Low-Cost Microbioreactor for High-Throughput Bioprocessing

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Abstract: The design of a microbioreactor is described. An optical sensing system was used for continuous measurements of pH, dissolved oxygen, and optical density in a 2 mL working volume. The K_La of the microbioreactor was evaluated under different conditions. An *Escherichia coli* fermentation in both the microbioreactor and a standard 1 L bioreactor showed similar pH, dissolved oxygen, and optical density profiles.

The low cost of the microbioreactor, detection system, and the small volume of the fermentation broth provide a basis for development of a multiple-bioreactor system for high-throughput bioprocess optimization. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* **72**: 346–352, 2001.

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INTRODUCTION

The human genome sequence is complete. While the sequencing of the human genome has been a mammoth task, many have pointed out that this effort pales in comparison to what lies ahead. The next step is to identify what turns these genes on and what proteins they express. Fermentation and cell culture will play a critical role in elucidating these factors. The method of choice to produce proteins is by cloning and expression in a suitable host and optimizing its production in a bioreactor. Assuming there are 50–100,000 human genes, these will first have to be cloned into various hosts (*E. coli*, yeast, CHO, BHK, and other workhorses). Then an enormous permutation of culture conditions will have to be evaluated to identify critical factors that turn these genes on. Following this, the identity of the proteins produced will have to be determined. Clearly, the ability to culture cells in controlled environments is crucial to this venture. The heart of any fermentation/cell culture (bioprocess) operation is an instrumented bioreactor capable of pH, temperature, and dissolved oxygen measurement and control. Bioprocess development is currently hampered by a paucity of high-throughput techniques to evaluate the effect of operational/nutritional parameters on cell growth and product formation in a systematic and statistically significant manner. Currently available instrumented bioreactors are expensive and bulky, thus making bioprocess development inefficient as large numbers of simultaneous experiments simply cannot be conducted. Recent advances in optical sensor technology provide a possible solution.

Traditionally, bioprocess technology has been critical to the development and availability of new drugs and vaccines. Additionally, bioprocesses are important in a wide variety of industries besides pharmaceuticals—food industry, ecology, water treatment, etc. (Arroyo et al., 2000; Bakoyianis and Koutinas, 1996; Bylund et al., 2000; Handa-Corrigan et al., 2000; López-López et al., 1999; McIntyre et al., 1999; Pressman et al., 1999; Yang et al., 2000). For bioprocess optimization in the pharmaceutical industry, significant numbers of fermentations are needed under varying environmental and nutritional conditions. This is expensive and time-consuming in practice, as this type of research is typically performed in shake flasks (with practically no control of the bioprocess parameters) or in small (1–3 L) bioreactors (Tholudur et al., 1999). In order to decrease the number of experiments required for optimization, mathematical modeling of the bioprocess is used (Alvarez-Ramirez et al., 1999; Boon et al., 1999; Cooney et al., 1999; Tholudur et al., 1999). However, this approach also requires a significant number of fermentations for establishing process parameters. It is sobering to contemplate the fact that bioreactor technology has changed little since the first successful bioproduct—penicillin. All the cell culture drugs approved to date (e.g., insulin, tPA, erythropoeitin, monoclonals, interferon, etc.) are based on bioreactor production. These were based on, at most, a few known genes. The task of going after several thousand target genes based on the human genome is truly staggering.

Thus a technology for fast, reliable, and inexpensive parallel bioprocessing is strongly desirable. One possible solution is to try to scaledown the volume of the bioreactor while preserving its control capabilities. Plate readers offer the opportunity for studies of parallel bioprocesses (Li et al., 2000), but they can read one or two parameters (absorbance

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and/or fluorescence) and are not equipped with chemical sensors or actuators for bioprocess control.

A key requirement for any bioprocess is the ability to measure process parameters, as well as to supply nutrients, oxygen, and pH correctors. Typically, pH, dissolved oxygen (DO), and optical density (OD) are monitored continuously. In small volumes (1–2 mL or less) it is difficult (or impossible) to use standard industrial probes because of their dimensions. There are miniaturized versions of these sensors (Liu and Neuman, 1982; Suzuki et al., 1991; Zhong et al., 1992) but their fabrication is rather sophisticated and they are expensive. Another problem is the fact that standard Clark-type oxygen probes consume oxygen (Lee and Tsao, 1979). In small volumes they will compete with respiring cells and distort the measurements.

One option for overcoming these problems is to use the emerging technology of optical chemical sensing (Bambot et al., 1994; Randers-Eichhorn et al., 1997; Xu et al., 1998). As these sensors are typically based on equilibrium principles, they do not interfere with the measured process. Their physical dimensions could be very small. Last, but not least, the cost of the measuring equipment (readout devices) is very low (Kostov et al., 1998, 2000) and the sensors themselves are sufficiently inexpensive to be disposable.

In this work, we describe an approach for scaling *down* the working volume of the bioreactor to 2 mL. The design and evaluation of the performance of the microbioreactor are presented. Three parameters—pH, DO, and OD—were continuously measured by means of optical sensors. A test fermentation was performed in the microbioreactor and the results were compared with those from a fermentation in a standard 1-L bioreactor. The results provide a basis for the development of highly parallel bioprocessing that can be successfully performed and monitored in small volumes.

MATERIALS AND METHODS

pH Measuring Channel

The excitation of the dye was performed consecutively by two light-emitting diodes (LEDs): blue LNG992CFBW (Panasonic, Secaucus, NJ), and UV NSHU590E (Nichia America, Mountville, PA). The emission of the blue LED was filtered with a 460 ± 15 nm bandpass filter (12.5 mm diameter); the UV LED was used without filtering. The fluorescence emission was observed by a large active area PIN photodiode S1223-01 (Hamamatsu, Bridgewater, NJ) through a 520 ± 5 nm interference filter. The filters used in all channels were from Intor (Sorocco, NM). The photodiode current was converted to voltage and amplified by transimpedance and lock-in amplifiers made in-house. The design of the amplifiers is described elsewhere (Kostov et al., 2000). The LEDs were switched on and off by transistor switches. LED control and the measurement of the output signal from the lock-in amplifier were done by the ADC/DAC board (described below). Calibration of the channel was performed using media with the dissolved indicator. The pH of the solution for the calibration was adjusted by titration with 1 M HCl or NaOH.

DO Measuring Channel

For excitation of the O_2 patch, a second blue LED was used. Its emission was also filtered with a 460 ± 15 nm filter. The detection of the emission from the sensor was performed by an avalanche photodiode module C5460 (Hamamatsu, Bridgewater, NJ) with a mounted 590 ± 20 nm bandpass interference filter. Additionally, the channel uses the red LED from the optical density channel as a reference for determination of the phase delays associated with the electronics. The phase measurements were made using an SR844 RF lock-in amplifier (Stanford Research Systems, Sunnyvale, CA). The output of the SR844 was measured by the ADC board. The LEDs were controlled by a special transistor driver.

The calibration curve was obtained by blending a controlled flow of air (supplied by an aquarium air pump) and pressurized nitrogen (Airgas Mid-Atlantic, Baltimore, MD). The percentage of oxygen was calculated as a function of the gas flow.

OD Measuring Channel

The optical density channel consisted of a red ultrabright LED LTL 4268 UR (Lite-on, Milpitas, CA) and photodiode detector (PIN photodiode coupled to transimpedance amplifier). An interference filter 600 ± 5 nm was positioned in front of the photodiode. The LED and the detector were placed on opposite sides of the cuvette, as shown in Figure 1. The output signal was measured directly from the ADC board. Calibration of the channel was performed using the seed culture. Out-of-calibration data were calculated using linear extrapolation.

Data Collection System

The data collection system recorded four parameters: intensities of HPTS fluorescence under UV and blue excitation, phase shift of the O_2 sensor fluorescence, and the intensity of the light transmitted through the media at 600 nm. The sensors are controlled and monitored by a 166 MHz Pentium computer equipped with a National Instruments PCI-6111E multiple input/output card. A program was written in Labview that sequentially enables each channel and measures the output of the sensor. The 370 nm LED is turned on and after a few seconds 500 intensity measurements are acquired in 5 sec and averaged. The 370 nm LED is turned off and the process is repeated for the 460 nm measurement of pH. For optical density, the ambient light is measured 1,000 times in 10 ms. The red LED is turned on and another 1,000 points are taken almost immediately. The red LED is then turned off and another 1,000 ambient light readings are

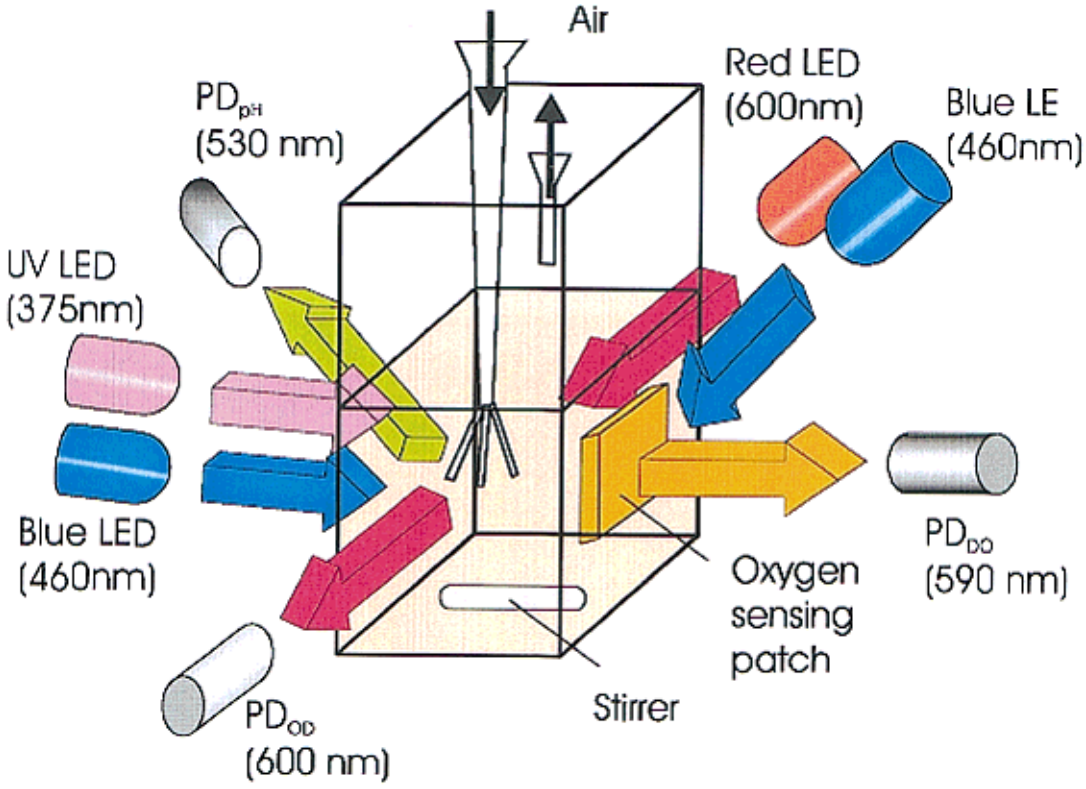

Figure 1. Cuvette-based microbioreactor. At the left cuvette wall, blue and UV LED together with 530 nm photodetector are used to measure pH; at the right cuvette wall, blue LED, oxygen sensing patch, and 590 nm photodetector are used to measure dissolved oxygen; red LED and 600 nm photodetector are used to measure optical density through the front and back wall. The air supply inlet and outlet are positioned at the corners of the cuvette. LEDs are fired in succession to prevent crosstalk (for clarity, figure not to scale).

taken. The process is repeated if the standard error is too high or if the ambient light measurements are substantially different. Dissolved oxygen is measured by turning on the blue LED, waiting 5 sec, and acquiring 100 phase measurements over 2 sec. Each measurement cycle takes just under 45 sec.

Fermentation in a 1-L Fermentor

The overnight seed culture consisted of a 0.5% inoculum of *Escherichia coli* strain JM105 frozen stock in LB media (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract, pH 7.2) incubated at 30°C with shaking at 260 rpm (model G24, New Brunswick Scientific, Edison, NJ). Fermentations were carried out in a New Brunswick BioFlo III fermentor containing 1 L buffered LB (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract, 4 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, pH 7.2) and 15 mL of 0.15 g/L sterilized solution of HPTS in deionized water. The fermentor was inoculated with 10% seed culture. Aeration, agitation, and temperature were controlled at 1 vvm, 300 rpm, and 25°C, respectively. Dissolved oxygen and pH were not controlled. Data of continuous readings were logged every 20 sec on a Mac II computer using a Strawberry Tree data acquisition system and Workbench software (Strawberry Tree, Sunnyvale, CA).

Optical density values representative of the bacterial growth were measured offline with a Hewlett-Packard 8452A Diode Array spectrophotometer at 600 nm.

Fermentation in the Cuvette

The cuvette and the oxygen sensing film were sterilized with 70% ethanol for 5 min. The cap with mounted inlet, outlet, and tubing was heat-sterilized. The sensing film was attached to the wall with sterilized grease to fit in the window in the black tape and the cuvette was sterilized again with ethanol. 2.25 mL of the same buffered LB media, 0.25 ml of the same seed culture, and 37.7 μL of 0.15 g/L sterilized solution of HPTS in deionized water were added and the cap was attached. The assembly was performed in a laminar flow hood. The assembled microbioreactor was transferred into the holder with the sensing system. Aeration and agitation were controlled at 2 vvm and 300 rpm, respectively. The fermentation was performed at room temperature of 25°C.

RESULTS AND DISCUSSION

Microbioreactor Design

The working vessel was a disposable polystyrene cuvette, 1 × 1 cm, with a total volume of 4 mL. To avoid contamina-

tion during the process, it was equipped with a silicone rubber cap. The cap had an inlet for air delivery and outlet for the exhaust air. They were positioned at the corners of the cuvette to avoid overlapping with the optical path for optical density measurements. The inlet was connected to an air sparger (Fig. 1). It was fabricated from a 100 mL plastic pipette tip. Three tubes with inner diameters of 0.25 mm were positioned at the end of the tip. The tubes were glued using epoxy resin. The outlet consisted of a short piece of a 16-gauge syringe needle. The air was supplied by an aquarium pump, passed through a regulator for low gas flow rates and filtered using a syringe filter (Millex-GV, 0.22 μm; Millipore, Bedford, MA). Stirring was executed by a small magnetic stir bar and magnetic stirrer. The K_La of the cuvette was adjusted to be approximately equal to the K_La of the 1-L fermentor operated at 300 rpm agitation and 1 vvm aeration (21 h^{-1}).

Positioning of the Measuring System

The cuvette was large enough to accommodate the required optics and electronics and no optical fibers were needed. The use of conventional optics allowed for increased signal levels, as practically all the excitation light is coupled to the sensors. All solid-state light sources and detectors, low-cost optical filters. and electronics were used. As some of the components (especially the filters) were bigger than the cuvette wall, more attention was paid to the proper spatial placement of the optical modules around the cuvette in order to avoid crosstalk between the channels. The positioning of the basic components for each channel is also shown in Figure 1.

pH Measurements

The pH measurements were performed using a ratiometric pH-sensitive dye: 1-hydroxypyrene-3,5,7-sulfonic acid, (HPTS; Sigma, St. Louis, MO), pKa = 7.2. A sterilized solution of HPTS was directly introduced into the culture media. Front-face geometry was used for fluorescence detection (Fig. 2A). The dye has two excitation peaks, 400 and 450 nm. When excited at these wavelengths, the ratio of the fluorescence emissions at 520 nm of the dye depends on pH. The ratiometric approach avoids interference from turbidity changes during the process and provides accurate measurements of pH. The longer wavelength is easily excitable using a blue LED; however, until recently there were no UV LEDs, which precluded the use of this indicator in low-cost systems. The pKa of HPTS (7.2) makes it appropriate for use with neutral-range bioprocesses. HPTS is a nontoxic indicator, used for blood gas measurements in vivo (Zhang et al., 1995). Its addition to the media did not influence cell growth. The calibration curve of the pH channel is shown in Figure 3A. The pH was calibrated by measuring the intensity ratio of the solution as the pH was changed and verified on a benchtop pH meter. The use of semiconductor light sources and detectors allowed us to design a very compact and low-cost detection system. To our knowledge, this is the first all-solid-state ratiometric instrument that operates in the UV/blue range.

One possible problem for future applications could be the range of the emission spectrum of HPTS—its emission maximum is positioned at 520 nm. With increased use of GFP (which possesses a very similar emission spectrum) for product quantification, it could be desirable to use pH sen-

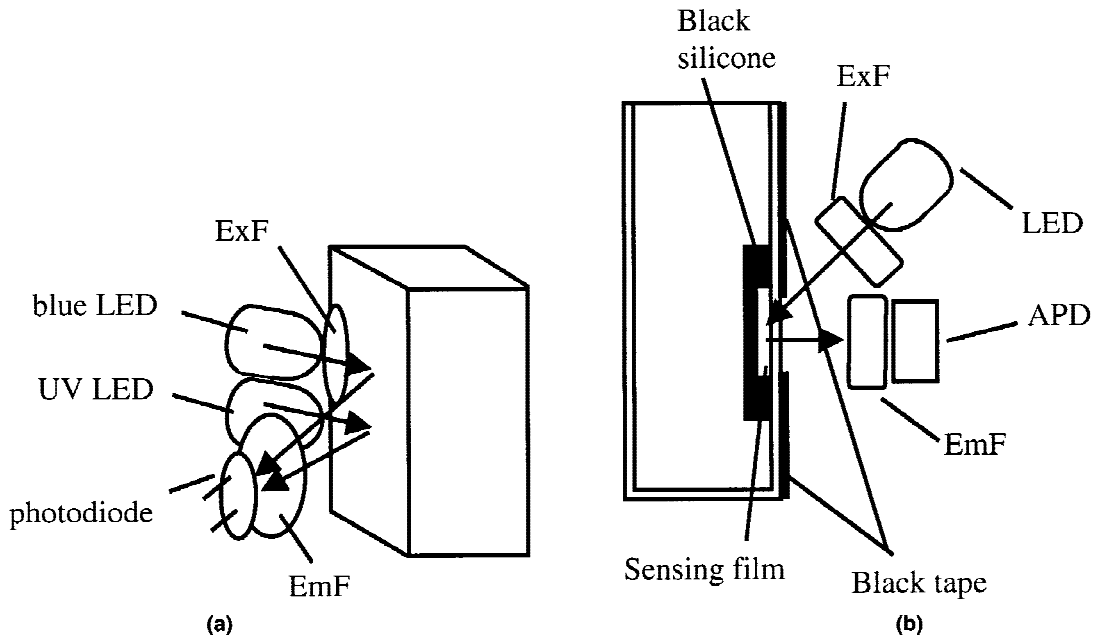

Figure 2. **a**: Optical configuration of the pH channel. **b**: Optical configuration of the oxygen sensing channel. LED — light emitting diode; APD — avalanche photodiode module; ExF — excitation filter; EmF — emission filter (for clarity, figure not to scale).

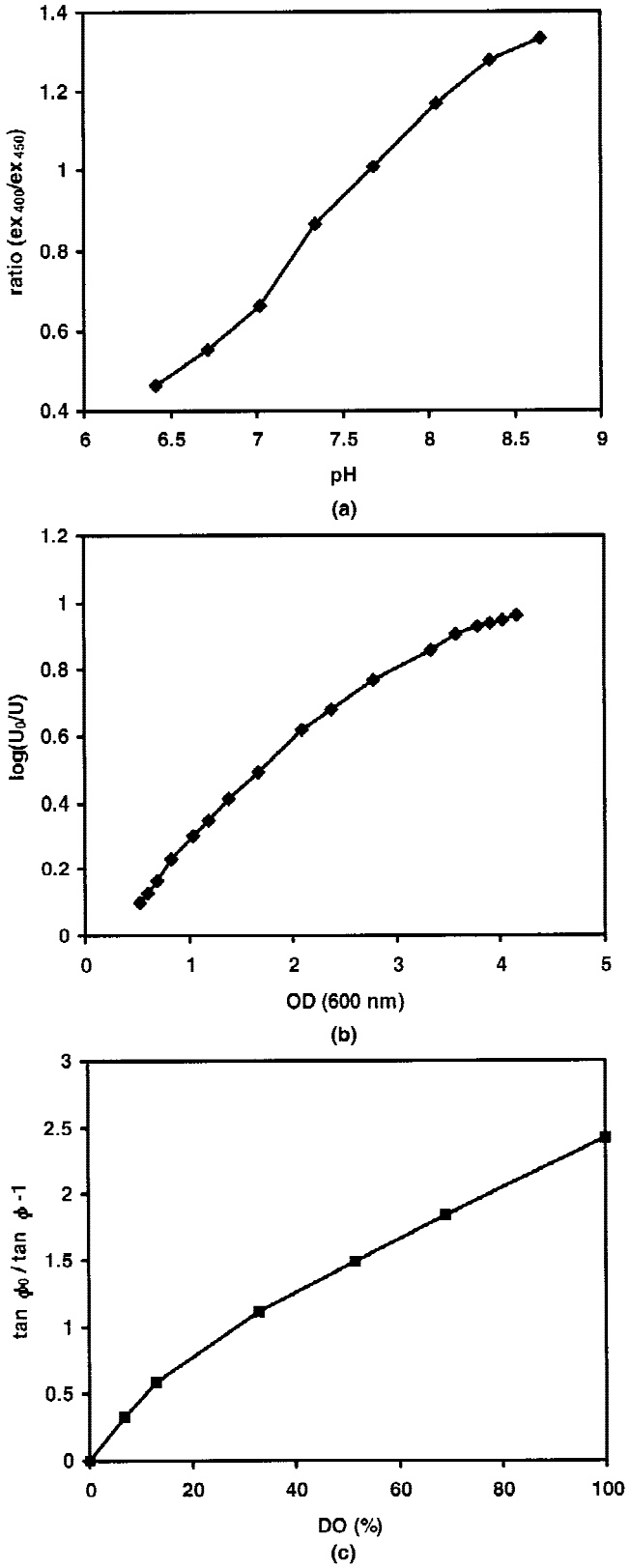

Figure 3. Calibration curves of the channels. **A**: pH channel. **B**: OD channel. **C**: DO channel.

sitive dyes that emit at longer wavelengths. While the approach of adding indicator directly to the culture medium worked for our proof-of-principle purposes, it is undesirable for long-term use. An immobilized indicator would be preferable for reasons of improved optical isolation, as well as eliminating undesirable interactions with growth medium components and/or cells.

OD Measurements

The OD channel was realized using the simplest possible means. The absorbance of the cell suspension was directly measured using a 600-nm LED. Its calibration curve is shown in Figure 3B. As expected, it does not follow Beer's law, and the sensitivity rapidly decreases with the increase in OD. The highest measurable optical density is limited by the noise of the photodetector. For this particular setup the maximum OD detected was approximately 9 (results not shown).

To ensure that pH and OD did not affect each other, their values were verified by offline measurements in the beginning and at the end of the process. The results are shown on the respective figures (Fig. 4A,C) with pH and OD profiles. Offline pH and OD values agreed very well with their online ones and demonstrate stable operation (no drift) over the period of operation.

DO Measurements

The DO channel was positioned on the opposite wall of the cuvette to the pH channel. It uses a ruthenium-based oxygen sensor, Ru(diphenylphenanthroline)$_3^{2+}$ immobilized in silicone rubber (Bambot et al., 1994). The optical configuration of the components is shown in Figure 2B. This silicone film was attached to the cuvette wall using silicone grease (high-vacuum grease; Dow Corning, Midland, MI). This successfully prevents the sensor patch from peeling off the wall and penetration of media between the film and the photodetector. The patch was covered by a layer of black silicone (GE 312A; General Electric, Waterford, NY) for optical isolation from the fermentation media. The wall of the cuvette was covered with black tape (with a window for the sensor) to prevent excitation of the media.

Oxygen detection was performed using frequency domain detection of ruthenium fluorophore lifetime. In this technique, the excitation light is modulated and the lifetime is measured by determination of the phase shift between excitation light and fluorescence emission. This is a well-established method for oxygen detection (Bambot et al., 1994) and relies on the reversible quenching of fluorescence emission due to oxygen binding. Its greatest advantage is the fact that the measurements are equilibrium based and do not consume oxygen. Calibration was achieved by using an air-nitrogen blending setup and recording the phase shift going from nitrogen to air. The calibration curve of the device is presented in Figure 3C.

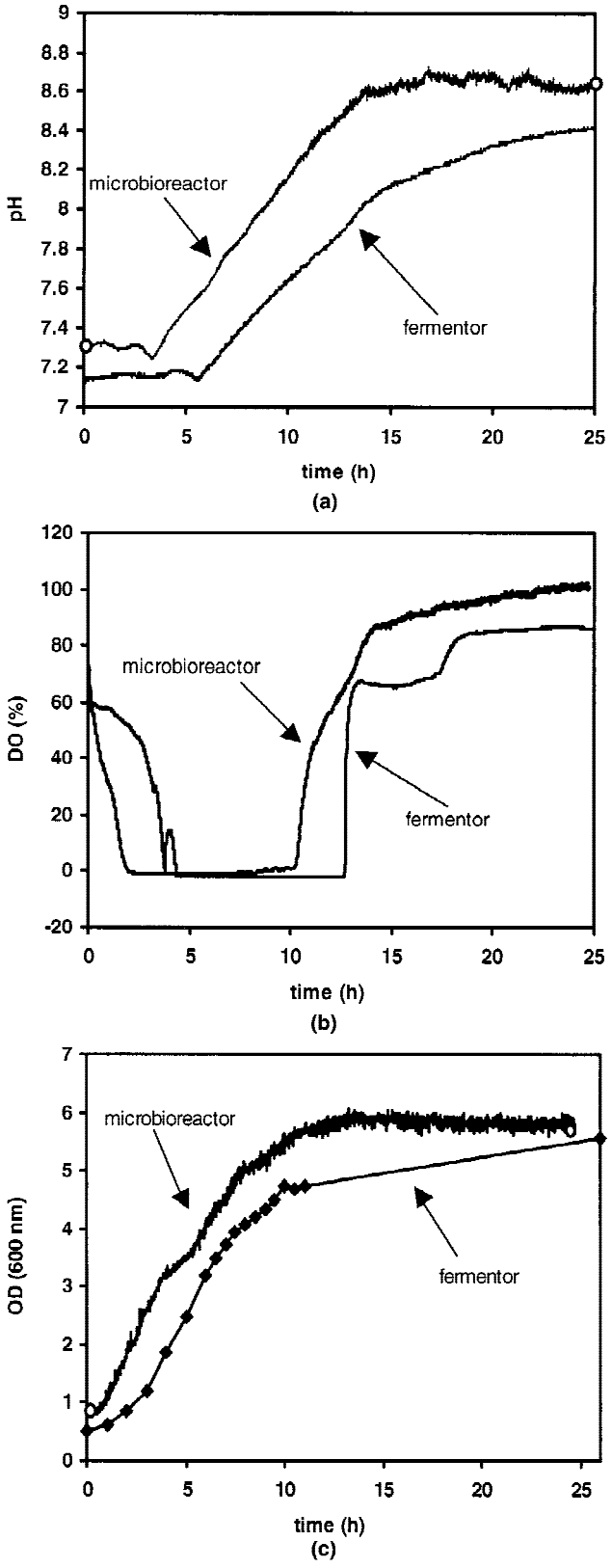

Figure 4. Time profiles of microbioreactor variables and comparison with a standard 1-L bioreactor. **A**: pH profiles. The circles in the beginning and at the end represent the pH values measured with a standard pH meter. **B**: DO profiles. **C**: OD profiles. The circles in the beginning and at the end of the process represent the values measured by spectrophotometer after dilution.

The increased thickness of the sensor resulted in an increased response time of the sensor—up to 2 min. While this is not a problem for bioprocess measurements, it created problems in measurements of the K_La. For this reason, K_La was determined using a sensor without the protective layer with a much shorter response time of 20 sec.

Preserving the value of K_La is a particular problem when scaling a bioprocess (up or down). In our case, it was determined that matching the air flow and the stirring rate resulted in K_La values that were substantially lower than the values obtained in the 1-L bioreactor ($K_La = 9.8\ h^{-1}$ in the microbioreactor; $21\ h^{-1}$ in the 1-L bioreactor). Furthermore, variation of the stirring rate had very little effect on K_La. One possible explanation of this finding is the difference in the positioning of the sparger and the stirrer. In a normal bioreactor, the stirrer is positioned in the escape path of the air bubbles. Stirring adds a significant horizontal component to their trajectory, increasing the total time of their presence in the solution. This effect is much less pronounced when the stirrer is below the sparger.

Another option for K_La variation is changing the air flow rate. The transfer coefficient of the microbioreactor was determined at 1, 2, and 3 vvm. K_La was $9.8\ h^{-1}$, $27.5\ h^{-1}$, and $44.4\ h^{-1}$, respectively. Transfer coefficients of the microbioreactor and the bioreactor were very close at an air flow rate of 2 vvm in the microbioreactor.

An additional advantage of the ruthenium complex is its long lifetime (microseconds)—this permits low frequency modulation (76 kHz in this case) which reduces the circuit cost to $100 or so. In contrast, short lifetime (tens of nanoseconds) fluorophores demand high-frequency modulation (tens to hundreds of MHz), where costs and complexity rise by an order of magnitude or more.

Microbioreactor Performance

The performance of the microbioreactor was finally tested by parallel fermentations of *E. coli* in the microbioreactor and a 1-L Bioflo III (New Brunswick Scientific) fermentor. Both were inoculated at the same time from the same seed culture after setting them to run under identical K_La—the fermentor was run at 300 rpm and 1 vvm aeration ($21\ h^{-1}$). This K_La was arbitrarily chosen as agitation/aeration above this level led to foaming in the microbioreactor. Clearly, this was more due to the ad hoc fabrication of the aeration system and an inefficient magnetic stir-bar served as the agitator. As mentioned above, oxygen delivery to a growing culture is the most critical parameter in bioreactor operation—this is largely due to the extremely poor solubility of oxygen in an aqueous phase (8 mg/L at 35°C in distilled water). Comparing the performance of two bioreactors (say, during scale-up) requires that all parameters be identical, especially the K_La, since a process with a higher K_La will invariably result in higher cell density due to greater oxygen availability. Comparison of the resulting profiles is shown in Figure 4. As can be seen, the profiles of pH, dissolved oxygen, and optical density are very similar in both pro-

cesses. The patterns of oxygen depletion during the exponential growth, as well as the recovery of the dissolved oxygen to 100% at the end of the process, are similar and reproducible in both cases. The correlation coefficient between the OD of the two fermentations was 0.984, indicating very similar growth profiles in the two cultures. However, all the specific points of the microbioreactor process occurred approximately 2 h earlier. Additional investigation showed that after 1 h of operation the microbioreactor heated up by approximately 3°C above the ambient temperature due to Joule heating (the cuvette was not equipped for temperature control). Thus, while the cells in the 1-L bioreactor were cultivated at 25°C, the microbioreactor process was at 28°C. Since the optimal growth temperature for *E. coli* is 37°C, the temperature elevation may explain the slightly increased cell growth rate and decreased lag time in the microbioreactor. One solution to the problem would be the use of a small thermoelectric device for temperature control.

This work has shown that a bioprocess could be successfully performed and followed in small volumes. The use of semiconductor light sources and detectors allowed us to design a very compact and low-cost detection system. All the parts used and the circuit components necessary to obtain a signal were obtained for a cost of less than $400. In the near future, we intend to develop feedback loops for temperature and air-flow control, use an immobilized pH indicator, and select an appropriate indicator with wavelengths that are different from GFP, so that it will be possible to simultaneously measure the cell mass and the product.

The sensors and actuators used allow for an even further decrease of the reactor volume.

Given the low cost of the detection system used (<$400), as well as the small volumes of the bioprocess, it seems possible to develop a system where many bioprocesses (96 or more) could be run in parallel for lower cost than one bench-scale bioreactor. This will greatly reduce both the cost and the development time for optimizing new or established bioprocesses and lead to high-throughput bioprocessing. In addition, it could dramatically improve microbial isolation and cultivation of new species, as enormous numbers of experimental conditions could be attempted on a massively parallel scale.

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