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METABOLIC MODELLING FOR THE CONTROL OF INTRACELLULAR PROCESSES IN PLANT CELLS CULTURES

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Abstract. Intracellular dynamics and accumulation of nutrients is an ongoing challenge in plant cells cultures, as it has been shown to affect secondary metabolites productivity and culture reproducibility. Using a kinetic model of plant metabolic pathways, it is observed that the stabilization of intracellular nutritional dynamics is desirable to increase culture productivity. This observation was used as a rationale to develop a perfusion feed profile for a bioreactor culture. Using mass balances and model-based estimation, it is possible to design a culture strategy to achieve the desired intracellular stabilization by feeding nutrients proportionally to global cell consumption rate. The development and implementation of this strategy is presented and discussed. The method presented here offers many advantages for the development of control applications for intracellular processes, as it only requires a reliable estimation of biomass and key nutrients consumption rates. As seen with plant cells cultures, the circumventing of intracellular dynamics will allow exploring new possibilities in bioprocess engineering.

1 Introduction

Plant secondary metabolism has been used for decades as a way of producing high value molecules such as dyes, pigments, antibacterial agents and anti-cancer drugs. The production of these molecules in a controlled environment is extremely important, especially if the product is used therapeutically. Achieving controlled conditions in bioprocesses is not a problem for basic variables such as pH, temperature and dissolved oxygen. Proper control of extracellular substrates concentrations is discussed in [1]. However in plant cultures, the control of extracellular states is not sufficient to ensure a proper operation, as the cells can accumulate significant intracellular nutrients reserves, which in turn affect productivity [2]. Thus, adequate feeding of medium is a critical problem in plant cells cultures [3]. To that end, a precise quantification of the system dynamics is required, as contradictory effects are reported when modulating plant cells cultures medium [2].

We propose here that a dynamic metabolic model [4-6] can be used to develop a feeding strategy for a perfusion bioreactor culture that will stabilize intracellular concentrations and thus allow a better control of the process and an increase in culture productivity. The theoretical aspects of manipulating intracellular variables by using extracellular states was explored in [7], however no implementation on a specific cellular system was performed.

2 Metabolic modelling

The dynamic metabolic model used in this study is based on previous work [4-6]. An overview of the model is presented in Figure 1, with a brief description of the approach in Section 7.1 and nomenclature in Section 7.2. Further details on model construction and calibration with experimental data can be found elsewhere [4-6]. As was seen in previously, this model can describe the major nutritional dynamics (extracellular and intracellular) of plant and hairy roots cultures. The cell line used in this study is the California poppy (*Eschscholtzia californica*) with the key products being a variety of alkaloids compounds (ALK in Figure 1).

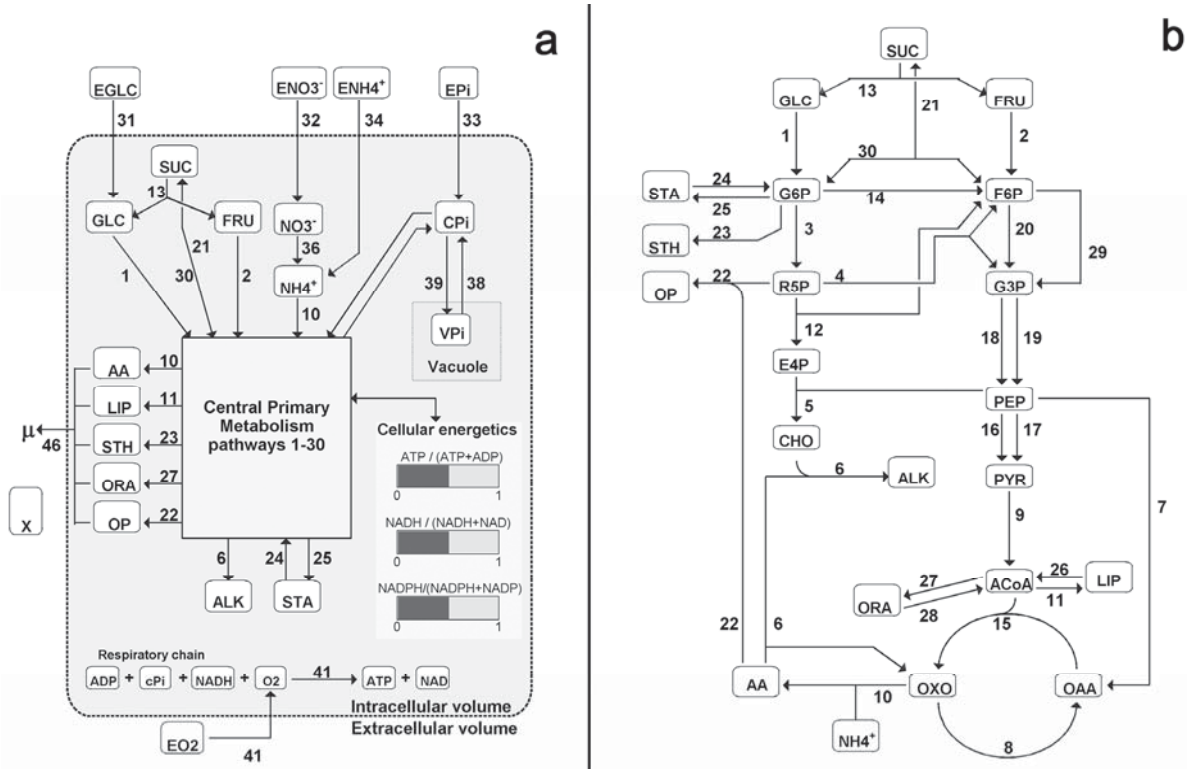

Figure 1. Dynamic metabolic model for plant cells – transport and storage of nutrients (EGLC, ENO3, ENH4, EPi) are shown in 'a' – central primary metabolism pathways are shown in 'b' – detailed nomenclature of the model's variable is provided in Section 7.2.

3 Perfusion bioreactor cultures to improve plant cells bioprocesses

Since plant cells usually have low growth rates ($\mu \approx d^{-1}$) and non-growth associated production of alkaloids, bioprocess design is critical. As was seen in a simulation study [8] the availability of carbon substrates (here glucose) at the intracellular level is critical to maintain the cells in favourable conditions for secondary metabolites production, an observation that is also supported by experimental results [2]. Thus, a feeding strategy for a bioreactor culture should aim at maintaining intracellular glucose concentration in order to stabilize cells in a productive state. However, this must be achieved in stationary phase, as it is the production phase for secondary metabolites.

The feeding of nutrients can be achieved in chemostat, fed-batch or perfusion culture. A chemostat would require continuous growth, which is not suitable for secondary metabolites production. A fed-batch operation would also dilute the biomass during the production (non-growth) phase. Thus, the perfusion culture seems to be the most appropriate design, given the constraints imposed by the slow growth and non-growth associated production. A lab-scale 3L perfusion bioreactor for plant cells was previously developed in our group and it will be used here to implement a culture strategy based on these premises. Further details on the culture system can be found in [9]. The perfusion process for a generic metabolic system is shown in Figure 2, with further details for the mass balances in Section 7.1.

3.1 Developing a feeding profile for a perfusion culture

From the metabolic system (Figure 1) and the culture system (Figure 2) it is possible to derive the feeding profile that will stabilize the intracellular glucose reserves. This can be achieved by feeding glucose proportionally to overall consumption in the bioreactor. To that end the mass balances and nutrient rates estimation from the model will be used. Equations (1) and (2) show the mass balances on intracellular and extracellular glucose (GLC and EGLC, respectively).

$$\frac{dGLC}{dt} = v_{31}(t) - v_1(t) - GLC \cdot \mu \tag{1}$$

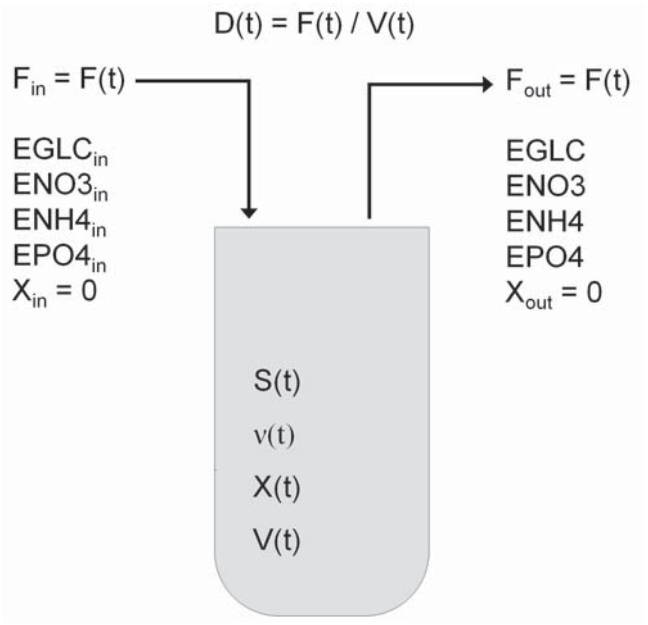

Figure 2. Perfusion bioreactor – *S* and *v* represents the states and fluxes of the metabolic system (see Section 7.1) – *X* is the biomass concentration and *V* is the volume – the manipulated variable is the liquid medium flow through the system *F* (feed rate in $L \cdot d^{-1}$) or *D* (dilution rate, d^{-1}).

$$\frac{d(EGLC)}{dt} = D(t) \cdot \left(EGLC_{in} - EGLC\right) - v_{31}(t) \cdot X(t) \tag{2}$$

Where v_{31} and v_1 refer to reactions in Figure 1 (uptake and intracellular consumption of GLC), μ is the growth rate, D is the dilution rate (in d^{-1}), $EGLC_{in}$ is the feeding concentration for glucose and X is the biomass concentration in the bioreactor (in $gDW \cdot L^{-1}$). Assuming a steady-state on EGLC (which would eventually be reached if the perfusion is to stabilize the system), it is possible to isolate v_{31} in (2) and replace it in (1).

$$\frac{d(GLC)}{dt} = \frac{D(t) \cdot [EGLC_{in} - EGLC]}{X(t)} - v_1(t) - GLC \cdot \mu \tag{3}$$

Then again, assuming that a constant concentration must be achieved for *GLC*, it will be possible to define a feed profile (*D(t)*) from (3):

$$D(t) = \frac{[v_1(t) - GLC \cdot \mu] \cdot X(t)}{[EGLC_{in} - EGLC]} \tag{4}$$

Assuming that the term $GLC \cdot \mu$ is negligible (as is the case in most metabolic systems [10]) and that a small residual EGLC would be present at high X, it is possible to simplify (4) to:

$$D(t) = \frac{v_1(t) \cdot X(t)}{EGLC_{in}} \tag{5}$$

where the unknown parameters are the biomass concentration (X) and GLC consumption (v_1), $EGLC_{in}$ being defined from the medium composition (here $EGLC_{in}$ = 160mM). Biomass concentration can be estimated by many techniques (turbidity, conductivity, O_2 consumption) so only an estimate of v_1 is required. From the model simulations (see Figure 3) it is observed that this reaction rate stabilizes at 2.5 $mmol \cdot gDW \cdot d^{-1}$. This constant value will thus be used as an estimate for v_1 in equation (5). This approach neglects the possible dynamics of glucose phosphorylation rate, but, as simulation results shows, this induces very little error on the overall process as, in the conditions of this study, this rate is relatively constant over a long period of time. However, quasi real-time Metabolic Flux Analysis (MFA) [11] would eventually allow the proper on-line estimation of reaction rates on this system and provide further ways of improving the control of intracellular processes.

3.2 Implementing the culture strategy through model simulation

Thus, a simulation of the metabolic model with the perfusion feed profile defined by (5) was implemented. In that simulation, cell growth is limited by feeding a medium without phosphate (EPi = 0). This allows a realistic simulation of the bioreactor system, as plant cells suspension can rarely be cultivated at densities higher than 20-25 gDW·L^{-1}. This is also in accordance with the literature on production media for plant cells, as these usually limit the cells in phosphate during the production phase [2].

Figure 3 shows simulations results obtained using the metabolic model and perfusion feed. As a comparison, a simulation with $D(t) = 0$ (dotted line) is also shown, which correspond to standard batch cultures usually performed with plant cells.

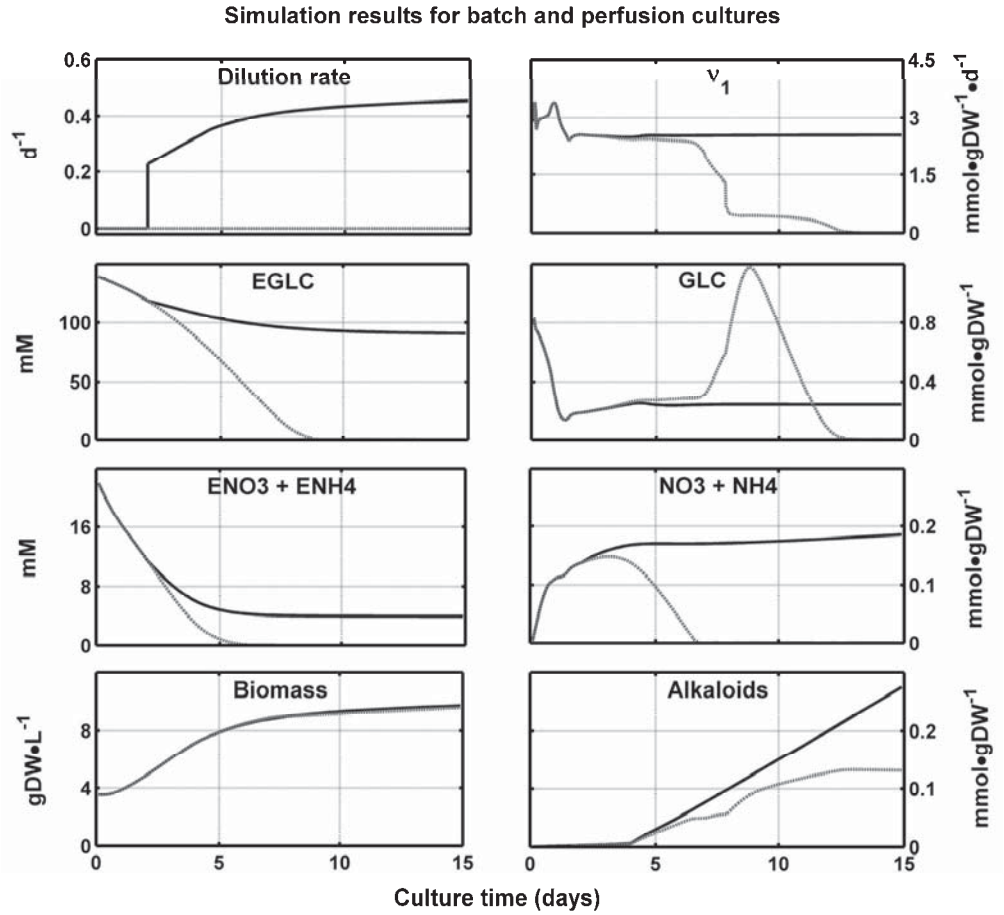

Figure 3 Simulation results. Full line is for a perfusion culture with feed rate defined by equation (5) and dotted (light grey) line is for a batch culture with $D(t) = 0$.

First, it is observed that the feeding described by (5) does stabilize intracellular GLC concentration. As comparison, in a batch culture with these conditions, EGLC is depleted after 8 days and intracellular GLC concentration cannot be maintained. A 'side-effect' of the feeding is the stabilization of intracellular nitrogen reserves (nitrate: NO3; and ammonium: NH4), as the medium GLC and NO3-NH4 are present in balanced proportions. The absence of phosphate in the feeding medium limits the growth in the perfusion culture and thus a similar biomass profile is observed in both conditions (i.e. the same amount of phosphate is available for both cultures).

The desired increase in productivity is achieved by this strategy, as can be seen from the alkaloids simulation results, where the simulation with stabilized intracellular GLC show a final product concentration 2.25 times higher than for a batch culture. The constant ALK production rate for the perfusion culture (Figure 3) shows that the metabolic system is stabilized in a productive state during the stationary phase. Thus, simulation results suggest that the proposed perfusion strategy is a promising way of improving the alkaloids production by this cell culture.

4 Experimental implementation

Experimental implementation of that feeding strategy was performed to evaluate the proposed approach,. A brief overview of the results for GLC and product concentration is given in Figure 4, with further description and results available in [6]. For practical reasons (cell death, product degradation etc.) the batch culture is usually harvested after 10-12 days and the perfusion culture was operated for a slightly longer time, with medium feeding being stopped after 13 days for a total culture duration of 15 days.

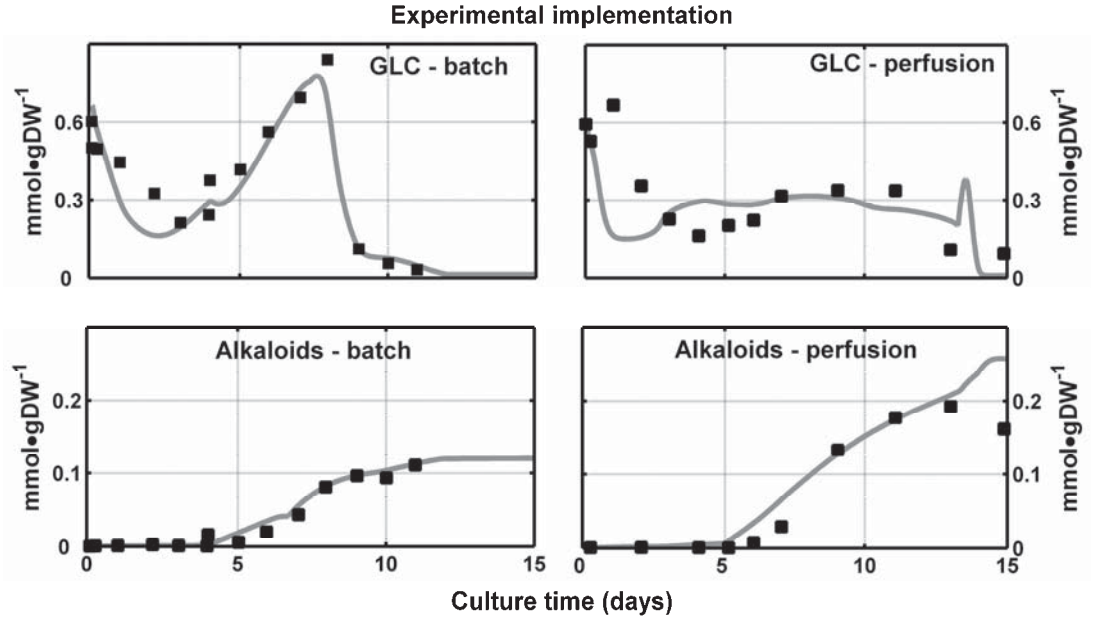

Figure 4 Experimental implementation of the culture strategy and comparison with simulation - batch results are presented on the left and perfusion results (not used for model calibration) are on the right – dots represent experimental data points and lines are simulation results.

These results show that stabilization of intracellular states is confirmed experimentally, as GLC concentration showed very little variation between days 2 and 11 of culture with a concentration of 0.28±0.07 mmol·gDW^{-1}. The increase in productivity that was observed from simulation is also confirmed experimentally, except for the last data point of the culture, where phenomena not considered in the model (product degradation and/or cell death) might be significant. Since the data points from the perfusion culture were not used for model calibration, the agreement between model simulation and experimental results in Figure 4 shows that the model has sufficient predictive capacity to be used for bioprocess design.

5 Conclusion

A dynamic metabolic model was used to develop a feeding profile for a plant perfusion bioreactor culture. This feed profile, when implemented by simulation, allowed achieving a proper stabilization of key intracellular state (glucose concentration), which showed to be critical in improving cell productivity.

First, the proposed approach shows that it is possible to manipulate the nutrients feeding rates in order to circumvent the intracellular 'open-loop' dynamics that might reduce the productivity in batch cultures. Further work on that should however consider the proper closed-loop control of intracellular processes, as the experimental techniques are becoming mature enough to provide adequate on-line measurements.

An experimental implementation also confirmed the soundness of the proposed perfusion bioprocess strategy. The stabilization of intracellular state was achieved, together with the predicted increase in productivity. The use of a dynamic model to analyze a cellular system and rationally design a culture strategy is thus proposed here as a way of achieving a better understanding and control of intracellular processes. This approach will eventually allow exploring new possibilities for bioprocesses development, as it will be possible to actually control critical intracellular events that lead to product formation.

Model simulation at the intracellular level to improve the production of biomolecules could also be pushed further. Optimization techniques could be used with different objectives (yield, production rate, product concentration, harvest time etc.) depending on the specific cellular system. This is already performed with standard methods like MFA, but not in closed-loop [10], and most of these approaches are limited to steady-state operation, which is not always achieved, depending on the cell culture method. However, the idea of using dynamic models of intracellular events is being applied to bioprocess optimization [12] and will definitely be part of the techniques for bioprocess improvement. Here we also suggest that not only dynamic modelling, but also closed-loop control of intracellular processes will be key for further improvements on bioprocesses. The example presented here for plant cells shows that the approach should definitely be considered.

6 References

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7 Appendix: model description

7.1 General modelling approach

The model is based on previous work [4-6]. In this study, the model is implemented in a 3L perfusion bioreactor and specific mass balances are required to describe the system correctly. The model can be described as a set of ordinary differential equation (ODE):

$$\frac{dS}{dt} = M \cdot v - \mu \cdot S \tag{A-1}$$

where S is a vector of the 41 metabolic species concentrations, M is the stoichiometric matrix of the metabolic system (built from the reactions of the model, as presented in [5]), v is the vector of the fluxes (reactions in Figure 1) and μ is the growth rate (reaction 46 in Figure 1). For extracellular states, the mass balance is different; especially in the case a perfusion bioreactor is used. Conversion of extracellular concentrations (mM) to intracellular concentrations (mmol·gDW^{-1}) has to be performed. The effect of medium feeding must also be considered. In that case, the mass balance can be written as follows:

$$\frac{dS_e}{dt} = v_T \cdot \frac{X}{V} + D \cdot (S_{in} - S_e) \tag{A-2}$$

where S_e is the extracellular states vector (EGLC, ENO3, EPi, ENH4), v_T is the vector of transport fluxes (v_{31} to v_{34} in Figure 1), D is the dilution rate (d^{-1}) and S_{in} is the vector of feeding concentrations for nutrients (defined from the medium composition).

The flux vector $v(t)$ is defined using biokinetics equations in the form of multiplicative Michaelis-Menten kinetics:

$$v_j = v_{\max,j} \cdot \prod_i \frac{S_i^{\alpha_i}}{K_{m,i}^{\alpha_i} + S_i^{\alpha_i}} \tag{A-3}$$

where j is the flux number (1 to 46 in Figure 1), i are the index of metabolites involved in reaction v_j and higher order reactions can be accounted for by adjusting the α_i term. As an example, the transport of glucose (EGLC) from the medium to the intracellular pool (GLC), reaction 31, is described by the following equation:

$$v_{31}(t) = v_{\max,31} \cdot \frac{EGLC}{K_{m,EGLC} + EGLC} \cdot \frac{ATP}{K_{m,ATP} + ATP} \tag{A-4}$$

Here, the intracellular ATP concentration is considered, as the co-transporter of an ion (Na^+) is used to pump GLC against its gradient in cells (indirect active transport). The complete construction of these equations, parameter estimation routines and performance of the modelling approach is presented and discussed in references [4-6].

7.2 Model states

Intracellular states

Name	Description	Name	Description	Units
AA	Amino Acids	NO3	Nitrate	mmol•gDW^{-1}
ACOA	Acetyl-CoenzymeA	O2	Oxygen	“
ADP	Adenosine Diphosphate	OAA	Oxaloacetate	“
ALK	Total alkaloids	OP	Organic Phosphates	“
ATP	Adenosine Triphosphate	ORA	Organic Acids	“
CHO	Chorismate	OXO	Oxoglutarate	“
CO2	Carbon Dioxide	PEP	Phosphoenolpyruvate	“
VPi	Vacuolar Pi	PPi	Pyrophosphate	“
CPi	Cytoplasmic Pi	PYR	Pyruvate	“
E4P	Erythrose-4-Phosphate	R5P	Ribulose-5-Phosphate	“
F6P	Fructose-6-Phosphate	STA	Starch	“
FRU	Fructose	STH	Structural Hexoses	“
G3P	Glyceraldehyde-3-Phosphate	SUC	Sucrose	“
G6P	Glucose-6-Phosphate	NAD	Nicotinamide Adenine Dinucleotide (oxidised)	“
GLC	Glucose	NADH	Nicotinamide Adenine Dinucleotide (reduced)	“
LIP	Lipids	NADP	Nicotinamide Adenine Dinucleotide Phosphate (oxidised)	“
NH4	Ammonium	NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)	“

Extracellular states

Name	Description	Name	Description	Units
EGLC	Glucose	EPi	Phosphate	mM
ENH4	Ammonium	EO2	Oxygen	“
ENO3	Nitrate			“

Other states

V	Liquid medium volume			L
X	Biomass			gDW•Flask^{-1}