10th International IFAC Symposium on
Computer Applications in Biotechnology
Preprints Vol.1, June 4-6, 2007, Cancún, Mexico

OUR AND CER ESTIMATION IN HIGH DENSITY MAMMALIAN CELL PERFUSION CULTURES

C. Goudar, K. Joeris, C. Cruz, C. Zhang and K. Konstantinov

Process Sciences, Bayer HealthCare, 800 Dwight Way, Berkeley, CA 94710, USA

Abstract: We present methods for real-time estimation of OUR and CER in high density mammalian cell perfusion cultures. These computations are based on global mass balances and do not require k_La data and reactor perturbations. The applicability of this approach was tested in a long-term CHO cell cultivation where pH, temperature and DO were varied over the course of the cultivation. Real time OUR and CER data enable real-time metabolic flux estimation and allow cell physiological state monitoring and manipulation through advanced bioreactor process control.

Keywords: cell culture, CER, metabolic flux analysis, OUR, process control

1. INTRODUCTION

Oxygen uptake and carbon dioxide evolution rates (OUR and CER, respectively) provide useful information on cell metabolism and physiology. Reliable estimation of these rates is desirable as they are indicators of changes in cellular metabolic activity Bonarius et al. (1995); Miller et al. (1987b); Ogawa et al. (1992); Ozturk and Palsson (1990); Philips et al. (1987); Ruffieux et al. (1998); Shi et al. (1993); Yamada et al. (1990). Oxygen uptake data are an indicator of cell density and metabolic rates such as glucose consumption and on-line OUR measurements have been used to design feeding strategies and control bioreactor operation Kyung et al. (1994); Zhou and Hu (1994). OUR information is also necessary for bioreactor design and scale-up given the low solubility of oxygen. This is especially important for high density perfusion cultivations that have high oxygen transfer requirements. Moreover, OUR and CER are required for metabolic flux analysis even in the simplest of mammalian cell bioreaction networks Balcarcel and Clark (2003). Robust OUR and CER estimation is thus critical for bioprocess development and is also important for monitoring and diagnosing manufacturing bioreactors.

The primary approaches that have been for insitu OUR estimation in mammalian cell cultures include the stationary liquid phase balance approach, the dynamic method, and the global mass balance (GMB) approach Ruffieux et al. (1998). CER estimation is more difficult than OUR because of the reversible dissociation of CO_2 into $H_2CO_3^*$, HCO_3^- and CO_3^{2-} in solution. The equilibria of these dissociation reactions are strong functions of pH, temperature and ionic strength that must be accounted for during CER estimation. There are only a few mammalian cell chemostat and batch studies where CER was estimated Bonarius et al. (1995); Frahm et al. (2002); Lovrecz and Gray (1994) and none in perfusion bioreactors. The use of bicarbonate buffered medium in mammalian cell culture further complicates CER estimation because of this additional abiotic CO_2 source.

In this study, we present methods to estimate OUR and CER in mammalian cell perfusion cul-

tures using global mass balances. While measurement of oxygen and carbon dioxide concentrations in the inlet and outlet gas streams is necessary, k_La data are not required and no reactor perturbations are necessary. Our approach allows real-time OUR and CER estimation that can also serve as indicators of cell density and nutrient consumption rates. Moreover, this data enables real-time estimation of metabolic fluxes providing useful insights into cell metabolism and physiology that can be used in advanced control strategies for optimal bioreactor performance.

2. THEORY

2.1 OUR Estimation

A combination of low oxygen solubility and high cell density make liquid stream oxygen contributions negligible and only gas phase oxygen balance equations are necessary for OUR estimation. Under steady-state conditions, there is no accumulation of oxygen in the bioreactor and oxygen uptake by the cells is the difference between the oxygen concentrations in the inlet and outlet streams

$$\mathrm{OUR} = \frac{F_{total}}{X_V^B V}(O_2^{in} - O_2^{out})10^3 \tag{1}$$

where OUR is the cell specific oxygen uptake rate (pmol/cell-d), F_{total} is the total gas flow rate (L/d), X_V^B the bioreactor viable cell density (10^9 cells/L), V the bioreactor volume (L) and O_2^{in} and O_2^{out} the inlet and outlet oxygen concentrations (mol/L), respectively.

2.2 CER Estimation

2.2.1. Bicarbonate System Dynamics in a Mammalian Cell Bioreactor Carbon dioxide sources in a perfusion system include cellular respiration, bicarbonate buffered medium and sodium bicarbonate when used as a base for pH control. Carbon dioxide produced by the cells dissolves in water to form carbonic acid

$$\mathrm{CO_{2(g)}} \leftrightarrow \mathrm{CO_{2(aq)}} \tag{2}$$
$$\mathrm{CO_{2(aq)}} + \mathrm{H_2O} \leftrightarrow \mathrm{H_2CO_3} \tag{3}$$

It is common practice in solution chemistry to combine the aqueous concentration of carbon dioxide and carbonic acid such that the above equations can be replaced by a single expression

$$\mathrm{CO_{2(g)}} + \mathrm{H_2O} \leftrightarrow \mathrm{H_2CO_3}^*; \qquad K_g = 10^{-1.47} \tag{4}$$

where $\mathrm{H_2CO_3}^* = \mathrm{CO_{2(aq)}} + \mathrm{H_2CO_3}$ and K_g is the equilibrium constant under standard conditions (T = 25 °C and ionic strength, $I = 0$). Further dissociation of $\mathrm{H_2CO_3}^*$ to $\mathrm{HCO_3^-}$ and subsequently to $\mathrm{CO_3^{2-}}$ can be described as

$$\mathrm{H_2CO_3}^* \leftrightarrow \mathrm{HCO_3^-} + \mathrm{H^+}; \qquad K_1 = 10^{-6.35} \tag{5}$$
$$\mathrm{HCO_3^-} \leftrightarrow \mathrm{CO_3^{2-}} + \mathrm{H^+}; \qquad K_2 = 10^{-10.33} \tag{6}$$

where K_1 and K_2 are the equilibrium constants under standard conditions.

For typical mammalian cell cultivations, however, the temperature is close to 37 °C and the ionic strength is ~0.1 M depending upon the composition of the medium. The rate constants must hence be corrected to be reflective of experimental conditions. The rate constants can be corrected for temperature using the Van't Hoff equation Snoeyink and Jenkins (1980)

$$K = K_{ref} \exp\left\{\left(\frac{\Delta H^0}{R}\right)\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right\} \tag{7}$$

where K and K_{ref} are the corrected and reference rate constants, respectively, at temperatures T and T_{ref}, ΔH^0 the standard enthalpy change for the reaction, and R, the gas constant. The corrected equilibrium constants were $10^{-6.30}$ and $10^{-10.48}$, respectively, at 37 °C.

To account for ionic strength effects, the activity coefficients were calculated using the Davies equation which is valid for ionic strengths < 0.5 M

$$\log\gamma = -Az^2\left\{\frac{\sqrt{I}}{1+\sqrt{I}} - 0.2I\right\} \tag{8}$$

where γ is the activity coefficient, A is a constant and I the ionic strength. The ionic strength of the medium used in this study was calculated as 0.115 M from the Debye-Hückel theory Morel and Hering (1993)

$$I = \frac{1}{2}\sum C_i z_i^2 \tag{9}$$

where C_i and z_i are charge and concentration of species i, respectively. The activity coefficients for $I = 0.115$M were estimated from Eq.(8) as 0.7747 and 0.3602, respectively, for species with charges 1 and 2. Incorporating the temperature corrected values of the equilibrium constants, Eqs.(5) and (6) can be rewritten in terms of the species concentrations and activity coefficients as

$$K_g = \frac{[\mathrm{H_2CO_3}^*]\,\gamma_{\mathrm{H_2CO_3}^*}}{[\mathrm{CO_{2(g)}}]\,\gamma_{\mathrm{CO_{2(g)}}}} = 10^{-1.60} \tag{10}$$

$$K_1 = \frac{[\mathrm{H^+}]\,\gamma_{\mathrm{H^+}}\,[\mathrm{HCO_3^-}]\,\gamma_{\mathrm{HCO_3^-}}}{[\mathrm{H_2CO_3}^*]\,\gamma_{\mathrm{H_2CO_3}^*}} = 10^{-6.30} \tag{11}$$

$$K_2 = \frac{[\mathrm{H^+}]\,\gamma_{\mathrm{H^+}}\,[\mathrm{CO_3^{2-}}]\,\gamma_{\mathrm{CO_3^{2-}}}}{[\mathrm{HCO_3^-}]\,\gamma_{\mathrm{HCO_3^-}}} = 10^{-10.48} \tag{12}$$

where K_g^c, K_1^c and K_2^c are the concentration based equilibrium constants and γ the activity coefficients of the various species. Activity coefficients

for the charged species were calculated from the Davies equation ($\gamma_{H^+} = \gamma_{HCO3^-} = 0.7746$, $\gamma_{CO3^{2-}} = 0.3602$) and $\gamma_{CO_{2(g)}} = \gamma_{H_2CO_3*}$ was estimated as 1.03 as described in Butler (1998). Substituting these values in Eqs.(10)-(12), the concentration based equilibrium constants K_g^c, K_1^c and K_2^c were calculated as $10^{-1.60}$, $10^{-6.07}$ and $10^{-10.04}$, respectively.

2.2.2. CO_2 Mass Balance Equations Recognizing that CO_2 produced by the cells can exist as both H_2CO_3* and HCO_3^-, it is convenient to combine them while deriving mass balance expressions. Designating $[H_2CO_3^*] + [HCO_3^-]$ as $[CO_2]_T$ to indicate total CO_2 and neglecting CO_2 accumulation in the bioreactor, we have

$$CER = \{[CO_2]_T\}_{in} - \{[CO_2]_T\}_{out} \qquad (13)$$

where $\{[CO_2]_T\}_{in}$ and $\{[CO_2]_T\}_{out}$ are the inlet and outlet concentrations, respectively. Sources of $[CO_2]_T$ include bicarbonate-containing cultivation medium, base ($NaHCO_3$ or Na_2CO_3) or CO_2 gas used for bioreactor pH control, and cellular metabolism. Removal mechanisms for $[CO_2]_T$ include the harvest and cell discard streams along with gaseous CO_2 stripping, either through sparging or membrane aeration. Quantifying contributions from the medium and base on a mol/day basis is straightforward as their carbonate concentrations and flow rates are known. The flow rate of CO_2 gas into the reactor will help determine the amount of CO_2 gas added to the reactor (this is seldom done when bicarbonate-containing medium is used). To determine $[CO_2]_T$ removal from the harvest and cell discard streams, bioreactor $[CO_2]_T$ must be known. This can be estimated from bioreactor pCO_2 measurements that are typically made using a blood gas analyzer.

3. MATERIALS AND METHODS

3.1 Cell Line, Medium and Cell Culture System

CHO cells were cultivated in perfusion mode with glucose and glutamine as the main carbon and energy sources. Experiments were conducted in a 15L bioreactor (Applikon, Foster City, CA) with a 12L working volume. The temperature was maintained at 36.5 °C and the agitation at 40 RPM. Under standard conditions, the dissolved oxygen (DO) concentration was maintained at 50% air saturation by sparging a mixture of oxygen and nitrogen (100 – 150 mL/min) through 0.5 μm spargers and the pH was maintained at 6.8 by the automatic addition of 0.3 M NaOH. The bioreactor was inoculated at an initial cell density of approximately 1.0 x 10^6 cells/mL and cells were allowed to accumulate to a steady-state concentration of 20 x 10^6 cells/mL. The steady-state cell density was maintained by automatic cell discard from the bioreactor.

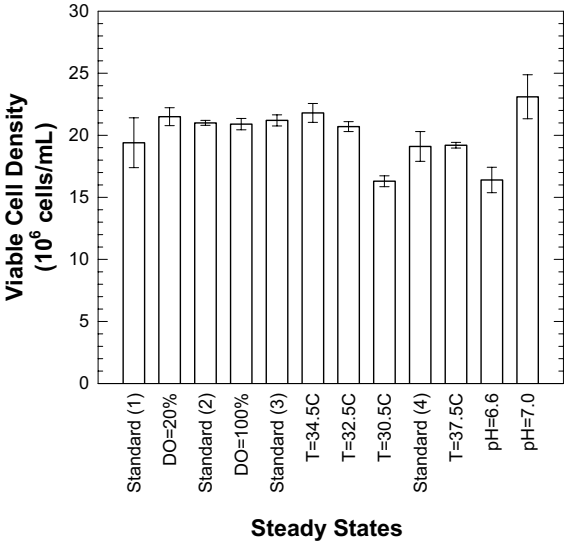

Fig. 1. Cell density averages for the different steady states during the course of the perfusion cultivation. For standard conditions, DO = 50%, T = 36.5 °C and pH = 6.8.

Bioreactor DO, temperature and pH were varied during the course of the cultivation to determine the operating ranges for these variables. The low and high values for DO were 20% and 100%, respectively (set point = 50%), while those for pH were 6.6 and 7.0, respectively. The temperature set point was 36.5 °C and was varied between 30.5 – 37.5 °C during the course of the experiment. Bioreactor conditions were maintained at each of these altered conditions for 10 days and data from the last 4 days were considered representative of each steady state. OUR and CER data presented in later sections are averages of these 4 days for each steady state.

3.2 Analytical Methods

Samples from the bioreactor were taken daily for cell density and viability analysis using the CEDEX system (Innovatis, Bielefeld, Germany). The samples were subsequently centrifuged (Beckman Coulter, Fullerton, CA) and the supernatants were analyzed for nutrient and metabolite concentrations. Glucose, lactate, glutamine and glutamate concentrations were determined using a YSI Model 2700 analyzer (Yellow Springs Instruments, Yellow Springs, OH) while ammonium was measured by an Ektachem DT60 analyzer (Eastman Kodak, Rochester, NY). The pH and DO were measured online using retractable electrodes (Mettler-Toledo Inc., Columbus, OH) and their measurement accuracy was verified through off-line analysis in a Stat Profile 9 blood gas an-

alyzer (Nova Biomedical, Waltham, MA). The same instrument also measured the dissolved CO_2 concentration. On-line measurements of cell density were made with a retractable optical density probe (Aquasant Messtechnik, Bubendorf, Switzerland), calibrated with CEDEX cell density measurements. Concentrations of oxygen and carbon-dioxide in the exit gas were measured using a MGA-1200 Mass Spectrometer (Applied Instrument Technologies, Pomona, CA).

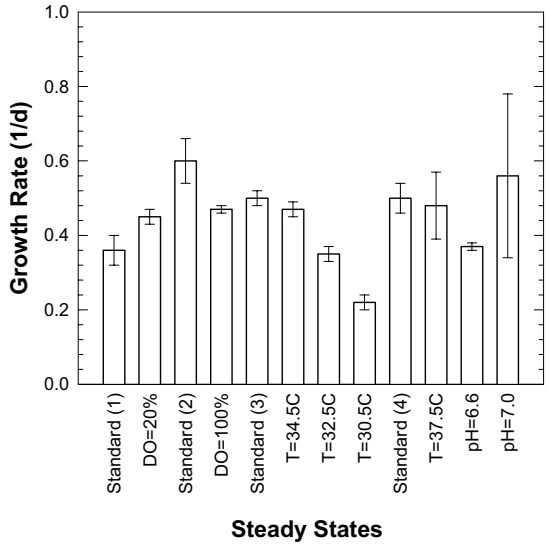

Fig. 2. Growth rate averages for the different steady states during the course of the perfusion cultivation. For standard conditions, DO = 50%, T = 36.5 °C and pH = 6.8.

4. RESULTS AND DISCUSSION

4.1 Cell Density and Growth Rate

The perfusion cultivation comprised of 12 steady states each of 10 day duration and average cell densities for each of these steady states are shown in Figure 1. The target cell density was 20 x 10^6 cells/mL with most values very close to the target. The exceptions were the T = 30.5 °C and pH = 6.6 steady states where growth rates were much lower than the other conditions (Figure 2). Temperature reduction caused an expected decline in growth rate as did pH reduction. No change in growth rate was seen when the DO was varied between 20 and 100%. Cell viability was greater than 95% in all cases (not shown).

4.2 OUR and CER Estimation

4.2.1. OUR, CER and RQ at Varying Operating Conditions OUR values at different DO, temperature and pH set points are shown in Figure

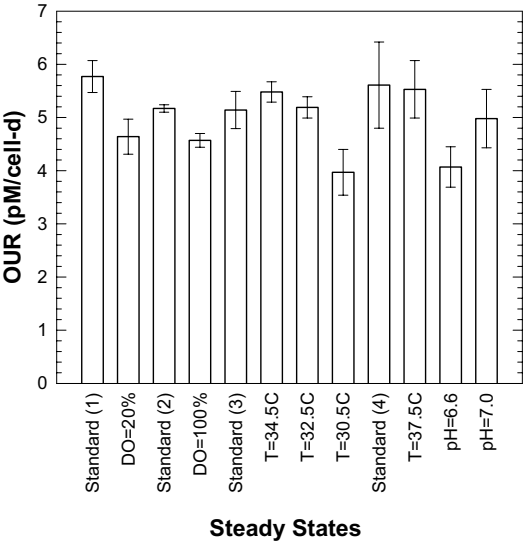

Fig. 3. Average OUR estimates from the mass balance method for the 12 steady states in the perfusion cultivation.

3. The values are averages over their respective steady states along with their associated standard deviations. While OUR values were mostly unchanged across most experimental conditions, they were lower at T =30.5 °C and pH = 6.6 where an overall reduction in growth (Figure 2) and metabolism (not shown) were observed. The lowest CER values of 4.02 and 4.15 pmol/cell-d were also observed at T = 30.5 °C and pH = 6.6, respectively, while those at other set points were relatively similar (Figure 4). RQ values estimated from the average OUR and CER values ranged from 0.96 - 1.18 (Figure 5) suggesting no impact of DO, temperature and pH set point changes on RQ.

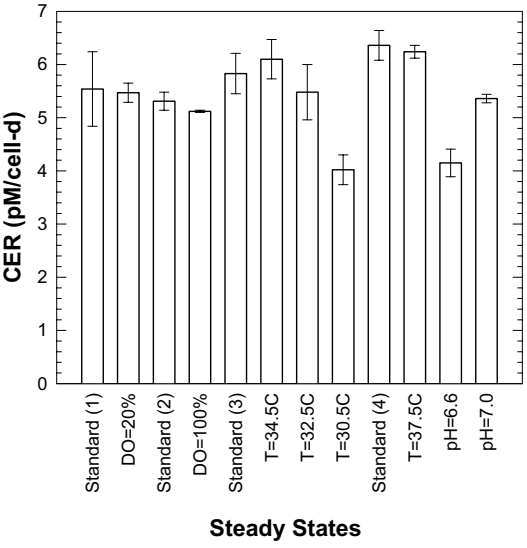

Fig. 4. Average CER estimates for the 12 steady states in the perfusion cultivation.

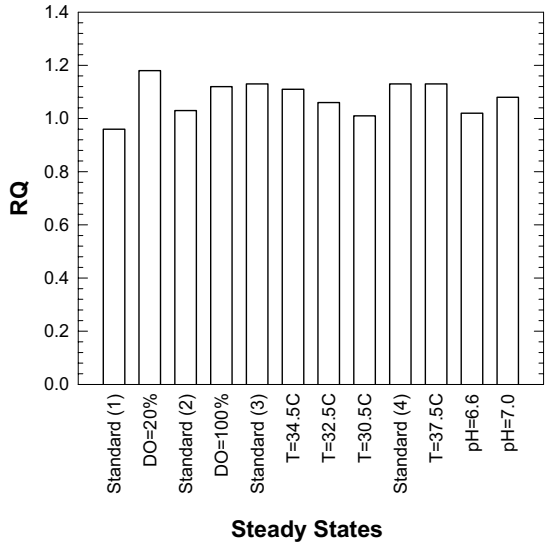

Fig. 5. RQ estimates for the 12 steady states in the perfusion cultivation.

4.3 Comparison with Literature Data

Changes to temperature and pH had the most effect on OUR and CER while DO in the 20 - 100% range had minimal effect (Figures 3 and 4). OUR values ranged from 3.97 – 5.77 pmol/cell-d and the low values of 3.97 and 4.07 were at T = 30.5 °C and pH = 6.6, respectively. Similar CER trends were seen with values of 4.02 and 4.15 pmol/cell-d at T = 30.5 °C and pH = 6.6, respectively (CER range was 4.02 – 6.36 pmol/cell-d). Published OUR values for mammalian cells are shown in Table 1 and are in the 0.55 – 10.7 pmol/cell-d range. Values for CHO cells obtained in this study were clustered in the middle of this range. CER values for hybridoma cells in chemostat culture were in the 9.9 – 11.1 pmol/cell-d range Bonarius et al. (1995) while those in batch culture varied between 1.2 and 8.4 pmol/cell-d Frahm et al. (2002). Our values for CHO cells were lower than the hybridoma chemostat data and closer to those observed in the batch hybridoma cultivation.

OUR	Reference
3.6 - 8.64	Backer et al. (1988)
5.62	Dorresteijn et al. (1996)
1.2	Fleischaker and Sinskey (1981)
4.56 - 9.6	Miller et al. (1987a,b, 1989a,b)
0.55 - 2.09	Ozturk and Palsson (1990)
7.92 - 8.88	Ramirez and Mutharasan (1990)
3.6	Singh (1996)
5.26 - 9.74	Yoon and Konstantinov (1994)
11.04	Zhou and Hu (1994)
5.52 - 10.08	Hiller et al. (1991)
10.1 - 10.7	Bonarius et al. (1995)
3.97 - 5.77	This Study

Table 1. Published OUR values for mammalian cells

Despite significant changes to OUR and CER at low temperature and pH, they were proportional such that RQ values were relatively unchanged. RQ values were close to unity (0.96 - 1.18, Figure 5) under all experimental conditions and the deviation from unity could be due to error in OUR and CER estimates. It is unlikely that cell metabolism was responsible for RQ changes because 1 mol of NADH accompanies 0.5 mol of CO_2 production and this NADH is oxidized by 0.5 mol of oxygen. While fatty acid synthesis can result in RQ values greater than unity Bonarius et al. (1995), it is unlikely that fluxes through these reactions are significant enough to cause an RQ increase on the order of 20%.

5. CONCLUSIONS

We have presented methods to estimate OUR, CER and RQ from mammalian cells in perfusion culture. These are based on global mass balance expressions and do not require k_La information and bioreactor perturbations. They are especially suited for perfusion systems where k_La values change over the course of the cultivation and the dynamic method is not applicable. Accurate CER estimation was possible by accounting for the dissociation of cellular CO_2 into $H_2CO_3^*$, HCO_3^- and CO_3^{2-} and the effect of temperature and ionic strength on the equilibria of the dissociation reactions. This CER estimation method is general and works when bicarbonate is present both in the medium and base. Since all necessary measurements can be made on-line, real time OUR and CER estimation is possible and data can be generated every second if necessary. In addition to providing information on cell physiology, this data can be used for real-time metabolic flux estimation resulting in improved understanding of cell metabolism. Since these advantages come at the expense of minimal analytical and computational effort, the OUR and CER estimation strategies presented in this study are useful both for bioprocess development and monitoring of manufacturing bioreactors producing licensed biotherapeutics.

(Chapter head:)*

Bibliography

M.P. Backer, L.S. Metzger, P.L. Slaber, K.L. Nevitt, and G.B. Boder. Large-scale production of monoclonal antibodies in suspension culture. *Biotechnol. Bioeng.*, 32:993–1000, 1988.

R. R. Balcarcel and L.M. Clark. Metabolic screening of mammalian cell cultures using well-plates. *Biotechnol. Prog.*, 19:98–108, 2003.

H.P. Bonarius, C.D de Gooijer, J. Tramper, and G. Schmid. Determination of the respiration quotient in mammalian cell culture in bicarbonate buffered media. *Biotechnol. Bioeng.*, 45: 524–535, 1995.

J.N. Butler. *Ionic Equilibrium: Solubility and pH Calculations*. John Wiley and Sons, New York, 1998.

R. C. Dorresteijn, K. H. Numan, de Gooijer C. D., J. Tramper, and E. C. Beuvery. On-line estimation of the biomass activity during animal-cell cultivations. *Biotechnol. Bioeng.*, 50:206–214, 1996.

R.J. Fleischaker and A. J. Sinskey. Oxygen demand and supply in cell culture. *Appl. Microbiol. Biotechnol.*, 12:193–197, 1981.

B. Frahm, H-C Blank, P. Cornand, W. Oelbner, U. Guth, P. Lane, A. Munack, K. Johannsen, and R. Pörtner. Determination of dissolved co2 concentration and co2 production rate of mammalian cell suspension culture based on off-gas measurement. *J. Biotechnol.*, 99:133–148, 2002.

G.W. Hiller, A.D. Aeschlimann, D.S. Clark, and H.W. Blanch. A kinetic analysis of hybridoma growth and metabolism in continuous suspension cultures on serum free medium. *Biotechnol. Bioeng.*, 38:733–741, 1991.

Y-S. Kyung, M. V. Peshwa, D.M. Gryte, and W. S. Hu. High density culture of mammalian cells with dynamic perfusion based on on-line oxygen uptake measurements. *Cytotechnology*, 14:183:190, 1994.

G. Lovrecz and P. Gray. Use of on-line gas analysis to monitor recombinant mammalian cell cultures. *Cytotechnology*, 14:167–175, 1994.

W.M. Miller, H.W. Blanch, and C.R. Wilke. A kinetic analysis of hybridoma growth and metabolism in batch and continuous suspension culture: Effect of nutrient concentration, dilution rate and ph. *Biotechnol. Bioeng.*, 32:947–965, 1987a.

W.M. Miller, C.R. Wilke, and H.W. Blanch. Effects of dissolved oxygen concentration on hybridoma growth and metabolism in continuous cultures. *J. Cell. Physiol.*, 132:524–530, 1987b.

W.M. Miller, C.R. Wilke, and H.W. Blanch. Transient responses of hybridoma cells to nutrient additions in continuous culture: I. glucose pulse and step changes. *Biotechnol. Bioeng.*, 33:477–486, 1989a.

W.M. Miller, C.R. Wilke, and H.W. Blanch. Transient responses of hybridoma cells to nutrient additions in continuous culture: Ii. glutamine pulse and step changes. *Biotechnol. Bioeng.*, 33:487–499, 1989b.

F.M.M. Morel and J.G. Hering. *Principles and Applications of Aquatic Chemistry*. John Wiley and Sons, New York, 1993.

T. Ogawa, M. Kamihira, H. Yoshida, H. Iijima, and T. Kobayashi. Effect of dissolved oxygen concentration on monoclonal antibody production in hybridoma cell cultures. *J. Ferment. Bioeng.*, 74:372–378, 1992.

S. Ozturk and B.O. Palsson. Effects of dissolved oxygen on hybridoma cell growth, metabolism, and antibody production kinetics in continuous culture. *Biotechnol. Prog.*, 6:437–446, 1990.

H.A. Philips, J.M. Scharer, N.C. Bols, and M. Moo-Young. Effect of oxygen on antibody production in hybridoma culture. *Biotechnol. Lett.*, 9:745–750, 1987.

O.T. Ramirez and R. Mutharasan. Cell cycle and growth phase dependent variations in size distribution, antibody productivity, and oxygen demand in hybridoma cultures. *Biotechnol. Bioeng.*, 36:839–848, 1990.

P. Ruffieux, U. Stockar, and I. Marison. Measurement of volumetric (our) and determination of specific (qo2) oxygen uptake rates in animal cell cultures. *J. Biotechnol.*, 63:85–95, 1998.

Y. Shi, D.D.Y. Ryu, and S.H. Park. Monoclonal-antibody productivity and the metabolic pattern of perfusion cultures under varying oxygen-tensions. *Biotechnol. Bioeng.*, 42:430–439, 1993.

V. Singh. On-line measurement of oxygen uptake in cell culture using the dynamic method. *Biotechnol. Bioeng.*, 52:443–448, 1996.

V.L. Snoeyink and D. Jenkins. *Water Chemistry*. John Wiley and Sons, New York, 1980.

K. Yamada, S. Furushou, T. Sugahara, S. Shirahata, and H. Murakami. Relationship between oxygen consumption rate and cellular activity of mammalian cells cultured in serum-free media. *Biotechnol. Bioeng.*, 36:759–762, 1990.

S. Yoon and K. Konstantinov. Continuous, real-time monitoring of the oxygen uptake rate (our) in animal cell bioreactors. *Biotechnol. Bioeng.*, 44:983–990, 1994.

W. C. Zhou and W. S. Hu. On-line characterization of a hybridoma cell culture process. *Biotechnol. Bioeng.*, 44:170–177, 1994.