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ABSTRACT: Several methods exist for increasing the scale of cell culture in the laboratory. While these methods provide significant increases in biomass, they are often prohibitively expensive for many laboratories. We have engineered a small-scale bioreactor with a novel means of introducing oxygen through the catalytic breakdown of hydrogen peroxide using a manganese oxide catalyst. We have also adapted and modified an existing assay for dissolved oxygen to be compatible with culture conditions. In this system we have been able to culture CHO cells at densities of up to 10^7 cells/mL without the use of automated feedback systems.

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KEYWORDS: dissolved oxygen; dissolved oxygen monitoring; CHO cells; bioreactor; microcarriers

Introduction

Cultured cells provide sources of biomolecules for isolation and purification in many modern experimental and industrial settings. To purify sufficient quantities of biomolecules from cultured cells, cultures often have to be performed on a large scale. A number of options currently exist for scaling-up cell culture, which range from liter-sized roller bottles to fluidized bed bioreactors and fermentors capable of 1,000 L volumes (Griffiths, 2001). One difficulty that exists in scaling cultures is the cost of production. This incurred cost prevents many smaller operations from achieving their full potential, and consequently creates a system less productive than possible.

Recently, many of the conditions required for both small-scale fed batch bioreactors and perfusion-based bioreactors have been refined (Butler, 2005; Hiedemann et al., 1994). Both suspension and adherent cell lines (CHO-K1, 293-T, MDCK, Hep-G2, and HeLa cells among others) have been used in these culture conditions to significantly increase cell growth and protein production (Goldman et al., 1998; Merten et al., 2001; Schoenherr et al., 2000). Under conditions of high-cell density (greater than 10^6 cells/mL), several culture parameters must be kept under tight control. The culture condition changes can be monitored through the use of real-time data collection and controlled for certain conditions (such as pH, glucose, and DO) (Hiedemann et al., 1998; Iding et al., 2000; Komives and Parker, 2003).

If these conditions could be managed in an economical manner it would be much more practical to run a small-scale bioreactor than numerous, smaller culture flasks. Because the testing apparatus for important cell culture conditions are often costly we sought an alternative way to test for two of the most commonly monitored components of culture media (glucose and dissolved oxygen). Previously, the use of small-scale bioreactor systems for smaller operations has not been an option due to the inability to ensure proper culture conditions without a real-time computer-controlled system.

Our solution uses a 1 L fed-batch spinner flask system in which CHO K-1 cells are cultured on macroporous microcarriers. In this paper, we describe an efficient and economic method of monitoring culture parameters, as well as the maintenance of optimal culture conditions. To accomplish this, we have developed a novel means of introducing oxygen to the system via the catalytic breakdown of hydrogen peroxide and have adapted a previously existing method (the Winkler dissolved oxygen assay) to the microscale range. We also had made modifications to the assay to remove or neutralize interfering substances. Using these novel methods we have been able to increase recombinant protein production several hundred-fold with a significant reduction in cost.

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Materials and Methods

Cell Culture

CHO K-1 cells ectopically expressing perilipin (Tansey et al., 2003) were obtained from lab stocks and cultured in T-75 (Corning, Acton, MA) flasks prior to introduction into the bioreactor system. Microcarriers (1.2 g) were equilibrated in media in a baffled 1 L glass spinner flask with two angled side arms (Corning). Prior to inoculation of the bioreactor, cells were detached from T-flasks using trypsin (Sigma, St. Louis, MO) (0.4 mL of 10× trypsin for 5 min), centrifuged at 200*g* for 5 min, and suspended in fresh media. The suspension culture was then introduced to the spinner flask dropwise while mixing at 30 rpm. The same medium was used for both T-flask and bioreactor cultures, consisting of Ham's F-12, supplemented with 10 mM L-glutamine, 5% fetal calf serum, 14 mM $NaHCO_3$, and 300 μg/mL Geneticin. Once introduced to the bioreactor system, viable cells both adhered to microcarriers and floated freely in suspension. All tissue culture media and chemicals were obtained from Gibco/Invitrogen (Carlsbad, CA) unless otherwise noted.

Preparation and Maintenance of Bioreactor

Prior to culture, the entire bioreactor assembly was coated with a silicone-based water repellant (Sigmacote, Sigma) to prevent cell adhesion to reactor surfaces and steam autoclaved for 20 min at 121°C. Cultisphere G Macroporous microcarriers (Percell Biolytica, Astorp, Sweden) were prepared according to the manufacturer's instructions. Culture conditions were tested at regular intervals throughout the day. At each sampling, mixing was ceased for 3 min to allow microcarriers to settle. Two separate aliquots were removed for further testing: 3 mL of suspension culture media from the upper region of the reactor, and 50 μL of the microcarrier-containing lower region. Cell counting on the upper region was conducted prior to further testing using a hemacytometer (Fisher Scientific, Pittsburgh, PA). Viability was measured using the Trypan blue dye exclusion method. Microcarriers were digested with 5 μL 10× trypsin/2 mM EDTA (Sigma) for 10 min. Glucose was measured using the Trinder glucose hexokinase assay (Bergmeyer, 1965), and maintained at 0.7–2.5 g/L. To compensate for glucose depletion, 0.55 M glucose in PBS was added dropwise, when required. pH was determined using a Fisher Accumet pH meter equipped with a AccuTupH probe. pH was maintained at 6.9–7.2 by the dropwise addition of 100 mM NaOH. Dissolved oxygen was measured using a modification of the Winkler method, and maintained at 2–4 mg/L (Winkler, 1888) and maintained as described below.

Modification of the Winkler Method and Dissolved Oxygen Testing

Media removed from the system at each interval was assayed for dissolved oxygen content. Removal of interfering substances was achieved by treating the sample with 50% (w/v) trichloroacetic acid (5% final concentration). The resulting solution was then centrifuged at 20,000*g* for 3 min. The supernatant was collected, neutralized with 150 μL 2 M KOH, and then used in a micro-scale Winkler method of detection. Briefly, 2.2 mL of neutralized media was placed in a 2 mL screw cap Eppendorf microfuge tube with 17 μL alkaline-iodide-azide (20 M NaOH, 0.9 M KI, 1% w/v NaN_3) (Asterberg, 1925) and 17 μL 2.15 M $MnSO_4$. The tube was inverted ten times, the precipitate allowed to settle, inverted once more, and again allowed to settle. Seventeen microliters of concentrated sulfuric acid was added to the solution, causing the precipitate to dissolve and a yellow color to appear. Iodometric titration with 0.0125 normal $Na_2S_2O_3$ was then performed as previously described (Winkler, 1888).

To verify the modifications made to the Winkler method, the modified method was compared to the full scale method as well as DO measurements made with a Vernier DO probe (Vernier Software and Technology, Beaverton, OR) using four different conditions (degassed distilled water, water equilibrated with air in a 5% CO_2 incubator, water saturated with sparged air, and water treated with O_2 using the oxygen generation system described below for 1 h).

Oxygen Source

O_2 was generated by the catalytic breakdown of 100 mL 1% H_2O_2 using 2–5 mg of MnO_2 catalyst and introduced to the system through gas permeable silicone tubing (0.063 I.D. × 0.125 O.D. inches, 85 cm length, Nalge Nunc International, Rochester, NY).

Oxygen Transfer Rate

OTR was measured using the sulfite oxidation method of Hermann et al. (2001). Both quantitative measurements (pH change) and qualitative observations (color change of the bromothymol blue indicator dye) were to determine the endpoint of the oxidation.

Results and Discussion

Generation of Oxygen Using the H_2O_2/MnO_2 System

O_2 gas was produced by catalytically cleaving 100 mL of a 1% solution of H_2O_2 with 2–5 mg MnO_2. In this manner, we were able to produce O_2 at a steady rate of 120 mL/h for at least 2 h (Fig. 1a), resulting in maintenance of DO levels in the bioreactor between 2–4 mg/L (50%–70 % saturation), as can be seen in Figure 4. DO concentration was determined using the modified Winkler method described below.

Results of Modifications Made to the Winkler Method for Dissolved Oxygen Testing

The Winkler method is a classic method of measuring dissolved oxygen in environmental field settings. It is highly

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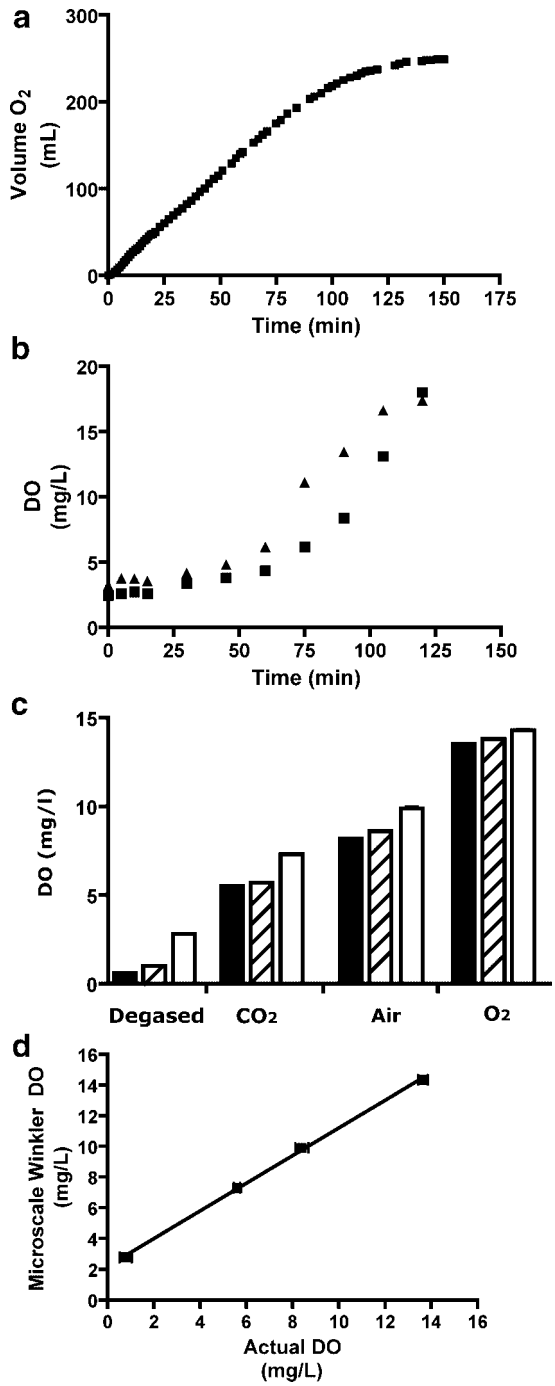

Figure 1. Oxygen generation and measurement. **a**: Production of O_2 gas for bioreactor by the catalytic breakdown of H_2O_2. Volume of O_2 produced (■) is plotted versus time. Oxygen production is linear through the first 2 h of the reaction. **b**: Measurement of DO using modified Winkler method. DO concentration (▲, media; ■, H_2O) is plotted versus time. Saturation rates for degassed H_2O and Hams F-12 media are comparable and show no significant differences in the modified microscale DO assay. **c**: Comparison of the modified Winkler method with the traditional large-scale and a DO probe. The three methods (solid bar represents DO probe measurements, hatched bar represents Winkler method measurements, and open bars represent the modified Winkler method) were used to assay four different conditions (see Materials and Methods). Each bar represents the average ± standard deviation of $n = 3$ measurements. **d**: Plot of DO measured using the standard Winkler method or DO probe versus modified Winkler method for each of the four conditions tested. The comparison resulted in a linear relationship between the two methods ($r^2 = 0.9995$).

accurate, but has several drawbacks. It requires large sample size (200 mL), is incompatible with high concentrations of organic matter, and is susceptible to error if the samples are mishandled prior to fixing DO. A microscale version of this assay was developed that employed only 2 mL of sample volume, making it feasible for use in a liter scale bioreactor system.

To verify that the modifications made to the Winkler assay have not compromised its integrity, we compared the microscale assay with the traditional large scale Winkler assay and DO measurements made with a DO probe. Each of these systems was used to assay degassed distilled water, water equilibrated with air in a 5% CO_2 incubator, water saturated with sparged air, and water treated with O_2 using the oxygen generation system described for 1 h (Fig. 1c and d). In each case, the microscale Winkler assay responded in lock step with other accepted methods but was systematically above the other readings by approximately 1.7 mg DO/L. However, the response of the microscale Winkler assay was linear throughout the assay range, enabling for a correction to bring it into complete accordance with the other methods (Fig. 1d).

We also compared deoxygenated media (Ham's F-12, without supplemental antibiotics, L-glutamine, bicarbonate, or serum) to water (Fig. 1b). Both the degassed media sample and the water sample were oxygenated using the H_2O_2/MnO_2 system and were able to absorb oxygen and saturated at similar rates.

To insure that media components did not interfere with the chemistry of the Winkler assay, we tested each of the major components in the media and compared the results obtained with deionized water (Table I). Most media components did not interfere with this assay at the concentrations typically used in cell culture. These included Ham's F-12 nutrient mixture, glucose, sodium bicarbonate, L-glutamine, and G-418 (geneticin sulfate). A notable exception to this was that penicillin-streptomycin did interfere with the assay, potentially due to the β-lactam ring of penicillin. These antibiotics were not used in the media of bioreactor runs to avoid interference with the assay. Serum was also found to interfere with the assay. To remove the interfering serum components, samples were treated with trichloroacetic acid to precipitate proteins and other organic

Table I. Substances tested for compatibility with the modified Winkler DO assay.

Media component	Concentration	Compatible	Compensation for assay compatibility
Calf serum	5%	No	TCA precipitation
Geneticin/G-418	300 μg/mL	Yes	—
Glucose	2 g/L	Yes	—
L-glutamine	100 mM	Yes	—
Ham's F-12	Per instructions	Yes	—
$NaHCO_3$	14 mM	Yes	—
Penicillin/Streptomycin	100 mM	No	Eliminated

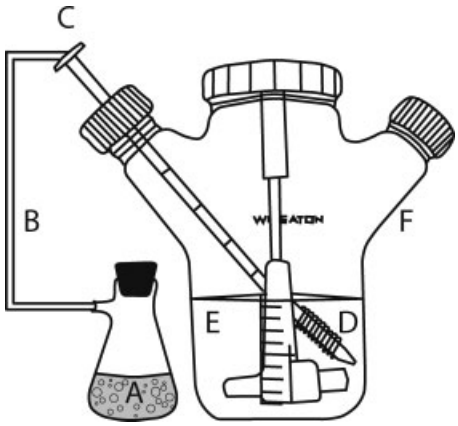

Figure 2. Schematic of bioreactor system. A, oxygen generation flask; B, Tygon tubing; C, sterile filter; D, oxygen-permeable silicone tubing; E, culture medium; F, 3 L glass spinner flask.

molecules. Trichloroacetic acid concentrations used in the precipitation did not interfere with the assay.

Collectively these data show that by incorporating these novel modifications we were able to successfully use the Winkler method to determine DO concentrations in our culture system.

Description of the New Culture System and Oxygen Generator

Cells were cultured in a 3-L Wheaton spinner flask kept in a 37°C, 5% CO_2 incubator (Fig. 2). The flask had two ports, one for sampling and one for O_2 introduction, and was equipped with a magnetic paddle for stirring. The flask contained 1 L of culture media, with ample headspace (2 L) for gas exchange to occur. The system was stirred at a spinning rate of 30 rpm. The oxygen transfer rate (OTR) of this system was determined using the method of Hermann et al. (2001). The observed rate was (0.00016 mmol/h). This is most likely due to the relatively large volume of media and relatively low stirring rate (30 rpm). This OTR did not appear to detrimentally impact cell growth, provided the DO concentration remained above the limits cited above.

The values observed for OTR in the present system are lower than those found in the literature for other small scale or microbioreactors (Hermann et al., 2001; Kensey et al., 2005; Maier et al., 2004; Mantzouridou et al., 2005), however, these studies focused on reactors with much smaller working volumes (typically under 75 mL) and under much more highly agitated conditions (upwards of 500 rpm). Given these differences it is understandable why a lower OTR was observed in this system and why O_2 supplementation is required for optimal cell growth at higher cell densities.

Culture Conditions

pH decreases as acidic wastes accumulate during cell growth. As the system equilibrated, its buffering capacity was reached and then exceeded as cell density reached the exponential phase (Fig. 3a). Ten milliliters of 100 mM NaOH was added dropwise over the course of several minutes to compensate for this decline in pH, and allowed the culture conditions to remain in the proper pH range (6.9–7.2). The addition of NaOH once per day provided a simple way of maintaining a suitable pH level.

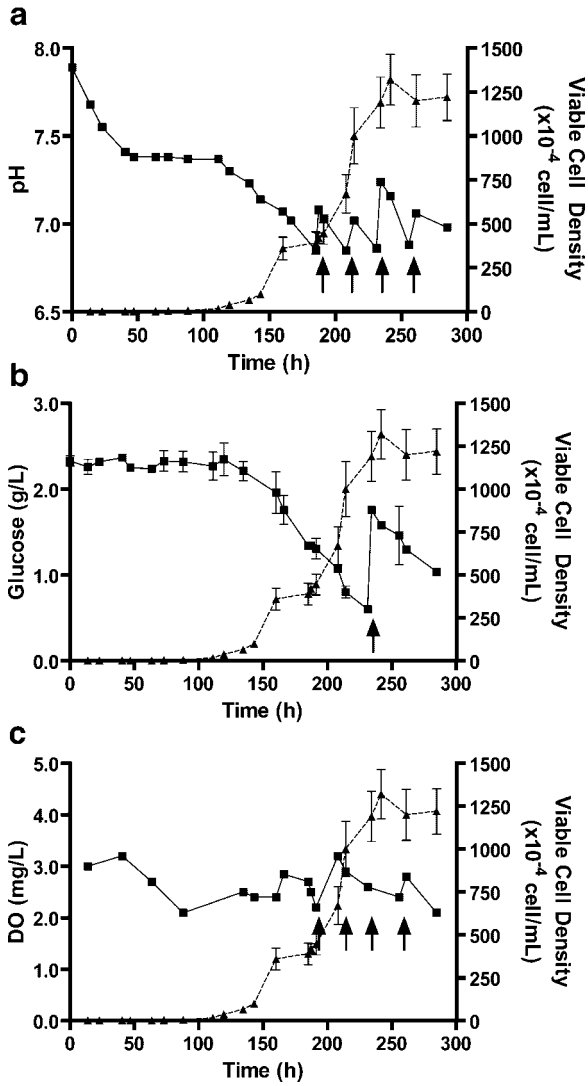

Figure 3. Effects of culture parameters on cell growth. **a**: Cell growth effects on pH levels. Viable cell density (▲) and pH (■) are plotted versus time. The arrows indicate the addition of NaOH. **b**: Glucose depletion and restoration. Viable cell density (▲) and glucose concentration (■) are plotted versus time. When glucose levels dropped below 0.7 g/L, glucose was added to the system as indicated by the arrow. **c**: Maintenance of DO levels. Viable cell density (▲) and DO levels in culture medium (■) are plotted versus time. DO levels were maintained at 2–4 mg/L (40%–80% saturation). Arrows indicate the introduction of DO into the system.

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The culture medium contained sufficient glucose to keep conditions at the desired level (approximately 1 g/L) until the exponential phase of cell growth was reached. When the glucose level dropped below 0.7 g/L, levels were restored through the dropwise addition of 10 mL 0.55 M glucose (Fig. 3b). This was generally required once or twice per bioreactor run, depending on cell seeding and growth levels.

DO levels in the bioreactor were maintained between 2–4 mg/L (50%–70 % saturation), as shown in Figure 3c. DO concentration was determined using the modified Winkler method described above. Oxygen was generated in a 125 mL side-arm Erlenmeyer flask which was connected to the bioreactor using Tygon tubing (Fig. 2). At the bioreactor interface, O_2 passed through a 22 mm Millipore Millex-GS 0.22 micron sterile filter before passing into the bioreactor oxygen solubilizer, an 85 cm length of oxygen permeable silicon tubing. Oxygen that did not dissolve into the media through this tubing was flushed into the headspace. This system provided a reasonable means of introducing DO to culture medium when cells reached higher cell densities ($\geq 10^6$ cells/mL).

When DO concentrations dropped below 2 mg/L, this system showed a distinct inhibition of cell growth compared to the system that was supplemented as described above (Fig. 4). When supplemental oxygen was added to the deprived system with retarded cell growth, the growth rate continued its exponential pattern and reached similar final cell densities as the system supplied with DO as described above.

Cell culture provides an excellent means of producing significant quantities of recombinant protein for further analysis and study. The proteins produced in mammalian cell culture offer several advantages to bacterial expression systems (including proper folding and post-translational modifications) (Wurm, 2004) but have traditionally been limited in scale for small academic laboratories. For most adherent cell lines (CHO, 293-T, MDCK, Hep-G2, HeLa) investigators are limited to using larger culture vessels, larger numbers of existing culture vessels, or opting to switch to more costly culture systems (such as roller bottles).

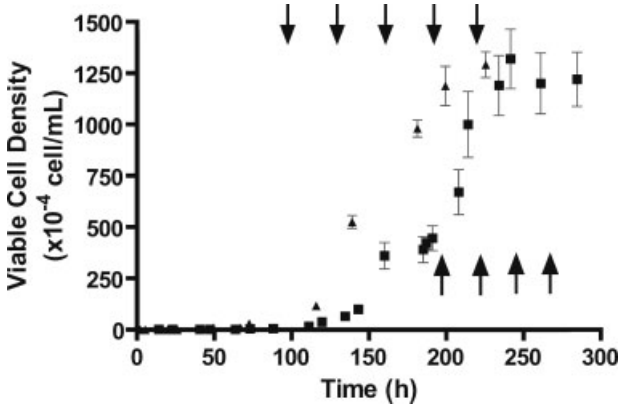

Figure 4. Availability of DO affects cell growth rate. Viable cell density for system with available DO at all times (▲) and a system in which DO was restricted (■) are plotted versus time. The arrows indicate the introduction of DO to each system. The restricted system did not receive supplemental oxygen until indicated.

At the other extreme, bioprocess engineers have adapted and developed bioreactors that can attain high-cell density (greater than 10^7 cells/mL) and range in scale from tens to tens of thousands of liters for the commercial production of biomolecules. Many of these have been scaled back to the 0.250–10 L scale and presently several excellent laboratory or pilot scale bioreactors are commercially available (for reviews see Hu and Piret, 1992; Kumar et al., 2004; Prokop and Rosenberg, 1989). While these systems offer advantages such as real-time control of DO, pH, and a wide range of metabolites (glucose, lactate, pyruvate, acetate, and amino acids among them), they come with a significant cost.

We have focused on three parameters critical for CHO cell growth (DO, pH, and glucose) and developed a fed batch spinner flask system in which cells were grown on macroporous microcarriers. A major concern at high-cell density is the concentration of dissolved oxygen present in the media. We have developed or modified methods for both the introduction of oxygen (which was generated by the catalytic breakdown of H_2O_2 and supplemented through porous silicon tubing) and the measurement of dissolved oxygen (via several modifications of the Winkler method to adapt both the scale of the assay and to remove or neutralize substances found in culture that would interfere with the assay).

Several methods were considered to introduce oxygen to the culture as cell density exceeded 10^6 cells/mL. Among these techniques were: sparging with air or pure oxygen, flushing the headspace with air or pure oxygen, or using a microfilamentous system (Kumar et al., 2004; Prokop and Rosenberg, 1989). The method we chose was to introduce oxygen by means of oxygen permeable silicon tubing. Our system provided 84.8 cm^2 of surface area through which the media could interact with the tubing. O_2 that did not diffuse into the media was flushed into the headspace above the media; however, this area was also in open exchange with the incubator gas (air enriched with CO_2 to 5%). It is unclear in our system how much gas exchange occurred through the tubing compared with the headspace but given the permeability of the tubing (7.96×10^{-7} cm^3 mm/s cm^2 cm Hg) and the oxygen gradient, we presume most exchange occurred before residual gas entered the headspace. Using this method, we avoided problems incurred by sparging including foaming, shear effects on cells, and stripping of CO_2, (which was required as a buffer in our culture conditions).

Oxygen for the DO supply system was generated in the incubator by the catalytic breakdown of H_2O_2 with MnO_2. While a cylinder of compressed oxygen with a regulator is the standard means of introducing oxygen to a bioreactor, our conditions required comparatively minor oxygen supplementation. Oxygen supplementation also requires

some means of insuring that the concentrations do not approach toxic levels. Under the conditions tested we never observed DO concentrations above 80% (4 mg DO/L), which could have deleterious effects on the cells. This system provided an easy, economical, and reliable means of supplementing DO in culture.

The Winkler method is a widely used method for the standardization of oxygen probes and for field studies. Typical difficulties encountered with the Winkler assay include interference from high concentrations of organic molecules and errors introduced by sample mishandling prior to fixing the DO. We have adapted this chemistry for the tissue culture laboratory. The assay itself was scaled down by a factor of more than 100 allowing for smaller volumes to be tested. Interfering substances were avoided (such as penicillin/streptomycin), neutralized, or removed by precipitation with trichloroacetic acid (as in the case of serum components). Errors due to sample handling still may complicate this assay. In each of our samples tested replicates were very precise with ranges typically from 0.2–0.3 mg/L. However, oxygen concentrations can be significantly effected by changes in temperature or storage conditions. Hence, it is of paramount importance to process the samples and fix the oxygen concentrations in as timely a means as is possible with this assay. Once proficient with the handling of samples we found little variability (<1%) from replicate measurements using the micro-scale Winkler assay.

The cost of a typical automated bioreactor system can easily range into tens of thousands of dollars. For this reason, smaller laboratories with fewer resources are typically limited in the scale of biomolecule production available to them. Provided that a laboratory has access to typical tissue culture facilities (CO_2 incubators, laminar flow hoods, and the like) the system we have proposed costs on the order of $400.00 for the initial set up and approximately $100.00 per subsequent batch for media, microcarriers, reagents, and chemicals. We have calculated that the average biomass produced in one reactor run equaled the amount generated from using 250 T-75 flasks or 120 150-mm petri dishes. Based on these calculations and the cost of only the plasticware used in culture, the bioreactor system becomes cost effective after only one to two runs.

In conclusion, we have developed a small-scale bioreactor for use in laboratory settings where traditional cell culture is failing to provide substantial quantities of cells or biomolecules. It is a readily available technology and scales up from T-flasks without difficulty. We have used this reactor on the 1 L scale to produce recombinant perilipin for further biophysical characterization (Londos et al., 2005; Tansey et al., 2004), but results in the literature indicate that it may be scalable (Dürrschmid et al., 2003; Griffiths, 2001). This system attains cell densities as high as those reported in the literature (10^7 cells/mL) at significant cost savings (Hu and Aunins, 1997). While the experiments we performed were run in fed batch mode, in theory could be used in other modes for secreted proteins. Using this system we have obtained consistent high-quality protein production at significant cost savings compared to a typical automated bioreactor.

Nomenclature

DO	dissolved oxygen
CHO	Chinese hamster ovary fibroblasts
PBS	phosphate buffered saline
TRIS	2-amino-2-hydroxypropyl-1,3-propanediol
OTR	oxygen transfer rate.

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