintenance. Recently, it was adapted for use with SIA[13] and characterized by monitoring on-line an aerobic yeast fermentation. The necessary characteristics of the method included short analysis time, permitting real-time control of the phosphate concentrations, and simplicity in the manifold to render it more robust. The final method consisted of two sampling schemes depending on the concentration of phosphate present in the medium. The sampling program automatically executes the routine for the "high phosphate" concentration; if the absorbance of the reaction product is below a threshold, the second "low phosphate" sampling routine is executed.

Initial monitoring of phosphate showed that phosphate depletion appeared to be correlated with the end of biomass growth (Figure 7a). As seen in the figure, at the point where the phosphate pool was exhausted (about 44 h), the biomass growth stopped at a level of 56 ± 8 g kg^{-1} ($n = 5$). Therefore, sustaining biomass growth by supplementing the fermentation with inorganic phosphate was attempted. Figure 7b shows the results when inorganic phosphate was added at 32 and 48 h into the fermentation. Biomass growth was sustained for a longer period of time, and the final total biomass reached a level of 77 ± 6 g kg^{-1} ($n = 4$). The analysis time, including sampling time and cleanup for the next assay, was 5 min. At low phosphate concentration when no sample dilution is necessary, the sampling frequency can be as fast as 25 h^{-1}.

The results of this study show the importance of phosphate control in a yeast fermentation. The ability to monitor phosphate on-line would allow the balance of cell growth and the production of the desired secondary metabolites to be attained.

Conclusions

The growth of the biotechnology industry over the past 20 years has impacted every aspect of daily life. Strides in genetic engineering have made it possible to use simple organisms such as yeast to produce peptides and proteins for human pharmaceutical use. Regardless of the frontiers conquered by molecular biology, limited progress has been made toward better methods of monitoring the status of these bioprocesses. The most significant contribution of the sequential injection assays developed is in the elimination of sample preparation for analysis.

Sequential injection analysis has the potential to have a great impact on the area of bioprocess monitoring and control. This is already evident by the wealth of literature, especially for on-line monitoring of amino acids, carbon sources, and some products.[16,37,44,45] It is a bigger step to eliminate sample preparation, and the assays here show the feasibility of such a step. As these methods move from benchtop fermentors to the industrial-sized bioprocesses, more parameters will be able to be tracked with near real-time speed, increasing the effectiveness of monitoring. The results shown here suggest that a direct sampling from the bioprocess is the best way to continuously measure a parameter in the bioprocess.

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