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Silicone-tubing aerated bioreactors for somatic embryo production

Reiner Luttman[1], Peter Florek[2] & Walter Preil[2*]
[1]*Polytechnical University of Hamburg, Lohbrügger Kirchstr. 65, D-21033 Hamburg, Germany;* [2]*Institute for Ornamental Plant Breeding, Bornkampsweg 31, D-22926 Ahrensburg, Germany (* requests for offprints)*

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Abstract

Bioreactors equipped with silicone tubings for bubble free oxygen supply are suitable for culture of embryogenic cell suspensions. The advantages of bubble free aeration systems over various devices for dispersion of air bubbles are the lack of foam formation and the possibilities of precise control of the desired oxygen set point. The specification of silicone tubing (length, diameter, wall thickness) has to be adapted according to the amount of embryogenic biomass to be produced in the bioreactor. Cell suspensions of *Euphorbia pulcherrima* were cultured in a 2 l bioreactor at 60% pO_2, supplied by a silicone tubing system of 155 cm length, 4.0 mm diameter and 0.4 mm wall thickness. The oxygen concentration decreased when the packed cell volume exceeded 14% (= 3.7 g l^{-1} cell dry weight), indicating the upper limit of oxygen supply by the silicone tubing. Mathematical considerations for membrane aerated bioreactors are presented with the intention of enabling a more precise definition for the configuration of silicone tube systems in different bioreactor types.

Introduction

Somatic embryogenesis has been recognized as the theoretically most efficient method for in vitro mass propagation of plants (Parrot et al. 1991; Gray & Purohit 1991; Durzan & Durzan 1991). The main advantage of somatic embryogenesis over axillary or adventitious shoot propagation is the possibility of a potentially unlimited production of single individuals with functional shoot and root poles. Furthermore, the induction of somatic embryos in liquid systems offers opportunities for process automation and control consequently resulting in reduction of manual labor and costs.

Bioreactors provide optimum conditions for growth and development of somatic embryos by regulation of chemical and physical environmental parameters. Therefore, they are superior to any other kind of culture vessels, for example Erlenmeyer flasks on gyratory shakers. Various types of bioreactors were described as suitable for plant cell growth (Ammirato & Styer 1985; Styer 1985; Panda et al. 1989; Cazzulino et al. 1991; Preil 1991; Denchev et al. 1992; Nishimura 1993). However, direct comparisons of effects of different bioreactor configurations on the cell growth and embryo development are rare (Chen et al. 1987; Taticek et al. 1990).

Although numerous plant species are able to form somatic embryos on agar solidified media or in liquid cultures, there is at present no system available that can replace the commercial standard micropropagation procedures using axillary or adventitious shoot regeneration. In most cases, the number and quality of produced embryos vary in a wide range due to still imperfect protocols for embryo induction, development or maturation. Additionally, in liquid systems hydrodynamic forces caused by agitators may damage the cell aggregates and developing embryos. Other problems may arise in bubble aerated vessels by foam formation and callus growth above the surface of the culture medium.

Since bioreactors for universal application do not exist, the configuration has to be adapted to the specific requirements of a given culture. Users of bioreactors that have been primarily developed for cultures of microorganisms or cell biomass production, often underestimate the influence of stirring and aeration devices on cell growth and differentiation processes. In any case, shear forces have to be minimized by using

slowly rotating large blade stirrers. Vibro mixers are suitable for agitating homogenous embryogenic cell suspension cultures, but they may damage aggregates and embryos of larger size.

At present most bioreactors are aerated by dispersion of air bubbles. The applied aeration rate must ensure that oxygen limitation does not occur. High aeration rates, however, were reported to inhibit the growth of cell suspension cultures (Hegarty et al. 1986) and amplify foam formation. The negative effects of air bubbles can be avoided by use of tubing made out of silicone (Kuhlmann 1987) or polypropylene (Lehmann et al. 1985; Piehl et al. 1988) which act as membranes of low diffusion barriers for gases like oxygen or carbon dioxide. This paper describes the theoretical considerations of membrane aerated systems and discusses its practical application.

Experimentally determined efficiency of silicone membrane aeration

Silicone tubing can be installed in the bioreactor with baskets, coils or any other kinds of devices (Preil 1991). For the experiments described here the silicone membranes were stabilized by steel springs inserted into the tubing (length: 155 cm; diameter: 0.4 mm; wall thickness: 0.4 mm) (Fig. 1). The oxygen concentration in the culture medium was regulated by means of pulse modulated dosage of oxygen supplemented to the circulating gas stream inside the tubing.

For mathematical calculation of the aeration device specifications (see below) the bioreactor was equipped with three mass flow controllers for O_2, N_2 and air (Fig. 2) ensuring a precise control of pO_2 with gas mixtures of air and nitrogen, or air and oxygen.

Cell suspensions of *Euphorbia pulcherrima* were semicontinuously cultivated over several months at 60 % pO_2. When the reactor vessel was inoculated with a packed cell volume (PCV) of 2 %, the adjusted pO_2 level could be kept constant for 180 h, (Fig. 3). However, after 210 h the oxygen concentration rapidly decreased when the PCV exceeded 14 % (= 3.7 g l^{-1} dry weight of cell mass) indicating the maximum amount of biomass that could be provided with oxygen by the silicone tubing of 155 cm length. The growth cycle was continued at 60 % pO_2 after cell harvest and addition of fresh medium.

In bioreactor cultures of *Clematis tangutica* with initial PCV of 5 %, the setpoint of 15 % pO_2 was kept constant for 3 months. Every two weeks spent medium was replaced by fresh. For this purpose the cell mass was allowed to settle and the supernatant was removed. After four weeks 1200 embryogenic clusters, 500 globular embryos and 700 embryos of the heart and torpedo stage were obtained per gram cell mass. One month later nearly all clusters had developed

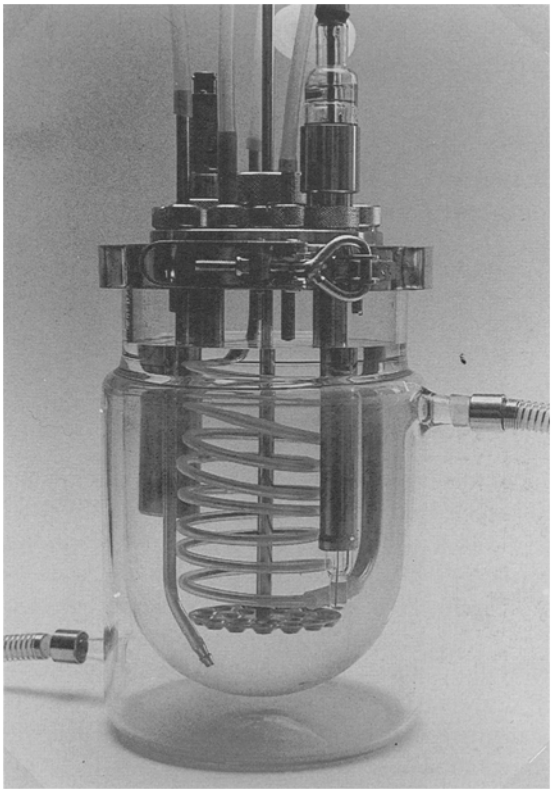

Fig. 1. 2 l bioreactor vessel (B. Braun Biotech Int.) equipped with silicone tubing for bubble free aeration, Vibro mixer, pH- and pO_2-elecrodes.

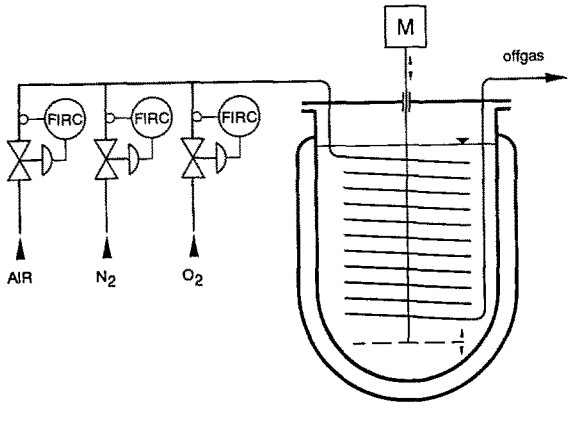

Fig. 2. Scheme of bioreactor membrane aeration using three mass flow controllers for different aeration lines.

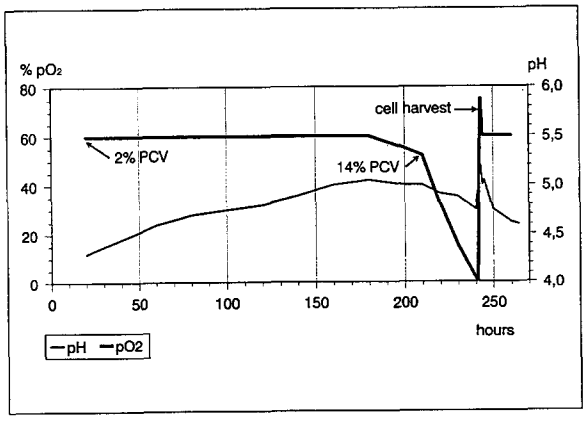

Fig. 3. Dissolved oxygen tension pO_2 (set point 60 %) and pH (not controlled) during cultivation of *Euphorbia pulcherrima* cells in a bubble free aerated bioreactor.

into globular embryos (Fig. 4 A). After three months the settled embryo fraction exceeded one third of the total suspension culture volume. Samples of embryos taken from the bioreactor every two weeks developed normally when plated on solid medium (Fig. 4 B).

These results indicate that high numbers of somatic embryos can be produced in bioreactors at constant low oxygen concentration (Weber and Preil, unpublished).

Mathematical description of membrane aerated systems

In comparison to gas/liquid transfer conditions in normal bioreactors, the oxygen transfer capability of membrane aerated systems is very low. The small amount of oxygen demand by tissue cultures needs a large mass transfer boundary layer. Therefore, a long membrane (e.g. 2 m) has to be installed in a liquid phase of relatively small size (e.g. 2 l).

The tubular aeration system can be defined as a plug flow reactor without any reaction in the gas phase but with mass transfer actions to the surrounding liquid phase. The liquid phase is assumed to be well mixed due to different agitation procedures. In the view of system theory, a combination of a distributed-parameter system (gas phase) and a lumped-parameter system (liquid phase) is the base of the following mathematical model (Luttmann et al. 1989).

In this paper, the main feature of the model is the description and experimental calculation of oxygen mass transfer capabilities in silicone membrane aerated systems (Kuhlmann 1987). Starting with the

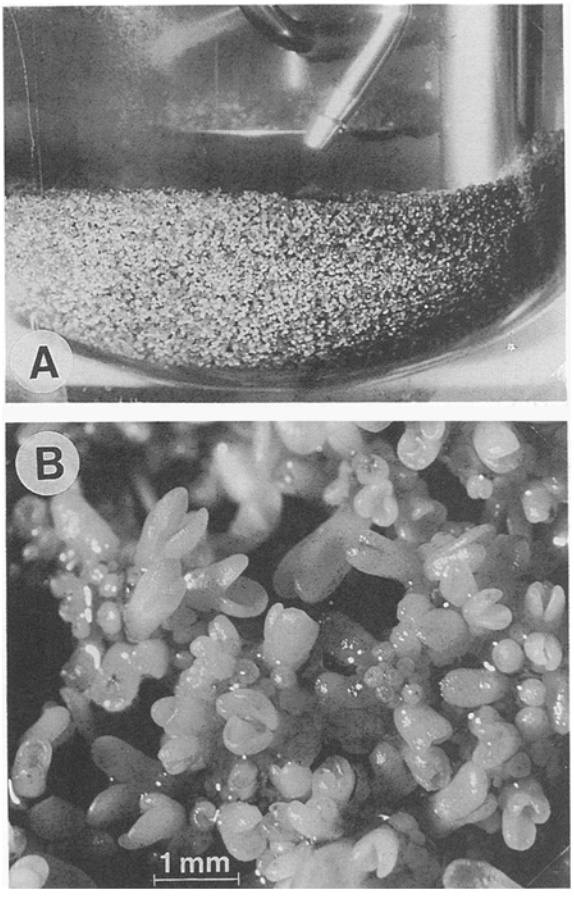

Fig. 4. Somatic embryos of *Clematis tangutica* settled at the bottom of the bioreactor vessel (*A*) and after plating on filter paper (*B*).

general gas phase balances and the oxygen liquid balance, the equations of oxygen supply are developed for experimental investigations.

Model equations of the gas phase

In Fig. 5 a volume element of the tubular membrane is shown. The mathematical problem is radial homogenous.

The space coordinate x [m] is normalized to the dimensionless parameter z,

$$z = \frac{x}{L_M}, \qquad (1)$$

by the length L_M of the membrane.

The mass balance of component I in the gas phase,

$$\frac{\partial}{\partial t}\left(n_G(z,t) \cdot x_{IG}(z,t)\right) = -\frac{\partial}{\partial z}\left(\dot{n}_G(z,t) \cdot x_{IG}(z,t)\right)$$

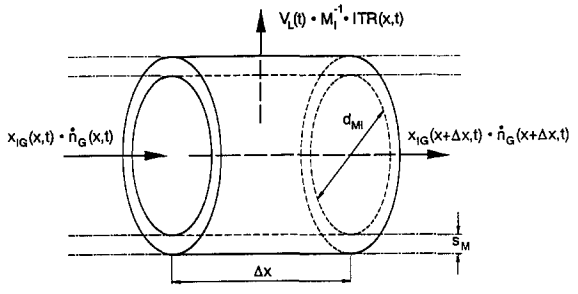

Fig. 5. Volume element of the tubular membrane.

$$-\frac{V_L(t) \cdot ITR(z,t)}{M_I}, \quad (2)$$

includes the unknown space and time dependent process variables,

$n_G(z,t)$ =: amount of gas moles in the cross sectional area, [mol]
$x_{IG}(z,t)$ =: mole fraction of component I in the gas phase [-]
$ITR(z,t)$ =: mass transfer rate of component I between gas and liquid phase [g l^{-1} h^{-1}]
$\dot{n}_G(z,t)$ =: average cross sectional molar flow velocity [mol h^{-1}]

the time dependent process variable,
$V_L(t)$ =: liquid volume [l]
and the constant parameter
M_I =: mole mass of component I [g mol^{-1}].

Equation (2) has to be computed for each component I, I = O_2, N_2, CO_2, ..., in the gas phase. The addition of all these equations leads to the quasi steady state continuity equation in order to calculate the unknown average flow velocity $\dot{n}_G$ (Luttmann et al. 1989).

Without loss of accuracy for experimental investigations, several assumptions and simplifications are shown for an analytical solution of eq. (2).

Cultivation processes in bioreactors are very slow. The system variables are defined in quasi steady state condition with

$$\frac{\partial}{\partial t}\left(n_G(z,t) \cdot x_{IG}(z,t)\right) \rightarrow 0. \quad (3)$$

The average molar flow velocity is assumed as space independent,

$$\dot{n}_G(z,t) \approx \dot{n}_G(0,t) = \frac{F_{GnE}(t)}{V_{Mn}}, \quad (4)$$

and calculated from the process parameter aeration rate with

$F_{GnE}(t)$ =: total aeration rate [l h^{-1}]
and the mole volume,
V_{Mn} = 22.412 l mol^{-1},
both defined in normalized conditions (index n).
The assumption 2 indicates an exhaust gas molar respiratory quotient RQ = 1.

The oxygen transfer rate, OTR, is described similar to gas bubble aerated bioreactors,

$$OTR(z,t) = K_L(t) \cdot \frac{A_M}{V_L(t)} \cdot \left[\frac{p_G(z,t) \cdot x_{OG}(z,t)}{H_{OL}(t)} - c_{OL}(t)\right], \quad (5)$$

and related to the liquid volume (Lydersen 1983). Equation (5) includes important process variables and parameters,

$OTR(z,t)$ =: oxygen transfer rate between gas and liquid phase [g l^{-1} h^{-1}]
$K_L(t)$ =: global oxygen transfer coefficient [m h^{-1}]
A_M =: membrane surface (boundary layer) [m^2]
$V_L(t)$ =: liquid volume [m^3]
$p_G(z,t)$ =: pressure of the gas phase [N m^{-2}]
$H_{OL}(t)$ =: Henry coefficient of oxygen in the cultivation broth [N m kg^{-1}]
$x_{OG}(z,t)$ =: oxygen mole fraction in the gas phase [-]
$c_{OL}(t)$ =: dissolved oxygen concentration in the liquid phase [g l^{-1}].

The pressure drop of the gas phase along the tube is very small in comparison to the amount of pressure at the gas entrance,

$$p_G(z,t) \approx p_G(0,t) = p_{GE}(t). \quad (6)$$

The aeration pressure p_{GE} is on-line measurable.

The Henry coefficent of oxygen in the culture medium, H_{OL}, is not really known in general, as it is dependent on different (time varying) medium conditions (Schumpe et al. 1982).
In most cases the values of O_2-solubility in water are used.

Inserting eq. (3) through eq. (6) into eq. (2) leads to a space-dependent differential equation for the oxygen mole fraction x_{OG} along the tube,

$$\frac{dx_{OG}(z,t)}{dz} = -\frac{K_L(t) \cdot A_M \cdot V_{Mn}}{M_{O2} \cdot F_{GnE}(t)} \cdot \left[\frac{p_{GE}(t) \cdot x_{OG}(z,t)}{H_{OL}(t)} - c_{OL}(t)\right], \quad (7)$$

with the known initial condition,

$$x_{OG}(0,t) = x_{OGE}(t). \qquad (7a)$$

The entrance oxygen mole fraction x_{OGE} is calculated from the O_2 carrying parts in the aerated bioreactor,

$$x_{OGE}(t) = \frac{F_{AIRn}(t) \cdot x_{OAIR} + F_{O2n}(t)}{F_{GnE}(t)}, \qquad (8)$$

with

$$F_{GnE}(t) = F_{AIRn}(t) + F_{O2n}(t) + F_{N2n}(t), \qquad (9)$$

including the variables,

$x_{OGE}(t)$ =: oxygen gas phase mole fraction at the entrance of the membrane [-]

$F_{In}(t)$ =: aeration rate of component I, I = AIR, O2, N2, defined in normalized conditions [l h^{-1}],

and the known oxygen mole fraction of air,

$x_{OAIR} = 0.2094$ [-].

It is recommended to use process specific (chemical engineering) parameters in order to get simple understandable equations.

Therefore, we introduce a theoretical maximum for oxygen offer rate,

$$Q_{O2Emax}(t) = \frac{F_{GnE}(t) \cdot M_{O2}}{V_{Mn} \cdot V_L(t)} \qquad (10)$$

in [g l^{-1} h^{-1}].

The parameter indicates the amount of oxygen mass flow in the membrane, which can be reached with the chosen total aeration rate and a pure oxygen gas phase.

The second important parameter is the theoretical maximum value of oxygen transfer rate,

$$OTR_{max}(t) = \frac{K_L(t) \cdot A_M \cdot p_{GE}(t)}{V_L(t) \cdot H_{OL}(t)}, \qquad (11)$$

in [g l^{-1} h^{-1}].

This parameter indicates how much oxygen is transferable from a pure oxygen gas phase with a pressure p_{GE} into an oxygen free medium. Both parameters are related to the liquid volume. Their quotient is the Stanton-Number,

$$St(t) = \frac{OTR_{max}(t)}{Q_{O2Emax}(t)}, \qquad (12)$$

well known in chemical engineering.

Finally, the transformation of the dissolved oxygen concentration c_{OL} into an equilibrium mole fraction in the gas phase,

$$x^*_{OG}(t) = \frac{c_{OL}(t) \cdot H_{OL}(t)}{p_{GE}(t)}, \qquad (13)$$

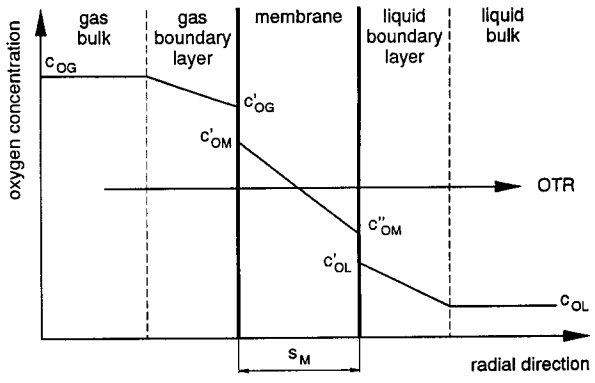

Fig. 6. Mass transfer behaviour across the membrane.

is introduced.

The rearrangement of eq. (7) with eqs. (12) and (13) leads to a first order equation in space,

$$\frac{dx_{OG}(z,t)}{dz} = -St(t) \cdot \left[x_{OG}(z,t) - x^*_{OG}(t)\right], \qquad (14)$$

which is simply solvable.

The solution of eq. (14), the space dependent behaviour of the oxygen mole fraction,

$$x_{OG}(z,t) = x^*_{OG}(t) + \left[x_{OGE}(t) - x^*_{OG}(t)\right] \cdot e^{-St(t)*z}, \qquad (15)$$

is the base for further formulations.

Investigation in gas/membrane/liquid mass transfer coefficients

Figure 6 illustrates the mass transfer behaviour across the membrane. Similar to gas/liquid interactions, a two film theory is the basis of the mathematical description (Bird et al. 1969).

The oxygen mass flux in [kg m^{-2} h^{-1}] between gas and liquid phase,

$$\begin{aligned} J_{GL}(z,t) &= \frac{OTR(z,t) \cdot V_L(t)}{A_M} \\ &= k_G \cdot \left[c_{OG}(z,t) - c_{OG}(z,t)\right] \\ &= \frac{D_M}{s_M} \cdot \left[c'_{OM}(z,t) - c''_{OM}(z,t)\right] \\ &= k_L \cdot \left[c'_{OL}(z,t) - c_{OL}(t)\right], \end{aligned} \qquad (16)$$

is controlled by the driving forces in the gas film in the membrane and in the liquid film.

It has to pass three mass transfer resistances with

k_G =: oxygen mass transfer coefficient in the membrane gas film[m h^{-1}]
$c_{OG}(z,t)$ =: oxygen concentration in the center of the gas phase [kg m^{-3}]
$c'_{OG}(z,t)$ =: oxygen concentration in the gas phase at the membrane boundary [kg m^{-3}]
D_M =: oxygen diffusion coefficient in the silicone membrane [m^2 h^{-1}]
s_M =: thickness of the silicone membrane [m]
$c'_{OM}(z,t)$ =: oxygen concentration in the membrane at the gas boundary [kg m^{-3}]
$c''_{OM}(z,t)$ =: oxygen concentration in the membrane at the liquid boundary [kg m^{-3}]
k_L =: oxygen mass transfer coefficient in the membrane liquid film [m h^{-1}]
$c'_{OL}(z,t)$ =: oxygen concentration in the liquid phase at the membrane boundary [kg m^{-3}]
$c_{OL}(t)$ =: oxygen bulk concentration in the well mixed liquid phase [kg m^{-3}].

The introduction of the ideal gas law,

$$c_{OG}(z,t) = \frac{p_{OG}(z,t)\cdot M_{O2}}{R\cdot T_G(t)} = \frac{p_G(z,t)\cdot x_{OG}(z,t)\cdot M_{O2}}{R\cdot T_G(t)}, \quad (17)$$

with
$p_{OG}(z,t)$ =: oxygen partial pressure in the kernel of the gas phase [N m^{-2}]
$T_G(t)$ =: temperature of the gas phase [K],
and Henry's law applied to the liquid phase,

$$c_{OL}(t) = \frac{p_{OL}(t)}{H_{OL}(t)}, \quad (18)$$

with
$p_{OL}(t)$ =: oxygen bulk partial pressure [N m^{-2}],
and Henry's law applied to the silicone membrane with a high oxygen solubility,

$$c_{OM}(z,t) = \frac{p_{OM}(z,t)}{H_{OM}(t)}, \quad (19)$$

with
$p_{OM}(z,t)$ =: oxygen partial pressure in the membrane [N m^{-2}]
$H_{OM}(t)$ =: Henry coefficient for oxygen solubility in silicone [Nm kg^{-1}] as well as the boundary layer equilibria,

$$p'_{OL}(z,t) = c'_{OL}(z,t)\cdot H_{OL}(t) = p''_{OM}(z,t) = c''_{OM}(z,t)\cdot H_{OM}(t), \quad (20)$$

and

$$p'_{OG}(z,t) = \frac{c'_{OG}(z,t)\cdot R\cdot T_G(t)}{M_{O2}} = p'_{OM}(z,t) = c'_{OM}(z,t)\cdot H_{OM}(t), \quad (21)$$

leads to a final description of the oxygen mass flux,

$$J_{GL}(z,t) = K_L(t)\cdot\left[\frac{p_G(z,t)\cdot x_{OG}(z,t)}{H_{OL}(t)} - c_{OL}(t)\right]. \quad (22)$$

The mass transfer resistance,

$$\frac{1}{K_L(t)} = \frac{1}{k_L} + \frac{H_{OM}(t)\cdot s_M}{H_{OL}(t)\cdot D_M} + \frac{R\cdot T_G(t)}{M_{O2}\cdot H_{OL}(t)\cdot k_G}, \quad (23)$$

is the sum of three resistances transformed to the liquid phase conditions and is generally a time varying parameter during cell cultivations.

The gas resistance is neglectable. In this case, the membrane/liquid mass transfer coefficient,

$$K_L(t) = \frac{k_L}{1+\frac{H_{OM}(t)\cdot s_M\cdot k_L}{H_{OL}(t)\cdot D_m}}, \quad (24)$$

is controlled by the membrane and liquid phase diffusion. The resulting coefficient is lower than those of conventional gas bubble aeration.

Oxygen mass balance in the liquid phase

The oxygen mass balance in the liquid phase combines the lumped parameter and the distributed parameter behaviour of the bioreactor.

The time derivation of dissolved oxygen concentration, c_{OL}, in the well mixed liquid phase,

$$\frac{dc_{OL}(t)}{dt} = \overline{OTR}(t) - OUR(t), \quad (25)$$

is influenced by the oxygen uptake rate of all active cells,

$$OUR(t) = q_{O/X}(t)\cdot c_{XL}(t), \quad (26)$$

with
OUR(t) =: volumetric oxygen uptake rate [g l^{-1} h^{-1}]
$q_{O/X}(t)$ =: cell specific oxygen uptake rate [h^{-1}]
$c_{XL}(t)$ =: active cell concentration (dry weight) [g l^{-1}]
and the average value of the space dependent oxygen transfer rate,

$$\overline{OTR}(t) = \int_0^1 OTR(z,t)dz. \quad (27)$$

The average OTR is given by integration of eq. (5) with inserted eqs. (6), (10) and (15),

$$\overline{OTR}(t) = Q_{O2Emax}(t) \cdot [x_{OGE}(t) - x^*_{OG}(t)] \cdot \left[1 - e^{-St(t)}\right]. \quad (28)$$

In normal process conditions, without stepwise changing of aeration rate or agitation speed (in the case of moving membranes), the oxygen supply can be assumed as a quasi steady state process,

$$OUR(t) = \overline{OTR}(t). \quad (29)$$

The assumption will be used to calculate the unknown oxygen transfer coefficient K_L and the unknown specific oxygen uptake rate $q_{O/X}$.

Equation (28) also indicates the upper limit of oxygen supply of cells with membrane aeration.

Assuming oxygen transfer limited growth conditions with $c_{OL} \rightarrow 0$, the oxygen uptake (transfer) rate,

$$OUR_{lim}(t) = \overline{OTR}_{lim}(t) = Q_{O2Emax}(t) \cdot x_{OGE}(t) \cdot \left[1 - e^{-St(t)}\right], \quad (30)$$

reaches its chemical engineering boundary.

Experimental verification of unknown process parameters

For identification of the unknown process parameters quasi steady state methods can be used as well as dynamic shift experiments (Sobotka 1982).

Quasi steady state method

The base of this method is eq (29),

$$OUR(t) = q_{O/X}(t) \cdot c_{XL}(t) = \overline{OTR}(t) = Q_{O2Emax}(t) \cdot [x_{OGE}(t) - x^*_{OG}(t)] \cdot \left[1 - e^{-St(t)}\right]. \quad (31)$$

Unknown parameters are,

$OUR(t)$ =: volumetric oxygen uptake rate [g l^{-1} h^{-1}]

$q_{O/X}(t)$ =: cell specific uptake rate [h^{-1}]

$St(t)$ =: Stanton-Number (including the oxygen transfer coefficient K_L) [-],

whereas the other parameters are known from measurements,

c_{XL} =: (active) cell dry weight (off-line) [g l^{-1}]

Q_{O2Emax} =: maximum oxygen offer rate (eq. (10) [g l^{-1} h^{-1}]

x_{OGE} =: oxygen mole fraction at the gas entrance (eq. (8)) [-].

The equilibrium mole fraction x^*_{OG} of the liquid phase depends on the dissolved oxygen tension pO_2, which is also measurable on-line.

The definition of pO_2,

$$pO_2(t) = \frac{p_{OL}(t)}{p_{OLcal}} = \frac{c_{OL}(t) \cdot H_{OL}(t)}{p_{Gcal} \cdot x_{OGcal}}, \quad (32)$$

with

$p_{OL}(t)$=: dissolved oxygen partial pressure [N m^{-2}]

p_{OLcal} =: 100 % pO_2 calibration pressure [N m^{-2}]

p_{Gcal} =: pressure of the gas phase during pO_2-calibration [N m^{-2}]

x_{OGcal} =: oxygen mole fraction of the gas phase during pO_2-calibration [-],

indicates the strong correlation with eq. (13),

$$x^*_{OG}(t) = \frac{p_{Gcal} \cdot x_{OGcal}}{p_{GE}(t)} \cdot pO_2(t). \quad (33)$$

The calculation of x^*_{OG} is possible without knowledge of the oxygen Henry-coefficient in the cultivation medium.

The identification procedure is carried out during a real process with a growing culture at $t = t_1$ (index-condition 1). At this point, $Q_{O2Emax1}$, x_{OGE1} and pO_{21} (x^*_{OG1}) are known from on-line measurements.

At first the Stanton-Number St_1 has to be calculated with the following operations:

- Change the aeration rate (index 2),

$$F_{GnE2} = \beta \cdot F_{GnE1}, \quad (34)$$

without changing the oxygen mole fraction and the pressure at the gas phase entrance. In this case

$$Q_{O2Emax2} = \beta \cdot Q_{O2Emax1}, \quad (35)$$

$$x_{OGE2} = x_{OGE1}, \quad (36)$$

and

$$p_{GE2} = p_{GE1} \quad (37)$$

holds true.

- Wait for a steady state condition and measure the pO_2 level (pO_{22}).

- Change the aeration rate (index 3)

$$F_{GnE3} = \frac{1}{\beta} \cdot F_{GnE1}, \tag{38}$$

without alteration of the entrance conditions p_{GE} and x_{OGE}.
- Wait for another steady state and measure pO_{23}.
- Return to the start point condition

$$F_{GnE4} = F_{GnE1}. \tag{39}$$

- Calculate x^*_{OG2} and x^*_{OG3} with eq. (33).

If we take into account the very slow growth process without changing oxygen uptake rate during the identification procedure ($pO_{24} \approx pO_{21}$),

$$OUR_3 = OUR_2 = OUR_1 \tag{40}$$

holds true and this method is useful for calculation of the unknown Stanton-Number St_1.

The introduction of an abbreviation

$$\alpha_i = x_{OGE1} - x^*_{OGi}, \tag{41}$$

and the insertion of the measured parameters in eq. (31) leads to

$$\frac{\alpha_2 \cdot \beta^2 \left(1 - e^{\frac{-St_1}{\beta}}\right)}{\alpha_3 \cdot \left(1 - e^{-\beta \cdot St_1}\right)} = 1 \tag{42}$$

Writing the Taylor series,

$$e^{-x} \approx 1 - x + \frac{x^2}{2} - \frac{x^3}{6}, \tag{43}$$

with

$$x_2 = \beta^{-1} St_1 \tag{44}$$

and

$$x_3 = \beta \cdot St_1, \tag{45}$$

the Stanton-Number is given by

$$St_1 = \frac{1.5 \cdot \beta \cdot (\alpha_2 - \alpha_3 \cdot \beta^2)}{\alpha_2 - \alpha_3 \cdot \beta^4} - \sqrt{\left[\frac{1.5\beta \cdot (\alpha_2 - \alpha_3 \cdot \beta^2)}{\alpha_2 - \alpha_3 \cdot \beta^4}\right]^2 - \frac{6 \cdot \beta^2 \cdot (\alpha_2 - \alpha_3)}{\alpha_2 - \alpha_3 \cdot \beta^4}}. \tag{46}$$

The unknown theoretical maximum mass transfer rate,

$$OTR_{max1} = St_1 \cdot Q_{O2Emax1} = \frac{K_{L1} \cdot A_M \cdot p_{GE1}}{V_{L1} \cdot H_{OL1}}, \tag{47}$$

represents a value for the mass transfer capacity of the membrane material.

However, the maximum capacity of the membrane aerated bioreactor ($c_{OL} \rightarrow 0$) is given by eq. (30),

$$OTR_{lim1} = Q_{O2Emax1} \cdot x_{OGE1} \cdot \left[1 - e^{-St_1}\right]. \tag{48}$$

The knowledge of the oxygen Henry-coefficient H_{OL} in the culture medium is a precondition for the calculation of the unknown oxygen mass transfer coefficient K_L from eq (46),

$$K_{L1} = \frac{St_1 \cdot Q_{O2Emax1} \cdot V_{L1} \cdot H_{OL1}}{A_M \cdot p_{GE1}}, \tag{49}$$

with the logarithmic membrane boundary layer,

$$A_M = \frac{(d_{Ma} - d_{Mi})}{\ln\left[\frac{d_{Ma}}{d_{Mi}}\right]} \cdot \pi \cdot L_M, \tag{50}$$

and

d_{Mi} =: inner membrane diameter [m],
d_{Ma} =: outer membrane diameter [m],
L_M =: length of the membrane [m].

With the identified Stanton-Number St_1, a determination of the volumetric oxygen uptake rate,

$$OUR_1 = Q_{O2Emax1} \cdot \left[x_{OGE1} - x^*_{OG1}\right] \cdot \left[1 - e^{-St_1}\right], \tag{51}$$

is possible as well as the identification of the specific oxygen uptake rate,

$$q_{O/X1} = \frac{OUR_1}{c_{XL1}}. \tag{52}$$

Result of a quasi steady state experiment

A growth experiment with plant cells is manipulated at a certain time t_1, in order to calculate the oxygen mass transfer coefficient K_L, as well as the volumetric oxygen uptake rate OUR, and the cell specific oxygen uptake rate $q_{O/X}$.

The membrane parameters,
$L_M = 1.55$ m
$d_{Mi} = 3.2$ mm
$d_{Ma} = 4.0$ mm
the observed process engineering parameters,
$V_L = 1.4$ l
$p_{GE} = 1.05$ bar
$F_{O2n} = 0.463\ l\ h^{-1}$
$F_{AIRn} = 0.137\ l\ h^{-1}$,

and the pO_2 calibration parameters,

$p_{Gcal} = 1.05$ bar

$x_{OGcal} = x_{OAIR} = 0.2094$,

are known, whereas the Henry coefficient of water,

$H_{OL} = 2.763 \cdot 10^6$ Nm kg^{-1}

is assumed to be true in the culture medium.

The cell dry weight at $t = t_1$, $c_{XL1} = 2.9$ g l^{-1}, is known from off-line measurements, whereas the controlled dissolved oxygen tension, $pO_{21} = 0.6$ (60 %) is available on-line.

The pO_2 control is stopped at $t = t_1$ and the process is manipulated manually. The decrease of both aeration rates with $\beta = 0.75$ brings at $t = t_2$ a new quasi steady state value, $pO_{22} = 0.585$, whereas the following increase with $\beta^{-1} = 1.33$ leads to another steady state at $t = t_3$ with $pO_{23} = 0.611$. The identification procedure of Stanton Number at $t = t_1$, described in the previous chapter, results in St = 0.027.

The calculated experimental parameters at $t = t_1$ are:

$OTR = OUR = 11.31 \cdot 10^{-3}$ g l^{-1} h^{-1}

$q_{O/X} = 3.9 \cdot 10^{-3}$ h^{-1}

$K_L = 3.49 \cdot 10^{-2}$ m h^{-1} and

$K_L \frac{A_M}{V_L} = K_La = 0.435$ h^{-1}.

These results, the given reactor parameters and the boundary process conditions of pure oxygen aeration with an aeration rate of 0.6 l h^{-1} and a pO_2 level of 0.6 (60 %) indicate the possible upper limit of oxygen uptake rate with 14.25 mg l^{-1} h^{-1}.

Dynamic methods for identification of oxygen mass transfer and oxygen uptake parameters

Dissolved oxygen balances

Besides quasi steady state methods, it is common to use stepwise changes in the aeration conditions in order to measure and to calculate the mass transfer and the oxygen uptake parameters (Vorlop 1989).

In order to discuss the real dynamic behaviour of the process, the general distributed parameter model has to be solved, but this mathematical overhead is without practical relevance. Therefore, we make some further simplifications:

- The gas residence time is short in comparison to the time constant of the oxygen transfer across the membrane.
- The same holds true for the first order lag of the pO_2 probe.
- In the case of cell free media, there is no oxygen uptake rate and carbon dioxide production rate.

Therefore the assumption for eq. (4),

$$RQ(t) = \frac{-CTR(z,t) \cdot M_{O2}}{M_{CO2} \cdot OTR(z,t)} = 1, \tag{53}$$

with

RQ(t) =: molar respiratory quotient [-]

CTR(z, t) =: carbon dioxide transfer rate [g l^{-1} h^{-1}]

holds no longer true.

In order to get useful experimental results, we assume,

$$x_{OG}(z,t) \approx x_{OGE}(t). \tag{54}$$

Without loss of accuracy, we assume that the gas phase is in a steady state condition, even with stepwise changes of gas components. So we obtain two different dynamic equations for the description of dissolved oxygen in the liquid phase.

During the experiments in cell free media, the dissolved oxygen balance in the liquid phase,

$$\frac{dc_{OL}(t)}{dt} = K_L(t) \cdot \frac{A_M}{V_L(t)} \cdot (c_{OLmax}(t) \cdot x_{OGE}(t) - c_{OL}(t)), \tag{55}$$

results in a simple stirred tank reactor equation without changing oxygen conditions in the gas phase.

The maximum solubility of oxygen in the culture medium,

$$c_{OLmax}(t) = \frac{p_{GE}(t)}{H_{OL}(t)}, \tag{56}$$

depends on the chosen pressure at the gas entrance, p_{GE}, and the actual Henry-coefficient, H_{OL}.

In the case of investigations during cultivation processes, eq. (25) with eqs. (26) and (28),

$$\frac{dc_{OL}(t)}{dt} = Q_{O2Emax}(t) \cdot \left[x_{OGE}(t) - \frac{c_{OL}(t)}{c_{OLmax}(t)}\right] \cdot \left(1 - e^{-St(t)}\right) - OUR(t), \tag{57}$$

is valid.

The oxygen uptake OUR is controlled by the dissolved oxygen concentration c_{OL}. If we assume the Blackman kinetics (Condrey 1982),

$$OUR(t) = \begin{cases} OURmax(t), & c_{OL}(t) \geqslant k_{Ocrit} \\ OURmax(t) \cdot \dfrac{c_{OL}(t)}{k_{Ocrit}}, & c_{OL}(t) < k_{Ocrit} \end{cases} \tag{58}$$

then the O_2 uptake rate is in first order below a critical oxygen concentration k_{Ocrit} and in zero order above this value.

The maximum oxygen uptake rate,

$$OURmax(t) = q_{O/Xmax} \cdot c_{XL}(t), \qquad (59)$$

increases with the growing biomass and is controlled by the maximum cell specific uptake rate $q_{O/Xmax}$.

The aim of the following experimental investigations is to calculate the unknown parameters K_L, OUR and $q_{O/Xmax}$.

Shift experiments in cell free media

In this case we use the bioreactor prepared for cultivation conditions but without inoculum. Two methods for identification of the mass transfer coefficient are available. The first one is the oxygenation of oxygen free medium. Here we start to aerate the bioreactor with nitrogen and a certain aeration rate F_{GnE} until the initial condition, an oxygen free medium,

$$pO_2(0) = 0, \qquad (60)$$

is reached.

The pO_2 probe is calibrated with the chosen gas phase entrance pressure p_{GE} and air (eq. (32)). Then we change to air aeration ($\tau = 0$). The step response of the pO_2 level is the solution of the rearranged eq. (55),

$$\frac{dpO_2(\tau)}{dt} = K_L \cdot \frac{A_M}{V_L} \cdot \left(1 - pO_2(\tau)\right), \qquad (61)$$

with the initial condition eq. (60), which results in

$$pO_2(\tau) = 1 - e^{-\frac{\tau}{T_L}}. \qquad (62)$$

The time constant,

$$T_L = \frac{V_L}{K_L \cdot A_M} = \frac{1}{K_L a}, \qquad (63)$$

includes the unknown oxygen transfer coefficient K_L.

The slope of the logarithmic graph,

$$\ln(1 - pO_2(\tau)) = -\frac{\tau}{T_L}, \qquad (64)$$

can be used in order to identify the unknown time constant T_L and to calculate K_La and K_L.

The second method starts with an oxygen saturated medium by air aeration and the initial condition,

$$pO_2(0) = 1. \qquad (65)$$

Then we change the aeration to nitrogen ($\tau = 0$) with

$$x_{OGE}(\tau) \equiv 0. \qquad (66)$$

The solution of eq. (55) results in

$$pO_2(\tau) = e^{-\frac{\tau}{T_L}}. \qquad (67)$$

The slope of the logarithmic graph,

$$\ln(pO_2(\tau)) = -\frac{\tau}{T_L}, \qquad (68)$$

can be used to identify T_L, K_La and K_L.

Shift experiments during cell cultivation

The dynamic response method is also useful for identification of the unknown parameters during real cultivation.

If we assume a certain total aeration rate F_{GnE} and an oxygen mole fraction x_{OGE} at $t = t_1$, we will define a new time scale ($\tau = 0$) and change the aeration to pure nitrogen with the same aeration rate,

$$F_{N2}(\tau) = F_{N2}(0) = F_{GnE}(t_{1-}). \qquad (69)$$

The measurement of pO_2 at $t = t_1$,

$$pO_2(t_1) = pO_2(\tau = 0) = pO_{20} \qquad (70)$$

is the initial value for the solution of eq. (57).

The description of oxygen reaction with Blackman kinetics (eq. (58)) results in two different solutions.

The step response,

$$c_{OL}(\tau) = c_{OL0} \cdot e^{-\frac{\tau}{T_M}} - T_M \cdot OUR_{max} \cdot \left(1 - e^{-\frac{\tau}{T_M}}\right), \qquad (71)$$

with the initial condition,

$$c_{OL0} = \frac{p_{Gcal} \cdot pO_{20}}{H_{OL0}}, \qquad (72)$$

and the time constant,

$$T_M = \frac{c_{OLmax}}{Q_{O2Emax} \cdot \left(1 - e^{-St}\right)}. \qquad (73)$$

is valid in the case of unlimited oxygen uptake ($c_{OL} \geq k_{Ocrit}$).

A comparison with the time constant with experiments in cell free medium, eq. (62), leads to

$$T_M = \frac{T_L}{1 + \sum_{i=1}^{\infty} (-1)^i \frac{St^i}{(i+1)!}}. \qquad (74)$$

The calculation procedure of the unknown maximum oxygen uptake rate is as follows:

- Use the dynamic response methods in cell free media in order to identify the time constant T_L.
- Calculate the volumetric oxygen mass transfer coefficient K_La (eq. (63)), the maximum solubility c_{OLmax}, (eq. (56)), the maximum oxygen transfer rate, OTR_{max}, (eq. (11)), the maximum oxygen offer rate, Q_{O2Emax}, (eq. (10)) and the Stanton Number, St, (eq. (12)).
- Calculate the time constant during cultivations, T_M, (eq. (73)).
- Transform the time varying measured dissolved oxygen tension, pO_2, into the dissolved oxygen concentration,

$$c_{OL}(\tau) = \frac{p_{Gcal} \cdot x_{OGcal} \cdot pO_2(\tau)}{H_{OL}}, \qquad (75)$$

  assuming the Henry coefficient H_{OL}.
- Prepare a graph OUR_{max} versus τ,

$$OUR_{max}(t_1) = \frac{c_{OL0} \cdot e^{-\frac{\tau}{T_M}} - c_{OL}(\tau)}{T_M \cdot \left(1 - e^{-\frac{\tau}{T_M}}\right)} \stackrel{!}{=} \text{constant}. \qquad (76)$$

  The upper constant limit of this graph is the unknown maximum oxygen uptake rate.
- Use the off-line measurement of cell dry weight $c_{XL}(t_1)$ in order to calculate the unknown maximum specific uptake rate $q_{O/Xmax}$ with eq. (59).

In the case of limited oxygen uptake rate ($c_{OL} < k_{Ocrit}$), the assumption of a time constant volumetric uptake rate no longer holds true.

The solution of eq. (57) results in

$$c_{OL}(\tau) = k_{Ocrit} \cdot e^{-\frac{\tau - \tau_{Ocrit}}{T_M^*}}. \qquad (77)$$

This equation is valid when c_{OL} passes k_{Ocrit} at $\tau = \tau_{Ocrit}$.

The time constant in this period,

$$T_M^* = \frac{T_M}{1 + \frac{T_M \cdot OUR_{max}}{k_{Ocrit}}}, \qquad (78)$$

is itself a function of the unknown maximum volumetric uptake rate.

Reactor design

The discussed methods are useful to calculate the length of the required membrane aeration line.

The aim of a process design is to avoid oxygen transfer limited growth conditions. Therefore, the dissolved oxygen tension pO_2 is controlled on a certain set point pO_{2w}.

The upper limit of oxygen demand during cultivation is given by

$$OUR_{opt} = q_{O/Xmax} \cdot c_{XLopt}, \qquad (79)$$

with

$q_{O/Xmax}$ =: maximum (unlimited) cell specific oxygen uptake rate [h^{-1}]

c_{XLopt} =: target cell concentration (dry weight) [$g\,l^{-1}$]

and

OUR_{opt} =: upper limit of volumetric oxygen uptake rate with controlled pO_2 [$g\,l^{-1}\,h^{-1}$].

The oxygenation potential of the membrane has to fulfill this oxygen demand. The chemical engineering boundary of pO_2 control is the aeration with pure oxygen ($x_{OGE} = 1$).

In this case

$$OUR_{opt} = Q_{O2Emax} \cdot \left[1 - x^*_{OGw}\right] \cdot \left[1 - e^{-St}\right] \qquad (80)$$

with

$$x^*_{OGw} = \frac{p_{Gcal} \cdot x_{OGcal} \cdot pO_{2w}}{p_{GE}} \qquad (81)$$

holds true.

The rearrangement of eq. (80) leads to the reactor design specification. The membrane length,

$$L_M = \frac{\ln\left(\frac{d_{Ma}}{d_{Mi}}\right) \cdot V_L \cdot Q_{O2Emax}}{(d_{Ma} - d_{Mi})} \cdot \frac{\left[-\ln\left(1 - \frac{OUR_{opt}}{Q_{O2Emax} \cdot \left[1 - x^*_{OGw}\right]}\right)\right]}{\pi \cdot K_L \cdot c_{OLmax}} \qquad (82)$$

includes the target parameter OUR_{opt}, the aeration condition Q_{O2Emax}, the oxygen solubility c_{OLmax} and the transfer parameter K_L, d_{Mi}, d_{Ma} as well as the reaction volume V_L.

The following design example is based on similar parameters as used in our plant cell experiment, described in section 'Result of a quasi steady state experiment',

$c_{XLopt} = 3.7\ g\,l^{-1}$

$q_{O/Xmax} = 3.9 \cdot 10^{-3}\ h^{-1}$

$pO_{2w} = 0.6\ (60\ \%)$

$p_{Gcal} = p_{GE}$

$x_{OGcal} = x_{OAIR} = 0.2094$

and the process target values,

$V_L = 1.4\ l$

$F_{GnE} = F_{O2nmax} = 0.6\ l\ h^{-1}$

$c_{OLmax} = 38 \cdot 10^{-3}\ g\ l^{-1}$

$d_{Ma} = 4.0 \cdot 10^{-3}\ m$

$d_{Mi} = 3.2 \cdot 10^{-3}\ m$

$K_L = 0.0349\ m\ h^{-1}$

The control of $pO_2 = 0.6$ is possible up to the cell concentration $c_{XLopt} = 3.7\ g\ l^{-1}$ with a calculated membrane length $L_M = 1.564$ m.

The control of pO_2 can be carried out by a constant total aeration rate F_{GnE} with a mixing of nitrogen and air at the beginning of the cultivation. The pO_2-controller changes to a mixing of air and oxygen when the oxygen demand of cells is rapidly increasing.

The upper limit is reached, when the pO_2 controller closes the air mass flow controller and aerates the bioreactor only with pure oxygen (Bellgardt et al. 1985).

Conclusion

The technical specification of membrane aeration devices can be calculated mathematically according to the specific oxygen requirement of a given suspension culture. Hydrophobic polypropylene membranes used by Piehl et al. (1988) have a higher oxygen transfer capability than silicone based systems. This may, however, lead to tiny air bubbles when the aeration pressure is raising above the bubble-point. High cell densities of 400 g l^{-1} fresh weight were achieved in a suspension culture of *Thalictrum rugosum* that was aerated with 30 % pO_2 by polypropylene gas-exchange membrane (Piehl et al. 1988).

Embryogenic cell suspensions usually do not reach as high cell densities compared with cultures for scaling up of pharmaceutically used biomass. Therefore, as silicone tubing is more easy to handle than polypropylene membranes, these are recommended for bioreactor production of somatic embryos. When calculating the maximum length of the silicone tubing, one has to consider, that plant cells tend to grow on surfaces and therefore, stagnant regions in the vessel have to be minimized. The distance between the silicone tubing and other devices inside the bioreactor should allow free floating of the cells, thus inhibiting the formation of callus colonies attached to the tubings or electrodes. Precise supply of different oxygen levels by use of membrane aeration in embryogenic suspension cultures will lead to more information on oxygen requirement during induction, development and maturation phase of somatic embryogenesis.

List of symbols

A_M:	membrane surface	[m^2]
c_{OJ}:	oxygen concentration in subsystem J	[$g\ l^{-1}$]
c_{OLmax}:	maximum oxygen solubility	[$g\ l^{-1}$]
c_{XL}:	cell (dry weight) concentration	[$g\ l^{-1}$]
CTR:	carbon dioxide transfer rate	[$g\ l^{-1}\ h^{-1}$]
d_M:	membrane diameter	[m]
D_M:	oxygen diffusion coefficient in the membrane	[$m^2\ h^{-1}$]
F_{GnE}:	total aeration rate in normalized conditions	[$l\ h^{-1}$]
F_{Kn}:	aeration rate of gas species K in normalized conditions	[$l\ h^{-1}$]
H_{OJ}:	Henry coefficient of oxygen in subsystem J	[$Nm\ kg^{-1}$]
ITR:	mass transfer rate of gas component I	[$g\ l^{-1}\ h^{-1}$]
J_{GL}:	mass flux of oxygen between gas and liquid phase	[$kg\ m^{-2}\ h^{-1}$]
k_J:	oxygen mass transfer coefficient in subsystem J	[$m\ h^{-1}$]
K_L:	global oxygen mass transfer coefficient	[$m\ h^{-1}$]
k_{Ocrit}:	critical oxygen concentration	[$g\ l^{-1}$]
L_M:	length of the tubular membrane	[m]
M_I:	mole mass of component I	[$g\ mol^{-1}$]
n_G:	gas moles in the cross sectional area	[mol]
$\dot{n}_G$:	average cross sectional molar flow velocity	[$mol\ h^{-1}$]
OTR:	oxygen transfer rate	[$g\ l^{-1}\ h^{-1}$]
OUR:	oxygen uptake rate	[$g\ l^{-1}\ h^{-1}$]
p_G:	total pressure in the gas phase	[$N\ m^{-2}$]
p_{OK}:	oxygen partial pressure in subsystem K, K = G, M, L	[$N\ m^{-2}$]
pO_2:	dissolved oxygen tension	[-]
$q_{O/X}$:	cell specific oxygen uptake rate	[h^{-1}]
Q_{O2Emax}:	maximum oxygen offer rate	[$g\ l^{-1}\ h^{-1}$]

R:	universal gas constant	[Nm K^{-1} $kmol^{-1}$]
s_M:	thickness of the membrane	[m]
St:	Stanton-Number	[-]
t:	process time	[h]
T_G:	temperature of the gas phase	[K]
T_L, T_M, T_M^*:	time constants	[h]
V_L:	liquid volume	[l]
V_{Mn}:	mole volume under normalized conditions	[l mol^{-1}]
x:	membrane coordinate	[m]
x_{IG}:	mole fraction of component I in the gas phase	[-]
z:	normalized membrane coordinate	[-]
α:	abbreviation parameter	[-]
β:	aeration rate ratio	[-]
τ:	shift experiment process time	[h]

Indices:

a:	outer diameter
AIR:	air
cal:	pO_2 calibration conditions
crit:	critical oxygen values
C, CO2:	carbon dioxide
E:	gas phase entrance
G:	subsystem gas phase
i:	inner diameter
I:	component I, I = C, N, O
J:	subsystem J, J = G, L, M
K:	individual aerated gas K, K = AIR, O2, N2
L:	subsystem liquid phase
max:	theoretical maximum value
M:	subsystem membrane
n:	normalized gas conditions
N,N2:	nitrogen
opt:	target value
O, O2:	oxygen
w:	set point value
X:	biomass
*:	equilibrium value
', '':	boundary layer value
—:	average value

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