ADCHEM 2006

International Symposium on Advanced Control of Chemical Processes
Gramado, Brazil – April 2-5, 2006

MONITORING THE PHYSIOLOGICAL STATE OF MAMMALIAN CELL PERFUSION PROCESSES BY ON-LINE ESTIMATION OF INTRACELLULAR FLUXES

O. Henry[1,2], A. Kamen[2], M. Perrier[1]

[1]École Polytechnique de Montréal, Montréal, Québec, Canada.
[2]Institut de Recherche en Biotechnologie, CNRC, 6100 avenue Royalmount, Montréal, Québec H4P 2R2, Canada
**Email of corresponding author: michel.Perrier@polymtl.ca*

Abstract: Rapid assessment of the cells physiological state during a culture is essential for bioprocess optimization and the design of effective control strategies. In this work, an approach was developed to provide an online estimation of the intracellular flux distribution of cells grown in perfusion cultures, based on a comprehensive metabolic network involving 40 biochemical reactions and 46 components. The specific uptake and production rates were evaluated from daily nutrient and metabolite concentration measurements, using an extended Kalman-filter for noise reduction and rate estimation. The biosynthetic rates were determined from an on-line estimate of the specific growth rate using a recursive least-squares method. Our results demonstrate that this approach allows monitoring of the cells metabolic activity and can be a useful tool for process development.

Keywords : monitoring, bioprocess, perfusion culture, metabolic flux analysis

1. INTRODUCTION

The design of efficient monitoring techniques and control strategies is instrumental for the rapid development and optimization of bioprocesses. For these methods to be truly effective in detecting the cellular response to environmental changes or operating conditions, they must be based on a profound knowledge of the cell metabolism. With proper assessment of the physiological state of the cells, the optimization can consist in selecting the appropriate operating conditions so as to maintain or drive the culture towards a desirable productive state (Konstantinov, 1996).

To date, most of the on-line monitoring tools derive knowledge on the physiological state indirectly, from extracellular measurements. Cell concentration and respiration are the parameters commonly measured on-line to monitor the metabolic activity of a culture. Flow injection analysis techniques have also allowed real-time monitoring of various nutrients and metabolites concentrations, such as glucose, glutamine, lactate and ammonia (Blankenstein, *et al.*, 1994; Siegwart, *et al.*, 1999).

However useful for monitoring a production, these process variables only give an incomplete estimate of the metabolic state of the cells. To complement the information of extracellular measurements, intracellular data must be considered as well. This constitute a challenging task, hampered by the lack of available measurements and the limited applicability of mathematical models, in turn due to the complexity of animal cell systems.

Advances in the development and application of techniques such as NMR, mass spectroscopy and radioactive tracers have permitted the development of more accurate metabolic model. Metabolic flux analysis is an approach that allows to take into account the intracellular reaction rates and has become an established tool to study the metabolism of cells. However, to date, metabolic flux analysis applied to animal systems was mostly confined to a descriptive tool, for *a posteriori* assessment of cellular response to culture changes. This can be mainly attributed to the large number of measurements required to carry the analysis (nutrients, metabolite products, cellular composition, etc).

In the present work, an approach was developed to provide daily estimates of the intracellular fluxes, based on on-line and off-line measurements and assuming a constant cellular composition. To illustrate this approach, the time courses of metabolic fluxes in two perfusion cultures performed under different feeding strategies were analyzed. Our results demonstrate intimate links between the intracellular flux distribution, culture productivity and the operating conditions. By allowing to monitor the physiological status of a culture, this approach can provide a systematic tool for process optimization and control.

2. MATERIALS AND METHODS

2.1 Cell Line, Bioreactor System and Culture Conditions

Experiments were conducted using the HEK-293 cells and a low-protein serum-free proprietary medium formulation. The cultures were performed in a controlled 3.5 L bioreactor equipped with 3 surface baffles and two marine impellers. The temperature was maintained at 37 °C and the pH was controlled at 7.2. The agitation was kept constant at 80 RPM and the dissolved oxygen was maintained at 40 % air saturation. Cells were retained in the reactor using a BioSep 10L acoustic filter (Applikon Inc., Foster City, Ca). The feed and harvest rates were controlled by two peristaltic pumps.

2.2 Analytical Methods

Viable and total cells were counted using a haemacytometer (Hausser Scientific, Horshaw, PA). Viability was assessed by dye exclusion method using erythrosine B. The Biolyzer (Kodak, New Haven, Connecticut) was used for the analysis of glucose, lactate and ammonia. Amino acid analysis was performed by HPLC. DNA analysis and dry weight estimation have been performed as described in Nadeau et al. (Nadeau*, et al.*, 2002). Extracellular proteins were assayed using the Dc Protein Assay (Bio-Rad, Hercules, CA) and total proteins were analysed using the BCA kit (Pierce, Rockford, IL).

2.3 Estimation of the Specific Growth Rate

An estimation of the growth rate is required to evaluate the biosynthetic rates. From a mass balance on viable cells, we obtain the following equation for a bioreactor operated in perfusion mode:

$$\mu = \frac{1}{X}\left(\frac{dX}{dt} + DX_H\right) \quad (1)$$

where D is the perfusion rate, X the concentration inside the bioreactor and X_H the concentration of cells in the outflow. In perfusion cultures, growth rate determination is particularly subject to noise due to the imprecision of cell counting and fluctuations in cell retention efficiency. This was addressed by using a biomass probe allowing real-time monitoring of the culture capacitance. The later is proportional to the concentration of viable cells. Based on this measurements, on-line estimation of the specific growth rate was performed using a discrete least-square estimator as described in (Bastin and Dochain, 1990). The algorithm is given by:

$$\hat{\mu}_t = \hat{\mu}_{t-1} + \Delta t \gamma_t X_{t-1}\left[X_t - X_{t-1} - \Delta t \hat{\mu}_{t-1} X_{t-1} + \Delta t D_{t-1} X_{Ht-1}\right] \quad (2)$$

where Δt is the sampling rate. The covariance (γ) is updated by the following equation:

$$\gamma_t = \frac{\gamma_{t-1}}{\lambda + T^2 X_{t-1}{}^2 \gamma_{t-1}} \quad (3)$$

The forgetting factor, λ, is a tuning parameter. The concentration of cells in the harvest (X_H) and the dilution rate (D) were re-evaluated at each sample and the values were assumed constant in between experimental measurements. The linear relation assumed between the capacitance signal and the cell concentration was also updated at each off-line measurement by linear regression on current and previous data points. This procedure allowed to re-calibrate on-line the biomass monitor system.

2.4 Nutrient uptake/Metabolite production rate estimation

In contrast with biomass, only few measurements of substrate and metabolite concentrations are available, typically once or twice a day. The specific uptake and production rates can be calculated using material balances around the bioreactor to yield the following equations:

$$\frac{dS}{dt} = -q_S X + D(S_{in} - S) \quad (4)$$

$$\frac{dP}{dt} = -q_P X + DP \quad (5)$$

where D is the perfusion rate, q_S and q_P are the specific consumption and production rates, S is the nutrient concentration in the reactor, S_{in} is the nutrient concentration in the feed and P is the metabolite concentration in the reactor.

Due to the large sampling rate, calculation using direct difference from current and past measurement is prone to error. Instead, an extended Kalman filter was employed for rate estimation and noise reduction. In the "continuous-discrete" form of the filter, the correction is made only at discrete times (t_k).

Prediction step:

$$\left.\begin{aligned} \frac{dS}{dt} &= qX + D(S_{in} - S) \\ \frac{dq}{dt} &= 0 \end{aligned}\right\} \; t_k \le t \le t_{k+1} \quad (6)$$

Whenever a new measurement is available, the predicted specific rate is corrected by a term proportional to the difference between the measured (S_M) and predicted (S) nutrient/product concentrations according to the following equation:

Correction step:

$$\begin{bmatrix} S \\ q \end{bmatrix}_F = \begin{bmatrix} S_M \\ q \end{bmatrix} + K(t)\left[S_M - \hat{S}\right] \quad (7)$$

where K is the filter gain optimized at each measurement time:

$$K = P(t)H^T(t)\left[H(t)P(t)H^T(t) + R\right]^{-1} \quad (8)$$

The error covariance is also updated according to:

$$P_F(t) = \left[I - K(t)H(t)\right]P(t) \quad (9)$$

The trade off between confidence in the measurements and confidence in the model is determined by the measurement error and the process model covariances (R and Q, respectively).

3. METABOLIC MODEL

The metabolic model used in this study was developed by Nadeau *et al.* (2002). The reactions considered in this biochemical network are shown in Table I. The metabolic model involves 46 components and 40 fluxes, which include the catabolism pathways of glucose, glutamine and 18 amino acids.

Evaluation of the metabolic flux vector F is performed using a weighted linear least-squares:

$$F = (M^T \psi^{-1} M) M^T \psi^{-1} q \quad (10)$$

where q is the vector of measured nutrient uptake and metabolite production rates, M contains the stoichiometric coefficients and ψ is the variance-covariance matrix associated with the measured rate.

Table I. Listing of biochemical reactions included in the metabolic network

1	Glc + ATP → Fruct6P
2	Fruct6P + ATP → 2 GAP
3	GAP → Pyr + 2 ATP + NADH + H^+
4	Pyr + NADH + H^+ → Lac
5	mPyr + CoA → ACCoA + NADH + CO_2 + H^+
6	Pyr + Glu → Ala + ∝KG
7	OAA + Glu → Asp + ∝KG
8	mGln + H_2O → mGlu + NH_3
9	2 Gln +5/4 Asp + Rib5P + ½ Gly + 27/4 ATP + 5/8 NADPH → ADNlARN + ¾ NADH + ¾ Fum + 2 Glu
10	mMal + 2ATP → mPyr + CO_2 + NADPH + H^+
11	Asp + Gln + ATP → Asn + Glu
12	Glu + ATP + 2 NADPH +2 H^+ → Pro
13	2 Gly → Ser + NH_3
14	G6P + ATP → 1/3 Rib5P + 2/3 Xu5P + 2 NADPH + H^+ + CO_2
15	R5P + 2 Xu5P → 2 Fruct6P + GAP
16	2 (9 Cit + 17 ATP + 9 CoA + 16 NADPH) → AcOLE + 18 OAA
17	Extracellular Proteins → ΣAA
18	ΣAA→ Intracellular Proteins
19	Lys + 2 m∝KG + NADPH → 2 AcCoA + 2 CO_2 + 4 NADH + 3 H^+ + $FADH_2$ + 2 mGlu
20	Leu + m∝KG + ATP → 3 AcCoA + mGlu + CO_2 + NADH + H^+ + $FADH_2$
21	Ile + m∝KG + ATP → SuCoA + AcCoA + CO2 + 2 NADH + 2 H^+ + $FADH_2$ + mGlu
22	Thr → CO_2 + 2 NADH + 2 H^+ + $FADH_2$ + NH_3 + mPyr
23	Val + m∝KG + ATP → SuCoA + 3 NADH + H^+ + $FADH_2$ + 2CO_2 + mGlu
24	Met + Ser + 2 ATP → Cte + SucCoA + NADH + H^+ + CO_2 + NH_3
25	Phe + O_2 + NADPH + H^+ → Tyr
26	Tyr + m∝KG + 2O_2 → Fum + 2 AcCoA +m Glu
27	Arg + m∝KG → mGlu + NADH + H^+
28	His → mGlu + NH_3
29	mOAA + AcCoA → Cit
30	Cit → m∝KG + CO_2 + NAPH + H^+
31	m∝KG → SuCoA + NADH + H^+ + CO_2
32	SuCoA → Fum + ATP + $FADH_2$
33	Fum → mMal
34	mMal → mOAA + NADH + H^+
35	NADH + ½ O_2 → 3 ATP
36	Pyr → mPyr
37	Glu + mAsp → mGlu +Asp
38	Gln → mGln
39	mMal + ∝KG → Mal + m∝KG
40	mGlu + mOAA → mAsp + m∝KG

4. RESULTS AND DISCUSSION

4.1 Growth Rate Estimation

Figure 1 illustrates one example of growth rate estimation from a raw capacitance signal (Fig 1A). Cells in the midexponential phase from a seed culture were inoculated at a concentration of $2x10^5$ cells/mL (Fig 1B). The culture was grown in batch mode for two days before the perfusion rate was initiated and kept constant at 1 vol/d. As evident from the reduced initial growth kinetics, the culture underwent a lag phase. The results demonstrate the ability of the estimator to track the changes in cellular growth as the cell entered exponential phase in a later stage (Fig 1C).

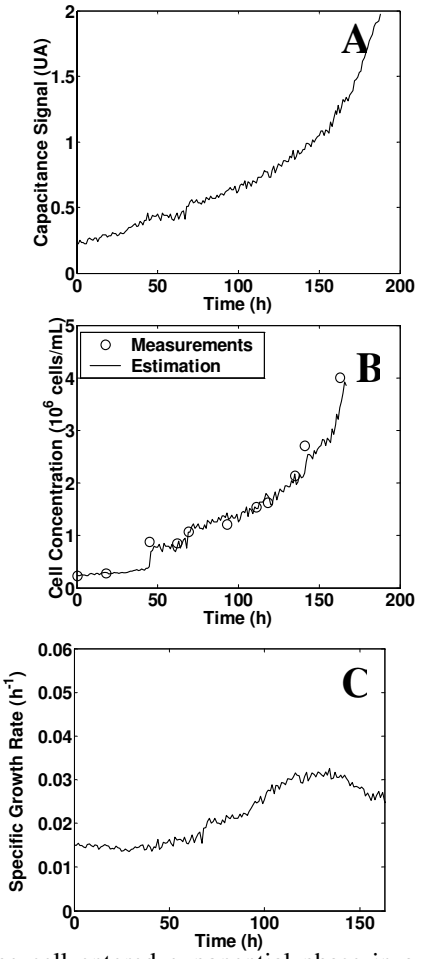

Fig.1. Real-time estimation of the specific growth rate (C) from on-line capacitance (A) and off-line cell count measurements (B) during a perfusion culture.

4.2 Estimation of Metabolic Fluxes

Based on the online estimate of the specific growth rate and the determination of specific uptake/production rates, the 40 intracellular fluxes of the metabolic network were computed online. Figure 2 illustrates how changes in cellular metabolism can be observed as they happen during the course of a culture. In this experiment, the perfusion rate was kept constant at 1 Vol/d throughout the culture (Fig 2A).

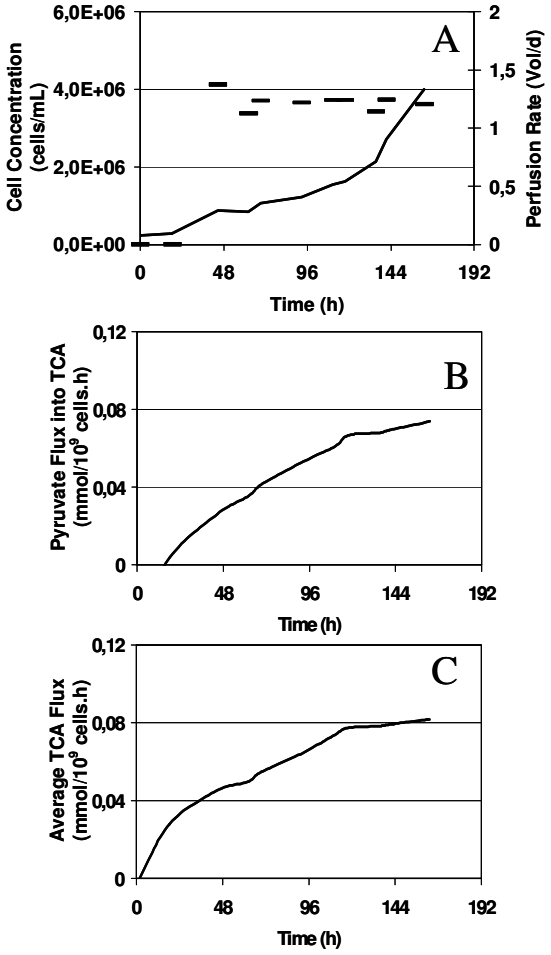

Fig.2. Estimation of intracellular fluxes during a perfusion culture. A) Cell concentration and perfusion rate profiles. B) Glycolytic flux. C) Pyruvate to TCA flux. D) Avergae TCA flux

The time profiles of selected intracellular fluxes are shown (Fig 2C & D). These key fluxes were identified as good indicators of the overall metabolic activity of the cells. Analysis of the flux distribution revealed that as the cell concentration increases over time, cells are shifting towards a more efficient utilization of the main substrate. However, the productivity of the cells tends to decrease with increasing cell concentrations (data not shown). Thus, it is critical to assess the physiological status of the cells and to maintain a culture in a favourable state. This can be accomplished through the manipulation of the perfusion rate as will be shown in the next section.

4.3 Effect of the Perfusion Rate

To evaluate the effect of the perfusion rate on the intracellular flux distribution, another experiment was conducted in which the feed rate was adjusted with increasing cell concentrations (Fig. 3A). This feeding strategy allowed to maintain relatively constant nutrient concentrations inside the bioreactor. In turn, the estimated intracellular fluxes remained very similar throughout the experiment (Fig. 3C & D). To investigate how this would affect the specific productivity, cells were harvested from the bioreactor at different cell concentrations (Fig. 3A) and inoculated into shake-flasks to measure their productivity. As shown in Fig 3D, no significant difference can be discerned in terms of cell specific productivity. These results demonstrate that cells can be maintained in a desirable productive state by manipulating the feed rate so as to meet their nutritional requirements.

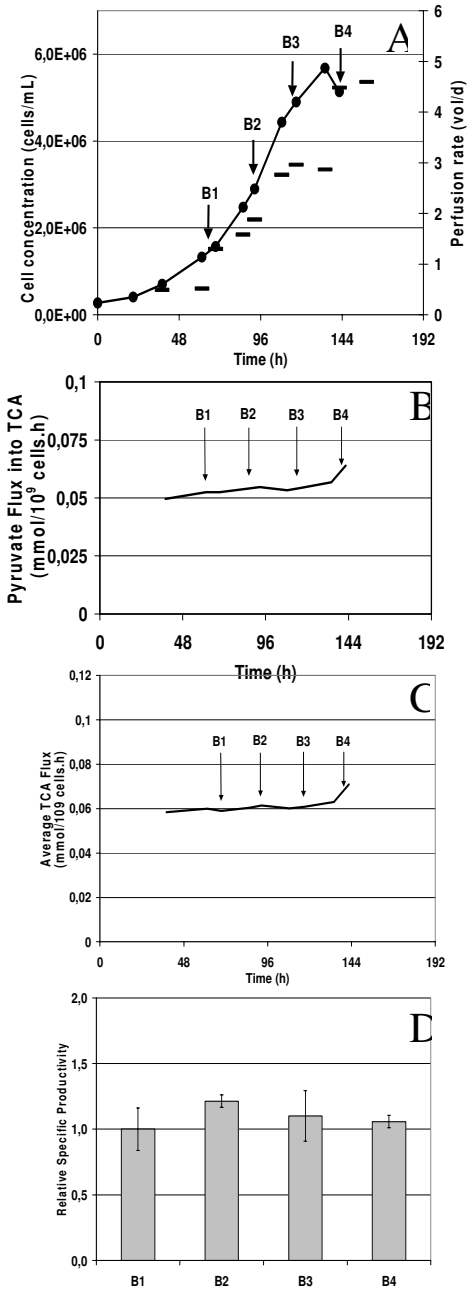

Fig. 3 A) Cell concentration and perfusion rate profiles. B) Pyruvate flux into TCA cycle. C) Average TCA flux. D) Relative cell specific productivity at different times during the perfusion.

CONCLUSIONS

The trend in bioprocess monitoring is towards strategies which are based on the physiological status of the organism in the bioprocess. An approach was developed to provide online estimates of metabolic fluxes of cells grown in perfusion mode. With a constant perfusion rate, intracellular fluxes were varying as cells were exposed to ever-changing environmental conditions (cell, nutrient and waste concentrations). However, by adjusting the perfusion rate with increasing cell concentrations, it allowed to maintain cells in a similar physiological status throughout a perfusion run. Monitoring the physiological state of the cells can be helpful to rapidly establish the conditions favouring the growth or resulting in enhanced productivities. Future efforts are aimed at incorporating this approach into a control scheme.

REFERENCES

Bastin, G., Dochain, D. 1990. On-line estimation and adpative control of bioreactors. Amsterdam, The Netherlands: Elsevier Science Publishers.

Blankenstein, G., Spohn, U., Preuschoff, F., Thommes, J., Kula, M.R., 1994. Multi-channel flow injection analysis biosensor system for on-line monitoring of glucose, lactate, glutamine, glutamate and ammonia in animal cell culture. Biotechnol. Appl. Biochem. 20, 291-307.

Konstantinov, K., 1996. Monitoring and Control of the Physiological State of Cell Cultures. Biotechnol. Bioeng. 52, 271-289.

Nadeau, I., Gilbert, P.A., Jacob, D., Perrier, M., Kamen, A., 2002. Low-protein medium affects the 293SF central metabolism during growth and infection with adenovirus. Biotechnol. Bioeng. 77, 91-104.

Siegwart, P., Cote, J., Male, K., Luong, J.H.T., Perrier, M., Kamen, A., 1999. Adaptive control at low glucose concentration of HEK-293 cell serum-free cultures. Biotechnol. Prog. 15, 608-616.