Biotechnology Letters **23:** 1841–1847, 2001.

Ensuring oxygenation of carrot cell cultures in a Couette viscometer during investigation of shear effects

Victor Wong[2], David Williams[1,*] & Christopher B. Colby[1]
[1]*Department of Chemical Engineering, Adelaide University, Adelaide, SA 5005, Australia*
[2]*Bioprocessing Technology Centre, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260*
[*]*Author for correspondence (Fax: +61 8 8303 4373; E-mail: dwilliam@chemeng.adelaide.edu.au)*

Received 7 September 2001; Accepted 21 September 2001

Key words: Couette viscometer, mathematical simulation, oxygen depletion, plant cell culture, shear sensitivity

Abstract

Mathematical simulation and experimental measurement of dissolved O_2 were performed for extended (up to 8 h) shear testing of *Daucus carota* (carrot) cell cultures in a conventional Couette viscometer (0.625 mm annulus). The results suggest O_2 depletion below critical levels for cell growth may occur. A novel design modification incorporating an O_2-permeable silicone-layer spun cast on a porous ceramic bowl was devised. It significantly improved oxygenation of the cell cultures, keeping dissolved O_2 near saturation.

Introduction

The Couette viscometer is popular as a shear device for shear sensitivity studies involving suspension cultures of plant or animal cells [for example, Rosenberg (1989), Zhong (1994), Wong *et al.* (2000)].

We are using a Coutte viscometer to study shear effects on the biological response of *Daucus carota* (carrot) cell cultures. The experiments involve shearing the cell cultures in the viscometer annulus for extended periods of up to 8 h at rotational speeds between 500 and 1200 rpm. Various biological indicators including mitochondrial activity and oxygen uptake rate are measured before and after the shear tests.

A conventional Couette viscometer comprising a solid stainless steel bowl and bob similar to that used by Rosenberg (1989) was constructed for our experiments. However, inconsistent results were observed in early data between shear tests of short and long duration that could not be attributed to shear effects alone. After investigations using both mathematical simulation and experimental measurement it was concluded that O_2 depletion was the likely cause.

It is well known that O_2 limitation has adverse effects on growth and differentiation of plant cells in suspended culture (Kessell & Carr 1972, Tate & Payne 1991). Kessell & Carr (1972) observed that carrot cell cultures have a critical dissolved O_2 concentration at about 10% saturation, below which cell growth becomes O_2 limited.

Furthermore, the potential for O_2 limitation to occur in laboratory-scale shear devices has been previously recognised. Wudkte & Schugerl (1987) incorporated an O_2-permeable membrane into the base of a Couette viscometer annulus in order to avoid O_2 limitation during shear testing of suspended cultures of *Stodoptera frugiperda* butterfly ovarian cells. For the same reason, Sun & Linden (1999) equipped the inner cylinder of a rotating wall vessel with an O_2-permeable membrane for shear testing of *Taxus cuspidata* plant suspension-cell cultures. However, both of these studies only appear to have assumed that O_2 limitation was important and might have taken place – they do not give details whether experimental testing or other investigations were performed to verify that O_2 limitation would have occurred, and if so, that it had been satisfactorily alleviated by modifying the shear device.

We report here our investigations to confirm the incidence of O_2 limitation in a conventional Couette viscometer during shear testing of carrot cell cultures. A novel design modification to the viscometer to over-

come the problem is presented and its performance in improving oxygenation of the carrot cell cultures is demonstrated.

Experimental methods

Cell culture and medium

The suspension cultures of *Daucus carota* (wild type) were a gift from the Commonwealth Scientific and Industrial Research Organization (Sydney, Australia). The cultures were maintained in 250 ml Erlenmeyer flasks containing 50 ml wild carrot medium and incubated in the dark at 27 °C on a gyratory shaker at 100 rpm. The cultures were subcultured every 2 weeks using a 10% (v/v) inoculum. The formulation of the medium includes 0.5 mg 2,4-dichlorophenoxyacetic acid l^{-1}, 3 mg thiamine l^{-1} and 20 g sucrose l^{-1}. The pH of the medium was adjusted to 5.4 before autoclaving.

Conventional Couette viscometer

The conventional Couette viscometer was similar to that used by Rosenberg (1989). The viscometer operated with a stationary inner bob and a rotating outer bowl. The outer bowl diameter was 101.6 mm and the annular gap (between outer bowl and inner bob) was 0.625 mm. The inner bob, outer bowl, top lid and base section of the viscometer were all constructed of Grade 316 stainless steel. A pulley wheel was attached to the base section of the viscometer and coupled to an external variable speed motor assembly for rotation of the outer bowl.

Modified Couette viscometer

To enhance oxygenation of suspended cultures in the annulus of the Couette viscometer, the stainless steel bowl was replaced with a bowl machined from a porous ceramic material. The inner surface of the ceramic bowl was lined with an O_2-permeable silicone polymer. The silicone lining was applied by a two-step spin-coating process. The ceramic bowl was fixed in a horizontal position and rotated at a speed of 2800 rpm. A 0.2 mm layer of an air-cured silicone (Silastic 732, Dow Corning) was applied first and allowed to cure completely. A second layer of a two-component silicone polymer (Silastic E, Dow Corning) was then applied over the first layer to form a smooth finish of constant thickness. The final composite thickness of the silicone lining was approximately 1.2 mm. This produced an annular gap between outer bowl and inner bob similar to that in the conventional design obtained with the stainless steel bowl.

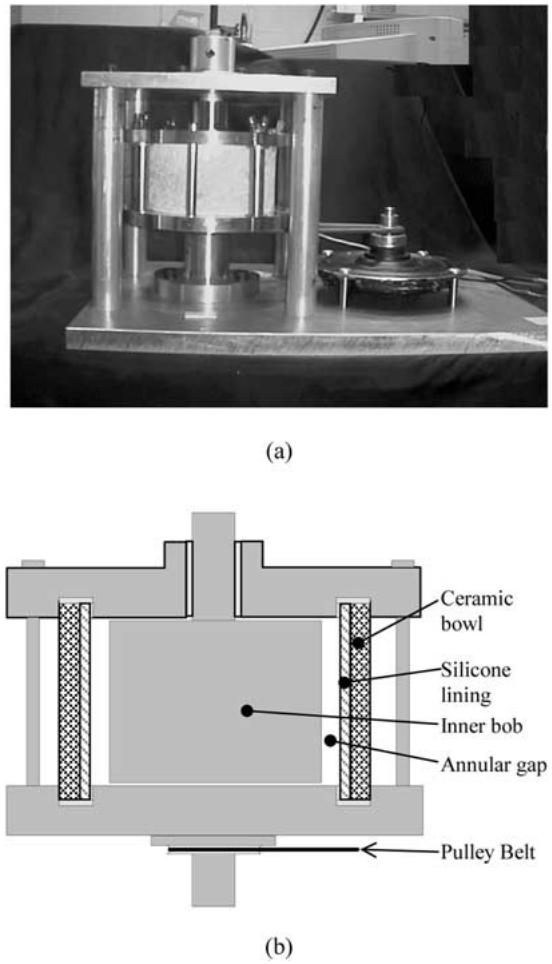

Fig. 1. Modified Couette viscometer: (a) photograph; (b) schematic diagram.

Figure 1 shows a photograph and schematic diagram of the modified Couette viscometer.

Viscometer sterilisation

All components of the conventional Couette viscometer were sterilised prior to use in a shear test by autoclaving at 121 °C for 20 min. Autoclaving at 121 °C for 20 min was also used for components of the modified Couette viscometer, except the ceramic bowl. The ceramic bowl was sterilised separately by multiple washes with 95% (v/v) ethanol followed by washes with sterile culture medium because of concern that re-

peated autoclaving might damage the porous ceramic material and/or silicone lining.

Sample preparation

Fourteen-day old (late growth phase) carrot cultures were pre-screened to aggregate sizes below 263 μm using stainless steel meshes and the cell density adjusted to approximately 0.05 g (fresh wt) ml^{-1} by re-suspension in air-saturated fresh culture medium prior to loading into the viscometer. The screening procedure was conducted in a laminar flow hood using aseptic techniques.

The pre-screened carrot cultures were pipetted into the viscometer annulus with wide-bore pipettes.

The time taken to load a carrot culture and secure the viscometer lid was approximately 2 min.

Shear tests

The viscometer containing the carrot cell culture was placed in a Perspex container. Air from a temperature-controlled air heater was circulated through the Perspex container to maintain the viscometer at 27 °C.

The pre-screened carrot cultures were sheared at a rotational speed of 1200 rpm for various periods of up to 8 h. The dissolved O_2 of the cell culture from the viscometer annulus was measured at the conclusion of each shear test as described in the following section.

Dissolved oxygen measurement

The small gap width of the viscometers made *in situ* measurements difficult as it was not possible to fit a standard dissolved O_2 probe into the annular gap. Instead, a sample of cell culture had to be removed from the viscometer and its dissolved O_2 measured.

The dissolved O_2 measurements were performed with a Rank oxygen electrode, Model 10 Dissolved Oxygen system (Rank Brothers, Cambridge, UK). The electrode was attached to the base of a 3 ml Perspex chamber maintained at 25 °C by a re-circulating water bath. Prior to dissolved O_2 measurement, the electrode was calibrated against air-saturated fresh culture medium.

At the conclusion of a shear test, the viscometer lid and inner bob was quickly and carefully removed and a sample of the cell culture pipetted from the viscometer into the Perspex chamber. Precautions were taken to ensure no air bubbles were trapped. The chamber was immediately capped with a Perspex plug.

The dissolved O_2 reading displayed an initial sharp drop as cell culture was pipetted into the electrode chamber. The dissolved O_2 level immediately following this sharp drop was taken to be an estimate of the 'average' dissolved O_2 concentration of the cell culture in the viscometer.

Mathematical modelling

Mathematical model for conventional Couette viscometer

In the conventional Couette viscometer the only free surface of the cell culture exposed to air is at the top of the annulus. During shear tests, O_2 from air will dissolve in the culture medium at this surface and diffuse through the culture medium down the annulus to replace dissolved O_2 being consumed by plant cells. The concentration of dissolved O_2 in the culture medium will vary with time and spatial position in the annulus, which may be described mathematically.

In our analysis, radial variations of dissolved O_2 were assumed negligible, and only changes with axial position (i.e., depth) in the annulus were considered. Furthermore, the effect of fluid spin in the annulus on O_2 transport was neglected.

The following partial differential equation was derived to describe the variation in dissolved O_2 concentration, c, of the culture medium in the conventional viscometer with time, t, and axial position (i.e., depth), z, from the bottom, $z = 0$, to top, $z = L$, of the annulus.

$$D\frac{\partial^2 c}{\partial z^2} + R_X = \frac{\partial c}{\partial t}, \tag{1}$$

where D is the dissolved O_2 diffusion coefficient in the culture medium; and R_X is the consumption rate per unit volume of dissolved O_2 by plant cells. It is assumed that D is not dependent on dissolved O_2 concentration.

Initial conditions (I.C.) and boundary conditions (B.C.) assumed to apply to Equation (1) prior to and during shear tests in the Couette viscometer were:

$$\text{I.C. } t = 0 : c(z, 0) = c^*, \tag{2}$$

$$\text{B.C. } z = 0 : -D\left.\frac{\partial c}{\partial z}\right|_{z=0} = 0, \tag{3}$$

$$z = L : -D\left.\frac{\partial c}{\partial z}\right|_{z=L} = \frac{k}{H \cdot R \cdot T}\left(c^* - c|_{z=L}\right) = \alpha\left(c^* - c|_{z=L}\right), \tag{4}$$

where k is the O_2 mass transfer coefficient from air to the free surface of the culture medium at the top of the annulus; H is the Henry's Law constant for solubility of O_2 in the culture medium, R is the gas constant, T is temperature, and c^* is the saturated dissolved O_2 concentration corresponding to the partial pressure of O_2 in air.

A Monod-type kinetic expression similar to that proposed by Cho & Wang (1990) was used to calculate the O_2 uptake rate by plant cells:

$$R_X = -q_{\max}\frac{c}{K_m + c}X, \tag{5}$$

where $q_{\max}$ is the maximum specific oxygen consumption rate of the carrot cells; K_m is the Monod constant for an O_2 substrate; and X is the cell concentration in the culture medium.

Mathematical model for modified Couette viscometer

The modified Couette viscometer has an O_2-permeable silicone lining through which O_2 diffuses into the culture medium from air in the pores of the ceramic outer bowl. In this situation, O_2 transport into the culture medium across the large contact surface area presented by the outer bowl would be much greater than that entering from the free surface at the top of the annulus, the effect of which may be neglected. Furthermore, the annulus gap width may be regarded as insignificant relative to the bowl diameter such that curvature effects across the annulus gap can be ignored. With these simplifications O_2 transport in the modified viscometer can be considered as a one-dimensional system in an identical way to the conventional viscometer but where O_2 transport is in the radial direction from outer bowl to inner bob (instead of from top to bottom of the annulus in the axial direction).

The variation in dissolved O_2 in the annulus of the modified viscometer was described by Equation (1), assuming z as radial position in the annulus, with the same boundary condition in Equation (3) applied at $z = 0$, now the outer surface of the inner bowl. The boundary condition for $z = L$, now the inner surface of the outer bowl and with L representing the annulus gap width, was changed to describe the rate of O_2 transport across the silicone lining from the air in the porous ceramic bowl into the culture medium. Assuming diffusion through the silicone lining as the sole resistance to O_2 transport (i.e., neglecting diffusive and/or convective resistances on bowl and culture medium sides) this boundary condition was:

$$\begin{aligned}\text{B.C. } z &= 1 : -D\left.\frac{\partial c}{\partial z}\right|_{z=L} \\ &= -\varepsilon\frac{D_s}{H\cdot R\cdot T}\frac{\left(c^* - c|_{z=1}\right)}{\Delta x} \\ &= \beta\left(c^* - c|_{z=1}\right),\end{aligned} \tag{6}$$

where D_s is the O_2 diffusion coefficient in the silicone lining; and $\varepsilon$is the porosity of the ceramic bowl.

Identical initial conditions to Equation (2), and the same kinetic expression for O_2 consumption, Equation (5), were also applied.

Numerical solution of model equations

Equation (1) is a partial differential equation with a non-linear term, R_X, which must be solved to obtain the time varying spatial concentration profiles for dissolved O_2 in the viscometer. An analytical solution for Equation (1) is not possible – it must be solved numerically.

An orthogonal collocation finite element method as described by Finlayson (1972) was used to discretise the spatial coordinate in Equation (1) and transform it into a system of ordinary differential equations (ODEs):

$$D\sum_{j=1}^{N+1} B_{j,k}c_j + R_k = \frac{\partial c_k}{\partial t}\ k = 1, 2, \ldots, N+1, \tag{7}$$

where B is the same matrix defined by Finlayson (1972).

The collocation points for Equation (7) were deduced from tables listed in Stroud & Secrest (1966).

The set of ODEs described in Equation (7) was reduced from $N + 1$ to N by applying the boundary condition at $z = L$ for $k = N+1$, which may be transformed into an algebraic equation by the orthogonal collocation finite element method to enable calculation of c_{N+1}. For the conventional Couette viscometer the algebraic equation derived from Equation (3) for c_{N+1} was:

$$c_{N+1} = \frac{\frac{\alpha}{D}c^* + \sum_{j=1}^{N} A_{N+1,j}c_j}{A_{N+1,N+1} + \frac{\alpha}{D}} \tag{8}$$

where A is the same matrix defined by Finlayson (1972).

The corresponding algebraic expression for c_{N+1} in the modified Couette viscometer was identical to Equation (8) but with β replacing α.

Table 1. Values of model parameters assumed for simulation of conventional and modified Couette viscometers.

Parameter	Symbol	Value
Annulus depth	L	6 cm
O_2 diffusivity in culture medium	D	2×10^{-9} m^2 s^{-1}
Plant cell concentration	X	0.005 g (dry wt) ml^{-1}
Saturated dissolved O_2 concentration	c^*	0.256 mmol l^{-1}
Maximum specific O_2 consumption rate	$q_{\max}$	0.0.017 mmol h^{-1} ml^{-1}
Monod constant	K_m	0.026 mmol l^{-1}
Mass transfer coefficient	k	0.161 m s^{-1}
Annulus gap width	L	600 μm
O_2 diffusivity in silicone (rubber) lining	D_s	4.1×10^{-9} m^2 s^{-1}
Ceramic bowl porosity	ε	0.3
Silicone lining thickness	Δx	1200 μm
Henry's Law constant	H	1.2×10^{-5} mmol l^{-1} Pa^{-1}
Temperature	T	300 K

The system of ODEs in Equation (7) for $k = 1, 2, \ldots, N$ and Equation (8) were encoded in Mathcad 5+ (Mathsoft Inc., Cambridge, MA) and solved simultaneously using a fourth-order Runge–Kutta algorithm.

Model parameters and assumptions

Values used for parameters in the mathematical models for simulation of conventional and modified Couette viscometers are summarised in Table 1.

The values for physical properties of the culture medium and the transport and solubility properties of dissolved O_2 were assumed to be the same as for pure water. The effect of fluid spin in the viscometers on O_2 transport in the culture medium was neglected.

Standard values for physical properties of air and the transport properties of gaseous O_2 were assumed.

No published value or method for directly calculating the mass transfer coefficient for O_2 from a stationary gas to a fluid in a rotating annulus could be found. The mass transfer coefficient was estimated from a generic correlation presented in Foust *et al.* (1980) for gas-phase mass transfer to a laminar fluid flowing over wetted walls and plates. A bowl diameter of approximately 10 cm and rotational speed of 1200 rpm were assumed for calculation of fluid velocity in the annulus.

The value of O_2 diffusivity in the silicone lining of the ceramic bowl of the modified viscometer was obtained from data published by Charati & Stern (1998).

The rates of O_2 uptake by carrot cells do not appear to have been previously studied. However, Payne *et al.* (1987) has reviewed O_2 uptake rates measured for other types of plant cells. The O_2 uptake rates ranged from 0.03 mmol O_2 h^{-1} g^{-1} (dry wt) for tobacco cells up to 3.6 mmol O_2 h^{-1} g^{-1} (dry wt) for sugar cane. Pepin *et al.* (1995) have also surveyed O_2 uptake rates published in the literature for various plant cells. They reported O_2 uptake rates of between 0.25 and 1 mmol O_2 h^{-1} g^{-1} (dry wt).

A maximum specific O_2 uptake rate of 0.33 mmol O_2 h^{-1} g^{-1} (dry wt) was assumed for the carrot cells in our study. Shear tests with carrot cultures were performed at a cell density of 0.005 g (dry wt) ml^{-1}, which gave a value for $q_{\max}$ of 0.0017 mmol O_2 h^{-1} ml^{-1}.

Kessel & Carr (1972) have investigated the critical O_2 concentration, where growth becomes O_2 limited, for carrot cells. A value of 0.04 mM or 16% saturation was obtained at a temperature of 26 °C. The Monod constant K_m would be of similar magnitude to this critical concentration. A value of 0.026 mM, approximately 10% of the saturation concentration at 27 °C, was assumed.

Results and discussion

Mathematical simulation of conventional viscometer

Figure 2 shows the axial concentration profiles for dissolved O_2 in the annulus of the conventional Couette

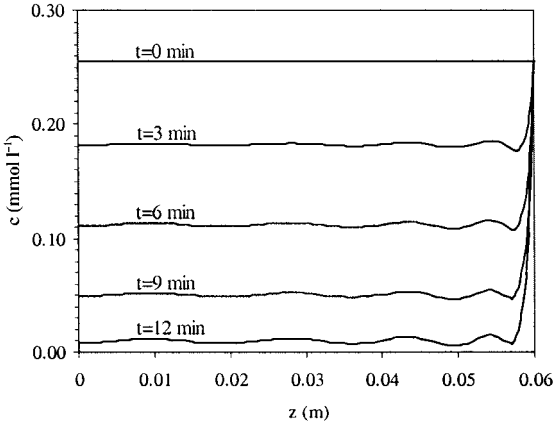

Fig. 2. Axial concentration profiles for dissolved O_2 in the conventional Couette viscometer predicted by mathematical simulation.

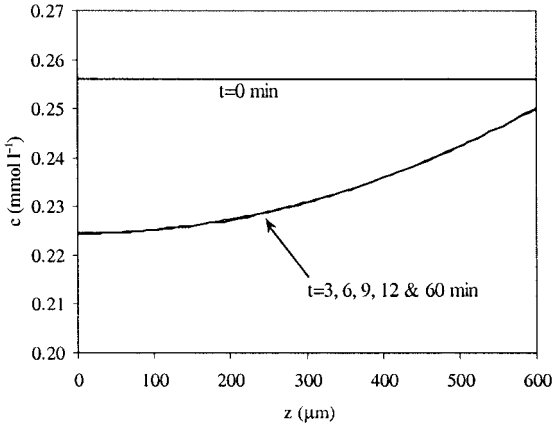

Fig. 3. Radial concentration profiles for dissolved O_2 in the modified Couette viscometer predicted by mathematical simulation.

viscometer at various time intervals predicted by simulations with the mathematical model. Nine internal collocation points (i.e., $N = 9$) were used to obtain a stable numerical solution with acceptable convergence (data not shown).

The model predictions indicate that dissolved O_2 during a shear test would decrease evenly throughout the entire annulus, except at the top (i.e., $z = L$) where dissolved O_2 would remain at or just below the saturation level. Figure 2 forecasts that dissolved O_2 in the annulus would fall below the critical concentration (0.04 mM) reported for carrot cells (Kessell & Carr 1972) after only a very short period, within 20 min.

Mathematical simulation of modified viscometer

The predicted radial concentration profiles for dissolved O_2 in the annulus of the modified Couette viscometer at various time intervals during a shear test are shown in Figure 3. A stable and converged numerical solution was obtained with 2 internal collocation points (data not shown).

Figure 3 predicts that the radial concentration profile for dissolved O_2 in the modified viscometer would rapidly arrive at a steady state, within 5 min. The lowest dissolved O_2 level in the annulus at this steady state would occur at the surface of the inner bob (i.e., $z = 0$) and have a value of approximately 0.2 mM, well above the critical O_2 concentration.

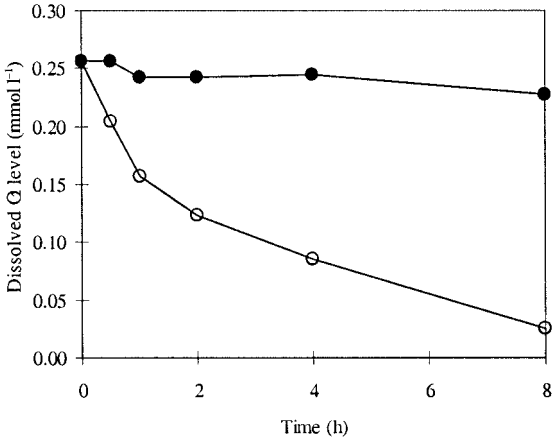

Fig. 4. Experimental measurements of 'average' dissolved O_2 concentration in conventional (○) and modified (●) Couette viscometers after shear tests with carrot cells at 1200 rpm

Experimental dissolved O_2 measurements with conventional viscometer

Figure 4 compares experimental measurements of the dissolved O_2 for carrot cultures during shear tests in conventional and modified Couette viscometers.

The (average) dissolved O_2 concentration of the cell culture in the annulus of the conventional viscometer can be observed to decrease gradually with shear test duration, and drops below the critical concentration of 0.04 mM at between 6 and 7 h. The experimental measurements indicate a much slower decline in dissolved O_2 than predicted by mathematical simulation. This discrepancy could be due to a number of factors. The real value of specific O_2 uptake by carrot cells could be less than that assumed for mathematical simulation. There may have been

re-oxygenation of samples during their transfer from viscometers to the Perspex sample cell where dissolved O_2 was measured, which would have increased the experimental value observed. The mathematical model did not take into account the effect of fluid spin on O_2 transport, which could have substantially increased the rate of O_2 diffusion through the culture medium.

Nevertheless, the experimental measurements show that dissolved O_2 in the conventional Couette viscometer could decrease to levels near or below the critical concentration for the carrot cells when the shear tests are performed over long periods.

Experimental dissolved O_2 measurements with modified viscometer

Experimental values of dissolved O_2 in the modified viscometer show an initial decrease with shear test duration but then appear to stabilise at around 90% saturation. The experimental measurements are consistent with predictions obtained by mathematical simulation.

Conclusions

Mathematical simulation and experimental measurement of dissolved O_2 in a conventional Couette viscometer with an annular gap of 600 μm indicate that O_2 depletion below critical levels for carrot cells could occur during shear tests of extended duration. By making the sidewalls of the Couette viscometer permeable to O_2 using a silicone-lined ceramic bowl, oxygenation was significantly improved and the potential for O_2 depletion avoided.

References

Charati SG, Stern SA (1998) Diffusion of gases in silicone polymers: molecular dynamics simulations. *Macromolecules* **31**: 5529–5535.

Cho MH, Wang SS (1990) Practical method for estimating oxygen kinetic and metabolic parameters. *Biotechnol. Prog.* **6**: 164–167.

Finlayson BA (1972) *The Method of Weighted Residuals and Variational Principles*. New York: Academic Press.

Foust A, Wenzel L, Clump C, Maus L, Anderson L (1980) *Principles of Unit Operations*, 2nd edn. New York: John Wiley & Sons.

Kessell RHJ, Carr AH (1972) The effect of dissolved oxygen concentration on growth and differentiation of carrot (*Daucus carota*) tissue. *J. Exp. Bot.* **23**: 996–1007.

Payne GF, Shuler ML, Brodelius P (1987) Large-scale plant cell culture. In: Lydersen BK, ed. *Large Scale Cell Culture Technology*. Münich: Hanser Publishers, pp. 195–229.

Pepin MF, Archambault J, Chavarie C, Cormier F (1995) Growth kinetics of *Vitis vinifera* cell suspension cultures: 1. Shake flask cultures. *Biotechnol. Bioeng.* **47**: 131–138.

Rosenberg MZ (1989) *The Hydrodynamic Shear Sensitivity of Suspension Cultured Plant Cells*. Ph.D. Thesis, Washington University.

Stroud AH, Secrest D (1966) *Gaussian Quadrature Formulas*. New Jersey: Prentice-Hall Inc.

Sun X, Linden JC (1999) Shear stress effects on plant cell suspension cultures in a rotating wall vessel bioreactor. *J. Ind. Microbiol. Biotechnol.* **22**: 44–47.

Tate JL, Payne GF (1991) Plant cell growth under different levels of oxygen and carbon dioxide. *Plant Cell Rep.* **10**: 22–25.

Wong V, Williams D, Colby C, Saint DA (2000) Correlation of O_2 uptake rate and mitochondrial activity in carrot cells cultures exposed to laminar fluid shear. *Biotechnol. Lett.* **22**: 1919–1924.

Wudtke M, Schugerl K (1987) Investigations of the influence of physical environment on the cultivation of animal cells. In: *European Society of Animal Cell Technology, the 8th Meeting Tiberias, Israel*. Butterworth & Co, pp. 297–315.

Zhong JJ, Fujiyama K, Seki T, Yoshida T (1994) A quantitative analysis of shear effects on cells suspension and cell culture of *Perilla frutescens* in bioreactors. *Biotechnol. Bioeng.* **44**: 649–654.