Title: A Simplified Implementation of the Stationary Liquid Mass Balance Method for On-line O.U.R Monitoring in Animal Cell Cultures

Short Title: Simplified OUR on-line monitoring based on stationary liquid mass balance

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ABSTRACT

BACKGROUND:

Compared to other methods, the stationary liquid mass balance method for O.U.R. determination offers advantages in terms of estimation accuracy and reduction of stress. However, the need for sophisticated instrumentation, like mass flow controllers and gas analysers, has historically limited a wider implementation of such method.

In this paper, a new simplified method based on inexpensive valves for the continuous estimation of O.U.R. in animal cell cultures is evaluated. The determination of O.U.R. values is based on the accurate operation of the D.O. control loop and monitoring of its internal variables.

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RESULTS

The method developed was empirically tested in 2-L bioreactor HEK293 batch cultures. O.U.R. profiles obtained by dynamic method, global mass balance method and the developed simplified method were monitored and compared. The results show how O.U.R. profile obtained with the proposed method better follows the off-line cell density determination. The O.U.R. estimation frequency was also increased, improving the method capabilities and applications.

The method's theoretical rationale was extended to the sensitivity analysis which was analytically and numerically approached.

CONCLUSIONS:

The results demonstrated to be not only a cheap method, but also a reliable alternative to monitor the metabolic activity in bioreactors in many biotechnological processes, being a useful tool for high cell density culture strategies implementation based on O.U.R. monitoring.

KEYWORDS OUR, on-line monitoring, Dissolved Oxygen control, Liquid mass balance

NOMENCLATURE

- O.U.R.: Oxygen Uptake Rate [$mol \cdot l^{-1} \cdot h^{-1}$]
- $k_L a$: Volumetric mass transfer coefficient [h^{-1}]
- H: Henry's constant [$l \cdot atm \cdot mol^{-1}$]

- K: Oxygen's dissolution constant for a given culture medium and reference temperature [°K]
- T_o: Oxygen's dissolution constant reference temperature [°K]
- T: Culture temperature [°K]
- P: Bioreactor's gas phase absolute pressure [atm]
- P_{atm}: Atmospheric pressure [atm]
- P_{S1}, P_{S2}: Relative pressure for Air/O_2 and the N_2 supplies respectively. Where will typically be the same. $P_{S1} = P_{S2} = P_S$ [atm]
- R_{Inlet}, R_{Outlet}: Equivalent pneumatic resistance shown by the gas filters of the bioreactor's gas lines (inlet and outlet respectively). Same type of filters gives $R_{Inlet} = R_{Outlet}$ [atm·lpm^{-1}]
- χ_{O_2} : Gas phase oxygen composition [%]
- pO_2: Bioreactor's gas phase oxygen partial pressure [atm]
- D.O.: Relative dissolved oxygen typically referred with respect to the concentration in equilibrium with the gas phase [%]
- C_L: Absolute dissolved oxygen concentration [mol·l^{-1}]
- C_L^*: Absolute dissolved oxygen concentration in equilibrium with the gas phase [mol·l^{-1}]
- $\overline{C_L^*}$: Average absolute dissolved oxygen concentration in equilibrium with the gas phase [mol·l^{-1}]
- C_L^{SP}: Absolute dissolved oxygen set-point [mol·l^{-1}]
- ctn: Arbitrary constant value [mol·l^{-1}]
- C_o: Initial dissolved oxygen concentration [mol·l^{-1}]
- e : Error signal [mol·l^{-1}]

- qO_2: Specific oxygen consumption rate [mol·cell^{-1}·h^{-1}]
- x_o: Cell seeding density [cell·ml^{-1}]
- t_d: Cell duplication time [h]
- α: Valves control signal. Duty cycle $\in$ (0…100) [%]
- K_p: Proportional gain [l·mol^{-1}]
- K_i: Integration gain [l·mol^{-1}·h^{-1}]

K_d: Differential gain [l·h mol^{-1}]

O.P.C.: Open Platform Communications

O.L.E.: Object Linking and Embedding (for process control)

Slpm: Standard liters per minute

- **INTRODUCTION**

Big efforts have been invested in the development of high cell density culture strategies for animal cell culture processes[1]. Implementation of such strategies in bioreactors requires the use of suitable monitoring systems for automated control and process optimisation. Therefore, approaches based on simple measurements of primary variables using cheap technologies easy to implement are of great interest.

Oxygen is a key substrate in animal cell metabolism[2,3] and the monitoring of its consumption known as oxygen uptake rate (O.U.R.) is a straightforward way to estimate viable cell density[4-6]. In addition, O.U.R. correlates well with the physiological state of cells[7].

Three different methods for the determination of O.U.R. in animal cell cultivation have been developed[8] : The dynamic technique based on the periodic measurement of the D.O. extinction profile in the liquid phase, this technique can be considered as a golden standard due to its simplicity and constitutes the operation fundamentals of some respiratory monitoring system like RAMOS[9-11]; the global mass balance which consists on analysing the differential χ_{O_2} between the bioreactor's gas inlet and outlet whilst the D.O. concentration is kept constant; and thirdly, the stationary liquid mass balance which is based on measuring the χ_{O_2} in the bioreactor's gas phase whilst the D.O. concentration is kept constant by controlling the oxygen supply according to the cell's consumption needs.

Due to its simplicity of implementation, the dynamic technique is by far the most commonly used method. Nevertheless, when coming to animal cell culture, it shows two considerable disadvantages: The necessary variation on the D.O. concentration increases the shear stress caused by bioreactor aeration system, which may affect negatively the cell growth and viability[12-14]. Moreover, the dynamic technique may not be compatible with the current trends in biopharmaceutical G.M.P. (Good Manufacturing Processes)[15,16,27], since the cyclic changes on dissolved oxygen concentration (from > 60% to < 25%) and aeration rates needed for the dynamic technique implementation can have significant effects on product quality and potency, especially with respect to glycosylation, post-transcriptional modifications and impurity profiles. In addition, the time resolution provided by the dynamic technique is very poor, typically not higher than 1 sample per 1-2 hours, depending

on the cell concentration. Alternatively, gas phase global mass balancing has several advantages: is a fully non-invasive method, there is no need for knowing the k_La value and yields a higher time resolution, increasing the density of accurate data obtained. In any case, global mass balancing has not been widely used due to the need for complex and expensive instrumentation like mass spectrometers and extremely accurate D.O. control systems.

The recent introduction into the market of zirconium dioxide-based oxygen analyzers[17], which are less expensive but still offer good measurement accuracy, is propitiating a wider use of such method for bioprocesses monitoring. However, at low cell densities the analytical error of such oxygen analysers is too close to the range of measurements, being the determination of O.U.R. not accurate enough during the initial stages of cultures. The stationary liquid mass balance method offers minimum cell stress and good estimation accuracy, but still has need of significant investment in mass flow controllers, as well as additional instrumentation to measure the oxygen concentration in the bioreactor's gas phase[18].

The novelty of the hereby presented work relies on a simplified embodiment of a stationary liquid mass balance method for continuous O.U.R. on-line estimation. The outstanding advantages of the new approach described in this work are its simplicity and the low cost associated to the equipment needed. The O.U.R. estimation is based on inexpensive proportional valves and the observation of the control loop signals. In

this way, there is no need for additional χ_{O_2} measurement on the bioreactor's gas phase.

EXPERIMENTAL

Description of the test setup

A Biostat B-plus bioreactor (Sartorius-Stedim, Germany) was used as the basis for the test setup (Figure 1), where the native D.O. control system, based on ON-OFF valves, was bypassed by means of two external simple P.W.M. (Pulse Width Modulated) proportional valves (VSO-Low Flow from Parker, U.S.) to regulate the inlet flow of the supply gasses (Air/O_2, N_2). The gas mixing and conditioning was performed in a humidification bottle and the valves were actuated by means of a custom driver connected to a Microsoft Windows based computer through a RS-232 interface. The computer was in charge of reading the D.O. data acquired by the Biostat B-plus using an O.P.C. client (OLE for Process Control), running the D.O. control loop and solving the O.U.R. estimation algorithm. To that end, customized external control software used was developed using LabView (National Instruments, U.S.). An additional gas line for CO_2 was directly applied to the humidification bottle in order to compensate the high initial pH of the medium and was kept constant along the fermentation at a flow rate of 0.05 slpm. The total flow of the gas mixture in the inlet was 0.4 slpm and was also kept constant during the experiments.

Figure 1

The Sartorius Biostat B-plus is commanded by a D.C.U. (Digital Control Unit), and consists in a polyvalent bioreactor system intended to be used with a 2-liter stirred tank. The unit is an embedded solution including all the basic probes and actuators to carry out main cultivation strategies (Batch and Fed-Batch). The D.C.U. can be operated by means of the control software (MFCS/win) through a remote computer connected to the D.C.U. via Ethernet.

On-line data exchange is provided by means of an O.P.C. Server, a software module intended to allow communication between different applications. So D.O. data is transferred to the custom controller (D.O. Control & O.U.R.) which generates the control signal to command the Pulse Width Modulator to drive the proportional valves. The need for a constant gas flow through the bioreactor imposes that the addition of the duty cycles, independently applied to both valves, has to be constant too. The key factor for an adequate O.U.R estimation relies on proper characterization of the valves used, as well as the ability to correct their non-linearity.

Finally, a Blusens analyzer connected between the bioreactor's gas inlet and outlet was used for pseudo-continuous monitoring of the differential χ_{O_2} across the bioreactor. An additional set of switching valves was necessary to sequentially measure the inlet and the outlet flows. The acquisition of the Blusens' data, as well as the valves actuation and the Global mass balance calculation, were carried out by the D.C.U.

For the implementation of the dynamic method the same setup was used, but the proportional valves were actuated as ON-OFF valves for periodically flushing the

bioreactor's gas phase with N_2 to generate a D.O. extinction profile as explained by Lecina *et al*. (2006)[7].

Bioreactor setup. Bioreactor aeration control & Mass transfer

A 2-liter bioreactor vessel equipped with 2 pitch blade stirrers (Biostat B-plus Sartorius-Stedim, Germany) was used for all the different set up for O.U.R. monitoring. For all cases bioreactor's setting were as follows: stirring rate of 100 rpm, a temperature of 37 ºC, a pH of 7.1 and an aeration rate of 0.35 slpm, with the exception of the gas mixing method (described in figure 1) used for D.O. control, which was involved in the estimation of O.U.R. values. Bioreactors were seeded at 0.2-0.3×10^6 cells·mL^{-1}. A CO_2 inlet gas, 1 M of HCl buffer (Sigma, USA) and 1 M of NaOH (Sigma, USA) buffer were used for pH control.

Moreover, in order to maintain the integrity of cell's membrane and to reduce the effects of shear stress, non-ionic surfactant Pluronic F-68 (Sigma, USA) was added to the culture medium at 0.2% (w/v). The k_La value was determined by means of the Van Riet's gassing out method and it was found to be 6.53 h^{-1} for PBS at 37 °C and 100 rpm.

It is important to emphasize the fact that the k_La value is susceptible of drifting along the experiment period, thus leading to an error on the O.U.R. estimation. Hence, it becomes of significant importance to previously measure the k_La value under realistic and representative conditions of those of the later experiment.

Method description and modelization

The proposed method was inspired by the O.U.R. estimation strategies applied in the monitoring of activated sludge reactors for environmental purposes. Where fast switching On-Off valves are P.W.M. commanded for pseudo-continuous O.U.R. estimation[20-22]. In our case, the gas phase oxygen molar fraction is regulated by means of two independent gas supplies (Oxygen and Nitrogen). Such regulation is performed by two P.W.M. driven valves allowing an accurate control of the D.O. concentration. Additionally, unlike for the dynamic method, O.U.R. will be estimated without generating a glimpse of cellular distress by simply observing the control loop internal signals.

Figure 2 shows the gas mixing station scheme, where the D.O. concentration in equilibrium with the gas phase is given by the Henry's law and the Air/O_2 flow through the corresponding valve is considered proportional with respect to the control signal's duty cycle α. Then, the averaged D.O. concentration in equilibrium with the gas phase can be written as shown by equation (*i*). H: Henry's constant [l·atm·mol^{-1}], P_{atm}: Atmospheric pressure [atm], P_{S1}, P_{S2}: Relative pressure for Air/O_2 and the N_2 supplies respectively [atm], R_{Inlet}, R_{Outlet}: Equivalent pneumatic resistance shown by the gas filters of the bioreactor's gas lines [atm·lpm^{-1}], χ_{O_2} : Gas phase oxygen composition[%], pO_2: Bioreactor's gas phase oxygen partial pressure [atm], $\overline{C_L^*}$: Average absolute dissolved oxygen concentration in equilibrium with the gas phase [mol·l^{-1}], α: Valves control signal. Duty cycle $\in (0 \ldots 100)$ [%].

$$\overline{C_L^*} = \frac{pO_2}{H}\Big|_{\substack{P_{S1}=P_{S2}=P_S \\ R_{Inlet}=R_{Outlet}}} = \frac{\left(\alpha \cdot \frac{P_S}{2} + P_{atm}\right) \cdot \chi_{O_2}/100}{H} \quad (i)$$

Figure 2

- As for any of the previously reported methods, O.U.R. estimation will also be derived from the mass balance, in this case over the liquid phase. If appropriate operating conditions are applied, the shown above control topology will behave as a causal linear and time invariant system, meaning that the mass balance equation allows to be written as a function of the duty cycle applied to the proportional valves, see equation (*ii*). O.U.R.: Oxygen Uptake Rate [mol·l^{-1}·h^{-1}], k_La: Volumetric mass transfer coefficient [h^{-1}], C_L: Absolute dissolved oxygen concentration [mol·l^{-1}], C_L^*: Absolute dissolved oxygen concentration in equilibrium with the gas phase [mol·l^{-1}], α: Valves control signal. Duty cycle $\in$ (0…100) [%].

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$$\frac{dC_L(t)}{dt} = k_L a \cdot \left(\alpha \cdot C_L^* - C_L(t)\right) - O.U.R. \quad (ii)$$

Equation (*ii*) matches the form of a first order linear differential equation that can be easily solved. In this case, O.U.R. was replaced by a simple exponential growing model like the one shown by equation (*iii*). qO_2: Specific oxygen consumption rate [mol·cell^{-1}·h^{-1}], x_o: Cell seeding density [cell·ml^{-1}], t_d: Cell duplication time [h]

$$O.U.R. = qO_2 \cdot x_0 \cdot e^{\frac{ln2}{t_d} \cdot t} \quad (iii)$$

- The solution to the linear differential equation (*ii*) is given by (*iv*). C_o: Initial dissolved oxygen concentration [mol·l^{-1}]

$$C_L(t) = \alpha \cdot C_L^*\left(1 - e^{-k_l a \cdot t}\right) + C_o \cdot e^{-k_l a \cdot t} + \frac{qO_2 \cdot x_o}{\frac{ln2}{t_d} + k_l \cdot a}\left(e^{-k_l a \cdot t} - e^{\frac{ln2}{t_d} t}\right) \quad (iv)$$

Hence, the corresponding transfer function can be expressed and solved by means of the Laplace transformation:

$$C_L(s) = L\{C_L(t)\} = \frac{\alpha \cdot C_L^*}{s(s + k_l a)} + \frac{C_o}{s + k_l a} - \frac{qO_2 \cdot x_o}{(s + k_l a) \cdot \left(s - \frac{ln2}{t_d}\right)} \quad (v)$$

Equations (*iv*) and (*v*) clearly show that the open loop transfer function is only dependent on the bioreactor's mass transfer capability; "disturbances" are also introduced by the evolution of the metabolic activity and the D.O. initial conditions. The control theory approach offers a useful way to write the mass balance equations taking into consideration the control loop external components. Therefore, if a constant value of the set point is considered, it can be easily demonstrated that the control loop's differential error equals the dissolved oxygen accumulation term. This is shown by equation (*vi*). Thus, if the accumulation term in equation (*ii*) is also

replaced by the differential error, a new expression of the O.U.R. described in function of the control loop variables α and e is obtained. See equation (*vii*).

$$\frac{de}{dt} = \frac{dC_L^{sp}}{dt} - \frac{dC_L}{dt}\bigg|_{C_L^{sp}=ctn} \Rightarrow \frac{de}{dt} = -\frac{dC_L}{dt} \quad (vi)$$

$$O.U.R. = k_L a \cdot \left(\alpha \cdot C_L^* - C_L\right) + \frac{de}{dt} \quad (vii)$$

Since the aim of the current approach is not just to estimate the oxygen consumption, but to keep a constant level of D.O., it will be necessary to match a good compromise between the control loop performance and the sensitivity of the control signal α with respect to the biological "disturbance". In other words, the control loop parameters need to be chosen according to a given transient response and stationary error. To that end, several simulations based on classical P-PI-PID control strategies were carried out. The control parameters were chosen in order to emphasize the effect of the oxygen consumption on the performance of each control strategy for an arbitrary cell line (data not shown). On one hand, it was found that the three control methods provided a good enough long term estimation of the O.U.R. On the other hand, pretty different stationary errors were observed. At a first glance, the logical control strategy selection was always PID. However, the presence of real unpredictable phenomena, such as variable inertia or unknown dead times, could easily produce unexpected behaviours, due to the action of the differential term, something highly undesirable due to the cells sensitivity to D.O. changes. Therefore, PI controller was found as the control strategy offering the best trade-off.

Under the stationary regime, when the control loop's error signal dwindles around zero, the different error sources can then be considered as independent, and the O.U.R. estimation error can be analytically approached by a Taylor's first order development, which its relative form follows the expression:

$$\frac{\Delta O.U.R.}{O.U.R.} = \sum_{i=0}^{n} \frac{\partial\left(ln(O.U.R.)\right)}{\partial x_i} \cdot \partial x_i = \sum_{i=0}^{n} \frac{\partial(O.U.R.)}{\partial x_i} \cdot \partial x_i \quad (viii)$$

As the control signal α is the outcome of a PID and the dissolved oxygen concentration in equilibrium with the gas phase is dependent on the Henry's law, the O.U.R. expression (*vii*) needs to be expanded considering the tuning parameters as well as the physical variables affecting the oxygen's diffusion through the medium:

$$O.U.R. = k_L a \cdot \left[\frac{P \cdot \chi_{O_2}}{H_0 \cdot e^{-K\left(1/T - 1/T_0\right)}} \cdot \left(K_P + K_i \cdot \int_0^t e \cdot d\tau + K_d \cdot \frac{de}{dt} \right) - C_L \right] + \frac{de}{dt} \quad (ix)$$

The sensitivity analysis was carried out considering only the most relevant sources of error $K_L a$ and C_L. Remaining variables were assumed to be constant or of an insignificant contribution. Subsequently, the overall uncertainty is given by the Taylor's first order development:

$$\frac{\Delta O.U.R.}{O.U.R.} = \frac{\partial O.U.R.}{\partial k_L a} \cdot \partial k_L + \frac{\partial O.U.R.}{\partial C_L} \cdot \partial C_L \ (x)$$

The first partial derivative is trivial and can easily be solved. However, $\partial O.U.R./\partial C_L$ which is the most interesting provided that offers the sensitivity with respect to the error related to the dissolved oxygen measurement, can only be solved by considering C_L time independent and applying the Schwarz theorem. The corresponding solutions are expressed as follows:

$$\frac{\partial O.U.R.}{\partial k_L a} = \frac{P \cdot \chi_{O_2}}{H_0 \cdot e^{-K\left(1/T - 1/T_0\right)}} \cdot \left[K_P \cdot \left(C_L^{sp} - C_L\right) + K_i \cdot \left(C_L^{sp} \cdot t - \int_0^t C_L \cdot d\tau \right) - \mathrm{K_d} \cdot \frac{d\mathrm{C_L}}{dt} \right] - \mathrm{C_L} \quad (xi)$$

$$\frac{\partial O.U.R.}{\partial C_L} = -k_L a \cdot \left[\frac{P \cdot \chi_{O_2}}{H_0 \cdot e^{-K\left(1/T - 1/T_0\right)}} \left(K_P + K_I \cdot t\right) + 1 \right] \quad (xii)$$

A straightforward interpretation of the equations above is not advisable due to the assumptions taken, especially for $\partial O.U.R./\partial C_L$, where their dependency with respect to the control loop action and the cell culture dynamics itself are not considered. Therefore, a local/derivative based sensitivity analysis was carried out applying the one-factor-at-a-time method. In order to avoid unrealistic results on the contribution of each error source, some previous considerations about the nature of the errors were taken into account. First, a major difference between $k_L a$ and C_L shall be noticed. Volumetric mass transfer can slowly evolve along the culture time due to the interaction of the cell concentration with the culture medium. C_L however, should show a faster fluctuation around a given set point adhering to some statistical distribution in addition to a bias error directly given by the instrument's accuracy.

Consequently, to feed the analysis, an exponential evolution of up to -10 % was assumed for k_La and a ±1 % constant measurement off-set was modeled for C_L. Since the numerical analysis was just focused on the assessment of the mean values no probability distribution function was considered for C_L.

The simulation was carried out for arbitrary but realistic animal cell line species, featured by a specific oxygen consumption of 0.5 pmol·cell^{-1}·h^{-1}, 24h of duplication time and initial cell concentration of 0.35×10^6 cell·ml^{-1}. The bioreactor was assumed to offer a nominal volumetric mass transfer of 40 h^{-1} under 21 % oxygen molar fraction aeration, at 1.1 atm of absolute pressure and 30 % D.O. set point. Finally, medium's D.O. saturation was calculated for an aqueous solution at 37°C assuming water equivalent oxygen solubility.

Figure 3 shows the 3-D profile for O.U.R. estimation uncertainties along the culture time and depending on both k_La. and C_L errors. It can be seen how the O.U.R. uncertainty approximately equals k_La uncertainty as cell density increases. Interestingly, O.U.R. estimation error tends to become less and less significant as the oxygen demands increases. Nevertheless, there is a clear limit related to the D.O. control settling time: a much higher uncertainties estimation than 10 % could be expected if O.U.R. is attempted to be estimated during the transient period. In other words, as far the control loop reaches the steady state and the D.O. concentration approaches the set-point, the O.U.R. estimation uncertainty approximately equals the relative drift experienced by the k_La along the culture time.

Figure 3

Cell line and culturing medium

The HEK293SF-3F6 cell line was kindly provided by Dr. A. Kamen (National Research Council of Canada). Cells were passaged at 2×10^5 cell/mL three times a week as previously reported[22]. Cell maintenance was performed in 125mL polycarbonate shake flasks (Corning Inc.) with 20mL of culture, and maintained at 37°C in a humidified atmosphere within a 5% CO_2 incubator (Steri-cult 2000 Incubator, Forma Scientific). Flasks were continuously agitated at 110 rpm on an orbital shaking platform (Stuart SSL110). Culture medium consisted in SFMTransFx-293 (HyClone, Thermo Scientific) supplemented with 4mM GlutaMAX (Gibco, Invitrogen), 5% FBS (v/v) (Sigma Aldrich) and 10% of Cell Boost 5 (80 g/L) (HyClone, Thermo Scientific).

Cell growth assessment and metabolite analysis

Cell number was determined by manual counting using a Neubauer hemocytometer and a phase contrast microscope (Nikon eclypse, TS100). Viability was assessed using the Trypan blue dye exclusion method. Glucose and lactate concentrations were measured using an automatic glucose and lactate analyzer (YSI, Yellow Springs Instrument, 2700 Select).

RESULTS

Three different methods for the on-line determination of O.U.R. in HEK293 cultures were implemented in the 2-liter bioreactor in order to compare the performance of such methods and how their implementation may affect other culturing parameters, as detailed in the Materials and Methods section.

In general terms, the different techniques for the determination of O.U.R. had no influence on cell culture development, as it was expected (Figure 4). In all cases, cell density reached values about 13-15×10^6 cells·ml^{-1} after 192-216 hours of culture. Those little differences may be related to little differences on inoculum cell densities, and are normally observed in cell cultures. Also, no significant differences in glucose and lactate concentration profiles were observed. Glucose was only exhausted after the exponential growth phase when the stationary liquid mass balance method was applied (Figure 4 C) while was even not depleted at all when the dynamic technique and gas analysers were used for O.U.R. determination (Figure 4 A and B). Those differences were not reflected on the cell density profiles, but in the lactate generation and accumulation. Differences in the maximum lactate concentration, about 2-fold increase, are consistent with the observation of different glucose consumption profiles while cell density reached was similar. It is relevant to state that the lactate concentration reached is not detrimental for cell growth in any case (other cultures reached concentration over 25mM without affecting cell growth, data not shown). This observation denotes that the aeration strategies used when implementing the different O.U.R. determination method has an effect on cell physiology and metabolism. The culturing conditions (pO_2 and pH) were kept nicely constant when the simplified method was used, but pO_2 and pH were affected when the dynamic technique was applied (depicted in Figure 5). Dynamic Technique

generates a pO2 fluctuation from 60% to 25% for each measurement, and during the analysis pH control is switched off. Since HEK293 metabolism are sensitive to lactate concentration and pH (and probably to other parameters such as pO_2 and shear stress), a change on metabolism[26] (from glucose consumption to co-consumption of glucose and lactate) was observed when culturing conditions were affected by the O.U.R. determination technique. In our hands we have recently observed differences in metabolism and in the total cell density reached (about 20%) when fed-batch cultures were performed using different monitoring systems. Again when the dynamic technique was implemented the fed-batch culture performance was negatively affected (metabolism altered) even the glucose set point control was kept therein the range set. Differently, when fed-batch nutrient feeding was commanded by the alkali buffer addition, those limitations were not observed (Unpublished data).

Regarding O.U.R. measurements, again similar profiles were obtained, except for O.U.R. calculated using the global mass balance by means of the Bluesens gas analyzer, that shows and erratic and a high dispersion in the measurements. As can be seen in the figure for the OUR calculated from the dynamic method and the stationary liquid mass balance using the valves controllers, only small differences were observed on the time in which the maximum was reached, what was in good concordance with the differences observed on cell density profiles as well. In those cases, in which the maximum cell density was reached 24 hours earlier, the maximum of O.U.R. profile was also accordingly anticipated. As it can be seen in Figure 4, cell density measurements and O.U.R. estimation showed parallel profiles for the dynamic and the stationary liquid mass balance methods. In two of three cases, a very short lag

phase (which corresponds to an adaptation time for cells to growth) was detected in both O.U.R. and cell density profiles during the first 24 hours post-inoculation. Afterwards cell density and O.U.R. profiles showed exponential curves, which corresponds to the exponential growth phase of cultures.

Interestingly, the end of the exponential growth phase was clearly detected by O.U.R. measurements, corresponding to the maximum of the O.U.R. profile, although the maximal cell density was reached about 48 hours later. Therefore, O.U.R. monitoring allowed detecting on-line the middle of the exponential cell growth reflected in the inflexion point in the O.U.R. curve. It is assumed that the inflexion point of the exponential growth phase corresponds to a suitable time to start feeding strategies in order to achieve high cell density cultures[7].

In the two cases where O.U.R. profile follow the cell growth, the maximum O.U.R. values were comprised therein the range about $1\text{-}1.25\times10^{-3}$ $mol\cdot l^{-1}\cdot h^{-1}$. When comparing O.U.R. profiles obtained by means of the different methods, the global mass balance using gas analyzers shows an erratic and a high dispersion in the measurements. The dynamic method, as has been mentioned before, showed an important measurement dispersion, whereas the O.U.R. determination by means of the stationary liquid mass balance using the valves control signals was more accurate.

Figure 4

The simplified implementation of O.U.R. estimation by stationary liquid mass balance using D.O. control loop signals showed an additional advantage in comparison to the dynamic method in terms of D.O. and pH control. Intrinsically to the dynamic method, the D.O. has to be risen up over 60% of saturation, and then the gas mixture inlet is discontinued including the CO_2 used for pH control. Under these conditions, D.O. and pH profiles showed higher oscillations as it can be seen in Figure 5. Values from 25 to 80% for D.O. and from 7 to 7.2 on the pH were observed (grey lines) when the dynamic method was implemented, whereas such parameters were almost constant for the stationary liquid mass balance method (black lines). The D.O. control for the latter strategy needed about 10 hours in order to reach the desired set point (50% of air saturation), which could be considered as the control stabilization time, but once the set point was reached it was kept constant along the fermentation (only 1-2 % of stationary error was observed). The peak of D.O. observed at 96 hours of culture corresponds to the replacement of the air flow for pure oxygen, so that the control needed a few hours to adapt the response to the gas composition change, and D.O, was stabilized again after few hours. Altogether made the D.O. and pH controls used for stationary mass liquid balance more suitable for those processes in which D.O. and pH disturbances should be avoided.

Figure 5

Eventually, O.U.R. values determined by means of the two successful methodologies were represented *versus* viable cell density obtained from the profile fitted to the experimental values (Figure 6). As it can be seen, both profiles were quite similar,

meaning that any of both methods can be used for the determination of O.U.R.. However, the profiles obtained using the dynamic method showed higher point dispersion due to the intrinsic estimation error of the method. On the contrary, the profile obtained with the stationary liquid mass balance was smoother and showed fewer transients, and therefore it would be a more suitable method to be used in future culture strategies based on OUR estimation.

Additionally, the specific oxygen consumption rates (qO_2) for HEK293 cells were evaluated from the linear part of the profiles, as the slope of the linear regressions. The estimated qO_2 values are compiled in the table endorsed to Figure 6. For the two different methods used for the evaluation of O.U.R. values, the estimated qO_2were of the same range to previously published values for HEK293 cells[16] (120-130 nmols O_2/ 10^6 cells h) indicating that all methods were suitable for the determination of O.U.R. Again, the coefficient of determination (R^2) obtained from the linear regression analysis for each data set, shows that the lowest variance between the experimental data and the linear adjustment for the data obtained with the stationary liquid mass balance method with a R^2 about 0.997 in comparison to the dynamic method (0.957).

Figure 6

DISCUSSION

A simplified stationary liquid mass balance method for the O.U.R. estimation has been proposed, implemented and tested, based on the knowledge of the D.O. control loop

variables and the volumetric mass transfer coefficient. The feasibility of the method was demonstrated for animal cells in a bench-scale bioreactor. Regarding the method's accuracy, any standard procedure to mimic a known profile of oxygen consumption has not been reported yet, unfortunately. Therefore, it was not possible to provide a measured value on the estimation error. Nevertheless, the method was empirically and successfully compared with the dynamic and the global mass balance methods, obtaining analogous results using the dynamic method, but clearly much less dispersed. Additionally, the sensitivity and uncertainty analysis were analytically and numerically approached. Ending conclusion is that as far the D.O. measurement error can be kept below reasonable limits (<5 %) and despite that during the D.O. settling time the O.U.R. estimation cannot be considered valid, once the set point is reached, the overall uncertainty trends to be mostly related just to the k_La drift as it happens for the dynamic method and the stationary liquid mass balance[23]. Interestingly, the simplified stationary liquid mass balance method offers outstanding advantages: 1) is the cheapest method tested in this work, 2) higher measurements frequency can be performed compared to others methods, 3) D.O. can stably been kept therein a narrow range and 4) eradicates the cell stress produced by gas flow pulses variations.

With the aid of this method it was possible to find a correlation between the O.U.R., the cell concentration[24] and the glucose consumption during the first phase of the culture. In a second phase, in which HEK293 were able to metabolize lactate as other human cell lines[25], a change in the O.U.R. profile, characterized by a decrease in the specific oxygen consumption rate was detected. Additionally, the off-line data available permitted a crucial observation, the fact that the turning point in the O.U.R.

graphs allowed anticipating the time of maximum viable cell concentration (≈48 hours before). The ability of HEK293 cells to consume endogenous lactate and glucose[26] opens up the possibility of defining different culture strategies (i.e. diauxic strategies). More interesting was that the metabolic change, from a phase in which glucose was consumed as a single carbon source to an other phase in which glucose and lactate were simultaneously consumed, was also reflected in the O.U.R. values obtained by the monitoring methods.

CONCLUSION

The feasibility of a new method based on inexpensive valves for the continuous estimation of O.U.R. in animal cell cultures has been demonstrated. The performance of said method (Simplified implementation of the stationary liquid mass balance) has been compared with respect to the Global mass balance and Dynamic methods. The proposed method showed obvious advantages in terms of time resolution, D.O. stability (lack of cell stress) and cost. Additionally, the results obtained could be applied to the optimization of high cell density culture strategies like fed-batch or continuous perfusion cultivation, in which the proposed method can be applied.

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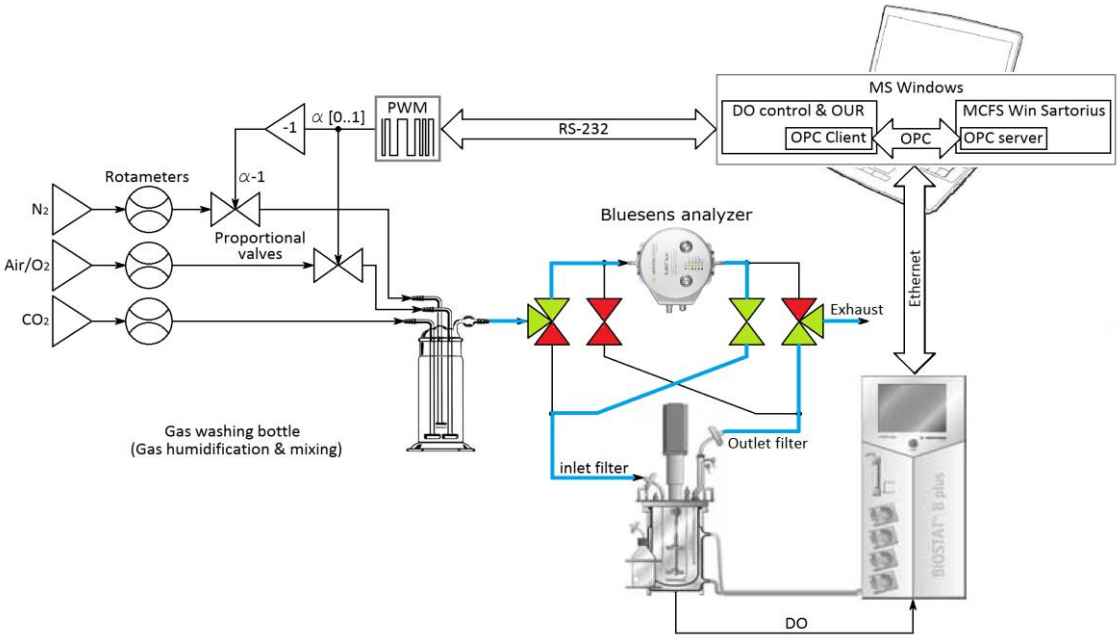


Figure 1

Block diagram of the test setup used for pO_2 control and O.U.R. on-line monitoring. In blue is also depicted the setup for counter measurement of O.U.R. using a gas analyzer (Global mass balance method)

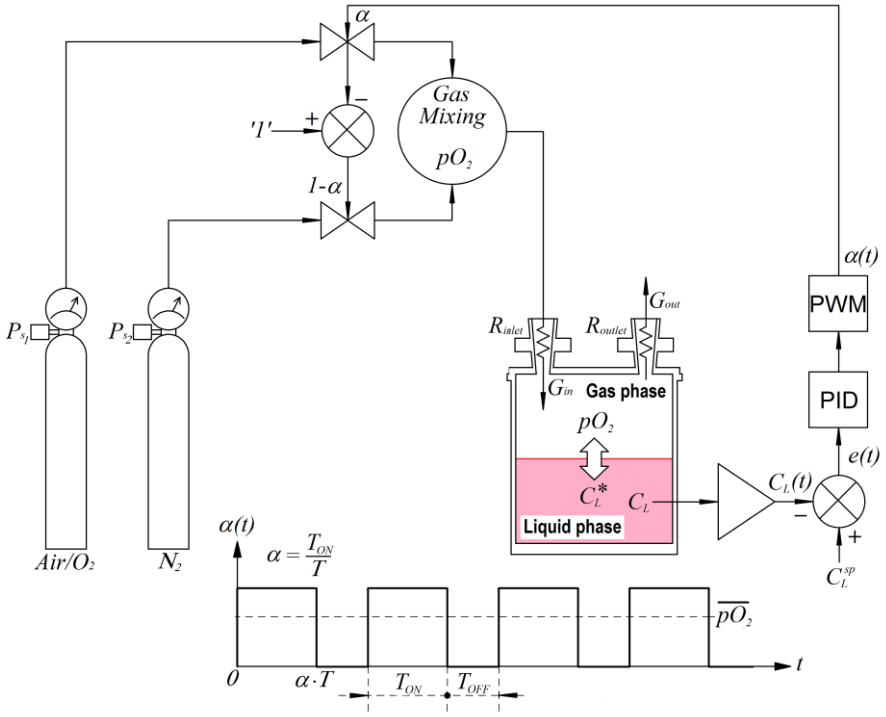


Figure 2

PID D.O. control loop implementation based on the PWM action of complementary proportional valves

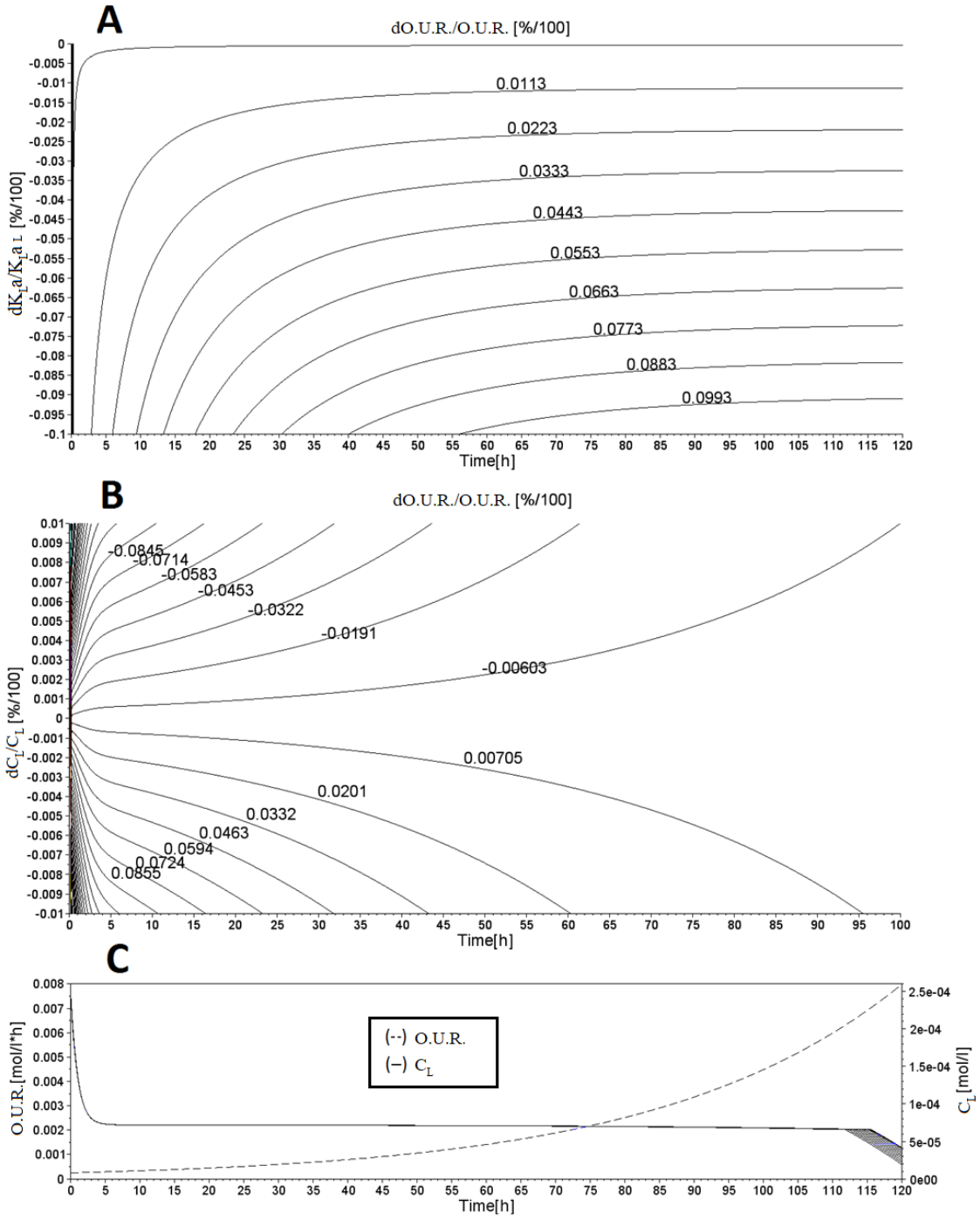


Figure 3

Analysis of O.U.R estimation uncertainty with respect k_La (A) and C_L (B) errors along the culture time. (C) O.U.R. reference profile & D.O. control performance

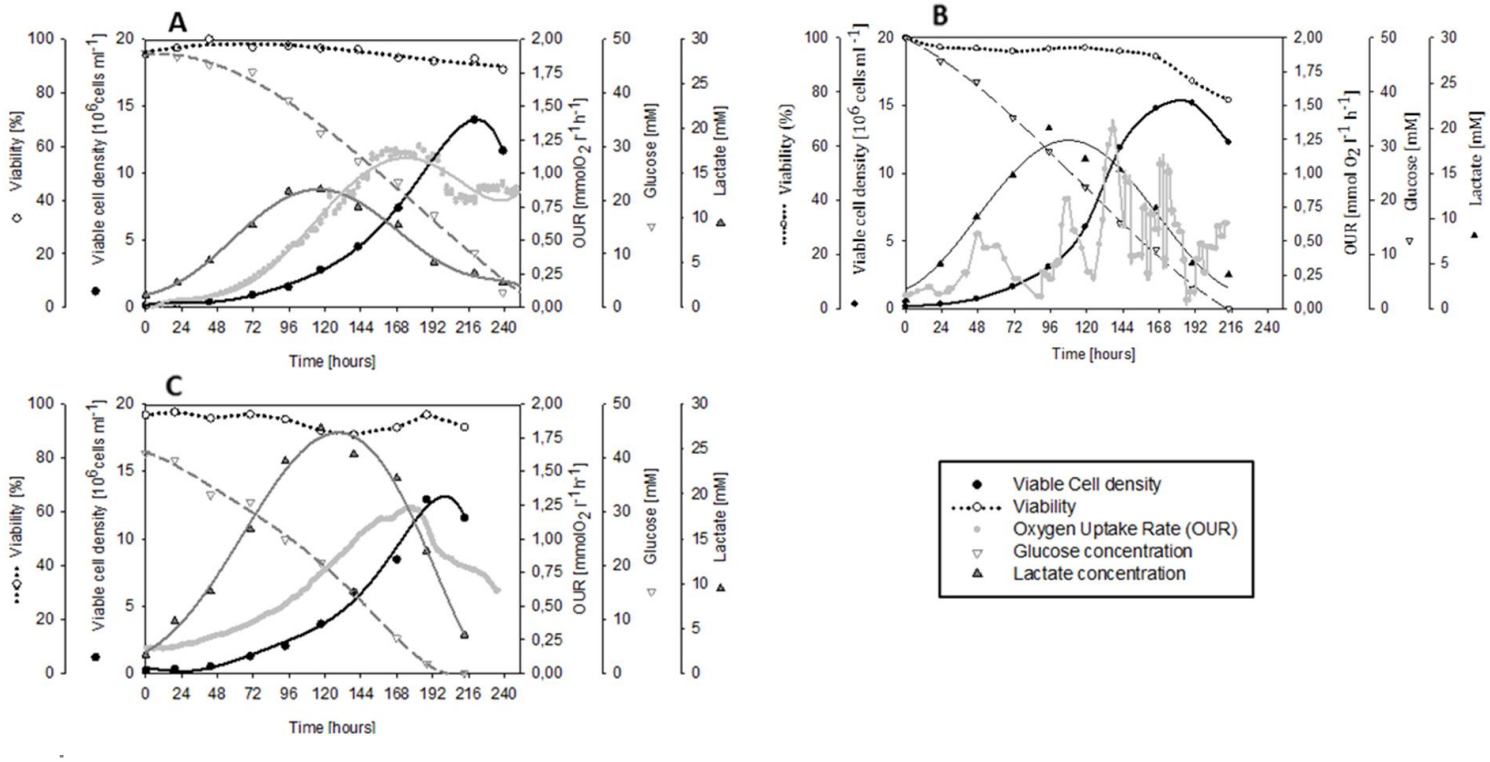


Figure 4

Comparison of the different O.U.R. estimation methods implemented in 2-liter bioreactor: A) Dynamic method, B) Global Mass Balance by means of gas analyzers and C) Simplified stationary Liquid Mass Balance

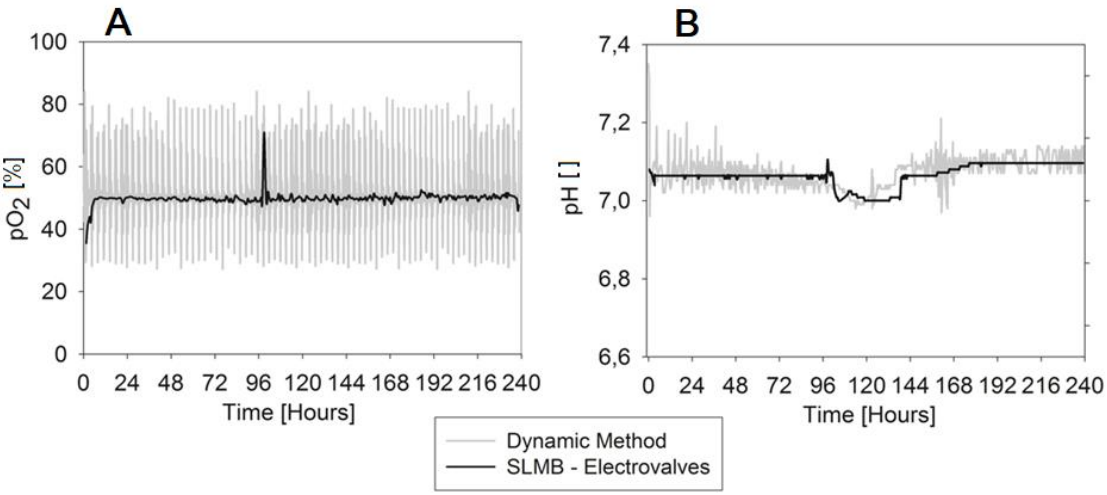
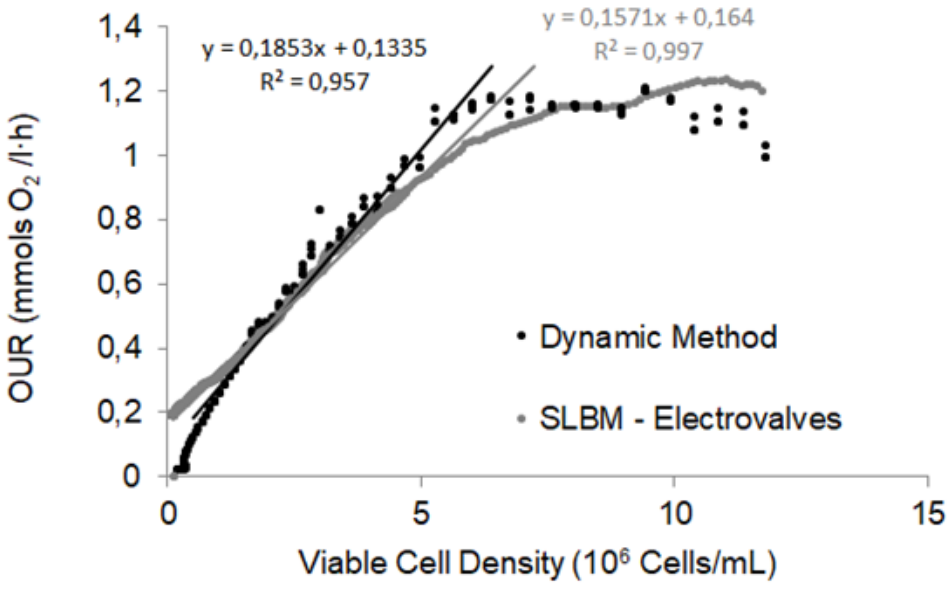


Figure 5

Effect of the method for O.U.R. estimation implemented on the main culture parameters. Comparison of (A) D.O. and (B) pH profiles obtained from batch cultures in which Dynamic method (grey line) and Simplified Liquid mass balance (black line) were implemented.



Monitoring System	qO_2 (mmols /10^9 cells ·h)	R^2
Dynamic Method	0,1853	0,957
SLMB - Electrovalves	0,1571	0,997

Figure 6

Comparison of the calculated specific oxygen consumption rate by correlating Viable Cell Density and O.U.R. profiles obtained by the different estimation methods