Modeling the Continuous Production of Monoclonal Antibodies in Perfusion Bioreactors Through a Dynamic Metabolic Flux Analysis (DMFA) Framework

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Abstract

In this work, a comprehensive Dynamic Metabolic Flux Analysis (DMFA) model is developed for monoclonal antibody (mAb) production in Chinese Hamster Ovary (CHO) cell perfusion culture. The framework integrates a kinetic, stoichiometric, and mass balance component to simulate and mechanistically link the temporal evolution of intracellular metabolism to the bioreactor conditions. The framework is calibrated to time-series metabolite data from scale-down semi-perfusion spin tube cultures grown under intensified conditions as a base case training dataset. Subsequent prediction of CHO cell performance in a continuous perfusion bioreactor offers agreement to a subset of measured extracellular species, demonstrating the model’s robustness arising from its mechanistic foundation. Perfusion process development and optimization are target model applications.

**Keywords:** Continuous biomanufacturing, perfusion, dynamic metabolic modeling

1. Introduction

Recombinant mAbs have transformed medicine since the 1980’s providing life-saving therapeutics for numerous diseases (Ecker et al., 2015). CHO cells are the preferred mammalian cell platform for mAb production given their robust growth in defined mediums, efficient transfectability, and innate capacity to induce complex post-translational modifications such as N-linked glycosylation (Trill et al., 1995; Wurm, 2004). Fed batch bioreactors have dominated upstream production in bioprocess pipelines. However, increasing global product demand, coupled with the emergence of an ever-expanding biosimilars market, have prompted the industry to transition towards continuous upstream manufacturing achieved through perfusion bioreactors (Walther et al., 2015). The resulting expansive design space and increased operational intensity relative to fed batch pose substantial challenges in the pursuit of continuous upstream bioprocess development and optimization. With respect to mAb production, modeling cell metabolism is imperative to assess nutrient utilization and physiologic objectives under varying operating setpoints (Reddy et al., 2023). Here, a DMFA framework is established to mechanistically link key parameters of perfusion bioreactors, including media composition, perfusion rate, and bleed rate, to CHO cell metabolism and bioreactor conditions.

1. **Materials and Methods**
	1. Semi-Perfusion CHO Cell Cultures

Semi-perfusion cultures of the CHO-K1 VRC01 cell line were seeded at 0.4×106 cells mL-1 with a starting volume of 9.4 mL in commercial basal media and supplemental L-glutamine in 50 mL vented spin tubes. Cultures were grown in a 5% CO2 humidified incubator at 37°C, 250 rpm shake speed, 19.05 mm orbital throw, and 90° rocking angle. 1.1 mL of culture broth was sampled every day from day 0 – 3, reducing the working volume to 5 mL by day 3. 0.1 mL of culture was used to quantify cell density, while the remaining 1 mL was centrifuged at 180 g for 5 minutes, 0.22 μm sterile-filtered, and saved for off-line metabolite analysis. On day 3, semi-perfusion at an effective perfusion rate of 1 vvd-1 was initiated by centrifuging cultures at 180 g for 5 minutes, removing the supernatant, and resuspending the cell pellet in 5.1 mL of pre-warmed perfusion media formulated to an in-house recipe. 1 mL of the supernatant was saved for metabolite analysis. 0.1 mL of resuspended culture broth was sampled for cell density quantification.

* 1. Analytical Methods

Viable cell density (VCD) was determined via the Trypan Blue exclusion method using a DeNovix CellDrop Fluorescence Cell Counter. Residual glucose, lactate, and ammonia concentrations were measured with a YSI 2950 BioAnalyzer. Amino acid concentrations were analyzed with an OPA/FMOC-derivatization protocol with an Agilent Poroshell HPH-C18 column on an Agilent HPLC 1260 Infinity II system. Daily titer was assessed via Protein A chromatography with a POROS A 20 μm column (4.6 × 100 mm, 1.7 mL) on an Agilent HPLC 1260 Infinity II system.

1. **Model Framework Development**

The mathematical framework is a structured-unsegregated DMFA model which integrates a kinetic, stoichiometric, and macroscopic mass balance component. The required inputs are the initial extracellular metabolite concentrations, media composition, process conditions, and a robust kinetic parameter set. The model inputs feed to the kinetic component to approximate the growth, death, and metabolite exchange rates. The exchange rates are subsequently incorporated as constraints to the stoichiometric component, functionalized through MFA to assess the intracellular flux distribution. The updated exchange fluxes from the resulting MFA solution are incorporated into the discretized perfusion bioreactor mass balances to predict the concentration of extracellular species at the next timepoint. Iteration over the sequential framework enables dynamic simulation of intracellular metabolism and bioreactor conditions.

* 1. Kinetics

The kinetic component describes the growth, death, and nutrient consumption and metabolite production rates as a function of the time-dependent extracellular concentrations in the bioreactor. The rate equations are formulated according to semi-empirical Monod kinetics, which consider the macroscopic importance of limiting substrates, and the biochemical relationships between nutrients, on cell growth and energetic functions to yield simple rate expressions. As an example, the growth rate, Eq. (1) and Eq. (2), was derived from the rules of multiple-substrate Monod kinetics and is a function of both limiting and inhibitory species. Time-series metabolite data from semi-perfusion cultures under intensified conditions was assessed to determine the key species, and thereby the form of the kinetic terms, to derive the growth rate expression.

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| $$μ=μ\_{max}\left(μ\_{Glc}+μ\_{Lac}\right) \left(μ\_{Asn}+μ\_{Asp}\right)\left(μ\_{Gln}+μ\_{Glu}\right)μ\_{Ser}μ\_{Arg}μ\_{Amm,I}$$ | (1) |
| $μ\_{i}= \frac{μ\_{max,i}\left[i\right]}{K\_{i}+\left[i\right]}$  | (2) |

The biochemical relationship between nutrients, such as glutamine and glutamic acid, and their ability to induce diauxic growth, was also considered in the overall growth rate formulation yet omitted here for brevity. In Eq. (1) and (2), $μ$ is the overall specific growth rate, $u\_{max,i}$ is the maximum growth rate on species $i$, $K\_{i}$ is the Monod constant for species $i $which physically represents the concentration of species $i $that will sustain half of the maximum growth rate on that substrate. Lastly, $[i]$ is the extracellular concentration of species $i$ in the bioreactor. The death rate is formulated in a similar manner. As an additional example, the rates for essential amino acid consumption are assumed to be proportional to the growth rate, implying that cells will only consume the minimal amount of an essential amino acid necessary to sustain metabolic functions (Chen et al., 2019).25 rate expressions were derived to encompass the growth, death, and exchange rates of 23 metabolites, yielding a set of 66 kinetic parameters.

* 1. Reduced Metabolic Network

The stoichiometric component is a network of 70 biochemical reactions and 43 metabolites representing the key metabolic pathways in CHO cells. The network was manually curated for prior fed-batch modeling work and includes the pathways for glycolysis, the TCA cycle, essential and nonessential amino acid metabolism, oxidative phosphorylation, the urea cycle, and biomass and antibody synthesis. Reactions for total carbohydrate metabolism, nucleotide synthesis, and lipid synthesis were lumped into the biomass synthesis reaction to reduce the network size. The stoichiometric coefficients for the mAb synthesis reaction were calculated from the amino acid sequence for the VRC01 antibody and include considerations of ATP requirements. The final reduced network has a rank of 43 and a condition number of 18.3. The network ensures physiologically consistent fluxes for the major pathways given the intracellular stoichiometric constraints and provides mechanistic insight towards resource allocation without the need for complex enzymatic rate expressions.

To functionalize the network, the pseudo steady state assumption (Zupke and Stephanopoulos, 1995) is applied to all intracellular metabolites yielding an overdetermined stoichiometric matrix. MFA is employed to solve for the flux value of each reaction and assess the distribution of the metabolic load on the cell at each simulated timepoint, taken as every 0.25 days; the duration over which the fluxes are assumed to remain constant. MFA quantifies the unknown intracellular fluxes, under the constraint of specified exchange fluxes, which can be experimentally determined, and is formulated as a quadratic programming problem, Eq. (3).

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| $Min \sum\_{i=1}^{n=24}\left(\frac{v\_{i}^{calc}-v\_{i}^{meas}\left(θ\right)}{v\_{i}^{meas}\left(θ\right)}\right)^{2}$ $s.t.          Sv=0$ $v^{lb}<v\_{exchange}<v^{ub}$ | (3) |

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$v\_{i}^{calc}$ is the calculated exchange rate of species $i$ scaled to flux units, as determined by solution to Eq. (3), $v\_{i}^{meas}$ is the measured exchange rate as determined by experiment, $S$ is the stoichiometric matrix resulting from steady state mass balances on each metabolite, $v$ is the vector containing both the intracellular and exchange fluxes, and $v^{lb}$ and $v^{ub}$ represent the lower and upper flux bounds on the exchange fluxes respectively. However, within the framework of DMFA, the measured exchange fluxes, $v\_{i}^{meas}$, are predicted from the kinetic rate expressions described in the previous section and therefore expressed as functions of the kinetic parameters, $θ$, thus integrating the kinetic and stoichiometric components.

* 1. Macroscopic Mass Balances

The macroscopic bioreactor mass balances on each measured species, link the dynamics between the intracellular and extracellular environments. Perfusion-specific mass balances account for the effect of the perfusion rate, bleed rate, and media composition on the bulk mass transport and cell growth dynamics of the bioreactor. These are three Critical Process Parameters (CPPs) of continuous upstream mAb production which have been explicitly included in the model. To capture all phases of bioreactor operation, the derivative term in the general mass balance is maintained, discretized via forward finite differences, and re-arranged to solve for the extracellular concentration of each measured species at the next time point, Eq. (4).

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| $C\_{i+1}=\frac{\left(DC\_{feed}+q\_{i}X\_{avg}\right)\left(t\_{i+1}-t\_{i}\right)+C\_{i}}{1+D(t\_{i+1}-t\_{i})}$  | (4) |

$C\_{i+1}$ is the concentration of any species at the next time point, $C\_{i}$ is concentration of any species at the present timepoint, $C\_{feed}$ is the concentration of any species in the media,$ D$ is the perfusion rate, $q\_{i}$ is the consumption or production rate of any species at the present timepoint, $X\_{avg}$ is the average viable cell density between the two timepoints, $t\_{i+1}$ is the next time point, and $t\_{i}$ is the present timepoint. The cell bleed, Eq. (5), is simulated as a discrete event to align with its intermittent application during experiments, and the concentration of each measured species after a cell bleed can be predicted accordingly.

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| $V\_{bleed}=V\left(1-\frac{X\_{sp}}{X\_{current}}\right)$  | (5) |

$V\_{bleed}$ is the required bleed volume, $V$ is the bioreactor working volume, $X\_{sp}$ is the VCD setpoint, and $X\_{current}$ is the presently measured VCD prior to engaging the bleed.

1. **Results and Discussion**

In addition to prior fed-batch work, time-series metabolic profiles of 24 species from scale-down semi-perfusion CHO cell cultures, as described in Section 2, provided the required data for model development and kinetic parameter estimation. Semi-perfusion cultures were grown without a cell bleed to approximate intensified process conditions characterized by industrially relevant VCDs, corresponding cell-specific perfusion rates (CSPRs), and nutrient depletion.

Intensified semi-perfusion induces a range of metabolic states across the culture duration. Starting from exponential growth coupled with large nutrient consumption rates, the culture approaches fed-batch dynamics ending in reduced net growth with high productivity, indicative of the stationary phase. The mechanistic framework of the model, provided by the reduced reaction network and mass balances, captures the range of metabolic states with a single set of kinetic parameter values (Figure 1.a).

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| a) | b) |
| A graph with numbers and a line  Description automatically generated | A graph of a number of objects  Description automatically generated |
| A graph of glucose  Description automatically generated | A graph showing the amount of glucose  Description automatically generated |
| A graph with black lines and white text  Description automatically generated | A graph of a graph showing the time and time  Description automatically generated with medium confidence |
| A graph of a model fit  Description automatically generated |  |
| Figure 1. Subset of CHO-K1 VRC01 cell metabolic profiles. a) Scale-down semi-perfusion experimental data and model fits. b) 1 L continuous perfusion bioreactor experimental profiles and model predictions. The solid vertical lines denote perfusion initiation. The dashed vertical line in b) indicates cell bleed initiation. |

While calibrated to only a single scale-down semi-perfusion dataset, subsequent model predictions of 1 L continuous perfusion bioreactor dynamics, under similar experimental conditions, followed to assess early performance of model extrapolation. Simulations of cell growth and metabolite profiles under mild process conditions, characterized by a low VCD and sufficient nutrient availability, agreed with previously collected data for a subset of species (Figure 1.b). The agreement between the predicted and experimental profiles can be improved, yet the general trends and magnitudes are maintained, highlighting the robustness of the DMFA framework to two significant differences between the datasets: process dynamics and the ensuing cell metabolic states. The difference between semi-perfusion, in the form of nearly instantaneous media exchanges, and continuous perfusion coupled with an intermittent cell bleed to control the cell growth rate at steady state, is captured through the mass balance component of the framework. The difference in metabolic states between the two processes, including the range of states observed in semi-perfusion, are explained by the underlying mechanistic foundation of the metabolic network, which provides physiologically meaningful constraints towards intracellular resource distribution.

1. **Conclusions**

The mechanistic basis of the DMFA framework enables quantitative assessment of CHO cell metabolism across perfusion bioreactor scales and operational modes with a minimal set of required inputs. Future work seeks model application towards process goals of industrial interest. Identification of key nutrients to reduce growth and increase productivity is of primary importance in perfusion bioreactors. Such information can be used to provide insight towards media reformulation and feeding strategies, and subsequently predict the corresponding minimum CSPR, translating to a reduction in operational costs. Increasing the framework’s complexity by defining a segregated structure to evaluate metabolic differences between heterogeneous cell populations in the bioreactor over the culture duration is an additional goal of future efforts.

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