Design and Performance of a 24-Station High Throughput Microbioreactor

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Abstract: Two prototype 24-unit microbioreactors are presented and reviewed for their relative merits. The first used a standard 24-well plate as the template, while the second consisted of 24-discrete units. Both systems used non-invasive optical sensors to monitor pH and dissolved oxygen. The systems were used to cultivate *Escherichia coli*. Both designs had their merits and the results obtained are presented. In addition, dissolved oxygen control was demonstrated at the milliliter scale and 24 simultaneously monitored fermentations were successfully carried out. These results demonstrated high quality high throughput bioprocessing and provide important insights into operational parameters at small scale. © 2005 Wiley Periodicals, Inc.

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INTRODUCTION

Microscale culture technology has evidenced a great deal of recent interest. The advantages of being able to replicate laboratory-scale stirred tank bioreactors at small scale are rather obvious, as one can economically conduct large numbers of cultivation experiments under controlled conditions. As a first step, we previously demonstrated the comparability of data from a liter and milliliter-scale *Escherichia coli* fermentation (Kostov et al., 2001).

Subsequently, several studies examining various designs and other aspects of microscale culture have been carried out. A number of research groups have used standardized well plates as reactors, and have added some type of sensing. The simplest used a standard plate reader to measure a pH sensitive dye in the growth medium (Girard et al., 2001). A more advanced system coated the well bottoms of 96-well plates with fluorescent oxygen sensors, again performing measurements with a commercial plate reader (John et al., 2003). The plate reader has a built-in shaker, but still can only

provide k_La values up to 40/h. With an improved shaker, the k_La could easily exceed 200/h (Hermann et al., 2002). Another promising study used optical measurements of dissolved oxygen and cell mass, and achieved k_La 's of 100–400/h (Lamping et al., 2003).

In other studies, Maharbiz et al. (2004) demonstrated an array of eight 250 μ L reactors based on printed circuit board technology that controlled temperature, pH, and measured optical density. Zanzotto et al. (2004) utilized a microbioreactor with volumes in the range of 5–50 μ L with integrated optical sensors for on-line measurement of optical density, dissolved oxygen, and pH during growth of *E. coli*. Elmahdi et al. (2003) measured pH during microscale fermentation by inserting a micro-pH probe into a 7 mL well in a microtiter plate. As mentioned earlier, Hermann et al. (2002) have characterized the mass transfer characteristics of well plates. Puskeiler et al. describe a 48-well milliliter scale bioreactor array. Finally, Doig et al. (2005) used three different microplates (24-, 96-, and 384-wells) with working volumes between 65 and 1,182 μ L to measure k_La during growth of *Bacillus subtilis*.

In this paper, we present the results from two prototype high throughput milliliter scale bioreactors. Both designs utilize individually driven impeller agitated units, and appear like miniature stirred tanks. One was based using a standard 24-well plate as the footprint, while the other employs a discrete miniature stirred tank design. In both cases, each unit had its independent agitator in the form of a DC or a stepper motor. A major reason for us to utilize the stirred tank approach is the large body of previous experience in design and use of such systems for cell and microbial culture and for the achievability of convective mixing. While agitated well plates do offer convective mixing, each well cannot be individually addressed. In terms of the footprint, the motivation was to utilize a 24-well plate architecture given its widespread commercial deployment. During the course of this design's testing and validation, it became apparent that

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a discrete miniature bioreactor design would also offer advantages and so both types were fabricated and tested as described next.

MATERIALS AND METHODS

Sensors and Instrumentation

In all cases, optical sensors were used to measure pH and DO as previously described (Kermis et al., 2002; Kostov et al., 2001). Cell mass was monitored by turbidity, and provision was also included to allow fluorescence intensity measurements of green fluorescent protein (GFP), which can be used to monitor protein expression and organism stress (Lu et al., 2003). The GFP sensor was not used in the studies reported in this paper and is mentioned for completeness. Figure 1 shows the generic scheme for the sensors and instrumentation. For the 24-well plate system, Figure 2a and b show the entire

Figure 1. Schematic of the positioning of the optical sensors around a single well in a 24-well microplate. The light source and the detectors are not drawn to scale. Interference filters are positioned in front of the LEDs and photodiodes (represented here by small disks). A pinhole is installed in front of every detector for better collection of the light (not shown). The spatial positioning is selected in such way that the excitation light from one channel creates minimum interference to the other channels. The glass optical guide GG extends below the liquid level to achieve constant light path length l for OD measurements. 1, excitation UV LED for the GFP channel; 2, detection photodiode for the GFP channel; 3, excitation blue LED for the oxygen channel; 4, detection photodiode for the oxygen channel; 5, 7, excitation blue and UV LEDs for the pH channel; 6, detection photodiode for the pH channel; 8, red LED for the OD channel; 9, detection photodiode for the OD channel; 10, the liquid level. For the discrete microbioreactor, the lightguide was discarded and OD was measured across the diameter of the vial (not shown). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 2. A: Measurement board and (B) patch placement in a microwell plate. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

board assembly and how the sensor patches were placed in each well. Further details are in Harms [2004].

24-Well Plate System

For the 24-well plate system, k_La 's were determined by the sulfite oxidation method as adapted for microtiter plates by Hermann et al. (2001, 2002). The reactors were filled with 1 mL of a solution of 0.5 M sodium sulfite, 0.012 M sodium phosphate, $<1 \mu\text{M}$ cobalt sulfate, and the pH indicator dye HPTS, adjusted to pH 8. Once all of the reactors were filled, agitation and aeration were started and held constant. As the sulfite is oxidized, the solution pH drops, causing an optical change of the HPTS. The k_La is inversely proportional to the time taken for this change, which ranged from 2 to 26/h.

Also for the 24-well plate system, *E. coli* JM105 was grown on LB media in the reactor system. Cells were taken from an overnight shake flask culture. The overnight culture (0.5 mL) was added to 12 mL of fresh media to provide

a starting OD of about 0.2. One milliliter of this culture was aliquoted into each reactor, after which the reactors were assembled and monitoring and controls were started. Six reactors were run simultaneously using wells B1 through B6. pH control was not used in this experiment. Since the well plate was not sealed, ethanol sterilization was used and the fermentations were run in a laminar flow hood.

Discrete Microbioreactor System

For the individual microbioreactor design, Figure 3a–c show the details of each microbioreactor, its instrumentation, and the entire assembly with 24-independent stirred vessels. The vessel used was a flat-bottomed transparent vial. The bottom of the vial has a footprint identical to that of a single well in a standard 24-well plate.

Figure 3. A: Sensor board, (B) patch placement, and (C) 24-discrete unit high throughput bioreactor.

In the discrete system, every bioreactor was equipped with an individual sterilizable cap. This allowed the individual units to be autoclaved and also permitted operation outside a laminar flow hood. Another significant difference from the plate design was the use of stepper motors for agitation control. At low speeds (10 rpm) the stepper motors offer greatest accuracy, while still offering maximum agitation speed of 1,000 rpm. A dedicated microcontroller for each motor allowed for individual rpm control of every bioreactor both through hardware and software. A third difference was that every bioreactor used a dedicated sensor board. All sensor boards (i.e., bioreactors) were sampled simultaneously. The total readout time of the sensors for all bioreactors was approximately 25 s. In contrast, the 24-well plate sensor board had a latency of several minutes between samples when all 24 stations are sampled, as the data were serially processed through a single bus.

Other than a strain variation (in this experiment, a GFP harboring *E. coli*), other culture conditions were the same as for the well plate experiment.

For data acquisition and control, LABView (National Instruments, Austin, TX) was used to write the routines in both systems.

RESULTS AND DISCUSSION

24-Well Plate Format

Oxygen Transfer

The results of k_La measurements in the 24-well plate system are shown in Figure 4. There is a wide scatter in observed k_La values, particularly above 1,000 rpm. Some scatter is certainly expected due to noise in the sensor, but most of the variation could be caused by differences between the reactors (due to difficulties in consistently making identical hand made units) and inequality of the sparge gas flow rates. The sparge gas distribution is governed primarily by the pressure drop through the sterile filters, which shows some variation, even from the same manufacturing lot.

Figure 4. k_La measurements in a 24-well plate microbioreactor.

The k_La curve appears to be flat until some critical agitation, above which k_La increases rapidly. If surface aeration is dominant (Humphrey, 1998), then increasing agitation would have a minimal effect until a vortex is formed. This vortex would increase the surface area, which would increase the k_La . The change in surface area has been measured photographically in shaken 96-well plates and similar k_La curves were generated (Hermann et al., 2002). Because the agitation speeds can be very high, up to 2,800 rpm, the baffling effect of the heater and needles for sparging, temperature monitoring, and cell mass measurement is not sufficient to prevent vortex generation.

The wide range of achievable k_La 's indicates that dissolved oxygen control is possible. However, the odd non-linear shape of the k_La curve and, more importantly, the high degree of scatter in the data, makes it difficult to find and tune a good oxygen control algorithm. Rather than performing the iterative testing of controller algorithms and settings, for later experiments, the control was set to the simplest method, namely on–off control, which provides proof of controllability but not accurate control.

In this implementation of on–off control, the agitation was switched between either a low speed (500 rpm) or a high speed (2,000 rpm). A traditional on–off controller would use zero as one of the agitation values, but that would prevent the reactor from being well-mixed, which would affect the readings from the oxygen sensor. As the oxygen sensor provides the input to the controller, this must be avoided. Bacterial cultures using this on–off control are discussed next.

Oxygen Control Versus Uncontrolled Cultures

Two sets of triplicate cultures were conducted to demonstrate oxygen control. Wells B1, B2, and B3 were run with on–off DO control and a setpoint of 20% as described earlier. Wells B4 and B6 were run without control. Well B5 experienced a temperature control malfunction and is not shown. Figure 5 shows DO and pH profiles from the 2-wells.

Reactors B4 and B6 were run without oxygen control at a constant agitation of 500 RPM. The dissolved oxygen profile shown in Figure 5a starts around 100% and approaches zero when the oxygen demand exceeds the oxygen supply, and then returns to 100% as the cells approach stationary growth phase. The curves are very similar between the two reactors, but B4 is oxygen limited for 10.2 h, while B6 is only limited for 8.6 h. This is a clear indication of a difference in k_La , even though the reactors were operated at identical agitation rates. Their pH profiles, shown in Figure 5b, show a slight drop and then rise.

Reactors B1, B2, and B3 were run with on–off oxygen control, with a setpoint of 20%.

Figure 6a shows a detailed view of the dissolved oxygen profile with oxygen control enabled. With the on–off control used here, the obvious oscillations are exactly as expected. These oscillations are caused entirely by the controller, and do not represent the behavior of the organism (Andersen

Figure 5. A: Dissolved oxygen profile from reactors B4 and B6. Both reactors were operated at a constant 500 rpm. B: pH profile of reactors B4 and B6. Both reactors were operated at a constant 500 rpm.

et al., 2001). The oxygen controller oscillates when the oxygen demand is greater than the oxygen supply at the low agitation but less than the supply at the high agitation, which also indicates rapid growth. An average oxygen concentration may help visualization of the data. As the period is about 10 min, a reasonable window for averaging is about 30 min. Because the magnitude of oscillation is very high, simple averaging is not a good choice. Instead, the data was passed through a 4th order Butterworth low-pass filter with a time constant of 30 min (15 samples). This has the advantage of smoothing the oscillations without needing to average an even multiple of the oscillatory period. The smoothed dissolved oxygen data are shown in Figure 6b and show that the control was successful in maintaining average DO close to the setpoint in all three microbioreactors.

While the smoothing makes it is easier to identify the major growth phases, it does not accurately represent the cells' true environment. Figure 6c shows a single cycle along with calculated values for the bulk oxygen concentration. The calculations assume a constant OUR and use previously calculated values of k_La (68 and 300/h) and patch

response time (100 s). They also include a 120 s dead time introduced by the software between measuring the phase of the oxygen patch and outputting the new agitation speed. The expected deadtime for the software is only about 60 s, but it should vary from well to well depending on the order of taking measurements. The model fits the data remarkably well. If this model is correct, it indicates that the patch response time and sampling interval are much too long for proper use of this control algorithm. The bulk oxygen concentration does not stay at an average level, but oscillates between an excess of oxygen and complete oxygen starvation.

A major issue with bioreactor scale-up is that as volumes increase, mixing becomes worse, and cells are subjected to transients in oxygen concentration and chemical composition, with frequent oscillations in substrate, product, and waste. While studies have attempted to reproduce this effect using multiple linked bioreactors (Sandoval-Basurto et al., 2005), the parallel miniature bioreactor system could be used to control the cells' microenvironment directly and rapidly. A change in mixing at a large scale could be simulated by a

Figure 6. A: Original and (B) filtered views of dissolved oxygen under on–off control. The filtering removes most of the oscillations, showing a slight downward trend between 2 and 6 h for all three reactors. C: Modeled behavior of a reactor with on–off control of dissolved oxygen. The oxygen levels in the patch closely resemble the measured data, but do not accurately represent the bulk dissolved oxygen due to the slow patch response. The cells may be oxygen limited for a significant fraction of the time, but it is not apparent from the measured data.

change in the control cycle time and sampling rate in the miniature system. With a proper setup, the system could resemble either a bench-scale fermenter or a production-scale fermenter, which could be greatly beneficial to bio-process characterization and operational qualification from a regulatory standpoint.

The DO controlled cultures had similar pH curves as shown in Figure 7. After 6.5 h, the consistently high dissolved oxygen concentration indicates that the cells stopped rapid growth. This observation is confirmed by the leveling off seen in the pH data. Compared to the uncontrolled cultures, the

Figure 7. pH profile of reactors B1, B2, and B3. The reactors used on–off control of oxygen, providing higher growth rates than reactors B4 and B6. Growth was completed at about 7 h.

experiments with DO control show greater consistency and repeatability in the fermentation. Clearly, improved control at the microscale is a necessary feature in order to replicate the utility of laboratory scale bioreactors.

24-Discrete Microbioreactors

The parallel plate architecture did present some drawbacks. A robotic sampler (available for both pH control and nutrient addition) was ineffective due to the large numbers of tubes and wiring harnesses that clogged the available space above the microwell plate. Furthermore, having all the sensors on a single board proved to be a major problem when a short circuit on one part of the board resulted in the entire board becoming non-functional. It was experiences such as this that led to the single microbioreactor and modular sensor board design shown in Figure 2.

Given the variations in sparged aeration that were observed, we decided to test operational consistency and sensor performance by operating the 24-discrete units also under surface aeration. As can be clearly seen in Figure 8a, the fermentations showed virtually identical oxygen profiles, other than minor variations. In each case, cell growth results in oxygen consumption that results in the oxygen sensor reading zero oxygen as the cells continue to grow microaerobically. Upon substrate exhaustion, respiration ceases and oxygen values revert to the equilibrium value seen prior to the fermentation.

The pH data corroborated the oxygen profiles. Deprived of oxygen, the cells grew microaerobically. Under such conditions, they produce acetate, which results in sharp pH drops and this is exactly what was observed. Later, the cells utilize the acetate as a carbon source, which results in a gradual pH rise until the end of the fermentation. One microbioreactor's pH channel (number 3) was noticeably noisier than the rest, but nevertheless showed a trend similar to the others. Microbioreactor 20 also did not perform like the rest and we believe that one of the LED's (pH is a ratiometric measurement using two LEDs) was not aligned correctly.

Another important observation needs to be noted here. In contrast to the microwell plate, the pH data are significantly more inconsistent in the discrete microbioreactors. We attribute this to the surface characteristics of the vessel. The bottom of a microwell plate was nearly perfectly flat. In contrast, the bottoms of the glass vials employed in the discrete design have a slight curvature and manifest irregularities that vary from vial to vial. Consequently, the light path of the excitation LEDs is variant, leading to inconsistent readings between the vials. These observations are being quantitatively documented and will be reported separately.

Finally, Figure 8c shows optical density data from the discrete microbioreactors. As can be seen, the data are highly scattered, so meaningful comparisons between microbioreactors are not possible. The trend for each individual trace shows the expected increase in optical density exhibited by a growing culture. Optical density did not work consistently—at this point in time, we believe that these measurements are too susceptible to minor changes in alignment and bubble interferences to be anything more useful than a relative measure of growth. In the case of GFP, the initial experiments used cells with very low expression levels and consequently

Figure 8. A: Dissolved oxygen profiles from 24 simultaneous fermentations conducted in individual microbioreactors. B: pH profiles from corresponding microbioreactors. C: Optical density profiles from 16 microbioreactors.

Figure 8. (Continued)

the signals were not much greater than the noise. Future experiments will address GFP measurements.

CONCLUSIONS

We have demonstrated an important advance in the further development of high throughput bioprocessing. The feasibility of controlled microbioreactor operation as well as the consistency of multiple experiments at the small scale has been demonstrated. It is hoped that these advances will lead to high throughput bioreactor systems that will be indistinguishable operationally from traditional larger scale bioreactors.

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References

- Andersen DC, Swartz J, Ryll T, Lin N, Snedecor B. 2001. Metabolic oscillations in an *E. coli* fermentation. *Biotechnol Bioeng* 75:212–218.
- Doig S, Pickering SCR, Lye GJ, Baganz F. 2005. Modelling surface aeration rates in shaken microtitre plates using dimensionless groups. *Chem Eng Sci* 60:2741–2750.
- Elmahdi I, Baganz F, Dixon K, Harrop T, Sugden D, Lye GJ. 2003. pH control in microwell fermentations of *S. erythrae* CA340: Influence on biomass growth kinetics and erythromycin biosynthesis. *Biochem Eng J* 16:299–310.
- Girard P, Jordan M, Tsao M, Wurm FM. 2001. Small-scale bioreactor system for process development and optimization. *Biochem Eng J* 7:117–119.
- Harms P. 2004. Parallel miniature bioreactors in a twenty-four well plate. Ph.D. Thesis, UMBC.
- Hermann R, Lehmann M, Büchs J. 2002. Characterization of gas–liquid mass transfer phenomena in microtiter plates. *Biotechnol Bioeng* 81:178–186.
- Hermann R, Walther N, Maier U, Büchs J. 2001. Optical method for the determination of the oxygen-transfer capacity of small bioreactors based on sulfite oxidation. *Biotechnol Bioeng* 74:355–363.
- Humphrey A. 1998. Shake flask to fermentor: What have we learned? *Biotechnol Prog* 14:3–7.
- John GT, Klimant I, Wittman C, Heinze E. 2003. Integrated optical sensing of dissolved oxygen in microtiter plates: A novel tool for microbial cultivation. *Biotechnol Bioeng* 81:829–836.
- Kermis HR, Kostov Y, Harms P, Rao G. 2002. Dual excitation ratiometric fluorescent pH sensor for noninvasive bioprocess monitoring: Development and application. *Biotechnol Prog* 18:1047–1053.
- Kostov Y, Harms P, Randers-Eichhorn L, Rao G. 2001. Low-cost microbioreactor for high-throughput bioprocessing. *Biotechnol Bioeng* 72:346–352.
- Lamping SR, Zhang H, Allen B, Shamlou PA. 2003. Design of a prototype miniature bioreactor for high throughput automated bioprocessing. *Chem Eng Sci* 58:747–758.
- Lu C, Bentley WE, Rao G. 2003. Observations of oxidative stress in aerobic *Escherichia coli* fermentations. *Biotechnol Bioeng* 83:864–870.
- Maharbiz MM, Holtz WJ, Howe RT, Keasling JD. 2004. Microbioreactor arrays with parametric control for high-throughput experimentation. *Biotechnol Bioeng* 85:376–381.
- Sandoval-Basurto EA, Guillermo Gosset, Francisco Bolivar, Octavio T Ramirez. 2005. Culture of *Escherichia coli* under dissolvedoxygen gradients simulated in a two-compartment scale-down system: Metabolic response and production of recombinant protein. *Biotechnol Bioeng* 89:453–463.
- Zanzotto A, Szita N, Boccazzi P, Lessard P, Sinskey AJ, Jensen KF. 2004. Membrane-aerated microbioreactor for high-throughput bioprocessing. *Biotechnol Bioeng* 87:243–254.