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Mathematical modelling and experimental validation for optimisation and control of mammalian cell culture systems

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Abstract

Monoclonal antibodies (mAbs) exhibit remarkable properties that make them suitable for a wide range of diagnostic and therapeutic applications. The main manufacturing platform used to produce mAbs is mammalian cell cultures due to their capacity for post-translational modifications, which are essential for the mAbs functionality (Zhu, 2012). Unfortunately, mammalian cell cultures present low yield, slow growth, and require expensive medium components. Model-based techniques could be industrially applied to overcome these limitations, e.g., implementing model-based optimisation strategies that identify feeding regimes to maximise mAbs titre in GS-NS0 cultures (Kiparissides *et al.*, 2011). However, existing feeding strategies depend mainly on glucose and glutamate supply neglecting the exhaustion of essential amino acids and cell's energy requirements not only for proliferation and maintenance but also for mAbs production.

In this work, cell and product compositions, and energy requirements for proliferation, maintenance and production, have been considered in the development of a novel dynamic predictive model for GS-NS0 cells producing cB72.3 mAbs, in modified DMEM medium supplemented with 10% serum or in a serum-free CD-Hybridoma medium. The model describes growth kinetics, nutrient metabolism, mAbs secretion and the adenosine triphosphate (ATP) balance based on glucose/amino acids energy metabolic networks, in batch and fed-batch cultures; and it successfully predicts the number cells, and the concentrations of ATP, glucose, amino acids and lactate throughout the culture.

The successful coupling of growth kinetics equations and stoichiometric balances, and the *in vitro/in silico* approach has enabled us to develop the first dynamic model that predicts the intracellular ATP content in mammalian cell cultures. Additionally, this experimentally validated model was utilised to design a tailor-made and low-cost supplemental medium and implement an optimised fed-batch schedule that maximises the mAbs production and extends the longevity of the culture. This integrated model-based approach has the potential to be applied for media development, upstream optimisation and the development of control strategies for a wide range of biopharmaceutical products.

Declarations

Declaration of originality

The work described in this thesis was carried out by the author (or in collaboration) in the Biological Systems Engineering Laboratory, Centre for Process Systems Engineering, Department of Chemical Engineering, Imperial College London, United Kingdom between October 2012 and October 2016. Any published and unpublished work used here has been cited in the text, and the list of references in the bibliography section at the end of this thesis.

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Ana Luz Quiroga Campano

*A mi hermosa familia,
por todos estos años que vivimos separados.*

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Nomenclature

AA	: Amino acid concentration [mM]
ADP	: Adenosine di-phosphate
ALA	: Alanine concentration [mM]
AMM	: Ammonia concentration [mM]
ARG	: Arginine concentration [mM]
ASN	: Asparagine concentration [mM]
ASN	: Asparagine concentration [mM]
ASP	: Aspartate concentration [mM]
ATP	: Adenosine triphosphate concentration [mM]
C _{pro}	: Proline coefficient
C _{ala}	: Alanine coefficient
C _{arg}	: Arginine coefficient
C _{asn}	: Asparagine coefficient
C _{asp}	: Aspartate coefficient
C _{glu}	: Glutamate coefficient
C _{AA}	: Amino acids coefficient
CO ₂	: Carbon dioxide
CoA-SH	: Coenzyme A
CYS	: Cysteine concentration [mM]
F _{in}	: Inlet flow [L/h]
F _{out}	: Outlet flow [L/h]
FAD	: Flavin adenine dinucleotide
Fin _{AA}	: Inlet flow of amino acids [L/h]
Fin _{GLC}	: Inlet flow of glucose [L/h]
Fin _{TRACE}	: Inlet flow of trace elements [L/h]
GDP	: Guanosine diphosphate
GLC	: Glucose concentration [mM]
GLC _{in}	: Glucose concentration in the supplemental medium for fed-batch cultures [mM]
GLC _{min}	: Minimum concentration of glucose [mM]
GLN	: Glutamine concentration [mM]
GLU	: Glutamate concentration [mM]
GLY	: Glycine concentration [mM]
GTP	: Guanosine triphosphate
H ₂ O	: Molecule of water
I	: Inhibitor concentration [mM]
k _{a,d}	: Constant for transition rate from early to late apoptotic.
K _{a,LAC}	: Monod saturation constant for apoptosis based on lactate [mM]
K _{ARG}	: Monod saturation constant for growth in arginine [mM]
K _{arg,lim}	: Monod inhibition constant for arginine limitation [mM]
K _{ASN}	: Monod saturation constant for growth in asparagine [mM]
K _{ASP}	: Monod saturation constant for growth in aspartate [mM]
K _{asp,lim}	: Monod inhibition constant for aspartate limitation [mM]
K _{d,amm}	: Monod saturation constant for death based on ammonia toxicity [mM]
K _{d,LAC}	: Monod saturation constant for death based on lactate toxicity [mM]
K _{d,lac}	: Monod saturation constant for death based on lactate toxicity [mM]
K _{deg,glu}	: Degradation rate of glutamine [1/h]
K _{GLC}	: Monod saturation constant for growth based on glucose metabolism [mM]
K _{glc,lim}	: Monod inhibition constant for glucose limitation [mM]
K _{GLN}	: Monod saturation constant for growth based on glutamine metabolism [mM]

Nomenclature

K_{GLU}	: Monod saturation constant for growth in glutamate [mM]
$K_{glu,lim}$	: Monod inhibition constant for glutamate limitation [mM]
K_I	: Monod inhibition constant [mM]
$K_{I, AMM}$	: Monod constant for metabolic inhibition from ammonia [mM]
$K_{I, LAC}$	: Monod constant for metabolic inhibition from lactate [mM]
$K_{ile,lim}$	: Monod inhibition constant for isoleucine limitation [mM]
$K_{leu,lim}$	: Monod inhibition constant for leucine limitation [mM]
$K_{lim,glu}$	: Monod saturation constant for death based on glutamine limitation [mM]
k_{lys}	: Specific cell lysis rate [1/h]
K_m	: Monod saturation constant [mM]
K_s	: Monod saturation constant for substrate S [mM]
K_G	: Rate of Golgi-to-medium antibody transport [1/h]
K_{MET}	: Saturation constant for preferential uptake of glutamate [mM]
LAC	: Lactate concentration [mM]
$M_{ala,glc}$	: Maintenance coefficient of alanine from glucose [mmol/Cell h]
$M_{lac,glc}$	: Maintenance coefficient of lactate from glucose [mmol/Cell h]
$M_{mAb,AA}$	: Antibody amino acids content [mmol/mg]
$M_{mAb,ala}$	: Antibody alanine content [mmol/mg]
$M_{mAb,arg}$	: Antibody arginine content [mmol/mg]
$M_{mAb,asn}$	: Antibody asparagine content [mmol/mg]
$M_{mAb,asp}$	: Antibody aspartate content [mmol/mg]
$M_{mAb,cys}$	: Antibody cysteine content [mmol/mg]
$M_{mAb,glc}$	: Antibody content of glucose [mmol/mg]
$M_{mAb,glu}$	: Antibody glutamate content [mmol/mg]
$M_{mAb,glu}$	: Antibody glutamine content [mmol/mg]
$M_{mAb,gly}$	: Antibody glycine content [mmol/mg]
$M_{mAb,pro}$	: Antibody proline content [mmol/mg]
$M_{mAb,ser}$	: Antibody serine content [mmol/mg]
$M_{mAb,trp}$	: Antibody tryptophan content [mmol/mg]
$M_{tca,AA}$	: Maintenance coefficient of biomass from energy from amino acids [mmol/Cell h]
$M_{tca,ala}$	: Maintenance coefficient of biomass from energy from alanine [mmol/Cell h]
$M_{tca,arg}$	: Maintenance coefficient of biomass from energy from arginine [mmol/Cell h]
$M_{tca,asn}$	: Maintenance coefficient of biomass from energy from asparagine [mmol/Cell h]
$M_{tca,asp}$	: Maintenance coefficient of biomass from energy from aspartate [mmol/Cell h]
$M_{tca,glc}$	: Maintenance coefficient of biomass from energy from glucose [mmol/Cell h]
$M_{tca,glu}$	: Maintenance coefficient of biomass from energy from glutamate [mmol/Cell h]
$M_{tca,pro}$	: Maintenance coefficient of biomass from energy from proline [mmol/Cell h]
$M_{X,ATP}$	: Maintenance energy requirement [mmol/Cell h]
MAB	: Monoclonal antibody concentration [mg/L]
MAB_{acc}	: Accumulated monoclonal antibody inside the cells [mg/Cell]
$M_{mAb,ATP}$	: ATP requirement for monoclonal antibody assembly [mmol/mg]
M_S	: Monoclonal antibody specific secretion rate [mg/Cell h]
n	: Exponential sensitivity factor
NAD	: Nicotinamide adenine dinucleotide
PRO	: Proline concentration [mM]
$Q_{ala,glc}$	: Specific consumption rate of glucose for alanine production [mmol/Cell h]
Q_{AMM}	: Specific production rate of ammonia [mmol/Cell h]
Q_{GLC}	: Specific consumption rate of glucose [mmol/Cell h]
$Q_{gly,ser}$	: Specific consumption rate of serine for glycine production [mmol/Cell h]

Nomenclature

Q_{LAC}	: Specific production rate of lactate [mmol/ Cell h]
$Q_{lac,glc}$	: Specific consumption rate of glucose for lactate production [mmol/Cell h]
$Q_{mAb,AA}$	: Specific consumption rate of an amino acid for mAb assembly[mmol/Cell h]
$Q_{mAb,ala}$	: Specific consumption rate of alanine for mAb assembly[mmol/Cel h]
$Q_{mAb,arg}$	: Specific consumption rate of arginine for mAb assembly[mmol/Cel h]
$Q_{mAb,asn}$	: Specific consumption rate of asparagine for mAb assembly[mmol/Cel h]
$Q_{mAb,asp}$	: Specific consumption rate of aspartate for mAb assembly[mmol/Cel h]
$Q_{mAb,cys}$	: Specific consumption rate of cysteine for mAb assembly[mmol/Cell h]
$Q_{mAb,glc}$	: Specific consumption rate of glucose for mAb assembly [mmol/Cell h]
$Q_{mAb,glu}$	: Specific consumption rate of glutamate for mAb assembly[mmol/Cel h]
$Q_{mAb,gly}$	: Specific consumption rate of glycine for mAb assembly[mmol/Cell h]
$Q_{mAb,pro}$	: Specific consumption rate of proline for mAb assembly[mmol/Cell h]
$Q_{mAb,ser}$	: Specific consumption rate of serine for mAb assembly[mmol/Cell h]
$Q_{mAb,trp}$	: Specific consumption rate of tryptophan for mAb assembly[mmol/Cell h]
$q_{mAb,x}$	: Specific antibody synthesis rate[mg/Cell h]
$Q_{tca,AA}$	: Specific consumption rate of amino acids for energy production through TCA cycle [mmol/Cel h]
$Q_{tca,ala}$	: Specific consumption rate of alanine for energy production through TCA cycle [mmol/Cel h]
$Q_{tca,arg}$	: Specific consumption rate of arginine for energy production through TCA cycle [mmol/Cel h]
$Q_{tca,asn}$	: Specific consumption rate of asparagine for energy production through TCA cycle [mmol/Cel h]
$Q_{tca,asp}$	: Specific consumption rate of aspartate for energy production through TCA cycle [mmol/Cel h]
$Q_{tca,glc}$	: Specific consumption rate of glucose for energy production through TCA cycle [mmol/Cell h]
$Q_{tca,glu}$	: Specific consumption rate of glutamate for energy production through TCA cycle [mmol/Cel h]
$Q_{tca,pro}$	: Specific consumption rate of proline for energy production through TCA cycle [mmol/Cel h]
$Q_{x,AA}$	: Specific consumption rate of an amino acid for proliferation [mmol/Cell h]
$Q_{x,ala}$	: Specific consumption rate of alanine for proliferation [mmol/Cel h]
$Q_{x,arg}$	: Specific consumption rate of arginine for proliferation [mmol/Cel h]
$Q_{x,asn}$	: Specific consumption rate of asparagine for proliferation [mmol/Cel h]
$Q_{x,asp}$	: Specific consumption rate of aspartate for proliferation [mmol/Cel h]
$q_{x,ATP}$	: Specific production rate of ATP [mmol/Cell h]
$Q_{x,cys}$	: Specific consumption rate of cysteine for proliferation [mmol/Cell h]
$Q_{x,glc}$	: Specific consumption rate of glucose for proliferation [mmol/Cell h]
$Q_{x,glu}$	: Specific consumption rate of glutamate for proliferation [mmol/Cel h]
$Q_{x,gly}$	: Specific consumption rate of glycine for proliferation [mmol/Cell h]
$Q_{x,pro}$	: Specific consumption rate of proline for proliferation [mmol/Cel h]
$Q_{x,ser}$	: Specific consumption rate of serine for proliferation [mmol/Cell h]
$Q_{x,trp}$	: Specific consumption rate of tryptophan for proliferation [mmol/Cell h]
Q_{MET}	: Saturation metabolic capacity due to growth on glutamate
S	: Substrate concentration [mM]
SER	: Serine concentration [mM]
STV	: Starvation factor
TRP	: Tryptophan concentration [mM]

Nomenclature

V	: Process rate [1/h]
V	: Volume [L]
v_{max}	: Theoretical maximum rate [1/h]
X	: Cell concentration [Cell/L]
X_a	: Concentration of apoptotic cells [Cell/L]
X_d	: Concentration of dead cells [Cell/L]
X_v	: Viable cell concentration [Cell/L]
X_0	: Initial cell concentration [Cell/L]
$Y_{ala,glc}$	: Yield on biomass from alanine from glucose [Cell/mmol]
$Y_{lac,glc}$	: Yield on biomass from lactate from glucose [Cell/mmol]
$Y_{tca,AA}$	: Yield of biomass from energy from amino acids [Cell/mmol]
$Y_{tca,ala}$	: Yield of biomass from energy from alanine [Cell/mmol]
$Y_{tca,arg}$	: Yield of biomass from energy from arginine [Cell/mmol]
$Y_{tca,asn}$	: Yield of biomass from energy from asparagine [Cell/mmol]
$Y_{tca,asp}$	: Yield of biomass from energy from aspartate [Cell/mmol]
$Y_{tca,glc}$	: Yield of biomass from energy from glucose [Cell/mmol]
$Y_{tca,glu}$	: Yield of biomass from energy from glutamate [Cell/mmol]
$Y_{tca,pro}$	: Yield of biomass from energy from proline [Cell/mmol]
$Y_{x,AA}$	: Yield on biomass from an amino acids [Cells/mmol]
$Y_{x,ala}$	: Yield on biomass from alanine [Cells/mmol]
$Y_{x,arg}$	: Yield on biomass from arginine [Cells/mmol]
$Y_{x,asn}$	: Yield on biomass from asparagine [Cells/mmol]
$Y_{x,asp}$	: Yield on biomass from aspartate [Cells/mmol]
$Y_{X,ATP}$	: Yield on biomass from energy [Cell/mmol]
$Y_{x,glc}$	: Yield on biomass from glucose [Cells/mmol]
$Y_{x,gln}$	: Yield on biomass from glutamine [Cells/mmol]
$Y_{x,glu}$	: Yield on biomass from glutamate [Cells/mmol]
$Y_{x,gly}$	: Yield on biomass from glycine [Cells/mmol]
$Y_{x,pro}$	: Yield on biomass from proline [Cells/mmol]
$Y_{x,ser}$	: Yield on biomass from serine [Cells/mmol]
$Y_{x,trp}$	: Yield on biomass from tryptophan [Cells/mmol]

Greek letters

β	: Constant
ϵ_2	: Endoplasmic reticulum glycosylation efficiency factor
μ	: Specific growth rate [1/h]
$\mu_{a,d}$	: Specific transition rate from early apoptotic to late apoptotic state [1/h]
$\mu_{a,LAC}$	: Specific apoptosis rate from lactate toxicity [1/h]
$\mu_{a,STV}$	: Specific apoptosis rate from starvation [1/h]
μ_d	: Specific death rate [1/h]
$\mu_{d,LAC}$	: Specific death rate from lactate toxicity [1/h]
$\mu_{d,max}$	: Theoretical maximum death rate [1/h]
$\mu_{d,STV}$	: Specific death rate from starvation [1/h]
μ_{lim}	: Specific death rate from nutrients limitation [1/h]
μ_{max}	: Theoretical maximum growth rate [1/h]
μ_{tox}	: Specific death rate from toxicity [1/h]
μ_a	: Specific apoptosis rate [1/h]
θ_i	: Yield of ATP from amino acid i [mmol/mmol]
λ	: Unit conversion factor [mg mAb/ mAb molecule]
Υ_1	: Constant for antibody production [h]
Υ_2	: Constant for antibody production

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List of Publications

Journal publications

- 1) Papathanasiou, M., Quiroga-Campano, A., M., Mantalaris, A., Pistikopoulos, “Advanced multi-parametric Model Predictive Control (mp-MPC) strategies towards integrated continuous biomanufacturing”. Submitted to Biotechnology Progress.
- 2) Quiroga-Campano, A., Papathanasiou, M., Mantalaris, A., Pistikopoulos, “A Predictive Model for Energy Metabolism and ATP Balance in Mammalian Cells: Towards the Energy-Based Optimisation of mAbs Production”. Computer Aided Chemical Engineering. Elsevier. 2016. 38. p:1581–1586.
- 3) Papathanasiou, M., Quiroga-Campano, A., Oberdieck, R., Mantalaris, A., Pistikopoulos, “Development of Advanced Computational Tools for the Intensification of Monoclonal Antibody Production”. Computer Aided Chemical Engineering. Elsevier. 2016. 38. p:1659–1664.

Journal publications in preparation

- 4) Quiroga-Campano, A., Mantalaris, A., Pistikopoulos, “Energy Medium design: A new platform for medium development”.
- 5) Quiroga-Campano, A., M., Mantalaris, A., Pistikopoulos, “ATP balance in Mammalian Cells”.

List of Publications

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- 1) Quiroga-Campano, A., Papathanasiou, M., Mantalaris, A., Pistikopoulos, E., (2016) “Energy-based medium design for mammalian cell fed-batch cultures”. AIChE Annual Meeting (San Francisco, CA, USA). Oral presentation.
- 2) Quiroga-Campano, A., Papathanasiou, M., Mantalaris, A., Pistikopoulos, E., (2016) “A predictive model for energy metabolism and ATP balance in mammalian cells: Towards the energy-based optimisation of mAbs production”. European Symposium on Computer-Aided Process Engineering (Portorož, Slovenia). Oral presentation.
- 3) Quiroga-Campano, A., Papathanasiou, M., Mantalaris, A., Pistikopoulos, E.(2016) “A predictive model for energy metabolism in mammalian cells”. Cell Culture (London, UK). Oral presentation.
- 4) Papathanasiou, M., Quiroga-Campano, A., Mantalaris, A., Pistikopoulos, E., (2015) “Advanced control strategies towards the intensification of monoclonal antibody production”. AIChE Annual Meeting (Salt Lake City, UT, USA). Oral presentation.
- 5) Papathanasiou, M., Quiroga-Campano, A., Mantalaris, A., Pistikopoulos, E., (2015) “Advanced computational tools to enhance continuous monoclonal antibody production”. Integrated Continuous Biomanufacturing II, (San Francisco, CA, USA). Oral presentation.

CHAPTER 1: Introduction

1 Introduction

In recent decades, the pharmaceutical industry has developed complex biological therapeutic drugs to meet medical needs of patients. In this respect, biotechnology has been the key discipline to produce intricate bio-products, enabling the manipulation and modification of living cells or organisms to create peptides, proteins, hormones, nucleic acids, blood factors, vaccines, antibodies, and other large molecules that cannot be synthesised by purely chemical processes. Monoclonal antibodies (mAbs) are part of this extraordinary group of new biologics and exhibit remarkable properties such as high homogeneity, specificity and affinity, and long elimination half-life in the human body. These properties make them suitable for a wide range of novel diagnostic and therapeutic applications; consequently, the mAbs market has been continuously growing and they are the highest selling class of biologics (Aggarwal, 2014). Additionally, the biopharmaceutical industry forecasts approximately 70 mAbs approved by 2020 with a global value of \$125bn (Ecker et al., 2015), and the most prominent pharmaceutical companies are investing about the 20% of their annual revenue in R&D, mainly in the development of new mAbs (Research in China, 2014).

Mammalian cell systems have been extensively used as manufacturing platforms to produce mAbs due to their capacity for human-like post-translational modifications (PTM) identical to native endogenous proteins, including glycosylation, the formation of disulphide bonds and proteolytic processing. These modifications are known to be essential for mAbs' pharmacokinetic and pharmacodynamic properties (Berlec & Štrukelj, 2013). Despite their successful entry in the biomanufacturing industry, mammalian cell cultures face significant challenges such as low yield, slow growth and high media costs. To enhance the expression of recombinant proteins using this platform, different approaches have been implemented such as cell engineering, vector design, screening tools, optimisation of culture medium composition and operation conditions, and development of new control strategies. Current optimisation strategies are typically empirical requiring a multitude of expensive, intensive and time-consuming experiments. Model-based approaches, such as model-based optimisation

strategies, seek to reduce unnecessary experimentation by rationally identifying feeding regimes to maximise mAbs titre in mammalian cell cultures (Kiparissides et al., 2015). However, existing feeding strategies rely mainly on glucose and glutamate supply, neglecting the exhaustion of other essential amino acids, which are the building blocks of the product. In addition, culture medium development has been based on stoichiometric balances considering only cell composition or metabolic profiling where the medium was designed based on the exhaustion of a limited number of nutrients (Xie & Wang, 1996; Sellick, 2011). Alas, the above-mentioned studies neglect the cell's energy requirements not only for maintenance and proliferation but also for mAbs production.

Since 1942, when Monod linked cell growth with the concentration of an energy source (Monod, 1942), and in 1960, when Bauchop & Elsdén linked cell growth to energy supply, adenosine triphosphate (ATP) has been linked to cell proliferation (Bauchop & Elsdén, 1960). Later, in 1969, Kilburn demonstrated the impact of maintenance energy demand in mammalian cell cultures, considering ATP usage in all major reactions that occur within the cell (Kilburn, 1969). These macroscopic concepts of cell yield and maintenance of energy were defined by Roels in the first mathematical model for the energetics of cell growth, where ATP consumption was a linear function of the specific growth rate (Roels, 1980). On the other hand, energy production was only linked to the oxidation of one substrate, glucose, until 1979 when Reitzer demonstrated that glutamine was the major source of energy in HeLa cells cultures and Zielke et al. calculated the contribution of glutamine oxidation to the energy requirement, which ranged between 30 and 50% in mammalian cells (Zielke et al., 1984). From this point, energy metabolism was established as a net of reactions that involve a wide number of nutrients including carbohydrates, lipids and amino acids as source of energy. The study of energy metabolic pathways led to the development of mathematical models to estimate energy production and consumption to establish the ATP balance in cell cultures. Xie & Wang developed a detailed material-balance model of animal cell metabolism based on stoichiometric reactions combined with a metabolic networks, using experimental data from batch and fed-batch cultures of hybridoma cells. In this attempt, the estimated biosynthetic ATP demand was

lower than the reciprocal ATP yield determined by the relationship between the specific ATP production rate and cell growth rate. Additionally, it missed non-growth-associated ATP demand, such as maintenance and product biosynthesis. Consequently, the final ATP balance was not achieved (Xie & Wang, 1996a).

In this work, the first dynamic model that predicts the ATP content in mammalian cell cultures is presented. The model considers energy demand for proliferation, maintenance and production, in GS-NS0 cell cultures producing cB72.3 mAbs in two different culture medium compositions, DMEM (10% serum) and serum-free CD-Hybridoma medium; and in two operation modes, batch and fed-batch. The model describes growth kinetics, glucose and amino acids metabolism, metabolic shifts, mAbs secretion and ATP production and consumption. The model accurately predicts the number of viable, apoptotic and dead cells, and the concentration of ATP, glucose, amino acids and lactate. Additionally, the experimentally validated model was used to design a supplemental medium that was subsequently used to implement an optimised fed-batch schedule, which maximised the mAbs production and extended the longevity of the culture. The integrated model-based approach presented here has the potential to be applied for media development, upstream optimisation and the development of control strategies for a wide range of biopharmaceutical products.

CHAPTER 2: Literature review

2 Literature Review

This chapter covers the scientific literature related to the biological system at hand, modelling techniques applied and computational tools used in the development of the present work. Firstly, mAbs technology and its production platforms are discussed. Then, energy metabolism in mammalian cells is reviewed in depth. Finally, an overview of mathematical model development methods, model-based optimisation techniques and control strategies is presented.

2.1 Monoclonal antibodies

The main function of the immune system is to protect animals from pathogens, biological or chemical substances that cause diseases in the host. The reactions of the host against the pathogen can be non-adaptive (physical, biochemical and cellular barriers) or adaptive defence mechanisms. The adaptive immunity includes a variety of cells and molecules involved in the degradation and eradication of harmful agents. The most important components of the immune system are lymphocytes, which are cells that synthesise Y-shaped glycoproteins called immunoglobulin or antibodies.

Antibodies consist of a symmetrical structure of two identical light and two identical heavy chains (H_2L_2), held together by strong non-covalent forces as well as disulphide bonds. Both chains consist of a constant domain (C) and a variable region (V) (Goding et al., 1996). Heavy chains vary between different classes and subclasses of antibody; IgG (Monomer), IgM (Pentamer), IgA (Dimer), IgE (Monomer) and IgD (Monomer), which have gamma, mu, alpha, epsilon and delta heavy chains, respectively. There are also two types of light chains, κ and λ . These different subclasses have specialised roles in immunity. These help to increase the diversity of immune-recognition by antibodies (George, 2000).

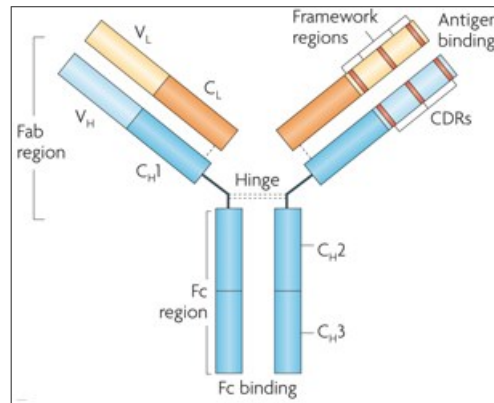


Figure 2.1: Basic structure immunoglobulin (Adapted from Hansel et al., 2010).

Immunoglobulins link an antigen and the immunological effector through the fragment antigen-binding site (Fabs) and fragment crystallisable (Fc), respectively. The enormous diversity of antibodies allows the immune system to recognize an equally wide variety of antigen shapes and sizes, while retaining a covalent link between the Fab domains and the conserved Fc region that interacts with a limited number of effector systems (Saphire et al., 2002). The hinge region, between the C_H1 and C_H2 (Figure 2.1), contains a single N-linked glycan at Asn²⁹⁷ in each of the two heavy chains, in IgG forms. These glycosylations play an important role modulating the binding affinity of the Fc receptor and conferring specific pharmacokinetic and pharmacodynamic properties to mAbs. Accordingly, the control of the mAbs glycosylation pattern is a critical requirement to ensure the safety and effectiveness of mAbs used as therapeutic drugs (Liu, 2014; Liu, 2015).

mAbs are one of the most important products of the biopharmaceutical industry due to their remarkable antigen-binding specificity and affinity properties, which allow their use in diagnosis and human therapy. The initial clinical studies with mAbs identified several areas that promise to make significant contributions, treatment of diseases such as neoplastic disease, lymphoma, breast cancer, inflammatory autoimmune diseases, leukaemia, asthma, psoriasis, Crohn's disease, transplant rejection and infectious diseases (Breedveld, 2000; George, 2000).

2.2 Product and expression systems

MAbs are derived from a single cloned B cell. Consequently, they are highly specific for a particular epitope, part of an antigen that is recognized by the immune system. The first technique applied for the development of new mAbs is the hybridoma technique (Kohler & Milstein, 1975). The first step of this protocol is to inject an animal with an antigen containing the specific epitope of interest. The immunization of the animal with the antigen stimulates B cells, which produce antibodies that recognize that antigen. Each B cell will produce a single type of antibody. The second step is to isolate the cells from the animal's spleen and mix them with myeloma cells, a type of cancer cell that grows continuously. The addition of chemical agents such as polyethylene glycol causes the two type of cells to fuse together to form immortalized hybridoma cells. The mixture of B cells, myeloma cells and hybridoma are cultured under conditions that allow the selection of the hybridoma cell. The third step is the screening where the hybridoma cells are separated into individual wells and tested for the ability to produce an antibody with a specific affinity. The final step is the amplification of the culture by clonal expansion and maintained indefinitely as cell lines that produce a unique monoclonal antibody (Secher & Burke, 1980).

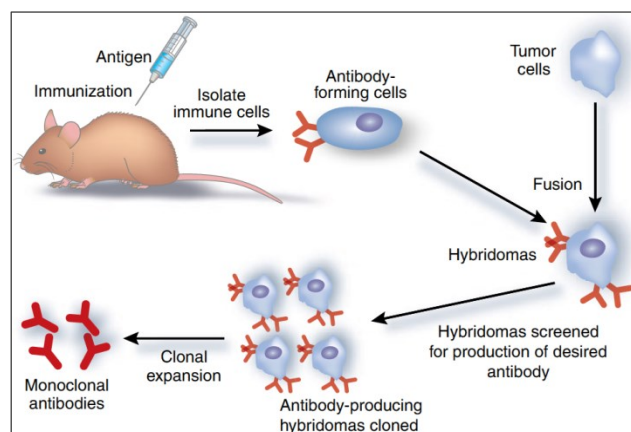


Figure 2.2: Production of mAbs by the hybridoma method (adapted from Michnick & Sidhu, 2008).

The choice of the expression system for the selected monoclonal antibody depends on the nature of the molecule, which can be a fragment or complete recombinant antibody.

The production of a selected intact immunoglobulin or mAb fragment (Figure 2.3), in high yield, is the main bottleneck in the product pipeline (Holliger and Hudson, 2005). Accordingly, the selection of the most productive transgenic host is critical (Zhu, 2012).

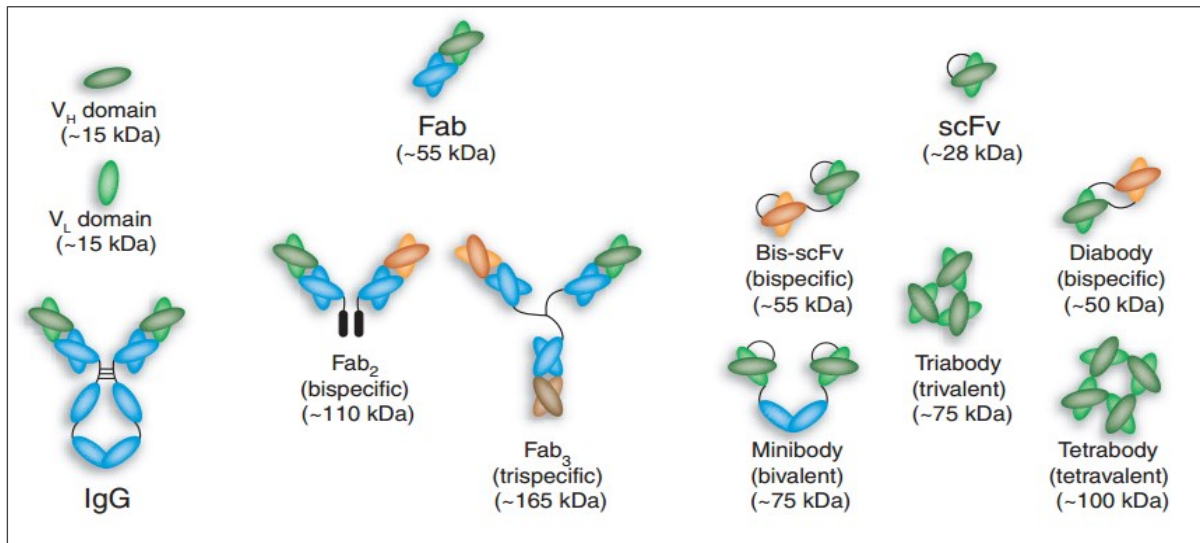


Figure 2.3: Schematic representation of different antibody formats, showing intact classic IgG molecule and a variety of antibody fragments (adapted from Holliger and Hudson, 2005).

2.2.1 Hosts

There is a wide variety of potential hosts for the production of mAbs including bacteria, yeasts, mammalian cell lines, plants, insects and animals. Bacterial expression systems are one of the most commonly used for producing recombinant antibody fragments, Fab and scFv fragments, diabodies and V domains (Figure 2.3)(Holliger and Hudson, 2005). This is due to the advantages of low transfection and clone selection costs, quick production cycle, easy in-process control and high productivity of these systems. Unfortunately, bacteria have a primitive machinery for folding and secretion of complex proteins, being unable to properly produce eukaryotic proteins. In the case of mAbs, which contain cysteine residues, disulphide bonds mismatch leads to the accumulation of insoluble and inactive forms of the product in the cytoplasm of bacteria. The active protein recovery, from inclusion bodies, requires complete

denaturation and renaturation of the product, a process that reduces the final yield of the total expressed protein. Nevertheless, simple recombinant proteins have been successfully produced in *E. coli* (Berlec & Štrukelj, 2013).

Table 2.1: MAbs production hosts (Schirrmann et al., 2008)

Host	Organisms	Advantages	Disadvantages
Bacteria	<i>E. coli</i> (Gram-negative) <i>Bacillus subtilis</i> (Gram-positive) <i>Lactobacillus paracasei</i> (Gram-positive)	High yields of antibody fragments	Formation of non-functional aggregates. Non-efficient denaturation and refolding. Production of non-glycosylated forms of antibody.
Yeast	<i>Saccharomyces cerevisiae</i> <i>Pichia pastoris</i>	Short generation time Ease of genetic manipulation Robustness Simple medium requirements	Premature transcriptional termination. Proteolysis of secreted proteins during high cell density fermentation. Inappropriate glycosylation of human glycoproteins (hyper-glycosylation)
Fungi	<i>Trichoderma reesei</i> <i>Aspergillus niger</i>	Ease of molecular cloning Simple upscaling Economic feasibility Functional IgG	Difficult transformation Proteolytic degradation Pathogenic contaminants
Insects cells	Cell lines Sf-9 and Sf-21 of <i>Spodoptera frugiperda</i> . Cell line High Five™ of <i>Trichoplusia</i> .	Large insertion capacities Post-translational modifications Economic feasibility	Poor technology development Insoluble aggregates formation. Proteolytic degradation
Mammalian cells	Chinese hamster ovary cells (CHO). Mouse myeloma NS0 cells. Baby hamster kidney (BHK) cells Human embryonic kidney (HEK-293) cells.	Correct folding, secretion and posttranslational modifications. Functional IgG forms	Expensive medium and cultivation requirements. Risk of unknown viral contamination.
Transgenic plants	<i>Agrobacterium tumefaciens</i> <i>Nicotiana tabacum</i>	Easy capacity expansion Low production costs. Easy storage (seed)	Expensive downstream process.
Transgenic animals	Goat Rabbit Mouse	Highly scalable	Complex transformation. Potential product immunogenicity (different glycosylation patterns)

Mammalian or plant cells are equipped with the transcriptional and translational machinery to produce large antibodies. Additionally, these cells have a sophisticated refolding mechanisms that operate in the endoplasmic reticulum and maturation compartments that are part of the Golgi apparatus (Verma et al., 1998). In animals, the most popular expression system is animal fluids, in particular, milk. This system shows high productivity, and harvesting is possible without killing the animal (rabbits, cows, pigs, sheep and goats). However, milk can carry human pathogens contamination and the complexity of its composition makes the purification process a laborious task (Zhu, 2012). In plants, unlike transgenic animals, there are minimal risks of contamination with human pathogens. However, the land requirements for cultivation and large-scale processing infrastructure for the product purification make this alternative expensive and laborious (Fischer et al., 2003).

The most prominent candidates for efficient expression of antibodies are mammalian cell lines such as Chinese hamster ovary cells (CHO-DHFR and CHO-GS), mouse myeloma lymphoblastoid like (NSO-GS and Sp2/0) cells, human embryonic kidney 293(HEK 293) cells and baby hamster kidney cells (BHK-21).

2.2.2 GS-NS0 expression system

The initial point in the development of NS0 cells line was the discovery of the induction of tumours, which secrete IgG1, by the injection of mineral oil into the intra-peritoneal region of BALB/c female mice. The tumour cells, MOPC21, were used to establish a continuous tissue culture line, which was re-cultured until they maintained a constant doubling time. The heterogeneous population of IgG1 secreting cells was called P3K. These cells were cloned giving rise to P3-X27, which derived 289-16 and P3-X27 cell lines. The 289-16 cell line was treated and cloned until they synthesise neither the original IgG1 nor its light or heavy chains, deriving the NS0 cell line (Barnes et al., 2000).

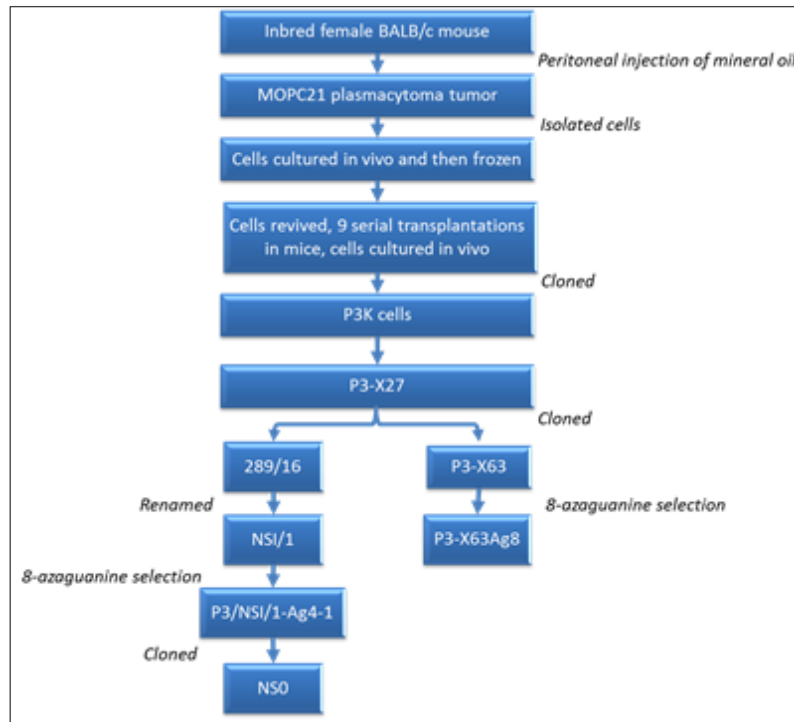


Figure 2.4 The sequence of events that led to the development of NS0 cell line (adapted from Barnes et al., 2000).

The NS0 cell line has a powerful internal machinery that allows the stable incorporation and expression foreign DNA. Furthermore, NS0 cells have the ability to process and secrete antibodies and polymeric proteins, grow in suspension cultures and exhibit stable and high production levels. Additionally, NS0 cells can easily adapt to a variety of derivation, selection and production environments. Therefore, they have been used as fusion partners for the production of hybridoma or transfectoma cells producing mAbs for a number of years (Barnes et al., 2000; Zhang 2010).

NS0 myeloma cells have been transfected with plasmids to produce different biologics and the most successful expression system is the glutamine synthetase gene expression system (GS-System).

Glutamine Synthetase (GS) is the enzyme that catalyses the reaction to synthesise glutamine from glutamate and ammonium. NS0 cells present insufficient GS activity, i.e., NS0 cells are phenotypically considered glutamine auxotroph and consequently they struggle to

survive without added glutamine. The transfection of NS0 cells with a plasmid vector that carries the GS gene and the product gene, in addition to methionine sulfoximine supplementation to inhibit endogenous GS activity, is one of the most successful and robust over-expression systems of biologics (Bebbington et al., 1992).

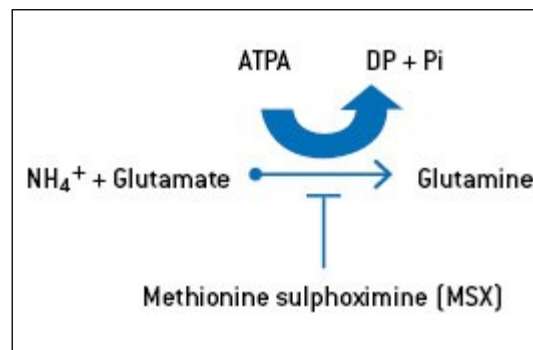


Figure 2.5: Principles of the GS gene expression system (Modified from Barnes et al., 2000)

GS-NS0 cells are highly efficient to produce, fold and secrete antibodies, and they have been adapted to grow in suspension, serum-free conditions, protein-free and animal-free cultures, which is particularly beneficial for large-scale production and attractive for the pharmaceutical industry (Spens & Häggström, 2005-2007; Zhou et al., 1997; Burky et al., 2006).

2.2.3 Mammalian cell platforms and challenges

The production of heterologous recombinant proteins in any expression system imposes a metabolic burden on the host cells. Comparisons between parental and derived clones have uncovered significant changes in cells metabolism, apoptosis and endogenous gene expression upon production of heterologous proteins. In some cases, the apparent growth rate is higher in transfected cells due to the expression of anti-apoptotic proteins (Ifandi & Al-Rubeai, 2005). In other cases, the apparent growth rate decrease and the viability drop faster in the transfected cells due to the earlier depletion of amino acids (building blocks of the heterologous protein) and reduction of the energy availability used for the product assembly (Niklas et al., 2011a;

Niklas et al., 2013). Wilkens & Gerdzen demonstrated that improvements in central cell metabolism through cell engineering, overexpressing or knocking down key genes, allow the cells to achieve similar cell densities than the parental cell, while using glucose efficiently, reducing lactate production and presenting higher ATP availability (Wilkens & Gerdzen, 2015).

2.3 Energy metabolism in mammalian cells

Mammalian cells need energy for proliferation and maintenance, with host cells having an extra requirement for the product biosynthesis. The primary source of energy is derived from the breakdown of nutrients into smaller molecules, a process also known as catabolism. The main metabolic pathways associated with the energy production, from nutrients such as glucose and glutamate, are the glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA cycle) and glutaminolysis, each covered in more detail below.

2.3.1 Glycolysis

Glycolysis is an ancient metabolic pathway, which has been preserved in evolution and consists of a linear and enzyme-catalysed chain of reactions that occurs in the cytoplasm and convert glucose into two molecules of pyruvate, each with three carbon atoms, and produce two adenosine triphosphate (ATP) molecules.

In normal mammalian cells, under anaerobic conditions, pyruvate is reduced to lactate, which is excreted into the extracellular medium through mono-carboxylate transporters (MCTs). On the other hand, in oxygen presence, glucose completes its oxidation and pyruvate enters the mitochondria to be oxidised to acetyl-CoA, which reacts with oxaloacetate to produce citrate, starting the TCA or Krebs cycle (Mulukutla et al., 2010; Zheng, 2012). In 1926, Otto Warburg observed that cancer cells consume high levels of glucose through glycolysis, even at normal oxygen tension, as consequence of the deregulation of the central metabolism (Warburg et al., 1926). Mammalian cell lines, used for therapeutic drugs production, are

derived from tumour cells, which rely on glycolysis to produce energy instead of completing the glucose catabolism through oxidative phosphorylation (OXPHOS).

The lack of progression of pyruvate into the TCA cycle results in an inefficient and inadequate supply of energy: firstly, because pyruvate still contains chemical energy that could be released by further oxidation, and secondly because pyruvate accumulation generates lactate formation and it has been reported that lactate inhibits cell growth due medium acidification and osmolality increase (Ozturk et al., 1991; Cruz et al., 2000).

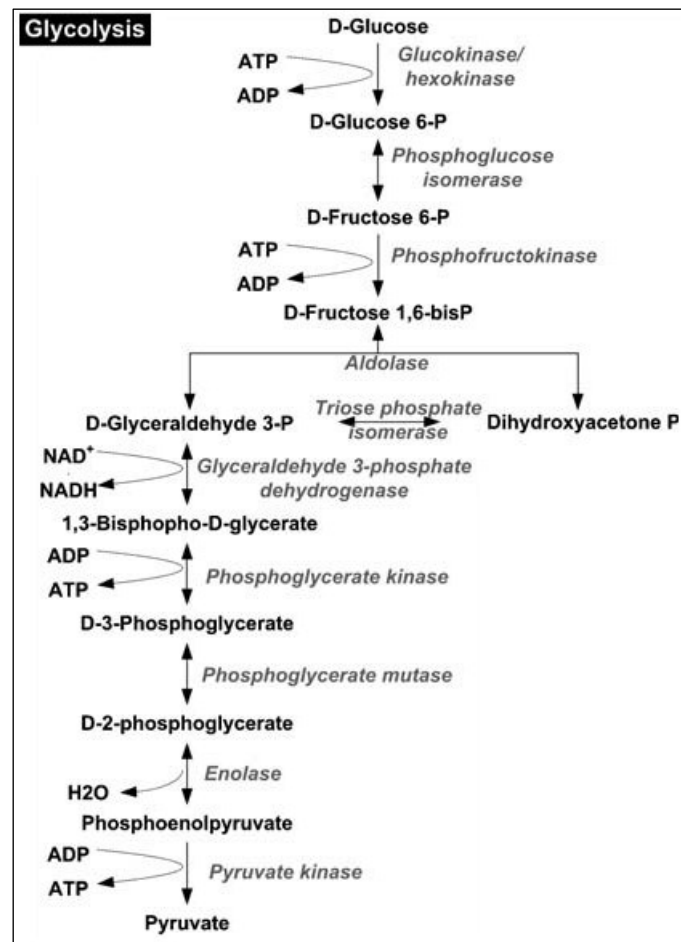


Figure 2.6: Glycolysis (adapted from Wamelink et al., 2008).

2.3.2 Pentose Phosphate Pathway

One way to produce more energy from anaerobic pathways is to follow the pentose phosphate pathway (PPP), which occurs in the cytosol. PPP reactions are separated in two segments, oxidative and non-oxidative phases. In the oxidative phase, glucose-6-P is irreversibly oxidised, generating nicotinamide adenine dinucleotide phosphate (NADPH), a molecule that plays an important role in energy production and in the oxidative stress response (Wang et al., 2014). In the non-oxidative phase, the pentose-5-phosphate is reversibly converted to intermediates of the glycolytic pathway through a series of rearrangements (Lennarz W., 2013).

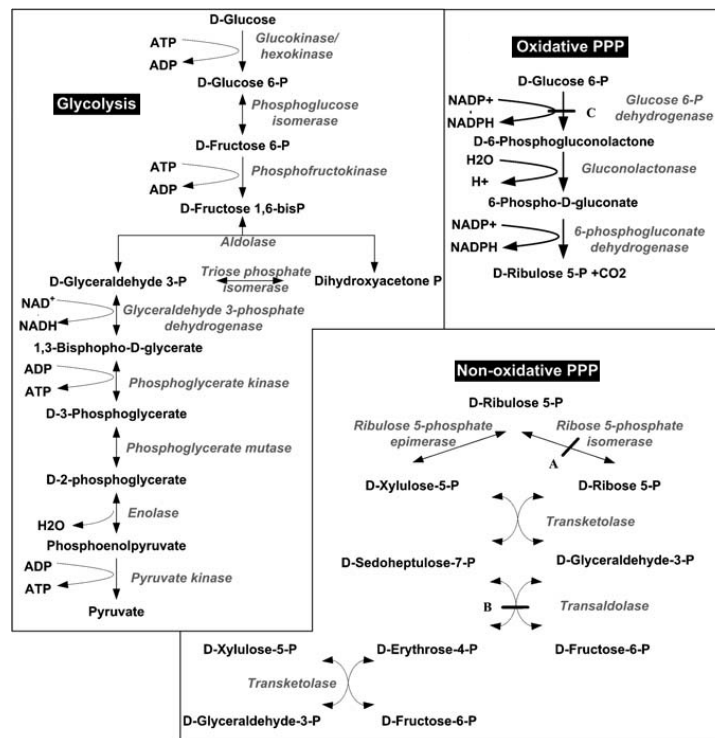


Figure 2.7: Pentose Phosphate Pathway (adapted from Wamelink et al., 2008)

In mammalian cells, 10–20% of glucose oxidation occurs via the PPP and the 80–90% of glucose oxidation is via glycolysis, producing lactate accumulation or entering into the TCA cycle (Wamelink et al., 2008).

2.3.3 Tricarboxylic acid cycle

In 1937, Hans Krebs and William Johnson introduced one of the key elements to complete the energy metabolism picture - the TCA cycle (Krebs & Johnson, 1937). This process starts with the incorporation of pyruvate into the mitochondrion, where it is transformed to acetyl-CoA by pyruvate dehydrogenase. Subsequently, the acetic acid subunit of acetyl-CoA is combined with oxaloacetate to form a molecule of citrate, where the cycle starts. In the cycle, three different energy precursors are produced: three molecules of nicotinamide adenine dinucleotide (NADH), one molecule of guanosine triphosphate (GTP) and one molecule of reduced flavin adenine dinucleotide (FADH_2). These are ultimately converted into ATP (Ngo et al., 2015).

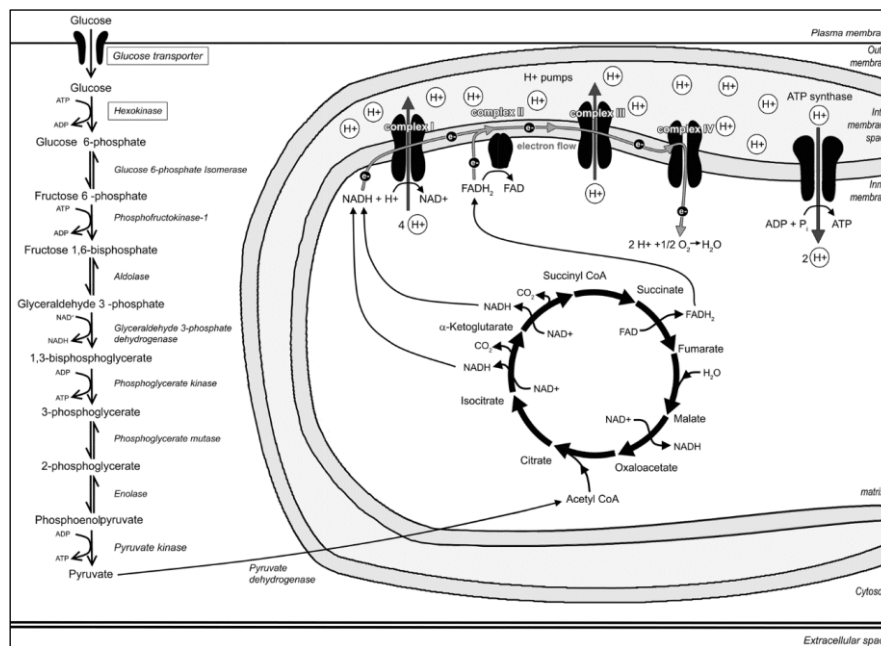


Figure 2.8: Glycolysis and tricarboxylic acid cycle (adapted from Ngo et al., 2015).

In tumour cells, the lack of aconitase, the enzyme that catalyses the stereo-specific isomerization of citrate to isocitrate, truncates the TCA cycle. Therefore, acetyl-CoA is exported from the mitochondrial matrix to the cytoplasm, and there it is used as a precursor for the synthesis of fatty acids, supporting biosynthesis and proliferation (Kroemer & Pouyssegur,

2008). In addition, tumour cells can metabolize glucose through the PPP to generate NADPH and ensure antioxidant defences against a hostile microenvironment. Furthermore, the metabolic reprogramming of these cells introduce the overexpression of glutaminase, the amidohydrolase enzyme that generates glutamate from glutamine, and glutamate is incorporated into the mitochondrion as an alternative substrate for the TCA cycle for ATP production. This pathway is called glutaminolysis (Yuneba et al., 2007). The activation of glutaminolysis and other metabolic changes, induced by oncogenes, may provide growth and proliferative advantages over normal cells (Yuneva, 2008).

2.3.4 Glutaminolysis

Glutamine can provide energy by aerobic oxidation that accounts for more than the 50% of the ATP produced, in high concentrations of glucose (Reitzer et al., 1978; Fitzpatrick et al., 1993).

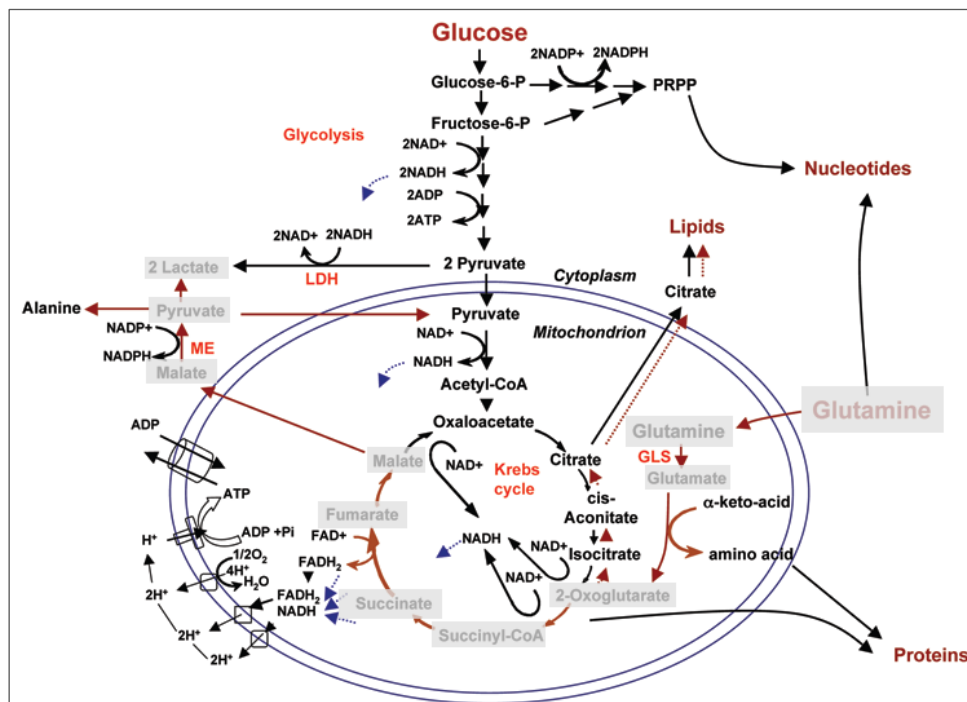


Figure 2.9: Glycolysis, tricarboxylic acid cycle and glutaminolysis (adapted from Yuneva et al., 2008).

Glutamine is not the only amino acid that can be incorporated into the TCA cycle. It has been reported that arginine, proline and histidine can be incorporated through α -ketoglutarate formation (Guoyao & Morris, 1998). Valine, methionine and isoleucine are incorporated through succinyl-CoA production (Wanders et al., 2012), phenylalanine and tyrosine are oxidised to fumarate, asparagine and aspartate can be introduced into the cycle through oxaloacetate formation; and lysine, leucine and isoleucine can be used to synthesise acetyl-CoA (Xie & Wang, 1996).

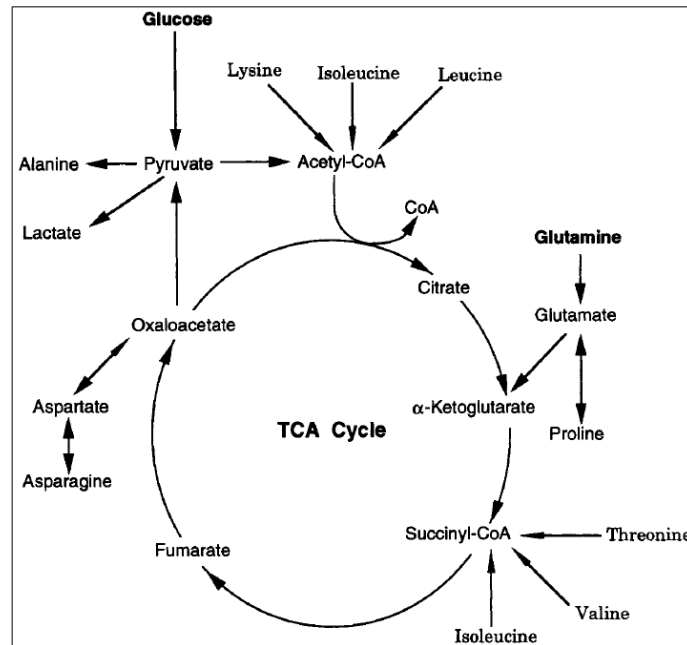


Figure 2.10: Schematic energy metabolism in mammalian cells (adapted from Xie & Wang, 1996).

The energy precursors produced by the amino acids metabolism are GTP, which transfers a phosphate group to ADP to form ATP; and NADH and FADH₂, high-energy molecules that are reduced by the inner mitochondrial membrane complexes that pump protons out into the mitochondrial intermembrane space producing a gradient. This gradient is the driving force to activate ATP synthase that generates usable energy in the form of adenosine triphosphate (Ngo et al., 2015).

2.3.5 Electron transport chain

NADH, which serves as a central intermediate in the processes previously described, is formed by the addition of a hydrogen nucleus and two electrons (a hydride ion, H^-) to nicotinamide adenine dinucleotide (NAD) leaving the hydride ion held in a high-energy linkage. Similarly, FADH_2 is formed by a molecule of flavin adenine dinucleotide (FAD) that accepts two electrons and two protons to become FADH_2 .

The electron transport chain (ETC) couples electron transfer between electron donors, NADH or FADH_2 , and an electron acceptor (O_2) with the transfer of H^+ ions (protons) across the mitochondrial inner membrane, building a proton gradient.

The established proton gradient built by the ETC drives the proton flow in ATP synthase that accompanies ATP synthesis from ADP and inorganic phosphate (P_i) (Yoshida et al., 2001).

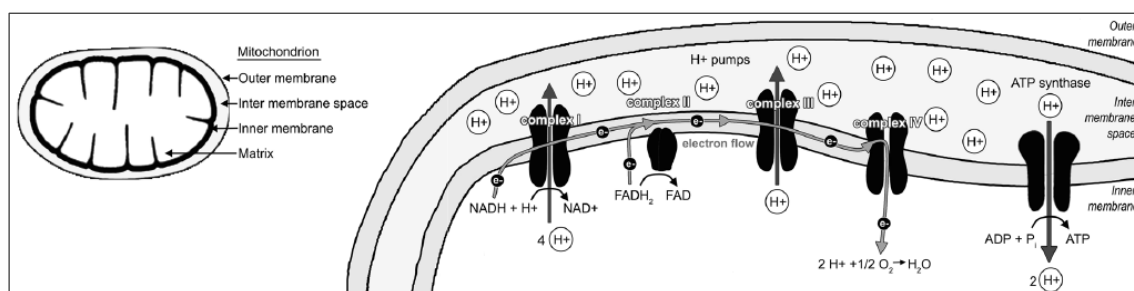


Figure 2.11: Glycolysis and tricarboxylic acid cycle (adapted from Ngo et al., 2015).

NADH produced in the cytoplasm cannot enter directly into the mitochondrial matrix to be re-oxidised. Therefore, to incorporate the reducing equivalents of NADH in the ETC, the cells use two transporters, one more efficient than the other. In the “malate-aspartate shuttle”, the efficient transporter, NADH reduces oxaloacetate in the cytosol producing malate, which is able to cross the membrane into the matrix, where it regenerates NADH by reducing NAD^+ , and then NADH donates electrons to the ETC where it can generate $2.5 \times \text{ATP}$, the same amount produced by mitochondrial NADH. The inefficient mechanism to use cytoplasmic NADH is the “glycerol 3-phosphate shuttle”, where the cytosolic NADH donates electrons to dihydroxyacetone-phosphate producing glycerol 3-phosphate, which deliver the reducing

power to FAD, generating only $1.5 \times \text{ATP}$ per NADH (Mulukutla et al., 2010; Nelson and Cox, 2013).

2.4 Adenosine Tri-Phosphate

Adenosine Tri-Phosphate (ATP) is a small coenzyme that consists of three components, one adenine base one ribose sugar and three phosphate groups. This molecule is the shared chemical intermediate linking cellular process that involves energy production and energy consumption. Energy is mainly produced by the ETC and primarily consumed by ATP hydrolysis. This reaction releases energy through the breakage of the phosphoanhydride bonds in ATP and it is catalysed by ATPase (standard free energy -30.5 kJ/mol) (Nelson and Cox, 2013).

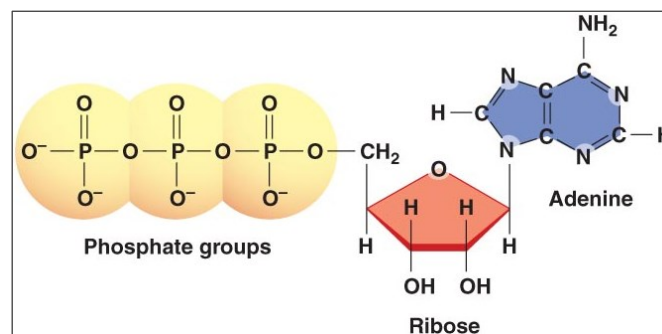


Figure 2.12: Adenosine tri-phosphate structure.

Energy production depends on the pathways previously described, which use nutrients such as glucose, glutamine and other amino acids to produce approximately 60%, 30% and 10% of the energy available, respectively (Xie & Wang, 1996b).

ATP availability is crucial to keep proliferation, production and viability at optimal levels in mammalian cell cultures. Bauchop & Elsdén concluded that the growth of an organism is a function of the energy yield of its catabolic processes; these findings related energy availability to the maximum cell density attainable, which is proportional to the maximum product concentration achievable (Bauchop & Elsdén, 1960). Kilburn et al. defined two types of energy

consumption: (i) biosynthetic consumption defined as the set of reactions related to the synthesis of monomers and the polymerization of cellular components required for proliferation; and (ii) maintenance consumption described as the set of reactions related to the reparation of degraded molecules or structures, molecules transport inside and outside the cell, active transport of ions against the concentration gradients and preservation of the internal cell organization (Kilburn et al., 1969). Stouthammer, in 1980, incorporated these definitions in a mathematical equation to express the overall rate of energy production.

Equation 2.1: ATP specific production rate (Stouthammer et al., 1980)

$$q_{x,ATP} = \frac{\mu}{Y_{x,ATP}} + M_{x,ATP}$$

Roels used this equation in a mathematical model for the energetics of cells growth on different substrates (Roels, 1980). This was the first attempt to balance ATP production and consumption equations. Xie & Wang published six papers, from 1993 to 1996, where they developed step-by-step a stoichiometric model for ATP balance in mammalian cells. First, they developed a mathematical model via analysis of nutrients requirements for proliferation, product biosynthesis and energy production. They used this model for medium design (Xie & Wang, 1994a). Then, they designed a fed-batch culture strategy and derived a material balance of nutrients (Xie & Wang, 1994b, Xie & Wang, 1994c, Xie & Wang, 1996b), and finally, developed a simplified metabolic network to calculate the intracellular ATP level. The energy metabolism was thoroughly studied but the challenge was unmanageable, the ATP demand was not in balance with the production, and the final ATP mass balance was not achieved (Xie & Wang, 1996a).

The number of ATP molecules is relatively constant inside viable cells after the adaptation period or lag phase (Sonderhoff et al., 1991). Moreover, culture viability crucially depends on ATP availability since it determines whether the cell death is via apoptosis or necrosis (Eguchi et al., 1997).

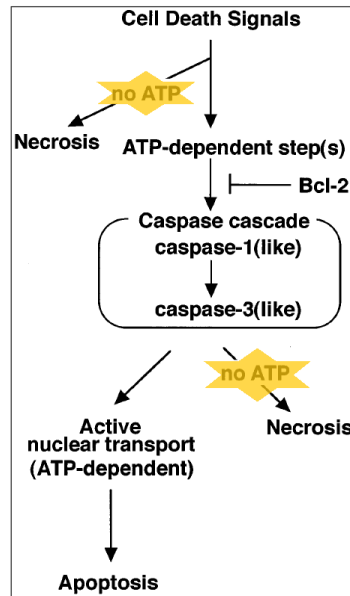


Figure 2.13: Death fate depending on intracellular ATP levels (adapted from Tsujimoto, 1997)

Apoptosis, or programmed cell death, is a highly controlled, energy-dependant and reversible process, whereas necrosis is uncontrolled, energy-independent and irreversible. Therefore, due to the apoptosis energy requirements, under ATP depletion, the cells die by necrosis (Figure 2.13), eliminating the possibility of viability recovery and reducing the productivity of the culture (Eguchi et al., 1997).

These findings demonstrate the importance of keeping the optimal ATP content in mammalian cell cultures for mAbs production. In order to keep the ATP content at a certain level, it is recommended to implement a culture strategy that constantly supply energy sources to cover the energy requirements of the host cells growing under the specific production platform conditions.

2.5 Operation modes: Batch, fed-batch and continuous

2.5.1 Batch cultures

In batch cultivation, the organisms are inoculated into a bioreactor under specific environmental conditions such as medium composition, agitation, aeration, pH and temperature, and the volume of the culture is constant since there is not medium addition or removal. The stages of a batch culture are characterized by the succession of variations in the apparent growth rate. The first stage, after inoculation, is the lag phase, where the growth rate is imperceptible and cells are adapting themselves to the new conditions. The second phase is the early exponential growth, a phase where the cells are fully adapted and they accelerate their growth reaching the next stage, exponential phase. In the course of the exponential growth phase, most of the cells are proliferating and so they consume nutrients at high rates. When nutrients and energy are scarce, the cells enter a retardation phase where the apparent growth rate decreases until it is zero, at which point it reaches the stationary phase. Afterwards, nutrients exhaustion or accumulation of toxic metabolites will lead the culture to the last phase, decline or death phase. The maximum concentrations of cells and product are directly linked to the concentration of energy sources in the culture medium at the beginning of the process (Monod, 1949).

In batch cultures, the culture medium composition is one of the most important considerations because it determines cell growth environment and physical conditions that have a crucial impact on proliferation, culture longevity, productivity as well as product quality (including glycosylation patterns). Moreover, the medium could stoichiometrically limit the maximum cell density (Xie & Wang, 1993a).

2.5.2 Fed-batch cultures.

In fed-batch cultures, one or multiple nutrients are fed into the system during cultivation. The most common approach to design a fed-batch culture is the development of a supplemental

medium followed by the design of a feeding strategy (Xie & Wang, 1993-1996; Zhang et al., 2004, Spens & Häggström, 2007; Shibuya et al., 2008).

The strategies for the development of supplemental medium varied from trial-and-error to stoichiometric models. Spens & Häggström (2007) established a chemically-defined, protein-free and animal-free supplemental medium for NS0 cells in fed-batch cultures by trial-and-error, progressively incorporating solutions of lipids, amino acids, vitamins and inorganic salts; and removing serum, replacing its most important components such as albumin (β -cyclodextrine), transferrin (ferric citrate), cholesterol (phosphatidilcholine plus synthetic cholesterol) and insulin (zinc). Zhang et al. and Altamirano et al., in 2004, used a more elaborate techniques designing the supplemental medium based on the nutrients specific consumption rate (Zhang et al., 2004; Altamirano et al., 2004). However, the most structured and sophisticated strategy was established many years before, in 1994, when Xie & Wang developed a stoichiometric model to design a supplemental medium to operate under low concentrations of nutrients and reduced toxic metabolites accumulation. The lack of information about the animal cell composition imposed the need for extra experimentation. Nonetheless, the efforts to implement the stoichiometric technique were successful. The final supplemental medium and feeding strategy led to a tight concentration range of amino acids throughout the culture (0.02-0.6 mM), extended the culture lifespan and increased the production 10-fold compared to the original batch operation mode. These results proved the potential of stoichiometric medium design to increase mAbs production. Later on, in 1996, the same group incorporated undialyzed fetal bovine serum (FBS) and trace metals to the supplemental medium extending the culture span 400 hours and producing 2,4 [g/L], increasing the mAbs production 47-fold compared to the original batch operation. Since then, stoichiometric medium design has been applied widely by different research groups (Xie & Wang, 1996b; Zhou et al., 1997a; Zhou et al., 1997b; Sauer et al., 2000; Burky et al., 2006; Li et al., 2009).

Once the supplemental medium composition is established, the next step is the design of a feeding schedule derived from a feeding strategy. The simplest and most common feeding

strategy attempts to avoid nutrients exhaustion, feeding the culture when any of its components is depleted (Xie & Wang, 1996b; Spens & Häggström, 2007; Shibuya et al., 2009). Sauer et al. applied a very different technique; they measured the concentration of glucose and fed the system to reach a set point or target concentration. This was the first successful control concept strategy applied in fed-batch cultures, but the constant nature of the set point and the dynamic behaviour of cell culture led to under and over-feeding of nutrients throughout the process (Sauer et al., 2000). Similar results were reported by Zhou et al., who developed a feedback nutrient feeding control strategy based on on-line oxygen uptake rate (OUR) measurements. In this case, the under-feeding led to starvation which triggered apoptosis, and the over-feeding induced accumulation of nutrients and toxic metabolites, increasing the osmolality, which reached toxic levels (>400 mOsm) (Zhou et al., 1997). Later on, to overcome the under/over-feeding issues, Zhang et al. and Altamirano et al. implemented feeding strategies based on the specific consumption rate of the nutrients to predict the nutrient requirements, using a simple mathematical equation, and supplying the precise amount of nutrients required by the cells (Altamirano et al., 2004; Zhang et al., 2004).

Supplemental medium design and feeding strategies have been commonly implemented by iterative trial-and-error processes. Nowadays, mathematical models and computational tools are used to assist these processes allowing the *in-silico* simulation of cultures under different medium compositions and diverse feeding schedules, reducing the number of experiments to be performed (Dhir et al., 2002; Kontoravdi et al., 2010; Kiparissides et al., 2015).

2.5.3 Continuous and perfusion cultures

In continuous and perfusion operation modes, fresh medium is added constantly. In continuous processes, the internal medium has the same compositions as the outlet, whilst in perfusion cultures, the cells are retained inside the bioreactor by an internal or external cell-retention device, and the removed broth is a cell-free outlet. In both configurations, the outlet contains the product in a relative low titre. The advantages of these systems are the steady state operation, reduced equipment size, streamlined process flow, low cycle times, and reduced

capital costs (Konstantinov & Cooney, 2014). One important consideration is the stability of the product as continuous and perfusion operations enable a significant decrease of the product residence time in the bioreactor.

Unfortunately, the implementation of biopharmaceutical process in continuous or perfusion requires high levels of on-line monitoring and control, which are currently not available.

Table 2.2: Advantages and disadvantages of different operation modes,
(Griffiths, 1992; Bibila & Robinson 1995)

Operation mode	Advantages	Disadvantages
Batch	Proven reliability Minimum control	Low productivity Changing conditions Toxic metabolite accumulation Cell debris accumulation
Fed-batch	Proven reliability Minimum control Extended culture period Flexibility for implementation in multipurpose facilities High volumetric productivity Reduction of waste	Changing conditions Toxic metabolite accumulation Contamination risk Cells debris accumulation
Continuous	Semi-homogeneous conditions Low toxic metabolites accumulation Less cell debris accumulation Enhanced production capability Reduced product hold-up Savings in equipment turn-around and the growing-up period.	Low yields Low titre Sophisticated control Increased start-up and cycle times Contamination risk High waste production
Perfusion	Semi-homogeneous conditions Low toxic metabolites accumulation Less cell debris accumulation Enhanced production capability Reduced product hold-up Savings in equipment turn-around and the growing-up period. High cell densities	Low titre Sophisticated control Increased start-up and cycle times Contamination risks High waste production Fouling of the retention device

2.6 Basal and supplemental medium design

Culture medium components include carbon sources, amino acids, vitamins, nucleotides, inorganic salts, buffers and serum. Some of these components are generally abundant, while others are often present in small amounts, e.g., metals that are essential for biological functions even though they are consumed at a low level (Xie & Wang, 1993a; Spens & Häggström, 2007; Shibuya et al., 2008). The complex nutritional requirements of animal cells impose challenges to the design of a medium to achieve optimal cell growth.

In 1954, Eagle proved that twelve and seven amino acids were essential and non-essential, respectively, for mammalian cell's survival (Eagle, 1954). In 1955, Eagle's group developed a minimal essential medium (MEM) and in 1958 a number of carbohydrates (D-fructose, D-galactose, D-mannose, D-ribose, trehalose, turanose) were found to be substitutes for glucose in supporting the growth of animal and human cell lines (HeLa, KB and H.Ep.) (Eagle et al., 1955-1958). Later, in 1994, Xie & Wang developed a culture medium and feeding strategy for fed-batch cultures of hybridoma cells CRL-1606, based on cell composition and stoichiometric analysis (Xie & Wang, 1994a). Shibuya, in 2008, generated a serum substitute for fed-batch hybridoma cells cultures (Shibuya et al., 2008) and Zhang and Robinson developed an animal-free, protein-free and chemically-defined medium for NS0 cell cultures (Zhang and Robinson, 2005). Currently, a vast variety of culture media is available for different mammalian cell cultures.

At the early stage of medium development, the goal is to determine all the essential nutrients for in vitro cell survival. The nutrient concentrations in the conventional medium are primarily determined experimentally through trial and error. This is a commonly used strategy for medium formulation and optimisation, which includes the selection of a basal formulation that is supplemented with particular components to cover the specific requirements of a given cell line (Zhang, 2005). The most abundant components of the basal medium are amino acids (Xie & Wang, 1996b; Spens & Häggström, 2007).

2.6.1 Special medium requirements

2.6.1.1 Amino Acids

Eagle described the essential amino acid for mouse fibroblast (L strain), their minimal vitamin and salt requirements, the important role of glutamine and the function of serum proteins. In his studies, a single amino acid was omitted from the medium and after a period (1 to 3 days), obvious degenerative changes occurred in the microscopic cells appearance and the cultures soon died when the amino acid removed was an essential amino acid. However, these cytopathologic effects of an amino acid deficiency were completely reversible. On the addition of the missing amino acid into the medium, the normal cellular structure was re-established within 1 to 3 days. Through this methodology, twelve amino acids proved to be essential for survival and growth: arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine. Additionally, seven amino acids were not found to be essential: alanine, aspartate, glutamate, glycine, hydroxyproline, proline and serine. In absence of these amino acids, the cells continued to proliferate after repeated subdivisions over a period of at least 6 weeks (Eagle, 1954).

In a subsequent research Eagle studied five human cell lines: a culture of cervical carcinoma cells, HeLa, and a cloned subline, HeLa-X3, the KB strain (carcinoma of nasopharynx), normal intestinal epithelium, and conjunctiva. The results showed that the intracellular concentration of valine, lysine, and threonine should exceed a critical threshold level (0.01 to 0.04[mM]) in order to initiate protein synthesis at a rate consistent with cellular survival and growth (Eagle et al., 1961).

In NS0 cells, glutamate, asparagine, histidine, leucine, isoleucine, methionine, valine and cystine have been noted to be rapidly exhausted (Keen & Hale, 1996). GS-NS0 cell lines, which are transfected with GS-containing vectors, do not require exogenous glutamine in the media to survive, but they require asparagine, as a metabolic source of ammonia, which is combined with glutamate to produce glutamine (Barnes et al., 2000). This is an example of how some non-essential amino acids may become essential depending on the selected expression system.

2.6.1.2 Serum

The key role of serum is to supply growth factors, hormones (insulin), binding proteins (albumin and transferrin) and components that provide hydrodynamic stress protection and protection against pH fluctuations (Xie & Wang, 1993a). However, serum also contains high concentration of proteins that hinder the downstream processing of the biologics and increasing the cost of the purification stages (Hesse & Wagner, 2000).

Serum, as an animal-origin substance, presents high physiological inconsistency that increases the variability from batch to batch in the production process (Raja et al., 2011), and introduces risks associated with prion protein transmission and viral contamination that threaten product safety. Accordingly, European regulations indicate that no compounds of animal origin should be used for the production of therapeutic drugs and urge manufacturers to always justify the use of materials of animal origin (Zhang and Robinson, 2005). From a research perspective, the unknown components of serum can greatly affect cell proliferation, alter culture performance and might affect the cellular metabolism reducing the reproducibility of experiments and consistency of experimental results (Shibuya et al., 2008). Therefore, due to safety, cost, consistency, efficiency and regulatory approval issues, both academic and industrial players are in favour of serum-free cultures.

The adaptation from serum-supplemented to serum-free cultures is a difficult task. To overcome adaptation issues, recombinant proteins and synthetic compounds have been developed to replace animal proteins commonly found in serum, namely lipoproteins, albumin, insulin and transferrin (Gorfien et al., 2000; Sauer et al., 2000; Spens & Häggström, 2005; Zhang and Robinson, 2005; Raja et al., 2011).

The most important advantage of animal-free and chemically-defined media is that they allow us to work under complete controlled conditions (Shibuya et al., 2008).

2.6.1.3 Cholesterol

Cholesterol is a 27-carbon molecule required by mammalian cells to maintain the membrane fluidity and acts as precursors for the biosynthesis of steroid hormones and

lipoproteins (Seth et al., 2005). Most animal cells can produce what they require from the most elementary nutrient supplies. Nevertheless, it was discovered that NS1 had become auxotrophs for cholesterol (Chen et al., 1986). The NS0 cell line is derived from NS1, which is a cholesterol-dependent cell line, and even when some cholesterol-independent variants have been reported (Keen & Steward, 1995), it is recommended to include cholesterol in the culture medium for a successful cell culture (2–6 mg/L) (Barnes et al., 2000).

Adding exogenous cholesterol to cell culture medium poses two disadvantages: (i) cholesterol is generally animal-derived, and (ii) it is difficult to supplement (Keen & Steward, 1995). Cholesterol addition issues include solubility, filterability, dispersion, physical stability, cell delivery kinetics, and container-vessel adhesion. To facilitate the addition of cholesterol, many technologies have been developed, such as vesicles, micelles, emulsions, micro-emulsions, carrier proteins, and carrier polymers such as cyclodextrin (Keen & Steward, 1995). The addition of complex protein solutions to high levels may inhibit cell growth, therefore, over-feeding should be avoided (Zhou, 1997).

The trend towards a chemically-defined media, completely free of animal-derived components, is driven by the biopharma awareness of potentially infectious adventitious agents into the production processes and the final products (Merten, 1999). For this reason, culture platforms that do not use exogenous cholesterol have been developed as a result of the adaptation of NS0 cells to grow in protein and cholesterol-free conditions (Barnes et al., 2000). Nowadays, synthetic non-animal cholesterol solutions are available to supplement hybridoma media formulations and promote cell growth and mAbs productivity in NS0 mouse myeloma and other cholesterol-dependent cell lines (Raja et al., 2011).

2.7 Model development

Mathematical models are used to assist the design of feeding strategies in fed-batch cultures through *in-silico* simulations under different feeding schedules, reducing the number of experiments to be performed (Kiparissides et al., 2015). However, cell culture processes are complex in nature and difficult to model. Challenges exist at all stages of model development: model formulation, identification and estimation of the parameters, and solution of the equations, which can become computationally intensive.

In general, the quantitative description of a bioprocess through a mathematical model involves three types of equations (Yahia et al., 2015):

- i. Mass balance equations: Depend on the reactor and they generally are a set of ordinary differential equations (ODEs) based on biological knowledge.
- ii. Rate equations: Describe the kinetics of various processes, usually as functions of intracellular parameters and the composition of the extracellular medium. They are empirical in nature and derive from Monod and Hill kinetics type equations.
- iii. Yield equations: Describe cellular metabolism and are based on material and energy balances. They relate amounts of compounds consumed and produced or, in differential form, rates of consumption and production. Although they may involve several assumptions about metabolism.

2.7.1 Model classification

Cell culture models are classified by Tsuchiya's framework (Tsuchiya et al., 1966). The main categories are structured/unstructured and segregated/unsegregated. The characteristics of each model type are summarized in Table 2.3.

Table 2.3: Major classification of biological models.

	Unsegregated	Segregated
Unstructured	Simplistic representation: homogeneous cell population comprised of single-component cells.	Cell population is viewed as heterogeneous (realistic); individual cells are represented as homogeneous , single component units (simplistic)
Structured	Cell population is viewed as homogeneous (simplistic); individual cells are represented as multicomponent units (Realistic).	Realistic representation: heterologous cell population comprised of multi-component cells.

Unstructured models do not take into account the inner structure of cells and thus acknowledge only implicitly the change of cellular physiological state with the environment (Fredrickson, 1976). Such models view the cell as a single unit, so their biological basis is limited, and their mathematical forms are generally phenomenological or empirical. Structured models enable the identification of a cell by assigning structure to it. The use of the term structure does not necessarily refer solely to the physical meaning of the term. Moreover, besides physical structure, which can be incorporated into the creation of intracellular compartments representing the various organelles; it can be incorporated through classification of the various biochemical species hence giving rise to biochemical structure (Tziampazis & Sambanis, 1994). Bibila & Flickinger presented a hybrid unsegregated/structured model introducing the compartmentalization of the cell related to RNA transcription and translation, mAbs assembly, maturation and secretion (Bibila & Flickinger, 1980). The compartments consisted of the nucleus, endoplasmic reticulum and Golgi apparatus (Bibila & Flickinger, 1980; Ho et al., 2006; Kontoradvi et al., 2010, Ho et al., 2011).

Unsegregated models view the population as consisting of identical "average cells" and use the same variable, e.g., biomass per unit volume, to describe the entire population. On the other hand, segregated models consider the differences of cells in the population: cell age, size, growth rate and metabolic state. Such segregated models are more representative and informative than unsegregated ones, but they are also more difficult to handle mathematically (Tsuchiya et al., 1966). This type of model has been used to study cell cycle dynamic in

mammalian cell cultures, in order to predict particular cell cycle changes (Garcia et al., 2013; Garcia et al., 2015).

A third distinction can be made between deterministic and stochastic (or probabilistic) models. In a deterministic model the outcomes are precisely determined through known relationships between states and events, i.e.; no randomness is involved in the development of future states of the system. In comparison, stochastic models use ranges of values for variables in the form of probability distributions (Tziampazis & Sambanis, 1994).

2.7.2 Modelling of mammalian cell cultures

Over 100 years ago, Michaelis & Menten published their now classic paper *Die Kinetik der Invertinwirkung* in which they showed that the rate of an enzyme-catalysed reaction is proportional to the concentration of the enzyme-substrate complex. Mammalian cells in culture present the same saturation type kinetics, i.e., the rate of the process (v) is limited by low substrate concentration (S), but the effect disappears when it increases and the process rate reaches its maximum value ($v_{\max}$) (Michaelis & Menten, 1913). This behaviour can be described by a Michaelis-Menten type equation (Michaelis & Menten, 1913):

Equation 2.2: Michaelis-Menten type equation (Tziampazis & Sambanis, 1994)

$$v = \frac{v_{\max}S}{K_m + S}$$

In mammalian cell models the metabolic response to substrates such as glucose (GLC), can be described by Michaelis-Menten type equations, in particular the specific growth rate. Monod, in 1949, successfully described the microbial-specific growth rate (μ) based on Michaelis-Menten equation:

Equation 2.3: Monod type equation for specific growth rate with glucose as substrate (Monod, 1949)

$$\mu = \frac{\mu_{\max}GLC}{K_{GLC} + GLC}$$

The effects of inhibitor components (I), which reduce the growth rate, can be modelled by analogy to the various types of inhibition of enzymatic reactions (Tziampazis & Sambanis, 1994), considering inhibition constant as a parameter (K_I). Of those, the simpler and easier to use is that of purely non-competitive inhibition:

Equation 2.4: Monod type equation for specific inhibition rate.

$$\mu = \frac{\mu_{max}}{1 + \frac{I}{K_I}}$$

These expressions can be modified to model data sets that are not accurately described by the equations. The equations modifications are usually related to the incorporation of additional terms, raising concentrations to various powers, and introducing a dependency of the parameters to other factors through empirical functions (Table 2.4).

Table 2.4: Specific growth rate formulations in unstructured dynamic models for mammalian cell cultures.

Equation	Description
$\mu = \mu_{max} \left[\frac{S}{K_S X^{-\beta} - S} \right]$	Growth rate based on serum (S) and cell concentrations (X) (Glacken et al., 1988)
$\mu = \mu_{max} \frac{S}{[K_S X O^{-\beta} - S]} \frac{K_{I,amm}}{[K_{I,amm} + AMM^2]} \frac{GLN}{[K_{gln} - GLN]}$	Growth rate based on cell, serum, glutamine (GLN) and ammonia (AMM) concentrations (Glacken et al., 1989)
$\mu = \mu_{max} \left[\frac{GLC}{K_{glc} - GLC} \right] \left[\frac{GLN}{K_{gln} - GLN} \right]$	Growth rate depends on two substrates: glucose (GLC) and glutamine (Tremblay et al., 1992)
$\mu = \mu_{lim} \mu_{inh}$ $\mu_{lim} = \mu_{max} \left[\frac{GLC}{K_{glc} - GLC} \right] \left[\frac{GLN}{K_{gln} - GLN} \right]$ $\mu_{inh} = \left[\frac{K_{I,lac}}{K_{I,lac} - LAC} \right] \left[\frac{K_{I,amm}}{K_{I,amm} - AMM} \right]$	Growth rates depend on nutrients availability (glucose and glutamine) and toxic metabolites inhibition (lactate (LAC) and ammonia) (Jang & Barford, 2000; Xing et al., 2010; Kontoradvi et al., 2010; Kiparissides et al., 2011)
$\mu = \mu_{max} \left[\frac{GLC - GLC_{min}}{K_{glc} + GLC - GLC_{min}} \right]$	Growth rates depend on glucose concentration (Ho et al., 2006)
$\mu = \mu_{max} \mu_{lim} \mu_{inh}$ $\mu_{lim} = \mu_{max} \left[\frac{GLC}{K_{glc} - GLC} \right] \left[\frac{GLN}{K_{gln} - GLN} \right] \left[\frac{ASN}{K_{ASN} - ASN} \right]$ $\mu_{inh} = \left[\frac{K_{I,lac}}{K_{I,lac} - LAC} \right] \left[\frac{K_{I,amm}}{K_{I,amm} - AMM} \right]$	Growth rates depend on nutrients availability (glucose, glutamine and asparagine (ASN)) and toxic metabolites inhibition (lactate and ammonia) (Karra et al., 2010)

The mathematical description of the specific cell growth rate usually includes the inhibitory effect caused by ammonia and lactate accumulation. However, high concentrations

of lactate and ammonia not only inhibit growth as consequence of the osmotic pressure increase produced by lactate accumulation (Omasa et al., 1991) and pH disruptions caused by ammonia accumulation (McQueen & Bailey, 1990 and Wu et al., 1993), but also induce high levels of necrosis (Mercille & Massie, 1994). The mathematical expression for specific death rate via necrosis are presented in Table 2.5.

Table 2.5: Specific death rate formulations in unstructured dynamic models for mammalian cell cultures.

Equation	Description
$\mu_d = \mu_{tox}\mu_{lim}$ $\mu_{tox} = \mu_{d,max} \left[\frac{1}{\mu_{max} - K_{d,lac}LAC} \right] \left[\frac{1}{\mu_{max} - K_{d,amm}AMM} \right]$ $\mu_{lim} = \left[\frac{K_{lim,gln}}{K_{lim,gln} - GLN} \right]$	Necrosis is induced by lactate and ammonia presence, and glutamine limitation. Death induced by toxicity (μ_{tox}) was linked to the maximum specific growth rate (Tremblay et al., 1992)
$\mu_d = \mu_{d,max} \left[\frac{1}{1 + \left(\frac{K_{d,amm}}{AMM} \right)^n} \right]$	Ammonia is considered the main inducer of necrosis. The factor n is greater than 1 to give a steeper increase of death rate as ammonia concentration increases (Jang & Barford, 2000; Kontoravdi et al., 2005-2007-2010; Kiparissides et al., 2011)
$\mu_d = \frac{K_{d,l}}{\mu_{max} - K_{d,T}LAC}$	In cultures where the GS-expression-system is used, ammonia formation is imperceptible. Therefore, lactate becomes the main necrosis inducer. In this case, the death rate was linked to the maximum specific growth rate (Ho et al., 2006; Kiparissides et al., 2010)
$\mu_d = \mu_{d,max} \left[\frac{1}{1 - \frac{K_{d,lac}}{LAC}} \right] \left[\frac{1}{1 - \frac{K_{d,amm}}{AMM}} \right]$	Necrosis is induced by lactate and ammonia presence (Xing et al., 2010)

Specific growth and death rates are combined to calculate the apparent growth rate (μ_d), which is used in the viable cell balance (Table 2.6).

Table 2.6: Viable and dead cells balance in unstructured dynamic models for mammalian cell cultures.

Equation	Description
$\frac{dX_v}{dt} = (\mu - \mu_d)X_v$ $\frac{dX_d}{dt} = \mu_d X_v$	Viable and dead cells balances in batch culture (Glacken et al., 1988)
$\frac{dX_v}{dt} = (\mu - \mu_d)X_v$ $\frac{dX_d}{dt} = \mu_d X_v - k_{lys}X_d$	Viable and dead cells balances in batch culture. In this case, the dead cells balance include a term related necrotic cells lysis ($K_{lys}X_d$)(Ho et al., 2006; Xing et al., 2010; Kiparissides, 2011)
$\frac{dX_v}{dt} = (\mu - \mu_d)X_v - \frac{F_{out}}{V}X_v$ $\frac{dX_d}{dt} = \mu_d X_v - k_{lys}X_d - \frac{F_{out}}{V}X_v$	Viable and dead cells balances in fed-batch cultures (Tremblay et al., 1992; Jang & Barford, 2000; Kontoravdi et al., 2007-2010)

Nutrient and metabolite balances present similar structures throughout the literature. The differences lay on the structure of the specific consumption rate or uptake rate (Q) (Tremblay et al., 1992; Ho et al., 2006; Kontoravdi et al., 2007; Kiparissides, 2011).

Table 2.7: Examples of nutrient mass balance based on glucose.

Equation	Description
$\frac{dVGLC}{dt} = -Q_{glc}VX_v$	Glucose balance, in batch cultures, depends on the specific consumption rate (Q_{glc}) (Ho et al., 2006; Kiparissides, 2011)
$\frac{dVGLC}{dt} = GLC_{in}F_{in} - GLCF_{out} - Q_{glc}VX_v$	In fed-batch cultures, the balance of nutrients depends on the specific consumption rate and also includes the effects of feeding ($GLC_{in}F_{in}$) and outlet ($GLC F_{out}$) (Tremblay et al., 1992; Kontoravdi et al., 2007-2010)

Toxic metabolites are not fed into the system and, therefore, their concentrations depend on their production. Lactate accumulation depends on the unbalance between the glycolytic pathway and the TCA cycle and ammonia production depends on the spontaneous degradation of glutamine and glutamine utilization (Tremblay et al., 1992; Kontoravdi et al., 2010; Kiparissides, 2011).

Table 2.8: Examples of toxic metabolites mass balances (Lactate and ammonia).

Equation	Description
$\frac{dLAC}{dt} = Q_{lac}X_v$ $\frac{dAMM}{dt} = Q_{amm}X_v + K_{deg,gln}GLN$	Toxic metabolites balances, in batch cultures, depend on their specific production rate (q_{lac} and q_{amm}) and, in the case of ammonia formation; it also depends on spontaneous degradation of glutamine (Kiparissides, 2011).
$\frac{dVLAC}{dt} = q_{lac}VX_v - F_{out}LAC$ $\frac{dVAMM}{dt} = q_{amm}VX_v + K_{deg,gln}VGLN - F_{out}AMM$	In fed-batch cultures, the balances include an extra term related to the effect of the outlet (F_{out}) (Tremblay et al., 1992; Kontoravdi et al., 2010)

The product balance presents different formulations depending on the assumptions established throughout the model development. The antibody expression rate has not been associated to proliferation, in mammalian cell cultures. Therefore, in general, the specific production rate (amount of mAb per cell per unit of time) multiplied by the concentration of viable cells is the term that contributes to mAb formation (Table 2.9) (Shirsat et. al., 2016).

Table 2.9: Examples of product balances.

Equation	Description
$\frac{dMAB}{dt} = q_{x,MAB}X_v - \frac{F_{out}}{V}MAB$	The product mass balance depends on the specific production rate ($q_{x,mAb}$) and the outlet (F_{out}) (Tremblay et al., 1992)
$\frac{dMAB}{dt} = q_{MAB} \left(\frac{GLN}{GLN + K_{gln}} \right) X_v - \frac{F_{out}}{V}MAB$	The mass balance includes a term related to the availability of one amino acid that is essential for the products assembly, glutamine (GLN) (Jang & Barford, 2000)
$\frac{dMAB}{dt} = q_{MAB}(\gamma_2 - \gamma_1\mu)X_v - \frac{F_{out}}{V}MAB$ $q_{MAB} = \varepsilon_2\lambda K_G[H_2L_2]_G$	The product formation depends on the glycosylation efficiency of the Golgi apparatus (ε) and the concentration of assembled light and heavy chains of the product ($[H_2L_2]$). This is an example of compartmentalization of mAbs production in a hybrid model (Kontoravdi et al., 2006)

The equations included in Table 2.4 to Table 2.9 correspond to dynamic models, which describe the evolution of the systems over an established time horizon, whereas static models capture specific instants along the process. Dynamic models consist of systems of differential algebraic equations (DAE), which computationally demanding compared to static models, which typically contain only algebraic equations (Kiparissides, 2011).

2.7.3 Evolution of dynamic models for mammalian cell cultures

Glacken et al. from 1987 to 1989 developed a dynamic model for CRL-1606 cells in batch cultures implementing new improvements in each publication. Most of the modifications applied were included to improve the accuracy of the model and based on experimental observations (Glacken et al., 1988; Glacken et al., 1989a; Glacken et al., 1989b). Tremblay et al., in 1992, improved Glacken's model and use it to find optimal feeding trajectories for a single feed stream containing both glucose and glutamine and for two separate feed streams, using a seventh order kinetic model for the optimisation. In this study, the criterion to be maximised was the total amount of monoclonal antibody obtained at the end of the fed-batch culture. The constraints were related to the control variables (feeding flow rates) and the culture volume (Tremblay et al., 1992). These studies paved the way for exploring model-based optimisation techniques.

Jang & Barford developed an unstructured kinetic model of macromolecular metabolism in cultures of murine hybridoma AFP-27 producing IgG1 antibody (Barford et al., 2000). The model describes the profile of macromolecules production and monoclonal antibody secretion, glucose and glutamine metabolism, toxic metabolites production, and the profiles of cell growth in batch and fed-batch cultures. Kontoravdi et al. modified Jang & Barford's model to include culture volume changes for the cases of fed-batch and continuous operation, and transformed the model into a hybrid model that includes a structured part, which describes the compartmentalization of antibody synthesis and secretion processes (Kontoravdi et al., 2005). The modified model consists of a total mass balance around the bioreactor and balances on the viable and total cell populations based on conventional assumptions such as negligible dilution effects and perfect mixing. Later, Kiparissides presented an integrated approach for the development of models of biological systems that consist of a biological model-building framework, its experimental validation and its application in model-based optimisation studies (Kiparissides et al., 2011). This holistic approach facilitated the optimisation of *in-vitro* cultures through *in-silico* simulations. Finally, in 2015, Kiparissides presented the implementation of a dynamic model-based optimisation for the design of a feeding strategy for GS-NS0 in fed-batch cultures. The model describes cell growth and monoclonal antibody formation and interlinks cellular growth rate with the availability of glucose and glutamate as nutrients.

2.8 Model analysis

Dynamic models for mammalian cell cultures contain a large number of parameters, whose values must be collected from the literature or experimentally estimated. The number of experiments needed to estimate all the parameters of a model can be vast or even unfeasible to perform. Therefore, the design of experiments must be focused on the most relevant parameters, i.e., it is required to analyse the model and determine which parameters have a significant effect on the output variable to be analysed.

Sensitivity analysis describes how the uncertainty on the model inputs influences the model output (Saltelli et al., 2000). There are two main classes of sensitivity analysis method: local and global definitions (Morio, 2011). A local analysis addresses sensitivity relative to point estimates of parameter values while a global analysis examines sensitivity with regard to the entire parameter range (Hamby, 1995).

2.8.1 Global sensitivity analysis

Global sensitivity analysis (GSA) is the only method that incorporates an extensive number of parameters, simultaneously and randomly varies all parameters, covers the entire range of the parameters values and provides quantitative results related to the contribution of the parameters to the output variance. The methodology estimates the sensitivity index of an individual parameter, varying all other parameters at the same time and taking into account the relative uncertainty of each input and revealing interactions between parameters (Saltelli et al., 1999). The importance of GSA of mathematical models for biological systems resides in: i) the extensive number of parameters that can be analysed in a simultaneous manner, ii) the wide ranges of the parameters values that depend on the culture operation conditions and iii) the identification of significant interactions between parameters. These properties of GSA allow us to acquire the deepest understanding of the studied biological system (Kontoravdi et al., 2005).

GSA Sobol's method measures the parameters' significance by estimating the total variance of the output. Firstly, the output variable is represented by the function $f(x)$ that depends on the parameters vector x . Subsequently, the function is decomposed into summands of different dimensions (ANOVA representation):

Equation 2.5: ANOVA representation (Sobol, 2001).

$$f(x) = f_0 + \sum_i f_i(x_i) + \sum_{i < j} f_{ij}(x_i, x_j) + \dots + f_{12\dots n}(x_1, \dots, x_n)$$

The total variance of $f(x)$ is defined as D :

Equation 2.6: Variance calculation (Sobol, 2001).

$$D = \int f^2(x) dx - f_0^2 = \sum_{S=1}^n \sum_{i_1 < \dots < i_S}^n \int f^2_{i_1 \dots i_S} dx_{i_1} \dots dx_{i_S} = \sum_{S=1}^n \sum_{i_1 < \dots < i_S}^n D_{i_1 \dots i_S}$$

Finally, the sensitivity index is defined as the contribution of each parameter or a subset of parameters to the total output variance:

Equation 2.7: Sensitivity indices (Sobol, 2001).

$$S_{i_1 \dots i_S} = \frac{D_{i_1 \dots i_S}}{D}$$

The values of the sensitivity index are non-negative and their sum is 1:

Equation 2.8: Sensitivity indices property (Sobol, 2001).

$$\sum_{S=1}^n \sum_{i_1 < \dots < i_S}^n S_{i_1 \dots i_S} = 1$$

S_{is} represents the first-order indices of individual parameters, while $S_{ik_1 \dots ik_m}$ ($1 \leq m \leq n-1$ and $1 \leq k_1 < \dots < k_m \leq n$) represents the sensitivity index of a specific set of parameters (Sobol, 2001).

Sobol et al., in 2007, presented a new theorem to estimate the average approximation error of the model output generated when a factor was considered non-influential and its value was fixed (Sobol et al., 2007). The theorem has been applied to reduce the loss of accuracy of the method, and implemented in toolboxes or packages in relevant software such as MATLAB®.

The graphical user interface-high dimensional model representation (GUI-HDMR) is a MATLAB® toolbox, which is freely available to execute GSA to evaluate and increase the

reliability of mathematical models. The set of parameters can be a Monte Carlo sample or experimental values and a quasi-random sampling method is implemented to guarantee the complete coverage of the input space (set of parameters in their entire range of values). The sensitivity indices are calculated automatically and can be directly used to explore the significance of individual parameters and the interactions between the parameters (Ziehn and Tomlin, 2009).

2.8.2 Parameter estimation

System identification and parameter estimation are important steps in model development. In general, system identification is a procedure that establishes possible model structures from experimental data and estimates values of parameters within that structure (Murray-Smith, 2015). In models for biological systems, the model structure is usually determined by first principles. Therefore, the problem becomes one of parameter estimation, rather than the complete process of system identification.

There are two different approaches for static parameter estimation, Bayesian or frequentist. In the Bayesian approach, the model parameters are considered random variables that require the assignment of a prior distribution; and its objective is to calculate the posterior distribution of the parameter vector given the observed data. On the other hand, in frequentist approaches, it is assumed that the parameter vector is constant and maximizes the likelihood of observed data, in other words, it makes the available data as likely as possible. A common example of frequentist method is the maximum likelihood estimation (MLE) (Gabor and Banga, 2015).

The formulation of a parameter estimation problem based on maximum likelihood estimation provides simultaneous approximation of parameters in the physical model of the process and the variance model of the measuring instrument, which can be constant (e.g. instrument accuracy), constant relative (e.g. analyser error) and heteroscedastic (e.g. instrument accuracy in addition to the analyser error).

MLE determines the unknown parameters values (θ) finding the values that maximise the probability of getting model predictions that match the experimental measurements, i.e., maximise the likelihood function (Φ), assuming independent, normally distributed measurement errors, ε_{ijk} , with zero means and standard deviations, σ_{ijk} .

Equation 2.9: Likelihood function (Pistikopoulos, 2011).

$$\Phi = \frac{N}{2} \ln(2\pi) + \frac{1}{2} \min_{\theta} \left\{ \sum_{i=1}^{NE} \sum_{j=1}^{NV_i} \sum_{k=1}^{NM_{ij}} \left[\ln(\sigma_{ijk}^2) + \frac{(z_{ijk} - x_{ijk})^2}{\sigma_{ijk}^2} \right] \right\}$$

N represent the total number of experimental measurements, θ is the set of model parameters to be estimated, NE depicts the number of experiments performed, NV_i is the number of variables measured in the i^{th} experiment and NM_{ij} is the number of measurements of the j^{th} variable in the i^{th} experiment. The variance of the k^{th} measurement of variable j in experiment i is denoted as σ_{ijk}^2 , while z_{ijk} is the k^{th} measured value of variable j in experiment i and x_{ijk} is the k^{th} model predicted value of variable j in experiment i (Pistikopoulos et al., 2011).

The MLE method has two limitations i) it can handle only measurement noise; and ii) for unstable systems, it might diverge.

Kalman filter algorithms or linear quadratic estimation (LQE) can be implemented within the structure of maximum likelihood output error to overcome the noise limitations. Kalman filtering is a two steps algorithm. In the prediction step, it produces estimates of the state variables and their uncertainties. These estimates are updated, when the next measurement is observed, using a weighted average, assigning more weight to estimates with lower uncertainty, recursively. The implementation of Kalman filter makes the MLE method more complex and computationally intensive (Murray-Smith, 2015).

The limitations of MLE for unstable systems can be overcome using stabilised output-error methods (SOEMs) by utilising measured states. The two categories are included in SOEMs: output-error methods (OEMs) and equation error methods (EEMs). The first one

minimises the error between the measured and model outputs produced by identical inputs and the second minimises the error in the state equation. Both methods assume that there is no process noise and EEM, in particular, requires accurate measurements of states and their derivatives for its stabilisation to enable estimation of the parameters of the unstable system directly. SOEMs belong to a class of mixed EEM and OEM methods for parameter estimation (Gopalratman and Raol, 1996).

Cell cultures are stable systems if we consider that the changes in the system are soft and any slight perturbation from fixed condition will cause a small response. Therefore, MLE methods are appropriate for parameter estimations for these specific systems.

2.9 Model-based optimisation

Unlike the chemical industry, the optimisation of biologics manufacture remains empirical, relying on costly and time-consuming experimentation (Burky et al., 2006; Spens & Häggström et al., 2007). However, over the recent years, the development of mathematical models that predicts the behaviour of biological systems have facilitated the implementation of model-based optimisation techniques, which take advantage of experimental data, fundamental biological knowledge and computational tools currently available (Koumpouras & Kontoravdi, 2012). The implementation of this integrated *in-vitro/in-silico* approach has demonstrated promising advantages and benefits (Banga et al., 2005).

Dynamic optimisation strategies have been implemented for the derivation of optimal feeding schedules to maximise the mAb titre, cell number and the integrals of mAbs or cell concentrations in fed-batch cultures (Dhir et al., 2000, Kiparissides et al., 2015).

In the formulation of the dynamic optimisation problem, it is necessary to determine the following list of settings:

Equation 2.10: Process model

$$x_i(t) = f_i(X_j(t), u(t), t)$$

$$i, j = 1 \dots n$$

Equation 2.11: Set of initial conditions

$$x_i(0) = x_{i,0}$$

Equation 2.12: One or multiple objective functions

$$\min_{u(t)} \text{ or } \max_{u(t)} = f_i(X_j(t), u(t), t)$$

$$i, j = 1 \dots n$$

Equation 2.13: Set of inequality constraints

$$g(X_j(t), u(t), t) \leq 0$$

Equation 2.14: Control variables

$$u(t)$$

Most process models for biological systems are highly non-linear and present constraints in the state and control variables. These features make the dynamic optimization a challenging problem to solve. Therefore, the selection of the method used to obtain the optimal operating control depends on the efficiency and robustness of the method.

The categories in the classification of the most promising strategies applied to solve dynamic optimisations problems are: dynamic programming, deterministic methods (indirect and direct methods) and stochastic methods.

Dynamic programming: These algorithms break down the original problem in simpler sub-problems or sequence of decision steps over time, which are solved once and their solutions are stored. The final solution for the original problem is the best solution combination of the sub-problems. These methods save time whilst requires storage space for the stored solutions.

Indirect approaches: Most of indirect methods use iterative procedures based on successive linearization to find a solution to the system of necessary conditions of optimality (quasi-linearization, control vector iteration and indirect shooting methods). The indirect shooting method or boundary conditions iteration transform of the original optimisation problem into a two/multi-point boundary value problem. When terminal or path inequality constraints are present, the resulting boundary value problems can be very difficult to solve. In

bioprocesses, inequality state constraints are common; therefore, indirect methods are not widely used in the field (Bryson and Ho, 1975).

Direct approaches: Direct methods solve optimisation problems discretizing the control problem, and then apply nonlinear programming (NLP) techniques to the resulting finite-dimensional optimization problem. The disadvantage of these methods is that they may provide suboptimal solutions due the discretization of the state and/or control variables. The most popular direct methods are control variable parameterization method (CVP), multiple-shooting approach and complete parameterization (CP) (parameterization of the state and control variables) (Schlegel et al., 2005).

Stochastic methods: These methods present two stages, the distributional assumption and the discretization. These algorithms are reliable because they enforce convergence to the global optimum (Banga et al., 1998).

Hybrid methods: Hybrid methods take advantage of the convergence speed and robustness, of stochastic and deterministic methods, respectively, feature that position the hybrid strategies on the first place for the optimisation of biological systems. However, the CVP method, which is a pure deterministic method, has presented good efficiency and robustness for the optimisation of fed-batch cultures, finding optimal solutions in less computation time than hybrid methods (Banga et al, 1998).

The criteria for the selection of the method used to solve the optimisation problem has to consider the efficiency and robustness of the solution.

2.10 Model-based control strategies

The most cutting-edge application of mathematical models is the development of model-based controllers for biological processes in order to keep culture variables within desired limits, eliminating the need for frequent sampling and operator intervention. The basic idea of model predictive control (MPC) is to solve an optimisation problem at each time point in order to minimise the deviation from the set-point by implementing the optimal values of the control variables. This sequence is repeated at regular time intervals, for the new state variable measurements, until the desired or set point values are obtained. Hence, the model must be implemented and validated in previous stages. For all model development work, suitable feedback from the experimental verification is required.

Dhir et al. proposed a model-based optimisation of glucose and glutamine feeding in hybridoma cell cultures. In that work, the model parameters were re-estimated in each sampling point, an optimisation was performed periodically and the bioreactor operated as batch between sampling times (Dhir et al., 2000). This was the first attempt to use model-based optimisation to improve fed-batch processes *in-silico*, reducing the number of experiments performed. Later on, Frahm et al. developed a control concept using an adaptive model for hybridoma cells, proving the promising potential of predictive models for the development of optimisation and control strategies (Frahm et al., 2002).

Nowadays, the challenge is to develop the required sensors for on-line monitoring, optimisation and control of biologic manufacturing processes. Currently, electrochemical and optical sensors enable upstream conditions monitoring in a physical and physicochemical manner (pH, temperature and DO online measurements). However, for the control of the product titre and quality it is necessary to measure cell density, major nutrients concentration, toxic metabolites level and product attributes such as activity, aggregation, glycosylation and impurities concentration. Despite the fact that there are advances in on-line sampling for at-line analysis (BioProfile analysers) and on-line probes (near-infrared spectroscopy technology), there is a wide gap between monitoring technologies and control techniques.

Filling this gap is the technological opportunity to drastically improve the sustainability of biopharmaceutical processes.

Table 2.10: Control strategies for fed-batch cultures.

Cell line	Operation mode (Control variable)	Control strategy (Output)	Reference
CRL-1606	Fed-batch (Nutrient feeding)	Manual calculation of nutrient demand (2.400 [g/L], 26-fold increase, 23 days, high yield , nutrients accumulation)	Xie & Wang, 1996b
NS0	Fed-batch (Nutrient feeding)	Manual calculation of nutrient demand (2.700 [g/L], 28 days, high yield, nutrients accumulation)	Zhou et al., 1997
4W4	Fed-batch (Glucose feeding)	On-line parameter estimation of a first principles model and model-based optimization in each sampling time (0.250 [g/L], 31% increase, 7 days, low productivity)	Dhir et al., 2000
NS0 and Sp2/0	Fed-batch (Nutrient feeding)	Manual calculation of nutrient demand (0.750 [g/L], 10-fold increase, 7 days, high yield)	Sauer et al., 2000
WuT3	Fed-batch (Nutrient feeding)	Off-line model-based control strategy. Prediction of the model were used to calculate nutrient requirements (0.350 [g/L], 6.3-fold increase, 15 days, precise control of nutrient concentrations)	Zhang et al., 2004
NS0	Fed-batch (Nutrient feeding)	Manual calculation of nutrient demand (2.640 [g/L], 13 days, high yield)	Burky et al., 2006
NS0	Fed-batch (Nutrient feeding)	Manual calculation of nutrient demand (0.625 [g/L], 11.4-fold increase, 12 days, amino acids accumulation)	Spens & Häggström, 2007
CRL-1606	Fed-batch (Nutrient feeding)	Manual calculation of nutrient demand (0.400 [g/L], 4-fold increase, 22 days, low yield)	Shibuya, 2008
GS-CHO and GS-NS0	Fed-batch (Nutrient feeding)	Constant glucose level. (0.207 [g/L] (GS-CHO) and 1.005 [g/L] (GS-NS0), 4-8 fold increase, 9 days, over and under feeding)	Fan et al., 2009

CHAPTER 3: Aim and objectives

3 Aim and objectives

The aim of this thesis was the development and experimental validation of a high fidelity model to predict the intercellular ATP content in mammalian cells. The ultimate goal was to use the predictive model for the energy-based optimisation of mAbs production.

The specific objectives were:

- 1) **Design, validation and testing of a mathematical model for the prediction of intracellular ATP content in GS-NS0 cells producing cB72.3 mAb (Chapter 5):** The high fidelity model was based on first principles, mass and stoichiometric balances, and Monod kinetics. GSA was applied to identify the subset of significant model parameters. Subsequently, this group of parameters was re-estimated using data from three independent replicates of a specific batch culture. Three batch experiments under different initial cell densities were performed, and their experimental results were compared to the model prediction for the validation and predictive capability testing of the model.
- 2) **Parameter estimation, validation and prediction capability testing of the model for cultures in serum-free conditions (Chapter 6):** The model was slightly modified to predict the uptake of some medium components, and subjected to GSA, parameter estimation, validation and testing.
- 3) **Model-based design of a supplemental medium and feeding strategy for fed-batch cultures (Chapter 7):** The model predictions were used for the design of a supplemental medium and a simple feeding strategy that ensured the presence of all amino acids in the culture medium.
- 4) **Model –based optimisation for fed-batch cultures (Chapter 7):** A dynamic optimisation approach was used for the derivation of an optimal feeding schedule using the supplemental medium composition previously developed. The experiment following the feeding strategy proposed by the dynamic optimisation was implemented for the validation of our approach.

CHAPTER 4: Materials and methods

4 Materials and methods

In this chapter, the methodology applied for the development of a mathematical model for batch cultures of GS-NS0 is presented. Additionally, the computational tools used for the model analysis and parameter estimation are designated. Finally, the materials used and methodologies implemented for the experimental model validation are described in detail.

4.1 Cell line, culture conditions and medium composition

4.1.1 Cell line and product

GS-NS0 mouse myeloma cells were used in this study (Lonza Biologics, UK). The cell line produces the chimeric monoclonal antibody cB72.3, which recognises the Tag-72 tumour marker in colorectal cancer.

4.1.2 Serum supplemented medium: Modified DMEM medium supplemented with 10% FBS

One litre of culture medium contained 820mL Advanced DMEM medium 1X (Invitrogen, UK), 100mL FBS (Invitrogen, UK), 20mL GS-Supplement 50X (Sigma-Aldrich, UK), 10mL MEM-vitamins solution 100X (Invitrogen, UK), 20mL MEM-amino acids 50X (Invitrogen, UK), 10mL MEM-non-essential amino acids 100X (Sigma-Aldrich, UK), 10mL Penicillin/Streptomycin 100X (Sigma-Aldrich, UK), and 10mL MSX(Sigma-Aldrich, UK) solution, final concentration 25 [μ M]. The pH was adjusted to 7.2 by adding drops of NaOH 1M using S20 SevenEasy™ pH meter (Mettler-Toledo). Finally, the medium was sterilised by filtration, aliquoted and stored at 4°C up to one week.

4.1.3 Chemically-defined: Modified CD-Hybridoma medium

One litre of culture medium contained 957mL CD-Hybridoma 1X (Gibco, UK), 20mL GS-Supplement 50X (Sigma-Aldrich, UK), 10mL Penicillin/Streptomycin 100X (Sigma-Aldrich, UK), 2mL Cholesterol Lipid Concentrate 250X (Gibco, UK), 1mL Serum

Replacement 50X (Sigma–Aldrich, UK), and 10mL MSX (Sigma-Aldrich, UK) solution, final concentration 25 [μ M]. The pH was adjusted to 7.2 by adding drops of NaOH 1M using a pH meter (Mettler-Toledo, UK). Finally, the medium was sterilized by filtration, aliquoted and stored at 4°C for up to one week.

4.2 Cryopreservation, cell bank development and expansion.

4.2.1 Cell bank development

One cryovial from the master cell bank was removed from the liquid nitrogen storage tank and thawed at 37°C in a water bath for 5min. The content of the cryovial was transferred to a 15ml Falcon tube (Corning, UK), combined with 10mL of pre-warmed fresh medium, gently mixed and centrifuged at 800rpm for 5min (Rotina 46R centrifuge, Hettich Zentrifugen, DE). The supernatant was discarded and the cells pellet re-suspended in 10mL of fresh medium and inoculated at 2×10^5 cells/mL in a 125mL Erlenmeyer flask (Corning, UK) with 30mL of working volume. Cells from cultures in mid-exponential growth were passaged four times and the final culture was processed to create a new working bank. The final culture was centrifuged at 800rpm for 5min, the supernatant was discarded and the cells pellet re-suspended in a medium mix containing: 90% (v/v) fresh medium (DMEM 10% FBS or CD-Hybridoma serum-free) and 10% (v/v) dimethyl sulfoxide (DMSO, Sigma-Aldrich, UK) to reach a final concentration of 1×10^7 [cells/mL]. Subsequently, 1mL aliquots were transferred to cryovials (Corning, UK) and stored in a Freezing Container (Nalgene, UK) at -20°C for 2h, transferred to a -80 °C freezer for 2h, and finally the cryovials were transferred to long term storage in the liquid nitrogen tank.

4.2.2 Cells expansion

For cell expansion, one cryovial from the working bank, containing cryopreserved cells, was removed from the liquid nitrogen storage and thawed at 37°C in a water bath for 5min. The content of the cryovial was transferred to a 15ml Falcon tube (Corning, UK), combined with 10mL of pre-warmed fresh medium, gently mixed and centrifuged at 800rpm for 5min (Rotina 46R centrifuge, Hettich Zentrifugen, DE), to remove the cryopreservation medium containing

DMSO. The supernatant was discarded and the cell pellet re-suspended in 10mL of fresh medium and inoculated at 2×10^5 [cells/mL] in a 125mL Erlenmeyer flask (Corning) with 25[mL] working volume. Cells from cultures in mid-exponential growth were passaged every 2 days at 2×10^5 [cells/mL] inoculation cell density, three times prior experiments.

4.2.3 Culture conditions

The cultures were performed in independent replicates (3). For flasks cultures, 1L Erlenmeyer flasks were used (Corning Sigma-Aldrich, UK) with 200mL working volume, agitated at 130rpm, incubated at 37°C and 5% CO₂ (V/V). For bioreactor cultures, the New Brunswick's BioFlo 110 Modular Bioreactor was used. The bioreactor has 1.3L total capacity and it was used with 500mL of working volume, agitated at 80rpm with low-shear marine blade Rushton impeller, 50% of air saturation, temperature controlled with PID controller and water jacket to 37°C set-point, pH controlled with PID controller and CO₂ injection to 7.2 set-point.

4.3 Detection of viable, early apoptotic and dead cells

The number of cells in different states was determined by flow cytometry using Guava ViaCount® assay (Merck Millipore, UK). The assay distinguishes the cells on the basis of differences in the permeability of two fluorescent dyes, a nuclear stain, which is low for debris and high for all cells; and a viability stain, which is negative for debris and viable cells, medium for early apoptotic cells and high for dead cells. Every 24 hours, 50µL were taken from the culture and subsequently incubated with 450µL of ViaCount® for 5 min at room temperature. The data was acquired using a bench-top flow cytometer, Guava EasyCyte™ (Merck Millipore, UK) and processed using GuavaSoft software (V 2.6) (Details in Appendix I).

The number of cells in each category depends on the stain levels: negative for viable cells, medium for early apoptotic cells and high for dead cells. Dead cell number include late apoptotic and necrotic cells, in both cases the stain penetrate inside the cells due to the loss of membrane integrity. The total number of cells represents the sum of viable, early apoptotic and dead cells.

4.4 Monoclonal antibody production

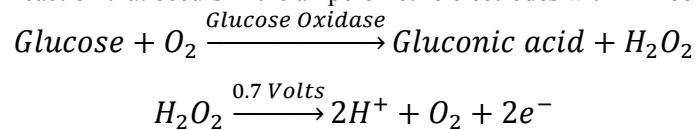
The product concentration was measured using HPLC techniques. Every day, 500µL of sample were collected from each flask and centrifuged for 5 minutes at 800rpm, the supernatants were filtrated through the 0.2µm filter and stored at -20°C prior to analysis. The mAb assay was performed with Agilent 1260 Infinity Quaternary LC, equipped with a RRLC in-line filter, 4.6mm, 0.2µm; and the Bio-Monolith Protein A 4.95mm x 5.2mm column, which has high affinity for the Fc region of polyclonal and monoclonal IgG-type antibody forms (Agilent Technologies, UK). The samples were processed at 30±0.8°C, flow of 1 mL/min and injection volume 50µL. The mobile phases are A (PBS buffer, pH 7.4) and B (0.5M acetic acid, pH2.6). The gradient elution program was: 0.5 min, 100% (A); 1 min, 100% (B); 1.5 min 100% (A). The UV diode-array detector (DAD) was used to detect the monoclonal antibody at a wavelength of 280 nm light absorption (reference at 360 nm for baseline drift).

The calibration curves were constructed by diluting a monoclonal antibody standard solution (1,000 [mg/L], Lonza Biologics, UK) (Appendix II)

4.5 Nutrient and metabolite analysis

Extracellular glucose and lactate concentrations were measured using the Nova BioProfile 400 Analyser (Nova Biomedical, UK). 1.5mL samples were collected from each flask at 24h intervals basis and centrifuged at 800rpm for 5 minutes. The supernatant was stored at -20°C prior to analysis. The Bioprofile has enzyme biosensors for the detection of glucose and lactate. These sensors, amperometric electrodes, have immobilised enzymes in their membranes and, in the presence the substrate and oxygen, they produce hydrogen peroxide (H₂O₂), which is then oxidised at a platinum anode held at constant potential. The resulting flow of electrons, and current change, is proportional to the sample concentration.

Equation 4.1: Reaction that occurs in the amperometric electrodes with immobilized enzymes.



4.6 Amino Acids Analysis

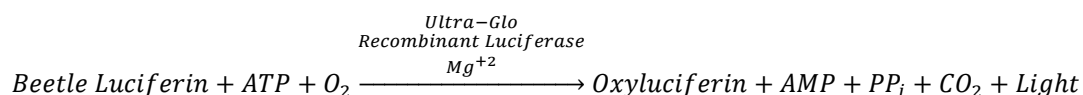
High performance liquid chromatography (HPLC) assay was used to determine the concentration of amino acids in samples collected every 24 hours. The samples were centrifuged 5 minutes at 800rpm. The supernatants were collected and filtrated through 10kDa cut-off ultrafiltration membrane (Microcon-10kDa, Merck Millipore, UK) at 22,000 rpm, 4°C for 1 hour and stored at -20°C prior to analysis. The amino acid analysis was performed with Agilent 1260 Infinity Quaternary LC, equipped with RRLC in-line filter, 4.6mm, 0.2um; and ZORBAX Eclipse Plus C18 LC, 4.6 mm, 100 mm, 1.8 µm column (Agilent Technologies, UK). The samples were processed at $40 \pm 0.8^\circ\text{C}$, flow of 1mL/min and injection volume 1.5 µL. The mobile phases were A (10mM Na₂HPO₄, 10mM Na₂B₄O₇, 0.5 mM NaN₃, pH 8.4) and B (45% acetonitrile, 45% methanol, 10% water [v/v/v]). The gradient elution program was: 0 min, 2% (B); 0.35 min, 2%(B); 13.4, min 57% (B); 13.5 min, 100% (B); 15.7, min 100% (B); 15.8 min, 2%(B), 16min. A UV diode-array detector was used to detect the primary amino acids, derivatized using o-phthalaldehyde (OPA), at a wavelength of 338 nm (reference at 390 nm) and the secondary amino acids, derivatized using 9- fluorenylmethyl chloroformate (FMOC), at a wavelength of 262 nm (reference at 324 nm).

The calibration curves were constructed by diluting an amino acids standard solution (AA standard 1 [nmol/µL], Agilent Technologies, UK). (Examples in Appendix III)

4.7 ATP Measurement

CellTiter-Glo® reagent (Promega, UK) was used to measure the concentration of ATP. The reagent induces cell lysis and generation of a luminescent signal proportional to the amount of ATP content in 100µL of the sample, which is directly collected from flasks and incubated with 100µL of Cell-Titer-Glo® reagent at room temperature for 10 minutes. The luminescence level, relative light units (RLU) was recorded using the Glomax microplate reader (Promega, UK).

Equation 4.2: Reaction for ATP detection.



The calibration curves were constructed by diluting an ATP standard solution in fresh medium at room temperature (ATP standard 10^{-7} [M], Promega, UK) (Appendix IV).

The intracellular ATP content was determined dividing the concentration of ATP [mmol/L or mM] by the concentration of viable cells [Cell/L]. Therefore, the units for intracellular ATP content are [mmol ATP/Cell]. It was assumed that early apoptotic and dead cells intracellular ATP content is negligible.

4.8 Statistical analysis

The analysis of variance (ANOVA) was applied to detect significant differences in the cellular ATP content at different stages of batch growth: lag, exponential, stationary and death phases, i.e., differences in cellular ATP content within days of culture.

The analysis applied consisted of two-way ANOVA, which compares the mean differences between groups that have been split into two independent variables or factors, in this case, batches with different initial cell densities and culture days (3 independent replicates).

When the ANOVA test detected interactions within groups, t-test were performed to discriminate which pair of groups presented significant differences.

4.9 Mathematical model development

The mathematical model was developed following the “Model Development Framework for Biological Systems” (Kiparissides et al., 2011) and implemented in gPROMS® 4.1.1 using a desktop computer with an Intel® Core™ i7 CPU.

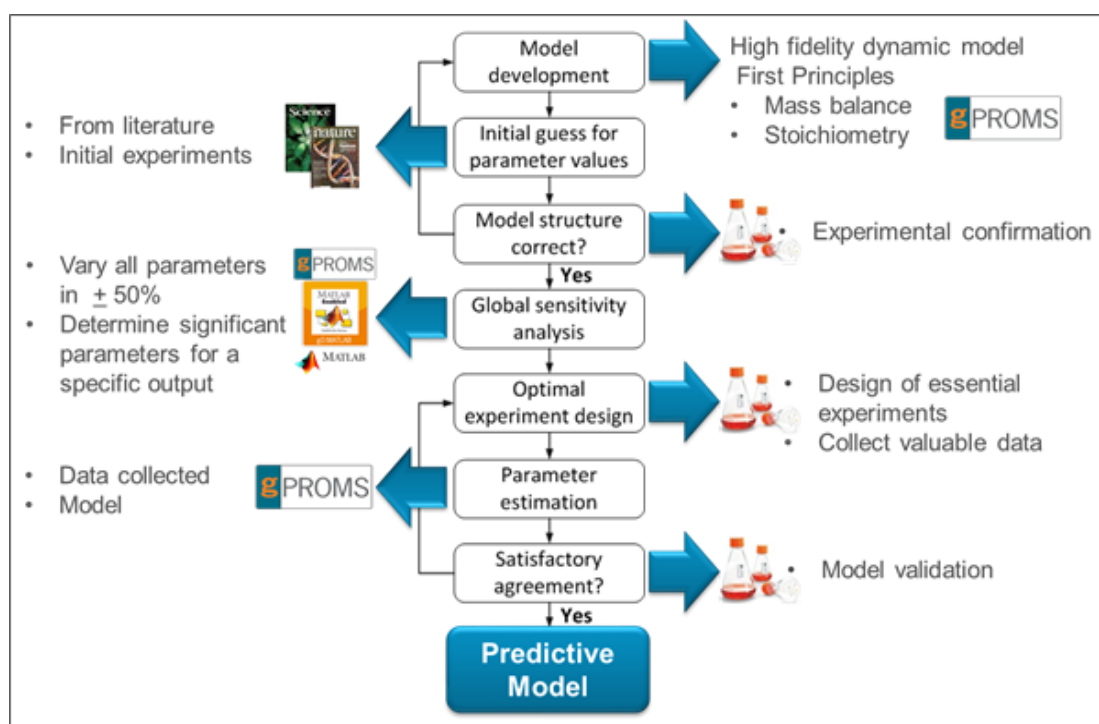


Figure 4.1: Model development framework.

The high fidelity models were developed base on mass balances, Monod-type kinetic equations and stoichiometric reactions.

4.9.1 Model analysis: GSA

The mathematical model development framework (Kiparissides *et al.*, 2011; Koumpouras & Kontoravdi, 2012) establishes that GSA must be performed to quantitatively measure the contribution of each parameter and their interactions to the output variance, and determine which the significant parameters are and need to be re-estimated. The model

parameters were varied from their initial value within a biologically feasible range ($\pm 50\%$). The sampling points were established using the `sobolset` function in MATLAB® R2015a, simulated using gPROMS® 4.1.1 and the data was transferred from gPROMS® to MATLAB via `go:MATLAB` tool. In MATLAB® the GUI-HDMR software was used to evaluate the output (viable cells, mAb titre and ATP concentration), at 288 simulation times (every 2 hours). The parameter whose sensitivity indices (SIs) values were higher than 0.1 were deemed significant and re-estimated, as SI values below 0.1 are considered the result of experimental noise (Sidolli et al., 2005).

4.9.2 Parameter estimation

The non-significant parameters were fixed to the initial value and the significant parameters were re-estimated using experimental data. The estimation of the parameters was performed in gPROMS 4.1.1, considering constant relative variance of the sensor equal to 10%.

**CHAPTER 5: Energy model for batch cultures of GS-NS0
cells in DMEM & 10% serum**

5 Energy model for batch cultures of GS-NS0 cells in DMEM & 10% serum

The first model that predicts the intracellular ATP content in mammalian cells is presented in this chapter, which covers the details of the model construction, analysis, parameter estimation, validation and prediction capability testing. The dynamic predictive model was developed for batch cultures of GS-NS0 in DMEM & 10% FBS, it is based on growth kinetics, nutrient metabolism, mAbs secretion and the production/consumption of ATP based on glucose/amino acids energy metabolic networks. The validated model successfully predicts the number of viable, apoptotic and dead cells, and the concentrations of ATP, glucose, 15 amino acids and lactate. The results from GSA, parameter estimation and model validation are extensively analysed and discussed. The validated model was used in the next chapters for medium development and fed-batch culture optimisation.

5.1 Introduction

ATP is vital for all cell functions, growth, proliferation, maintenance and production. Mammalian cells maintain a relatively permanent intracellular level of energy or ATP content, which is required to operate in optimal conditions (Reitzer et al., 1979). ATP levels are slightly higher during exponential growth and viable cells contain more than two orders of magnitude greater specific intracellular ATP levels than non-viable cells (Sonderhoff et al., 1991). The energy homeostasis is preserved by the energy metabolism and its understanding is essential to control the intracellular ATP content.

Nutrient starvation induces apoptosis and reduces the intracellular ATP content, which determines cells' death fate, via apoptosis (25-70% of the viable intracellular ATP content) or by necrosis (less than 15% of the viable intracellular ATP content) (Eguchi et al., 1997, Lieberthal et al., 1998). During apoptosis nuclear fragmentation, chromatin condensation and formation of apoptotic bodies disrupt the machinery to produce and secrete heterologous proteins. Throughout necrosis, the loss of the membrane integrity, swelling and the drastic

morphological changes make the product assembly and its secretion unfeasible. Therefore, apoptosis and necrosis have detrimental effects on mAbs productivity. However, apoptosis is a reversible process where the supply of nutrients can revert the starvation effect (Eagle, 1954), whilst necrosis is irreversible. Hence, the energy availability becomes a key factor in maintaining the viability cultures. The development of a mathematical model to predict the intracellular ATP levels will allow the estimation of energy sources requirements that need to be replenished to maintain the optimal energy levels to avoid premature apoptosis and necrosis by starvation, preserve the culture viability, extend the culture longevity and increase the mAbs productivity.

5.2 Aims and objectives

The aim of this chapter is the formulation of a mathematical model based on first principles, mass balances, stoichiometric equations and modified Monod kinetics that allows a precise calculation of intracellular ATP content in batch cultures of GS-NS0 cells. The objectives to achieve this aim were:

- 1) **Design of a mathematical model for the prediction of intracellular ATP content in GS-NS0 cells producing cB72.3 mAb:** The high fidelity model was based on first principles, mass and stoichiometric balances, and Monod kinetics.
- 2) **Identification of significant model parameters:** GSA was applied to identify the subset of significant model parameters.
- 3) **Parameter estimation:** The subset of the model significant parameters was re-estimated using data from three independent replicates of a specific batch culture.
- 4) **Validation and prediction capability testing of the model:** Three batch experiments under different initial cell densities were performed, and their experimental results were compared to the model prediction for the validation and predictive capability testing of the model.

5.3 Methodology

Model categories are structured/unstructured and segregated/non-segregated. The first classification consists in the internal compartmentalization of the cell, e.g., the model explains processes that take place in different units inside the cell. The second classification is related to the heterogeneity of cell culture population, for instance, if a cell culture is segregated, it is composed of cells in different stages of growth, conversely, an unsegregated model assumes a homogeneous cell population (Sidoli et al., 2004). An unstructured and non-segregated model has been developed to describe batch cultures under the conditions given Chapter 4 and the model assumes ideal CSTR conditions.

5.3.1 Cell growth

The total balances on viable (X_v [Cells/L]), early apoptotic (X_a [Cells/L]) and dead cells (X_d [Cells/L]) are function of the specific growth rate (μ [h^{-1}]), apoptotic rate (μ_a [h^{-1}]) and dead rate (μ_d [h^{-1}]), respectively. The number of apoptotic cells depends on the apoptosis rate and the transition rate from early apoptotic to late apoptotic or dead cells ($\mu_{a,d}$ [h^{-1}]) (O'Connor et al., 2006). This transition is characterised by the constant $k_{a,d}$ that implies a directly proportional relation between the apoptotic rate and transition rate, similar to the equation presented by Ho et al., that relates lysis rate to death rate (Ho et al., 2006).

Equation 5.1: Mass balance for viable cells.

$$\frac{dVX_v}{dt} = VX_v(\mu - \mu_a - \mu_d)$$

Equation 5.2: Mass balance for apoptotic cells.

$$\frac{dVX_a}{dt} = VX_v\mu_a - VX_a\mu_{a,d}$$

Equation 5.3: Transition rate from early apoptotic for late apoptotic or dead cells.

$$\mu_{a,d} = k_{a,d} \mu_a$$

Equation 5.4: Mass balance for dead cells.

$$\frac{dVX_d}{dt} = VX_v\mu_d + VX_a\mu_{a,d} - VX_dk_{lys}$$

The growth rate equation comprises of four terms that describe the GS-NS0 system metabolism: (i)The maximum theoretical growth rate (μ_{max}) for GS-NS0 cells in DMEM medium,(ii) Monod term for growth in glucose and (iii) glutamate or arginine, and (iv) Monod term for growth inhibition by lactate concentration.

Equation 5.5: Specific growth rate.

$$\mu = \mu_{max} \left(\frac{GLC}{K_{GLC} + GLC} \right) \left(\frac{GLU}{K_{GLU} + GLU} + \frac{ARG}{K_{ARG} + ARG} \right) \left(\frac{1}{1 + \left(\frac{LAC}{K_{I,LAC}} \right)^n} \right)$$

Glucose has been commonly considered as the only limiting substrate for cell growth, but mammalian cells proliferation is also limited by the absence of essential amino acids, which cells are unable to synthesise and must always be available in the culture medium for cells' survival (Eagle, 1954). For the GS-expression-system, cells produce glutamine, amino acid that is absent in the medium for cells selection, from glutamate. When glutamate is depleted the cells can still grow by shifting their metabolism, producing glutamine and glutamate from arginine. In consequence, cell proliferation is diminished but not zero until arginine is exhausted. In Figure 5.1 (A), the profile of the total cell number reflects the effect the of glutamate and arginine depletion in the cells proliferation. In Figure 5.1 (B), the concentration profile of the amino acids glutamate and arginine depicts the depletion times, 48 and 96 hours of culture, respectively.

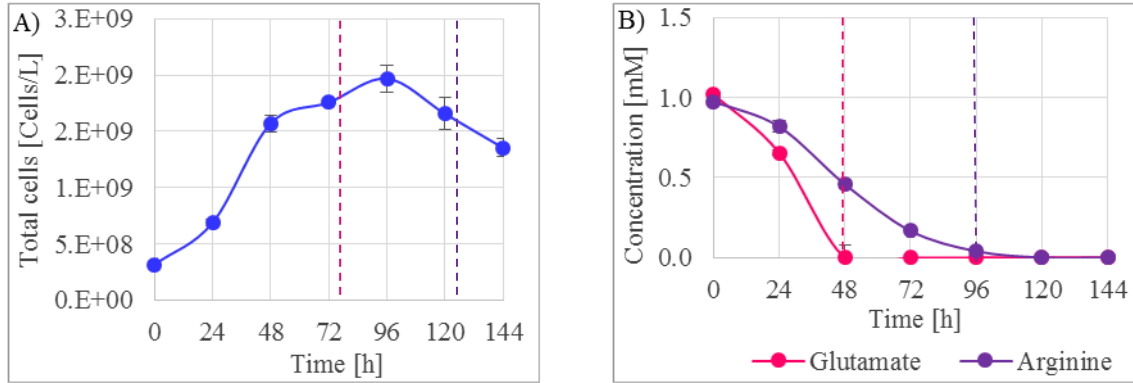


Figure 5.1: Effect of glutamate and arginine depletion in batch cultures. Experimental results for (A) Total cells concentration and (B) Glutamate and arginine concentrations.

The last term in the equation corresponds to the modified Monod formulation of growth inhibition by lactate. Ozturk et al. presented a compilation of the lactate inhibition Monod constant ($K_{I,LAC}$), which values varies from 10 to 55 [mM], in mouse hybridoma cells (Ozturk et al., 1991).

5.3.2 Apoptosis and cell death

Amino acid starvation is one of the conditions that induce apoptosis (Franěk & Šrámková, 1994, Simpson et al., 1998). In GS-NS0 cultures in DMEM medium supplemented with 10% FBS, glutamate and aspartate are exhausted early in the culture triggering apoptosis. This event occurs even when both amino acids are replaced by arginine and asparagine, respectively. When leucine and isoleucine are exhausted at the end of batch cultures, the depletion of these nutrients has a significant impact in the system because these are essential amino acids, and they cannot be synthesised from any precursor molecule. The term STV relates to the limitation of glucose and amino acids, the depletion of which activates apoptosis or death.

Equation 5.6: Starvation term.

$$STV = \left(\frac{K_{glc,lim}}{K_{glc,lim} + GLC} + \frac{K_{glu,lim}}{K_{glu,lim} + GLU} + \frac{K_{asp,lim}}{K_{asp,lim} + ASP} + \frac{K_{leu,lim}}{K_{leu,lim} + LEU} + \frac{K_{ile,lim}}{K_{ile,lim} + ILE} + \frac{K_{arg,lim}}{K_{arg,lim} + ARG} \right)$$

The specific apoptosis rate is composed of two terms. The first takes into account the limitation of glucose and amino acids throughout the culture (Simon and Karim, 2001) and the second considers the effect of lactate.

Equation 5.7: Specific apoptosis rate.

$$\mu_a = \mu_{a,STV}STV + \mu_{a,LAC} \left(\frac{1}{1 + \left(\frac{K_{a,lac}}{LAC} \right)^n} \right)$$

The specific necrosis death rate is expressed as a function of the same starvation term. In this case, the limitation of amino acids is considered as a limitation of energy sources that triggers the reduction of intracellular ATP concentration, which is a determinant for the type of cell death, apoptosis or necrosis (Eguchi et al., 1997) and the second term is related to the toxic effect of lactate concentration. Omasa et al. presented a review of the Monod constant for lactate toxicity ($K_{d,LAC}$) and the exponential factor (n), which describes the steepness of the effect of lactate concentration on the death rate (Omasa et al., 1991), e.g., n was estimated to be 2 for mouse-mouse hybridoma CRL-1606 (Kontoravdi et al., 2010).

Equation 5.8: Specific death rate.

$$\mu_d = \mu_{d,STV}STV + \mu_{d,lac} \left(\frac{1}{1 + \left(\frac{K_{d,lac}}{LAC} \right)^n} \right)$$

5.3.3 Glucose and lactate mass balances

5.3.3.1 Glucose mass balance

The stoichiometric analysis developed by Xie & Wang was the first model that considered nutrient uptake in a separated form. In this case, the specific consumption rate of glucose has been divided into four different terms: proliferation, mAbs assembly and energy production (Xie & Wang, 1993). The last term, energy production, has been separated into two expressions, one that takes in account the amount of glucose that produces energy through

glycolysis and produces alanine and lactate, and the other takes into account the amount of glucose that completes its oxidation via TCA cycle.

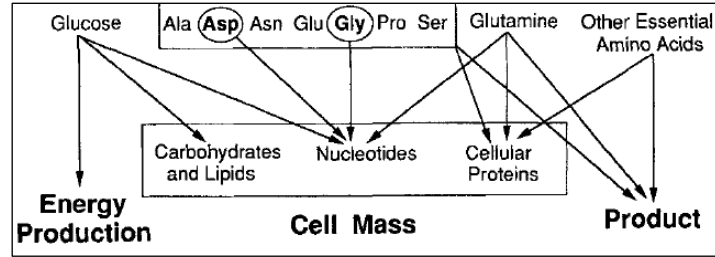


Figure 5.2: Analysis of animal of nutrients uptake (adapted from Xie & Wang, 1993).

Equation 5.9: Glucose mas balance.

$$\frac{d(V GLC)}{dt} = -VX_v(Q_{x,glc} + Q_{mAbs,glc} + Q_{lac,glc} + Q_{ala,glc} + Q_{tca,glc})$$

The specific consumption rate of glucose to generate structural components, $Q_{x,glc}$, takes into account the cell composition. The carbohydrates cell composition varies between cell lines and culture conditions. The values reported in the literature are 7.05%, 4.12% and 6.4% for CRL-1606 (Zupke & Stephanopoulos, 1994), murine hybridoma cell line (Bonarius et al., 1995) and CRL-1606 (Xie & Wang, 1996), respectively.

Equation 5.10: Specific consumption rate of glucose for biosynthesis of structural components.

$$Q_{x,glc} = \mu/Y_{x,glc}$$

The second specific consumption rate is related to the carbohydrates that are part of the product glycosylations, $Q_{mAb,glc}$. The N-linked glycosylations occur at Asn²⁹⁷ on the Fc region in the CH₂ domain of the monoclonal antibody structure (Hamm et al., 2013). The differences in the glycosylation pattern or glycosylation heterogeneity in the same clone under different culture conditions have been well documented (Hossler et al., 2009; Jimenez et al., 2011).

Hamm et al. presented the most common glycosylation patterns in NS0 cells, G1F and G2F. The pattern G2F consist of one fucose (C₆H₁₂O₅), four N-acetyl-glucosamine (C₈H₁₅NO₆), three mannose (C₆H₁₂O₆) and two galactose (C₆H₁₂O₆) (Hamm et al., 2013).

Therefore, 20 molecules of glucose per molecule of antibody were considered as the glucose requirement for mAbs assembly (1.36×10^{-4} [mmol of glucose/mg of mAb]).

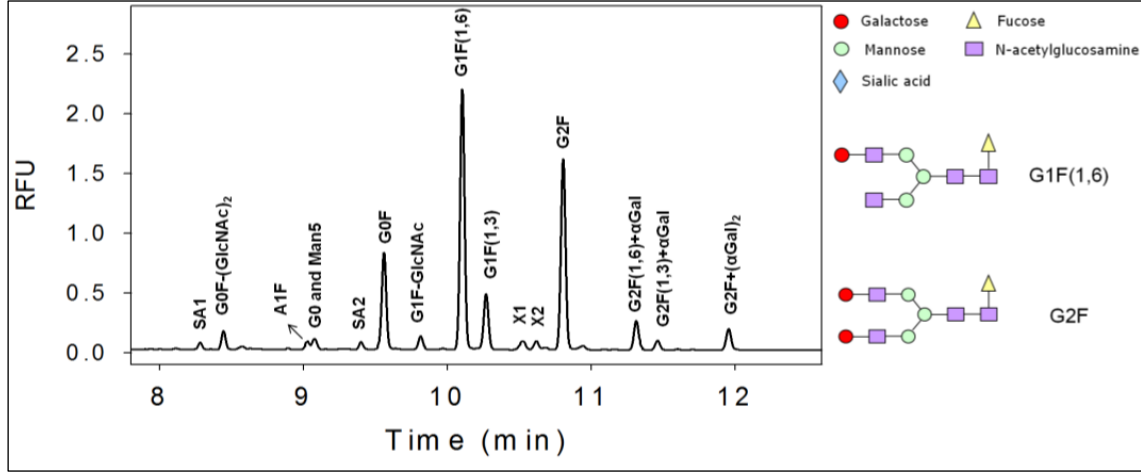


Figure 5.3: Most common glycosylation patterns in NS0 cells. Relative fluorescence units (RFU) (Hamm et al., 2013).

The terms $q_{mAb,x}$ and $M_{mAb,glc}$ are the specific antibody synthesis rate (mg/Cell h) and the mmol of glucose needed per milligram of product (1.36×10^{-4} [mmol/mg]), respectively.

Equation 5.11: Specific consumption rate of glucose for product biosynthesis.

$$Q_{mAb,glc} = q_{mAb,x} M_{mAb,glc}$$

Glucose is used to produce energy through two main pathways, glycolysis and the TCA cycle. Assuming that pyruvate is not accumulated, a molecule of glucose could produce lactate, alanine or enter into the TCA cycle through acetyl-CoA formation. The terms $Q_{lac,glc}$ [mmol/Cell h] and $Q_{ala,glc}$ [mmol/Cell h] depict the glucose specific consumption rate to produce lactate and alanine, and the term $Q_{tca,glc}$ [mmol/Cell h] describes the glucose specific consumption rate to produce energy through the TCA cycle. All these terms have the same structure that consists of one term related to proliferation and a second term related to maintenance (Jang & Barford, 2000; Kontoravdi et al., 2007-2010; Xing et al., 2010; Kiparissides et al., 2010-2011).

Equation 5.12: Specific consumption rate of glucose through glycolysis that forms lactate.

$$Q_{lac,glc} = \begin{cases} 0 & [ARG] = 0 \\ \frac{\mu}{Y_{glc,lac}} + M_{glc,lac} & [ARG] > 0 \end{cases}$$

Equation 5.13: Specific consumption rate of glucose through glycolysis that forms alanine.

$$Q_{ala,glc} = \left(\frac{\mu}{Y_{ala,glc}} + M_{ala,glc} \right)$$

Equation 5.14: Specific consumption rate of glucose to produce energy through TCA cycle.

$$Q_{tca,glc} = \left(\frac{\mu}{Y_{tca,glc}} + M_{tca,glc} \right)$$

5.3.3.2 Lactate mass balance

Lactate metabolic shifts are well documented in NS0 and CHO cell lines (Khoo & Al-Rubeai, 2009; Ma et al., 2009; Dorka et al., 2009; Wilkens et al., 2011; Luo et al., 2011; Mulukutla et al., 2012, Kyriakopoulos et al. 2013). In Figure 5.4, the results for lactate concentration from an exploratory experiment show that lactate is continuously produced until 96 hours of culture, when arginine is exhausted and the cells enter into stationary phase. Lactate shift is represented in Equation 5.12 where lactate production is zero when arginine is exhausted.

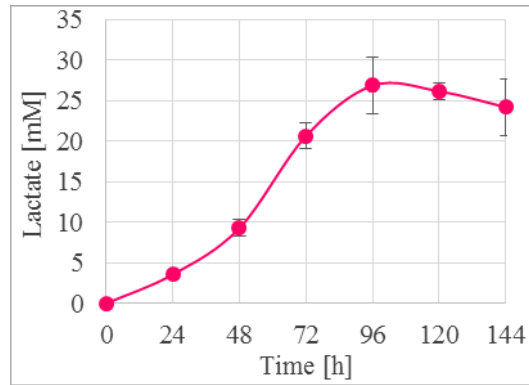


Figure 5.4: Experimental results for lactate concentration.

Per molecule of glucose that completes only glycolysis and form lactate the yield would be 2 molecules of the by-product ($Y_{lac,glc}$ equal 2). Thus, the material balance for lactate is:

Equation 5.15: Mass balance of lactate.

$$\frac{d(V LAC)}{dt} = VX_v 2Q_{lac,glc}$$

5.3.4 Mass balance of “Energy Amino Acids”

The mass balances of all the amino acids involved in energy production (glutamate, arginine, proline, histidine, valine, methionine, phenylalanine, tyrosine, leucine, isoleucine and lysine) have the same structure (Equation 5.16). In the mass balance equation AA is the amino acid concentration and the specific consumption rate has been separated into three different terms (Xie & Wang, 1993): specific consumption rate for proliferation ($Q_{x,AA}$), for mAbs production ($Q_{mAb,AA}$) and for energy production ($Q_{tca,AA}$).

Equation 5.16: Mass balance of amino acids involved in energy production.

$$\frac{d(V[AA])}{dt} = -VX_v(Q_{x,AA} + Q_{mab,AA} + Q_{tca,AA})$$

The specific consumption rate of amino acids for proliferation or proliferation uptake depends on the growth rate and the yield of biomass per amino acid ($Y_{x,AA}$ [Cell/mmol]). Kontoravdi et al., in 2007, presented an unstructured mathematical model describing cell's growth and death kinetics and the metabolism of glucose and 19 amino acids for HEK-293 and CHO cell cultures (Kontoravdi et al., 2007). In this attempt to develop a model library for mammalian cell cultures, the same term for the proliferation uptake was successfully included.

Equation 5.17: Specific consumption rate of amino acids for proliferation.

$$Q_{x,AA} = \frac{\mu}{Y_{x,AA}}$$

To calculate the yield of biomass per amino acid ($Y_{x,AA}$ [Cell/mmol]). The average of mammalian cells composition was collected from the literature (Sheikh et al., 2005). The dry

cell weight (DCW) was experimentally calculated and its value is 4.55×10^{-10} [g/Cell]. The methodology applied is in Appendix V (Bonarius et al., 1996).

Table 5.1: Yield of nutrients on biomass (Sheikh et al., 2005).

Amino acid	Cell composition [mmol/g DCW] (Sheikh et al., 2005)	Yield ($Y_{x,AA}$) [Cell/mmol]
Alanine	0.6	3.66E+09
Arginine	0.377	5.83E+09
Aspartic acid	0.359	6.12E+09
Asparagine	0.288	7.63E+09
Cysteine	0.145	1.52E+10
Glutamic Acid	0.386	5.69E+09
Glutamine	0.322	6.83E+09
Glycine	0.538	4.09E+09
Histidine	0.143	1.54E+10
Isoleucine	0.324	6.78E+09
Leucine	0.564	3.90E+09
Lysine	0.570	3.86E+09
Methionine	0.138	1.59E+10
Phenylalanine	0.219	1.00E+10
Proline	0.313	7.02E+09
Serine	0.430	5.11E+09
Tyrosine	0.182	1.21E+10
Threonine	0.386	5.69E+09
Tryptophan	0.044	5.00E+10
Valine	0.416	5.28E+09

The specific consumption rate of amino acids for mAbs production or production uptake depends on the specific antibody synthesis rate ($q_{mAb,x}$ [mg/Cell h]) and the product composition ($M_{mAb,AA}$ [mmol/mg]). Xie & Wang also included this terms, in a slightly different manner, considering both parameters as constants (Xie & Wang, 1993).

Equation 5.18: Specific consumption rate of amino acids for production.

$$Q_{mab,AA} = q_{mab,x} M_{mab,AA}$$

To calculate the product composition ($M_{mAb,AA}$ [mmol/mg]), the average IgG forms composition was collected from the literature (Quek et al., 2010).

Table 5.2: Product composition calculation.

Amino Acid	mAb composition [mg/mg mAb]	Molecular weight of AA [mg/mmol]	$M_{mAb/AA}$ [mmol AA/mg mAb]
Alanine	0.041	71.08	5.77E-04
Arginine	0.031	156.19	1.98E-04
Aspartic acid	0.038	114.1	3.33E-04
Asparagine	0.047	115.09	4.08E-04
Cysteine	0.024	103.14	2.33E-04
Glutamic Acid	0.06	129.12	4.65E-04
Glutamine	0.046	128.13	3.59E-04
Glycine	0.039	57.05	6.84E-04
Histidine	0.028	137.14	2.04E-04
Isoleucine	0.034	113.16	3.00E-04
Leucine	0.065	113.16	5.74E-04
Lysine	0.07	128.17	5.46E-04
Methionine	0.015	131.19	1.14E-04
Phenylalanine	0.054	147.18	3.67E-04
Proline	0.065	97.12	6.69E-04
Serine	0.088	87.08	1.01E-03
Tyrosine	0.041	163.18	2.51E-04
Threonine	0.099	101.1	9.79E-04
Tryptophan	0.036	186.21	1.93E-04
Valine	0.079	99.13	7.97E-04

Finally, the specific consumption rate of amino acids for energy production or energy uptake is represented by $Q_{tca,AA}$, which consists of two terms one of them related to the amino acids uptake to produce energy for proliferation ($Y_{tca,AA}$ [mmol/Cell]), and the other one associated with maintenance ($M_{tca,AA}$ [mmol/Cell h]). The parameters $Y_{tca,AA}$ and $M_{tca,AA}$ do not change from one amino acid mass balance to the other, the only parameter that changes is C_{AA} . The initial value of the parameters in Equation 5.19 are not available in the literature and were calculated based on the exploratory experiment results, fixing C_{glu} to 1.

Equation 5.19: Specific consumption rate of amino acids for energy production.

$$Q_{tca,AA} = C_{AA} \left(\frac{\mu}{Y_{tca,AA}} + M_{tca,AA} \right)$$

5.3.4.1 Glutamate mass balance

Glutamate mass balance includes the requirements of glutamate as a substrate to produce glutamine, in GS-system (Barnes et al., 2000). The terms related to glutamine requirements for cell structures and product assembling have been included in the proliferation and production uptakes equations (Equation 5.21 and Equation 5.22).

Equation 5.20: Glutamate mass balance.

$$\frac{d(V[GLU])}{dt} = -VX_v(Q_{x,glu} + Q_{mab,glu} + Q_{tca,glu})$$

Equation 5.21: Proliferation uptake.

$$Q_{x,glu} = \frac{\mu}{Y_{x,glu}} + \frac{\mu}{Y_{x,glu}}$$

Equation 5.22: Production uptake.

$$Q_{mab,glu} = M_{mab,x}(M_{mab,glu} + M_{mab,glu})$$

Equation 5.23: Energy uptake.

$$Q_{tca,glu} = C_{glu}Q_{tca,AA}$$

5.3.4.2 Arginine and proline mass balances

Glutamate is required to produce glutamine and it is also one of the main energy sources in mammalian cells. Therefore, glutamate availability is critical for cell survival and product assembly.

In Figure 5.5, the experimental results for (A) glutamate, arginine and proline concentrations, (B) total number of cells, (C) intracellular ATP concentration and (D) mAb concentration are presented. In Figure 5.5 (A) it is possible to observe that glutamate was depleted after 48 hours of culture. However, the growth rate was diminished but not zero (Figure 5.5 B), the cells did not present significant changes in the intracellular ATP content (Figure 5.5 C), and the product was continuously secreted throughout the culture (Figure 5.5

D). It was also observed that the specific growth rate was zero when arginine was depleted and its consumption increased when glutamate was exhausted.

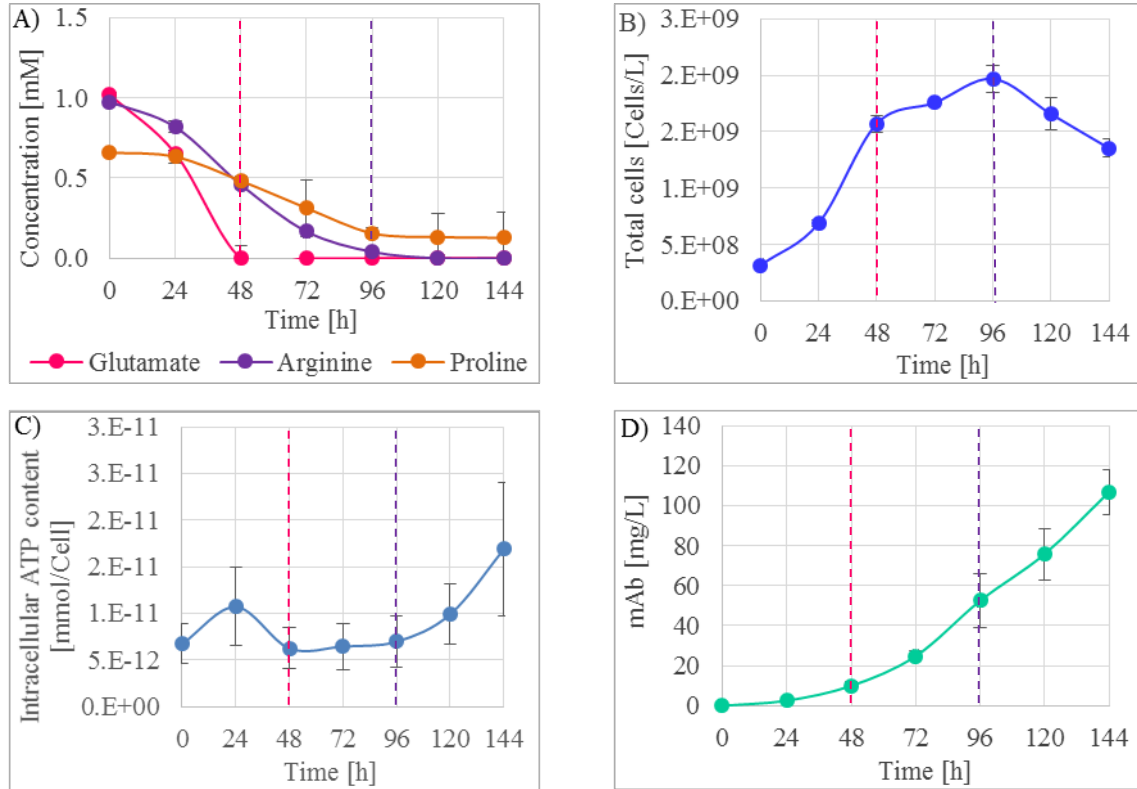


Figure 5.5: Effect of glutamate and arginine depletion in the proliferation rate, intracellular ATP content and product secretion. Experimental results for (A) Glutamate, arginine and proline concentrations, (B) Total cells concentration, (C) Intracellular ATP content and (D) Monoclonal antibody concentration.

In this work, metabolic shifts in arginine and proline metabolism have been incorporated. The assumptions are: (i) glutamate depletion activates the incorporation of arginine into the mitochondrion, arginase II synthesises the production of ornithine, nitric oxide synthase (OAT) catalyses the production of Δ -L-pyrroline-5-carboxylate and pyrroline-5-carboxylate dehydrogenase (P5CD) synthesises glutamate (Figure 5.6)(Li et al., 2001); (ii) glutamate depletion activates apoptosis and proline oxidase (POX), a mitochondrial enzyme, which catalyses the oxidation of proline to Δ -L-pyrroline- 5-carboxylate and glutamate synthesis (Chien-an et al., 2006).

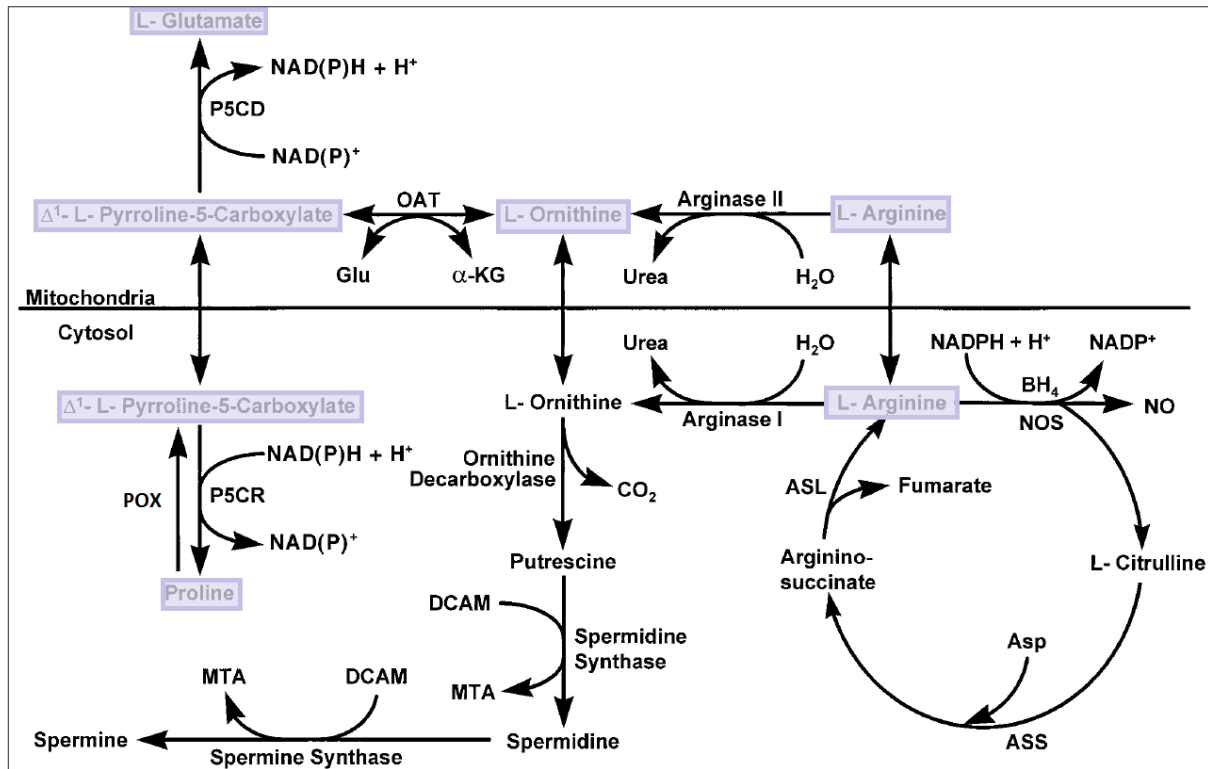


Figure 5.6: Arginine metabolism in mammalian cells. ASL, argininosuccinate lyase; Asp, aspartate; ASS, argininosuccinate synthase; BH_4 , tetrahydrobiopterin; DCAM, decarboxylated 5-adenosylmethionine; Glu, glutamate; MTA, methylthioadenosine; NO, nitric oxide; NOS, nitric oxide synthase; OAT, ornithine aminotransferase; P5CD, pyrroline-5-carboxylate dehydrogenase; P5CR, pyrroline-5-carboxylate reductase; α -KG, α -ketoglutarate. (Adapted from Li et al., 2001).

Metabolic shifts have been widely documented in metabolic flux analysis studies. It has been observed that cells adapted their metabolism and experience three metabolic states: an initial state characterized by a high glycolytic activity and lactate production; a second with balanced glycolysis and TCA cycle; and the third with glutamine/glutamate limitation alanine consumption (Figure 5.7). These metabolic shifts and states have been observed in batch, fed-batch and continuous cultures (Gambhir et al., 2003; Niklas et al., 2011b; Dean & Reddy, 2013).

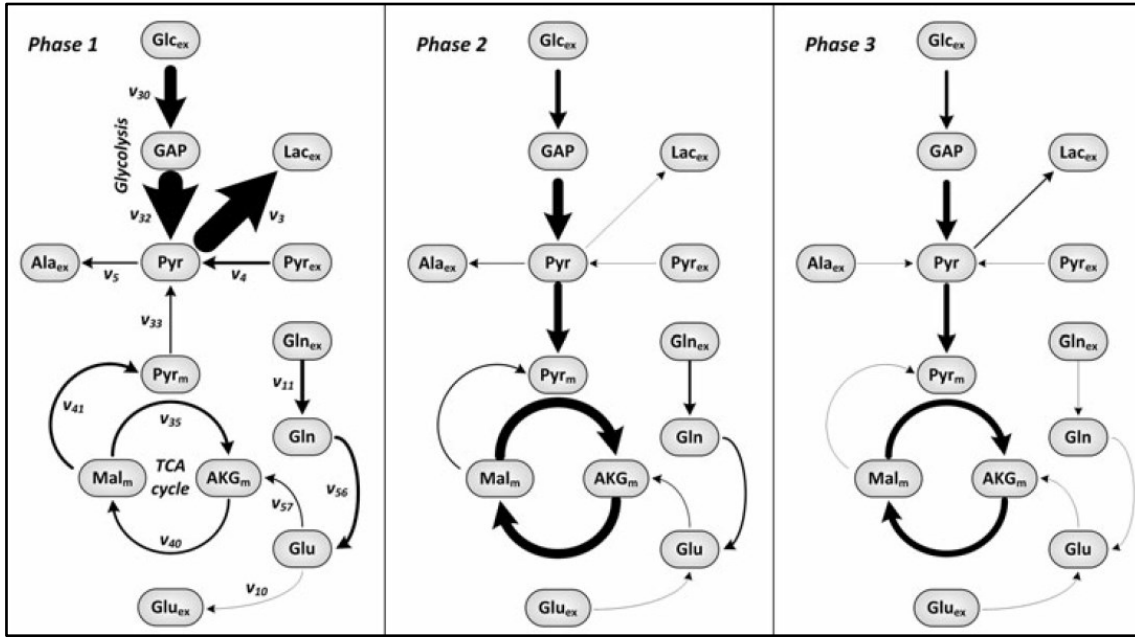


Figure 5.7: Metabolic flux distribution of AGE.HN cells in batch cultures in each metabolic states (Niklas et al., 2011b).

In glutamine/glutamate limitation, the cells adapt their metabolism to produce glutamine through arginine and energy incorporating proline into the TCA cycle (Quek et al., 2010). These two metabolic shifts were included in the arginine and proline mass balances.

Equation 5.24: Arginine and proline mass balances.

$$\frac{d(V ARG)}{dt} - V X_v (Q_{x,arg} + Q_{mab,arg} + Q_{tca,arg})$$

$$\frac{d(V PRO)}{dt} = -V X_v (Q_{x,pro} + Q_{mab,pro} + Q_{tca,pro})$$

Equation 5.25: Proliferation uptake of arginine and proline.

$$Q_{x,arg} = \begin{cases} \frac{\mu}{Y_{x,arg}} & [GLU] > 0 \\ \frac{\mu}{Y_{x,arg}} + \frac{\mu}{Y_{x,glu}} + \frac{\mu}{Y_{x,gln}} & [GLU] = 0 \end{cases}$$

$$Q_{x,pro} = \frac{\mu}{Y_{x,pro}}$$

Equation 5.26: Production uptake of arginine and proline.

$$Q_{mab,arg} = \begin{cases} M_{mab,x} M_{mab,arg} & [GLU] > 0 \\ M_{mab,x} (M_{mab,arg} + M_{mab,glu} + M_{mab,gln}) & [GLU] = 0 \end{cases}$$

$$Q_{mab,pro} = M_{mab,x} M_{mab,pro}$$

Equation 5.27: Energy uptake of arginine and proline.

$$Q_{tca,arg} = C_{arg} Q_{tca,AA}$$

$$Q_{tca,pro} = \begin{cases} 0 & [GLU] > 0 \\ C_{pro} * Q_{tca,AA} & [GLU] = 0 \end{cases}$$

5.3.4.3 Aspartate and asparagine mass balances

Asparagine also presents a metabolic shift. Aspartate depletion does not present any effect in the growth rate or productivity, but a significant change in asparagine uptake is observed when aspartate concentration reached 0.2 [mM]. Quek et al., observed the same change in Sp/02-derived hybridoma cells, and included the asparagine degradation pathway instead of the synthesis pathway given that the observed uptake of asparagine exceeded the biosynthetic demand (Quek et al., 2011). In Figure 5.8, the effect of low concentrations of aspartic acid (under 0.2[mM]) in arginine uptake is presented.

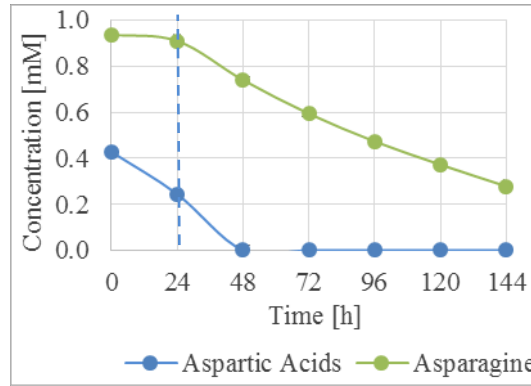


Figure 5.8: Change in asparagine uptake under low concentration of aspartate. Experimental results for aspartate and arginine concentration profiles.

The metabolic shift was incorporated in Equation 5.29 and Equation 5.30.

Equation 5.28: Aspartate and asparagine mass balances.

$$\begin{aligned}\frac{d(V[ASP])}{dt} &= -VX_v(Q_{x,asp} + Q_{mab,asp} + Q_{tca,asp}) \\ \frac{d(V[ASN])}{dt} &= -VX_v(Q_{x,asn} + Q_{mab,asn} + Q_{tca,asn})\end{aligned}$$

Equation 5.29: Proliferation uptake of arginine and proline.

$$\begin{aligned}Q_{x,asp} &= \frac{\mu}{Y_{x,asp}} \\ Q_{x,asn} &= \begin{cases} \frac{\mu}{Y_{x,asn}} & [ASP] > 0 \\ \frac{\mu}{Y_{x,asn}} + \frac{\mu}{Y_{x,asp}} & [ASP] = 0 \end{cases}\end{aligned}$$

Equation 5.30: Production uptake of arginine and proline.

$$\begin{aligned}Q_{mab,asp} &= M_{mab,x}M_{mab,asp} \\ Q_{mab,asn} &= \begin{cases} M_{mab,x}M_{mab,asn} & [ASP] > 0 \\ M_{mab,x}(M_{mab,asn} + M_{mab,asp}) & [ASP] = 0 \end{cases}\end{aligned}$$

Equation 5.31: Energy uptake of arginine and proline.

$$\begin{aligned}Q_{tca,asp} &= C_{asp}Q_{tca,AA} \\ Q_{tca,pro} &= \begin{cases} 0 & [ASP] > 0 \\ C_{asp} * Q_{tca,AA} & [ASP] = 0 \end{cases}\end{aligned}$$

5.3.4.4 Alanine mass balance

Alanine and lactate metabolic shifts have been documented as simultaneous phenomena (Ma et al., 2009; Luo et al., 2011). In Figure 5.9 (A), alanine shows a notorious metabolic change after 48 hours, while lactate, in Figure 5.9 (B), is continuously produced until 96 hours of culture, when the cells enter into stationary phase. The changes are not simultaneous and alanine shift seems to be affected by glutamate depletion, while lactate shift seems to be affected by arginine exhaustion (Equation 5.12).

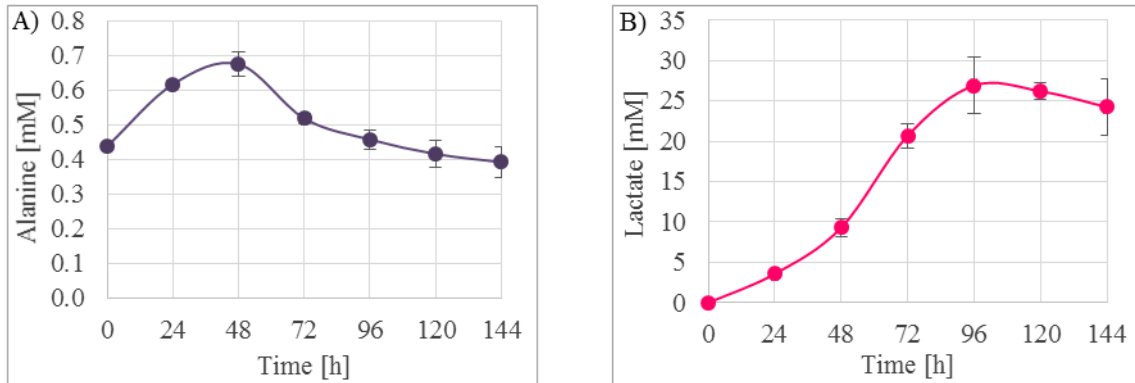


Figure 5.9: Metabolic shifts. Experimental results for (A) alanine concentration profile and (B) lactate concentration profile.

The material balance for alanine is more complex compared to other amino acids mass balances due to the incorporation of all the metabolic shifts detected in the batch culture. It has been reported that alanine shift is related to glutamate depletion (Niklas et al., 2011b) and it has been assumed that arginine replaces glutamate biosynthetic requirements and proline replaces glutamate energy requirements, when glutamate is exhausted (Li et al., 2001; Chien-an et al., 2006). These shifts have been incorporated in the term for the alanine specific consumption rate (Equation 5.35).

Equation 5.32: Mass balance of lactate.

$$\frac{d(V[ALA])}{dt} = VX_v(2Q_{ala,glc} - Q_{x,ala} - Q_{mab,ala} - Q_{tca,ala})$$

Equation 5.33: Proliferation uptake.

$$Q_{x,glu} = \frac{\mu}{Y_{x,ala}}$$

Equation 5.34: Production uptake.

$$Q_{mab,ala} = M_{mab,x}M_{mab,ala}$$

Equation 5.35: Energy uptake.

$$Q_{tca,ala} = \begin{cases} 0 & [GLU] \wedge [ARG] > 0 \\ \frac{(\theta_{glu}Q_{tca,glu} - \theta_{tyr}Q_{tca,tyr} - \theta_{phe}Q_{tca,phe} - \theta_{pro}Q_{tca,pro})}{\theta_{ala}} & [GLU] = 0 \wedge [ARG] > 0 \\ \frac{(\theta_{glu}Q_{tca,glu} - \theta_{tyr}Q_{tca,tyr} - \theta_{phe}Q_{tca,phe} - \theta_{pro}Q_{tca,pro})}{\theta_{ala}} + \frac{\theta_{arg}Q_{tca,arg}}{\theta_{ala}} & [GLU] \wedge [ARG] > 0 \end{cases}$$

It has been assumed that alanine is incorporated into the TCA cycle to cover the energy requirements that have not been covered by tyrosine, phenylalanine and proline metabolic shifts. When arginine is exhausted alanine, and possibly lactate, is incorporated into the TCA cycle via acetyl-CoA to supply the energy required. Metabolic shift of alanine and other intermediates linked to the glycolytic pathway or the TCA cycle (citrate, isocitrate, and succinate) have been reported. In particular, it has been shown that the shifts occur downstream of pyruvate, but upstream of fumarate in the TCA cycle (Ma et al., 2011).

5.3.5 Monoclonal antibody production

The amount of monoclonal antibody accumulated inside the cells, MABacc [mg/Cell], depends on the specific antibody synthesis rate ($q_{mAb,x}$ [mg/Cell h]) and the secretion rate (M_s [mg/Cell h]). Apoptosis induces organized destruction of the cytoskeleton and apoptotic bodies formation (Vives et al., 2003), and cellular death triggers Golgi apparatus fragmentation (Mukherjee et al., 2007). These events induce the passive and unspecific release of the product in the culture medium (Al-Rubeai & Emery, 1990), to capture this phenomenon the accumulation term has been included in the mass balance of monoclonal antibody concentration (MAB [mg/L]). Thus, the accumulation of the mAb in the medium would be a result of the addition of the mature antibody secretion from viable cells and the unspecific release of the accumulated antibody by apoptosis and necrosis fragmentations.

Equation 5.36: mAb accumulation inside viable cells.

$$\frac{d([MAB_{acc}])}{dt} = q_{mAb,x} - M_s$$

Equation 5.37: Monoclonal antibody mass balance.

$$\frac{d(V[mAb])}{dt} = VX_v M_S + VX_v (\mu_a + \mu_d) MAB_{acc}$$

5.3.6 ATP balance

Viable mammalian cells require constant intracellular ATP availability for a variety of essential activities. Therefore, production and consumption of energy must be in balance. The main energy production metabolic pathways are glycolysis and the TCA cycle, which begins with the transfer of a two-carbon acetyl group from acetyl-CoA to an acceptor compound (oxaloacetate) to form citrate. Acetyl-CoA is derived from glucose, lysine, leucine, isoleucine, phenylalanine and tyrosine, while oxaloacetate can be produced by the oxidation of glutamate, glutamine, arginine, valine, threonine, methionine, phenylalanine, tyrosine, aspartate and asparagine. Figure 5.10 depicts the energy metabolism network used in the present work (Xie & Wang, 1996; Follstad et al., 1999; Dorka et al., 2009; Zhao et al., 2009; Wilkens et al., 2011; Niklas et al., 2011b; Mulukutla et al., 2012; Nicolae et al., 2014).

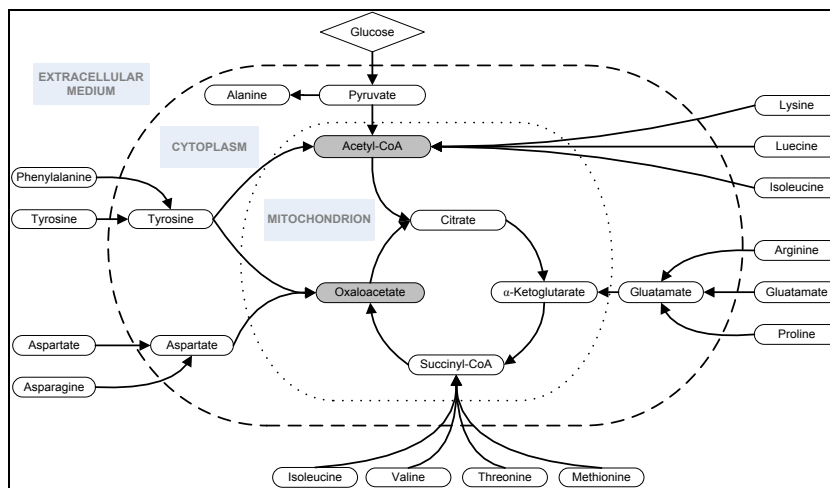


Figure 5.10: Simplified energy metabolism pathways.

The stoichiometric macro-reactions for ATP production from glucose and amino acids are described in Table 5.3.

Table 5.3 Significant macro-reactions for glucose and amino acids catabolism.

Nutrient (i)	Stoichiometric reactions	Reference
Glucose	$Glucose + 2ADP + 2Pi \Rightarrow 2Lactate + 2ATP + 2H_2O$	(Xie & Wang, 1996)
Glucose	$Glucose + 2ADP + 2Pi + 2NAD^+ + Glutamate \Rightarrow 2Alanine + 2ATP + 2NADH + 2H^+ + 2H_2O + \alpha Ketoglutarate$	(Xie & Wang, 1996)
Glucose	$Glucose + 2CoASH + 2ADP + 2Pi + 4NAD^+ \Rightarrow 2AcetylCoA + 2ATP + 2NADH + 2H^+ + 2NADH_{(M)} + 2H_2O + 2CO_2$	(Xie & Wang, 1996)
Alanine	$Alanine + CoASH + NAD^+ + \alpha Ketoglutarate \Rightarrow AcetylCoA + CO_2 + NADH_{(M)} + H^+ + Glutamate$	(Nelson and Cox, 2013)
Lysine	$Lysine + 5NAD^+ + FAD + CoASH \Rightarrow 2Acetyl\ CoA + 5NADH + FADH_2 + 2CO_2 + 2NH_2-$	(Xie & Wang, 1996)
Leucine	$Leucine + 2NAD^+ + FAD + 3CoASH + 2ATP \Rightarrow 3Acetyl\ CoA + 2NADH + FADH_2 + ADP + Pi + NH_2-$	(Xie & Wang, 1996)
Isoleucine	$Isoleucine + 4NAD^+ + 2FAD + CoA \Rightarrow AcetylCoA + Oxaloacetate + 3NADH + NADH_{(M)} + FADH_2 + FADH_{2(M)} + NH_2-$	(Xie & Wang, 1996)
Phenylalanine	$Phenylalanine + 2CoA + NADH + NAD^+ \Rightarrow 2Acetyl - CoA + Oxaloacetate + NAD^+ + NADH_{(M)} + NH_2-$	(Nelson and Cox, 2013)
Tyrosine	$Tyrosine + 2CoA + NAD^+ \Rightarrow 2Acetyl - CoA + Oxaloacetate + NADH_{(M)} + NH_2-$	(Nelson and Cox, 2013)
Glutamate	$Glutamate + 2H_2O + 4NAD^+ + FAD + GDP + Pi \Rightarrow Oxaloacetate + CoA + NADH + 3NADH_M + FADH_{2(M)} + 2CO_2 + GTP$	(Xie & Wang, 1996)
Arginine	$Arginine + 3H_2O + 5NAD^+ + FAD + GDP + Pi \Rightarrow Oxaloacetate + CoA + 2NADH + 3NADH_M + FADH_{2(M)} + 2CO_2 + GTP$	(Nelson and Cox, 2013)
Proline	$Proline + 2H_2O + 5NAD + FAD + GDP + Pi \Rightarrow Oxaloacetate + CoA + 2NADH + 3NADH_M + FADH_{2(M)} + 2CO_2 + GTP$	(Nelson and Cox, 2013)
Aspartate	$Aspartate + \alpha Ketoglutarate \Rightarrow Oxaloacetate + Glutamate$	(Xie & Wang, 1996)
Asparagine	$Asparagine + H_2O + \alpha Ketoglutarate \Rightarrow Oxaloacetate + Glutamate + NH_4^+$	(Xie & Wang, 1996)
Valine	$Valine + 5NAD^+ + 2FAD + ATP + GDP \Rightarrow Oxaloacetate + 4\ NADH + NADH_M + FADH_2 + FADH_{2(M)} + CO_2 + NH_2- + ADP + GTP$	(Xie & Wang, 1996)
Threonine	$Threonine + 2\ NAD^+ + FAD + CO_2 + GDP + Pi \Rightarrow Oxaloacetate + NADH + NADH_M + FADH_{2(M)} + NH_2- + GTP$	(Xie & Wang, 1996)
Methionine	$Methionine + 2ATP + NAD + FAD + 2H_2O + GDP \Rightarrow Oxaloacetate + PP_i + NADH + NADH_M + FADH_{2(M)} + NH_4 + 2ADP + GTP$	(Nelson and Cox, 2013)
TCA Cycle	$AcetylCoA + 2H_2O + GDP + Pi + 3NAD^+ + FAD \Rightarrow CoASH + GTP + 3NADH_{(M)} + FADH_{2(M)} + 2CO_2$	(Nelson and Cox, 2013)

(C) The molecule is produced in the cytoplasm and (M). The molecule is produced inside the mitochondrion.

The stoichiometric macro-reactions contribute with the stoichiometric coefficient values (θ_i) and the differential equations that describe the mass balances of cells, nutrients and metabolites, contribute with the specific consumption rate of nutrients to produce energy ($Q_{tca,i}$). The coupling of the stoichiometric macro-reactions and the mass balances allow us to calculate the ATP balance.

Equation 5.38: ATP balance.

$$\frac{d(V \text{ ATP})}{dt} = VX_v \left(\sum_{i=1}^n \theta_i * Q_{tca,i} \right) - VX_v \left(\frac{\mu}{Y_{x,ATP}} + M_{x,ATP} + M_{mab,ATP} q_{mab,x} \right)$$

The first term describes the ATP production. This term considers the sum of the energy contribution from each nutrient, which is the stoichiometric coefficient (θ_i) multiplied by the specific consumption rate of the corresponding nutrient ($Q_{tca,glc}$ and $Q_{tca,AA}$). The values for the stoichiometric coefficients are found in Table 5.4. The values of the coefficients were calculated based on the assumption that acetyl-CoA is the limiting reagent, regarding the oxaloacetate regeneration. Additionally, it was considered that acetyl-CoA is used to produce other macromolecules such as fatty acids and only the 70% enters into the TCA cycle (Nicolae et al., 2014). Thus, the stoichiometric coefficients consider one cycle per molecule of acetyl-CoA that enters into the TCA cycle. The stoichiometric coefficients for ATP production from cytoplasmic NADH and FADH₂ are 1.5, while from mitochondrial NADH and FADH₂ are 2.5 and 1.5, respectively (Mulukutla et al., 2010; Nelson and Cox, 2013).

The second term, in the ATP balance equation, depicts ATP consumption, which is expressed as the addition of three terms. The first, ATP utilization for proliferation ($\mu/Y_{x,ATP}$), the second, ATP requirements for non-growth-associated processes such as maintenance ($m_{x,ATP}$) (Kilburn et al., 1969; Roels et al., 1980; Xie & Wang, 1993-1996) and the third, ATP consumption for mAbs synthesis and processing, including mRNA synthesis, proofreading, assembly and modifications, $m_{mAb,ATP}$ (4.306 [mol ATP/mol AA]) (Sheikh et al., 2005).

Table 5.4: Stoichiometric coefficients.

Nutrient	Product	ATP (C)	NADH (C)	NADH (M)	FADH ₂ (C)	FADH ₂ (M)	GTP	ATP from TCA	ATP Balance	θ_i
Glucose	2 Lactate	2							2	2
Glucose	2 Alanine	2	2						5	5
Glucose	2 Acetyl-CoA	2	2	2				20	30	24
Alanine	Acetyl-CoA							10	10	7
Lysine	2 Acetyl-CoA		5		1			20	29	23
Leucine	3 Acetyl-CoA	-2	2		1			30	32.5	23.5
Isoleucine	Acetyl-CoA Oxaloacetate	-1	3	1	1	1	1	10	20	17
Glutamate	Oxaloacetate			3		1	1		10	10
Arginine	Oxaloacetate			4		1	1		12.5	12.5
Proline	Oxaloacetate			4		1	1		12.5	12.5
Histidine	Oxaloacetate			3		1	1		10	10
Phenylalanine	Acetyl-CoA Oxaloacetate		-1	3	1	1		10	19	16
Tyrosine	Acetyl-CoA Oxaloacetate			3	1	1		10	20.5	17.5
Aspartate	Oxaloacetate								0	0
Asparagine	Oxaloacetate								0	0
Valine	Oxaloacetate	-1	4	1	1	1	1		11.5	11.5
Threonine	Oxaloacetate	-1	1	1	1	1	1		7	7
Methionine	Oxaloacetate		1	1		1	1		6.5	6.5

(C) Cytoplasmic and (M) mitochondrial.

5.4 Model analysis: Global sensitivity analysis

The final model, the Energy Model, consisted of 24 differential and 71 algebraic equations, with 96 variables and 42 parameters. The parameters' uncertainty was established to be $\pm 50\%$ and the sampling interval was 30 minutes. The scenarios were generated using the sobolset function in MATLAB and the values of the output variables were obtained simulating the scenarios in gPROMS through the tool go:MATLAB, which makes the connexion between MATLAB and gPROMS. The graphical representation of the generated scenarios is in Figure 5.11.

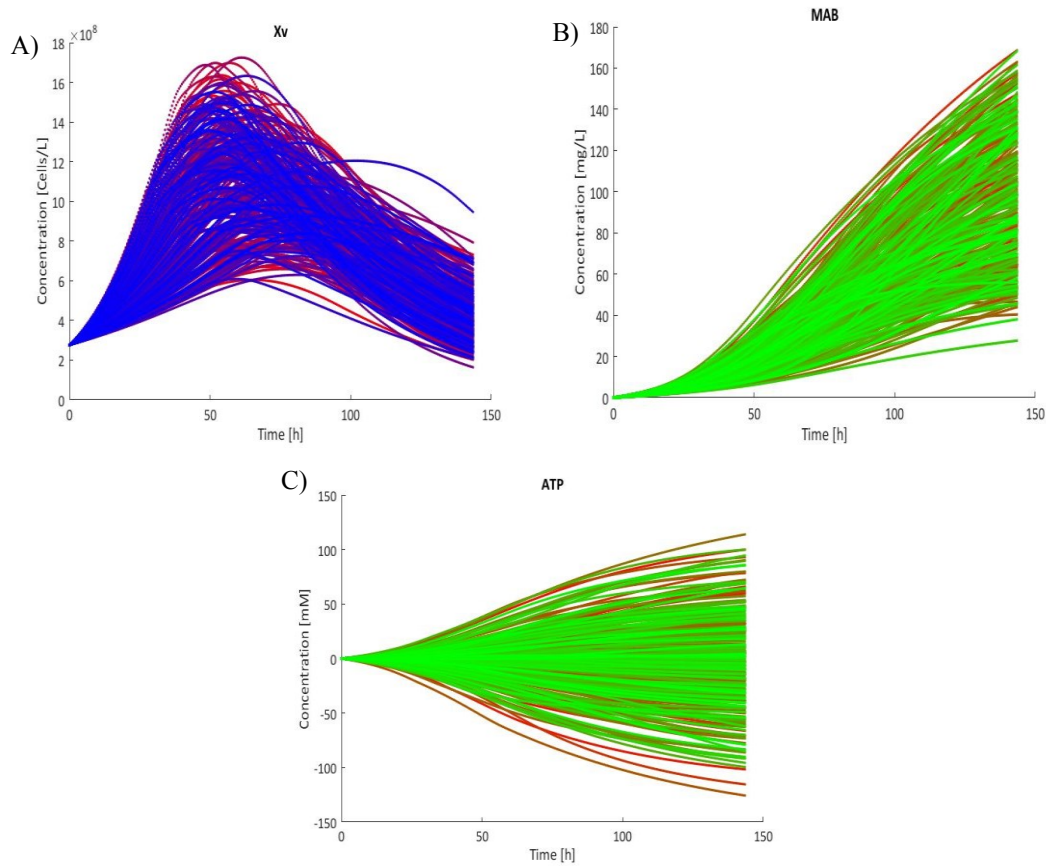


Figure 5.11: Graphical results obtain through gPROMS simulations of the scenarios generated with sobolset function in MATLAB. A) Viable cells output B) mAb output C) ATP output.

GSA was performed based on the scenarios presented in Figure 5.11, in MATLAB® R2015a, using the GUI-HDMR toolbox that calculates the sensitivity index for each parameter. The parameters with sensitivity index values above 0.1 were considered as significant; the graphical results are in Figure 5.12.

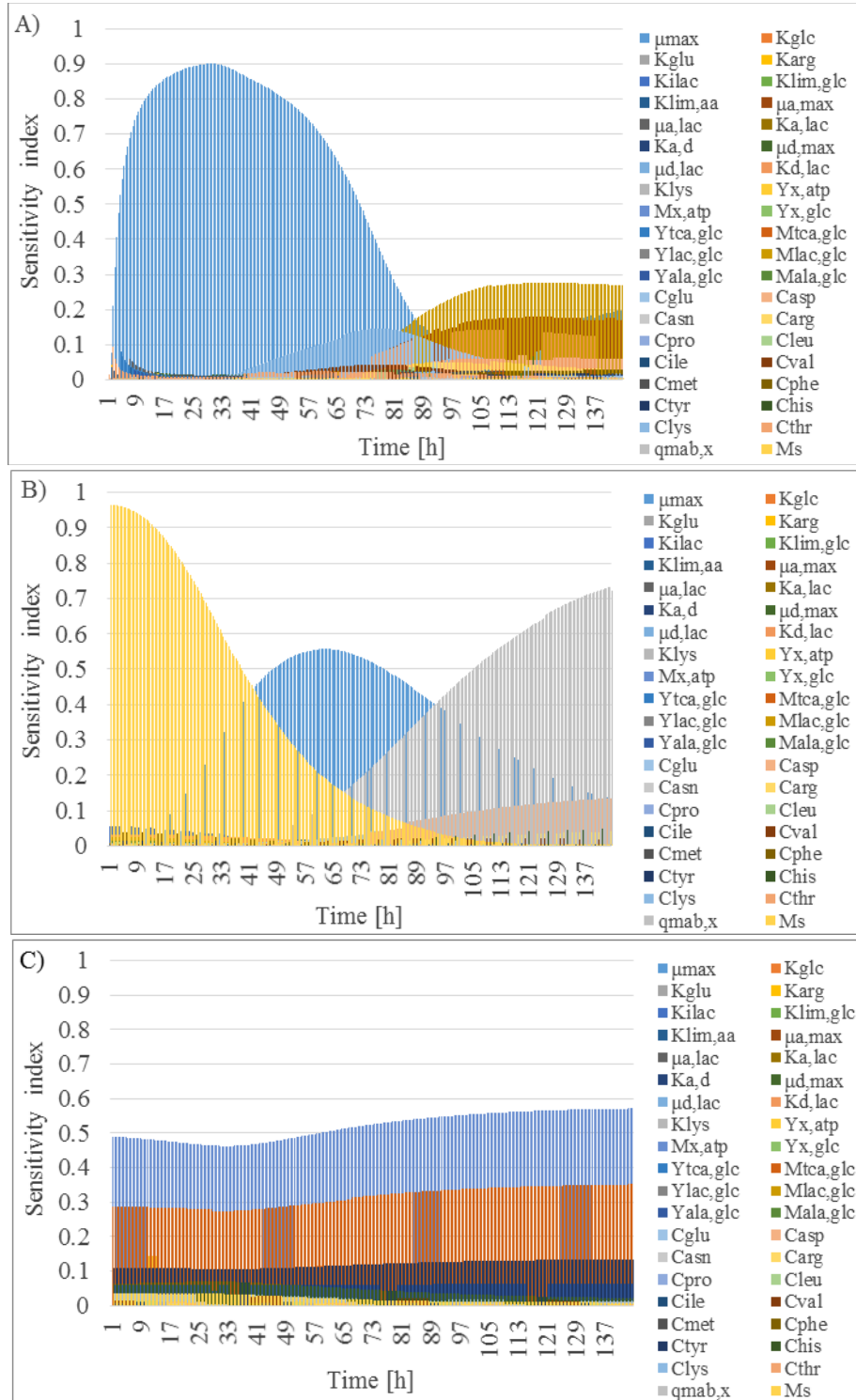


Figure 5.12: Global sensitivity analysis results. Sensitivity index for A) Viable cells (X_v) B) Monoclonal antibody (MAB) C) Adenosine tri-phosphate (ATP)

The results from the model analysis were clear and conclusive. The viable cell concentration (X_v) is sensitive to the theoretical maximum growth rate of GS-NS0 cell in DMEM medium & 10% FBS, and the predominance of this parameter sensitivity index is observed exactly during the exponential growth (24-72 hours), when the cells are growing faster. The mAb concentration (MAB) is also sensitive to this parameter, but other two parameters are predominant, M_s and $q_{mAb,x}$. At the beginning of the culture (0-48 hours), the mAb' concentration is highly sensitive to the M_s . This result is in accordance to the high cell's viability at the beginning of the culture when the only source of mAbs is its secretion from viable cells and there is not unspecific mAbs release via cells fragmentation. On the other hand, towards the end of the culture the mAbs concentration is sensitive to $q_{mAb,x}$, which is related to the accumulation of product inside the cells. This is in accordance to the fragmentation phenomenon that occurs at the end of the culture, when the mAbs concentration will depend on the accumulated mAbs inside the cells that is released as result of the apoptosis blebbing, and loss of the cell membrane integrity throughout the death stage (Al-Rubeai & Emery, 1990). The last studied output was the ATP concentration, it presented a stable GSA profile indicating a consistent sensitivity to the parameters $Y_{x,atp}$ and $M_{x,atp}$, energy requirements for proliferation and maintenance, respectively. This result is in accordance with the literature, where the ATP balance has been commonly expressed using exclusively two terms associated with these specific parameters (Kilburn et al., 1969; Roels et al., 1980; Xie & Wang, 1993-1996).

The GSA results demonstrate the impact of the development of mathematical models based on first principles. For instance, the change of the parameters significance over time reflect the dynamic of batch cultures, which pass through different stages: lag phase, early, mid and late exponential growth; stationary phase and death; and it is possible to observe which parameters are more relevant in each stage. Consequently, a mathematical analysis provides relevant biological information of the system in study.

5.5 Parameter estimation

The non-significant parameters (sensitivity index below 0.1) were fixed to the initial value and the 11 significant parameters were re-estimated using dynamic experimental data (Appendix VIII, Batch 2). The parameter estimation was performed in two groups: Group 1: $\mu_{\max}$, $\mu_{a,stv}$, $K_{d,lac}$, $M_{lac,glc}$, C_{glu} , $Q_{mAb,x}$, M_s , C_{asp} and C_{tyr} ; and Group 2: $Y_{x,atp}$ and $M_{x,atp}$. The method applied to perform the parameter estimation was the Minimum Likelihood Estimation, which is available in gPROMS, and the analysis were performed considering that the constant relative variance of the sensors was equal to 10% (Group 1) and 25% (Group 2). For Group 1, the t-values showed 95% accuracy for most of the parameters, the parameter C_{tyr} presented a t-value (0.46) lower than the reference (1.68), indicating that more experiments are required to estimate this parameter. The Lack of Fit test showed that the weighted residual (3.67×10^6) was larger than Chi-squared value (6.75×10^5), indicating that the model may be an inadequate representation of the physical system. This can be a consequence of the poor estimation of the parameter C_{tyr} and the low value assigned to the constant relative variance. On the other hand, for Group 2, the t-value for both parameters showed 95% accuracy. The Lack of Fit test showed that the weighted residual (1.69) was lower than Chi-squared value (11.07), indicating good fit of the model to the experimental data.

Table 5.5: Re-estimation of significant parameters.

Significant parameters	Initial Value	Final Value	Units
$\mu_{\max}$	0.020	0.022	h^{-1}
$\mu_{a,stv}$	0.0015	0.0014	h^{-1}
$K_{d,lac}$	12.0	13.2	mM
$M_{lac,glc}$	2.5E-11	2.3E-11	mmol/Cell h
C_{glu}	1	1.1	-
$Q_{mAb,x}$	9.0E-10	9.9E-10	mg/Cell h
M_s	5.0E-10	5.2E-10	mg/Cell h
C_{asp}	0.500	0.46	-
C_{tyr}	0.020	0.019	-
$Y_{x,atp}$	6.9E+07	6.9E+07	mmol/Cell
$M_{x,atp}$	1.1E-09	1.1E-09	mmol/Cell h

5.6 Results and discussion

In this section, the results from the experiments performed for parameter estimation (Batch 2), validation (Batch 3) and prediction capability testing (Batch 1 and Batch 4) are presented in the same graphs to facilitate the visualization of similarities and differences. These experiments were performed under different initial cell densities and fixing the other variables (culture volume, medium composition, temperature, agitation and pH). The experiments for parameter estimation was performed under the initial cell density recommended by the cell's master bank provider 2×10^8 [Cells/L] (Lonza Biologics, UK). The validation experiment was performed using an initial cell density slightly higher than the recommended concentration 3×10^8 [Cells/L]. Finally, the prediction testing experiments were performed under lower (half) and higher (twice) initial cell densities than the recommended concentration, 1×10^8 [Cells/L] and 4×10^8 [Cells/L], respectively.

5.6.1 Growth: Viable, apoptotic and death cells

In Figure 5.13, the experimental results and model predictions are presented. The model predicts the concentration of viable (Figure 5.13 A), total (Figure 5.13 B), apoptotic (Figure 5.13 C) and dead cells (Figure 5.13 D) of the cultures with the lowest initial cells densities (Batch 1 and 2), but it does not capture the second sampling point of viable and dead cells for cultures with higher initial cell densities. However, the model accurately predicts the total number of cells; this means that during the lag phase, cultures with initial cell concentration higher than 2×10^8 [Cells/L] face more difficulties in the adaptation process and a higher proportion of cells die. These discrepancies are more acute for cultures with 4×10^8 [Cells/L] initial cell concentration, where the concentration of dead cells is underestimated from the second sampling point until the end of the culture. These observations suggest that a term related to cell death during the lag phase should be included in the model to improve its predictions accuracy for dead cell at high initial cell densities.

5.6.2 Monoclonal antibody production.

In Figure 5.14, the experimental results and model predictions for mAbs production are presented. The model predicts the monoclonal antibody concentration at the beginning and end of each culture, but it slightly overestimates it from 72-120 hours of culture. These discrepancies could be attributed to a detrimental effect of the glutamate and aspartate exhaustion in the mAbs mRNA translation, between 48-72 hours. The addition of a mathematical expression that depicts the effect of amino acids limitation in the mAbs assembly could be incorporated to improve the model predictions.

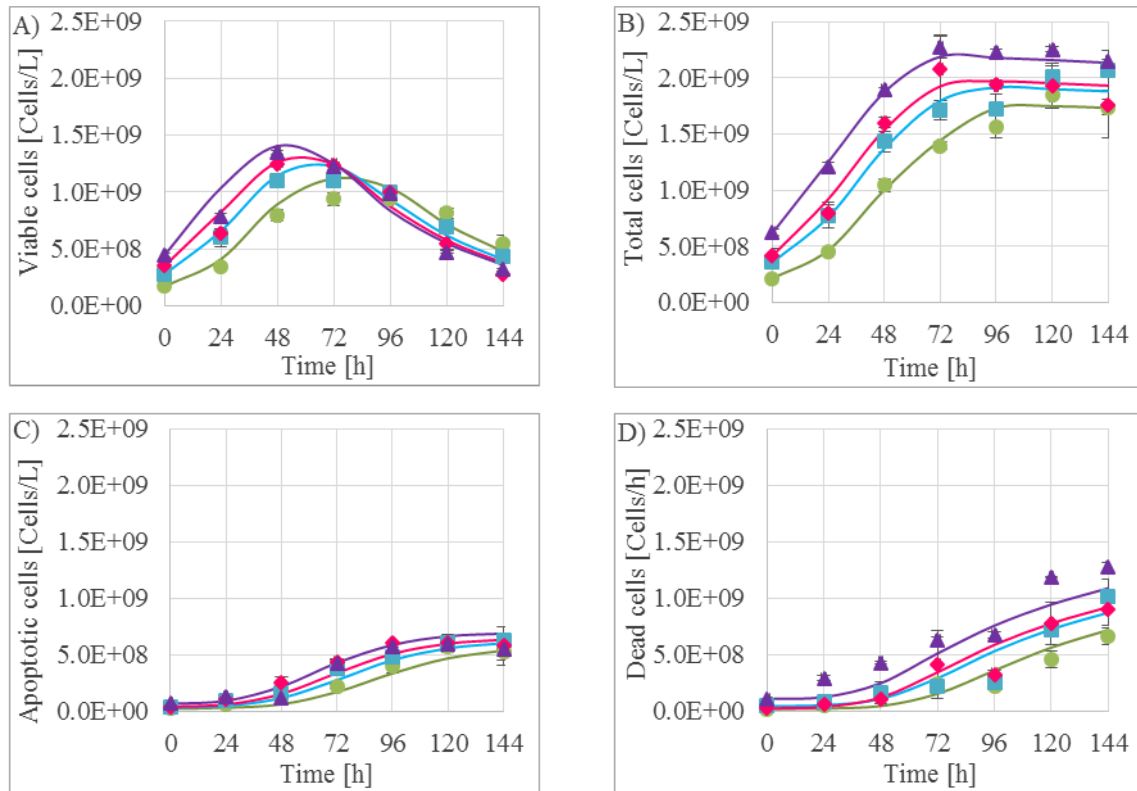


Figure 5.13: Comparison of experimental results (dots) and model predictions (lines) for (A) Viable cells, (B) Total cells, (C) Apoptotic cells, and (D) Dead cells. (○-green) Batch 1, (□ - blue) Batch 2, (◇- pink) Batch 3 and (Δ-purple) Batch 4. Model agreement within 95% confidence interval.

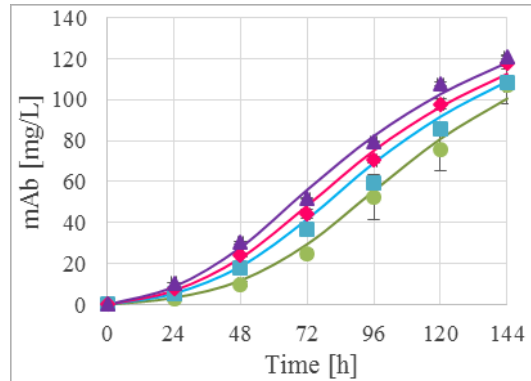


Figure 5.14: Comparison of experimental results (dots) and model predictions (lines) for mAbs concentration. (○-green) Batch 1, (□ - blue) Batch 2, (◇- pink) Batch 3 and (Δ-purple) Batch 4.

5.6.3 Glucose metabolism and lactate production

NS0 mouse myeloma cell line, as most tumour cells, relies on aerobic pathways such as glycolysis to produce ATP instead of completing the glucose catabolism through the mitochondrial oxidative phosphorylation (Warburg et al., 1926) and this accelerated glycolysis induce the production and accumulation of lactate, a toxic metabolite.

In the Energy Model, it has been considered that glucose is consumed to produce lactate, alanine, and energy, and there is not pyruvate accumulation. In Figure 5.15, the experimental results and model prediction for (A) glucose and (B) lactate are presented. The model predicts the glucose consumption profile and lactate production, which reach a plateau after 96 hours of culture, when arginine is exhausted and the growth rate is zero.

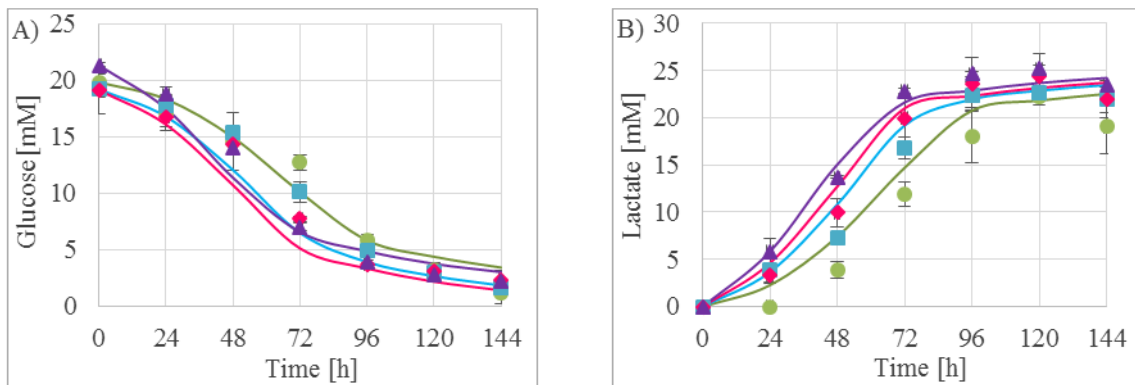


Figure 5.15: Comparison of experimental results (dots) and model predictions (lines) for (A) Glucose and (B) Lactate. (○-green) Batch 1, (□ - blue) Batch 2, (◇- pink) Batch 3 and (Δ-purple) Batch 4.

5.6.4 Amino acid metabolism

5.6.4.1 Glutamate consumption and metabolic shifts

In NS0 cells the level of endogenous glutamine synthetase activity is low, for this reason the GS-system has been used has a selection marker to promote the growth of transfected cells over the non-transfected or non-producer cells (Barnes et al., 2000). Regarding the expression system, glutamate is the main source of glutamine used for the biosynthesis of cellular structures and product. However, it has been reported that glutamate is not only used as a biosynthesis precursor, but also as one of the main energy sources in mammalian cell cultures, contributing with more than 50% of the ATP produced (Reitzer et al., 1979; Fitzpatrick et al., 1993; Altamirano et al., 2001). In the Energy Model, glutamate is consumed for proliferation, mAbs assembly and energy production, and to produce the amount of glutamine required for biosynthesis.

In Figure 5.16, the experimental results and model predictions for the concentration of amino acids are presented: (A) glutamate, (B) arginine, (C) proline, (D) histidine, (E) tyrosine and (F) phenylalanine. In the figure, it is possible to observe the fit of the model predictions to the experimental results, and the similarities between the amino acids concentration profiles trends.

From the total consumption of glutamate, 8% was used to produce the antibody, 53% to generate biomass and 39% entered into the TCA cycle to produce energy precursors for the electron chain transport (NADH and FADH₂). Between 48-72 hours of culture, glutamate was exhausted. However, the cells still require it for glutamine production, proliferation, mAbs assembly and energy production. Hence, in the Energy Model, metabolic shifts have been incorporated.

Metabolic shifts have been extensively reported in NS0 and CHO cultures (Ma et al., 2009; Mulukutla et al., 2012; Luo et al., 2012; Toussaint et al., 2016) and in the model, it is considered that glutamate exhaustion activates the uptake of phenylalanine, tyrosine, histidine and proline to produce energy and arginine to produce glutamine and glutamate required for proteins biosynthesis. It is important to remark that phenylalanine and histidine uptakes did not present an increase after glutamate exhaustion and their consumption was restricted to their

stoichiometric requirements for proliferation and mAbs assembling. Histidine concentration presented a sharp reduction in Batch 4 (Figure 5.16 D), the culture with the highest initial concentration. Even when phenylalanine was not included in the metabolic shift its uptake has been overestimated by the model (Figure 5.16 F). This indicates that the cell and product composition should be re-calculated.

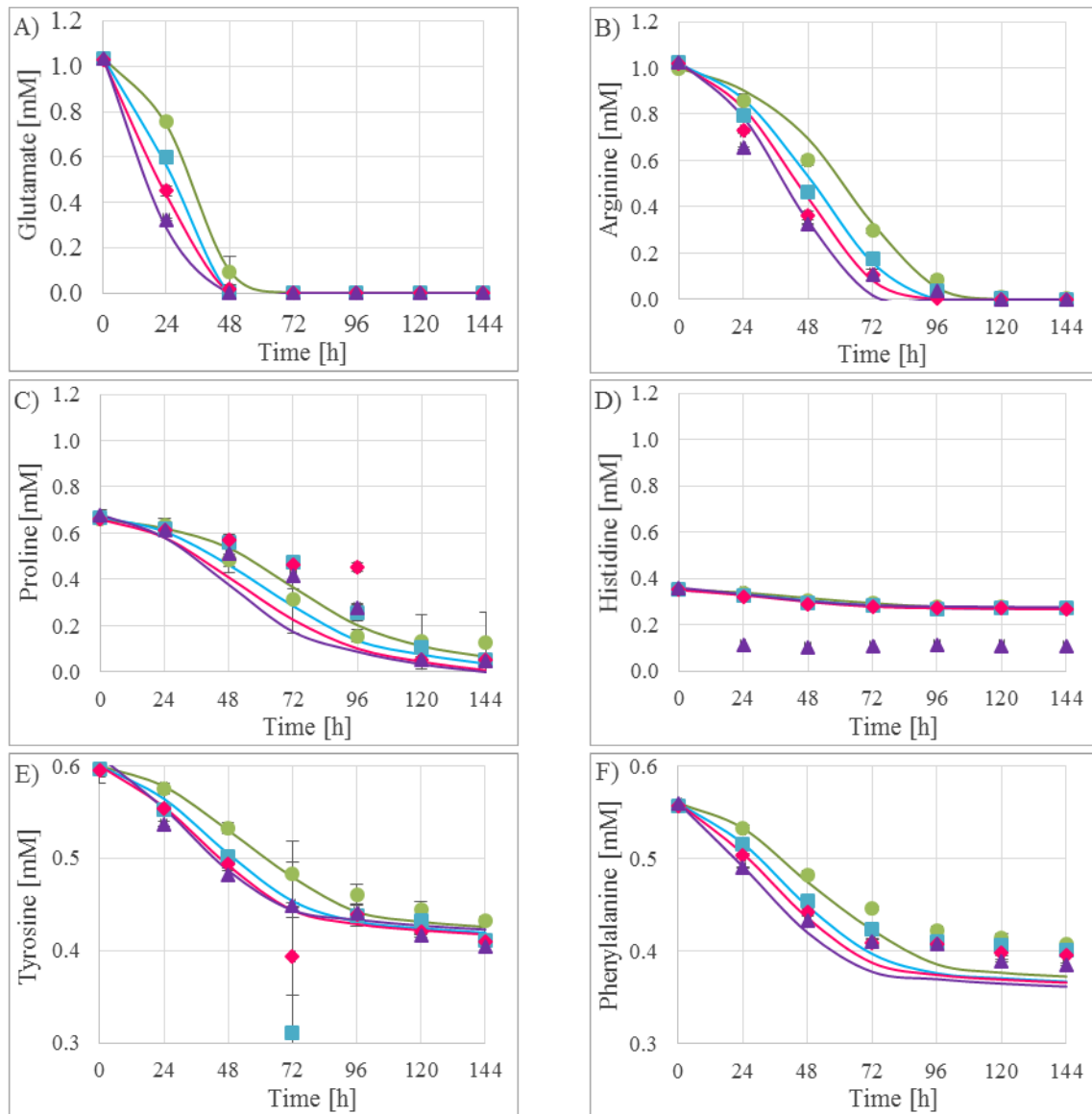


Figure 5.16: Comparison of experimental results (dots) and model predictions (lines) for (A) Glutamate, (B) Arginine, (C) Proline, (D) Histidine, (E) Tyrosine and (F) Phenylalanine. (o-green) Batch 1, (□-blue) Batch 2, (◇-pink) Batch 3 and (Δ-purple) Batch 4.

In Figure 5.17, the experimental results and model prediction for alanine concentration are presented. In addition to the expected metabolic shifts, a switch in alanine concentration profile, from accumulation to decrease was also detected. Tyrosine and proline uptakes support the energy production when glutamate is exhausted. However, their uptakes are not sufficient to replace the total ATP production from glutamate, consequently, the alanine switch was incorporated as activation of alanine uptake to produce acetyl-CoA, which enters into the TCA cycle and replace glutamate as an energy source (Ma et al., 2009).

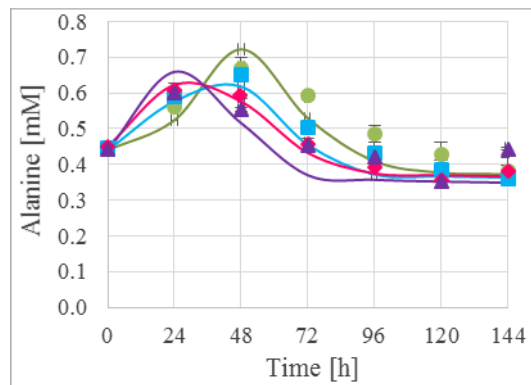


Figure 5.17: Comparison of experimental results (dots) and model predictions (lines) for alanine. (o-green) Batch 1, (□ - blue) Batch 2, (◇- pink) Batch 3 and (Δ-purple) Batch 4.

5.6.4.2 Aspartate consumption and metabolic shift

In Figure 5.18 A, aspartate is depleted between 48-72 hours of culture, following the same trend as glutamate. In this case, the model proposes that asparagine is used to supply the exact amount of aspartate required for biosynthesis. This assumption is validated when the experimental results and model predictions are compared (Figure 5.18 B).

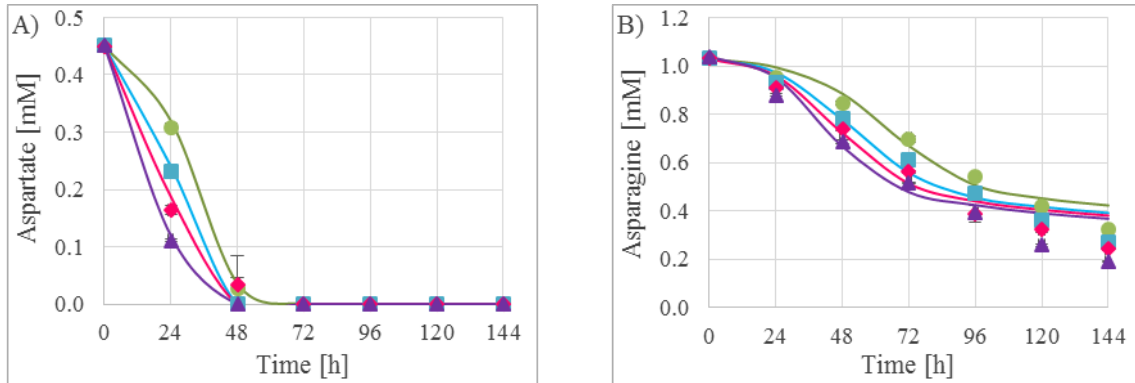


Figure 5.18: Comparison of experimental results (dots) and model predictions (lines) for (A) Aspartate and (B) Asparagine. (○-green) Batch 1, (□ - blue) Batch 2, (◇- pink) Batch 3 and (Δ-purple) Batch 4.

In Figure 5.18 B, asparagine uptake seems to suffer another metabolic shift after 96 hours of culture. Henceforth, the model under-predicts asparagine uptake. In this phase of the culture, arginine has been depleted and some essential amino acids present low concentration, consequently, it was not possible to identify what could be the cause of the second metabolic shift of asparagine.

5.6.4.3 Essential amino acids exhaustion.

The exhaustion of the essential amino acids leucine and isoleucine has an important impact in the mammalian cell cultures. This event coupled to arginine exhaustion induces incremental lysine and asparagine uptake. However, the activation of apoptosis and necrosis processes collapse the system and the viability decrease to 50%. In Figure 5.19, the experimental results and model predictions for the concentration of amino acids are presented: (A) valine, (B) threonine, (C) methionine, (D) lysine, (E) isoleucine and (F) leucine.

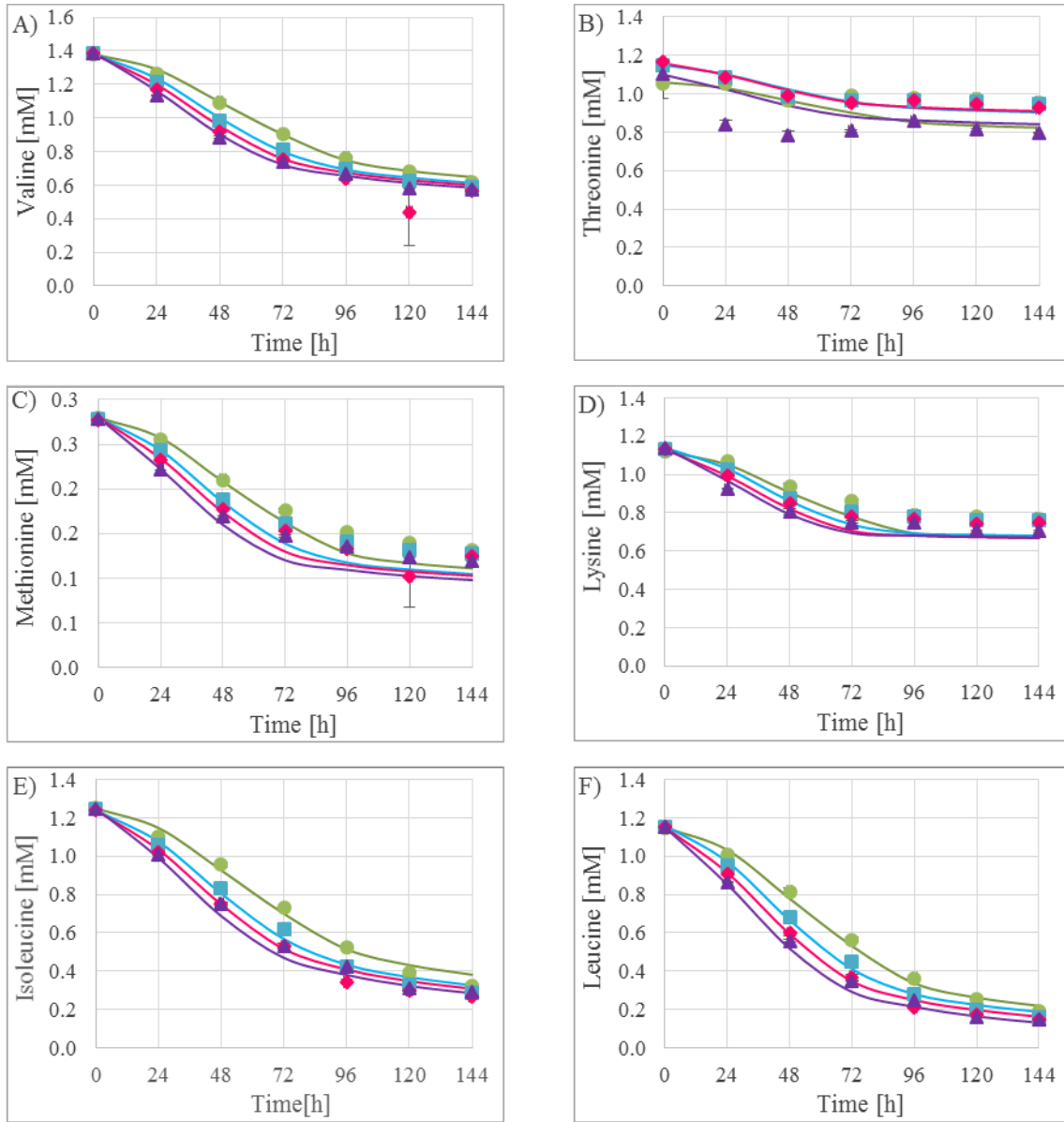


Figure 5.19: Comparison of experimental results (dots) and model predictions (lines) for (A) Valine, (B) Threonine, (C) Methionine, (D) Lysine, (E) Isoleucine and (F) Leucine. (o-green) Batch 1, (□ - blue) Batch 2, (◇- pink) Batch 3 and (Δ-purple) Batch 4.

5.6.5 ATP Balance

The prediction of the total energy available in the culture is based on the precise prediction of viable, apoptotic and dead cells concentrations, nutrients uptakes and product concentration. Therefore, the successful prediction of the ATP profile depends on the accurate estimation of 19 variables.

Figure 5.20 displays the ATP concentration from experimental results (dots), model predictions (solid line) and the 95% interval (grey area), which was calculated in excel using the function CONFIDENCE.T. The Student's t-distribution was used because of the low number of replicates (3). The graphical representation of the standard deviation of the experimental data (error bars) and 95% confidence interval of the model predictions (grey area).

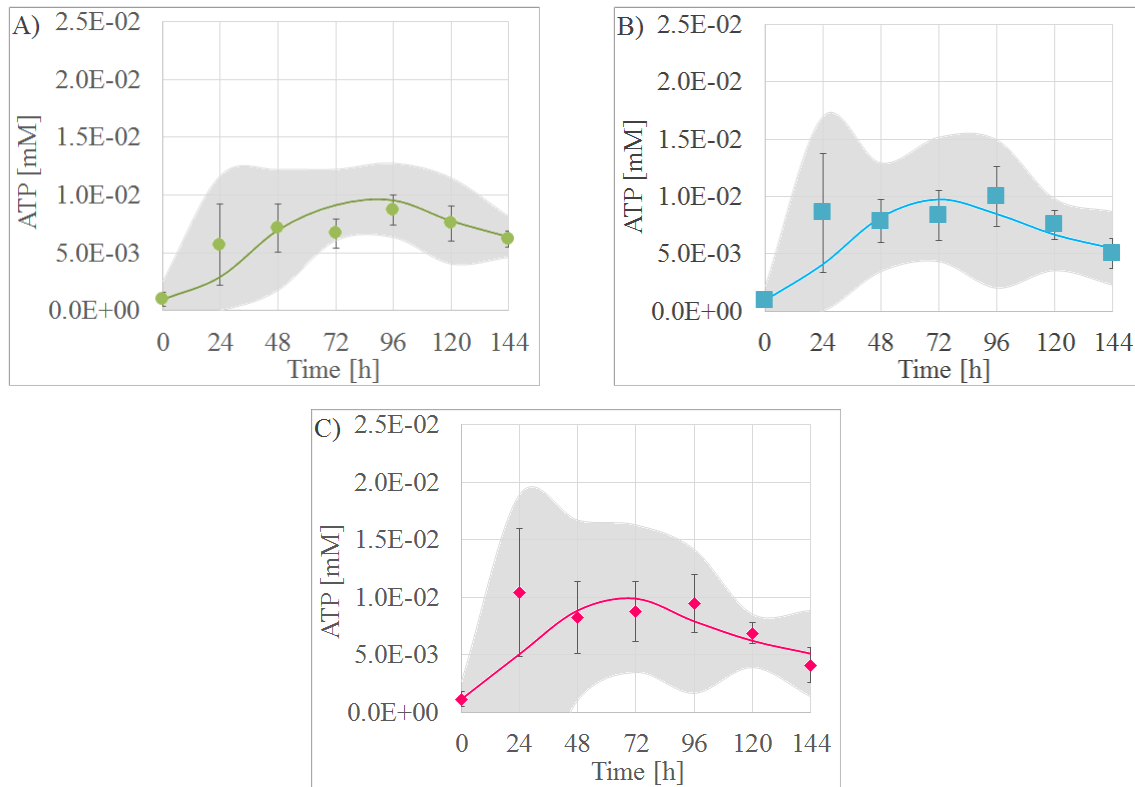


Figure 5.20: Comparison of experimental results (dots) and model predictions (lines) for ATP concentration in (A) Batch1, (B) Batch 2 and (C) Batch 3.

The measurement of intracellular ATP content to estimate the number of viable cells is an established and reliable technique to evaluate proliferation in mammalian cell cultures. To corroborate that the ATP content per viable cells is constant, ANOVA analysis was performed using data from experiment Batches 1, 2 and 3 (Table 5.6).

Table 5.6: ATP per cell measurements [mmol/Cell].

Experiment/Time	0hrs	24hrs	48hrs	72hrs	96hrs	120hrs	144hrs
Batch 1	4.30E-12	5.86E-12	3.72E-12	3.65E-12	3.85E-12	6.27E-12	8.87E-12
Batch 1	8.28E-12	1.30E-11	7.94E-12	8.30E-12	8.66E-12	1.27E-11	1.92E-11
Batch 1	7.62E-12	1.33E-11	7.04E-12	7.41E-12	8.40E-12	1.08E-11	2.25E-11
Batch 2	5.05E-12	7.90E-12	4.98E-12	7.28E-12	9.09E-12	1.13E-11	1.12E-11
Batch 2	1.81E-12	7.69E-12	7.05E-12	5.43E-12	7.90E-12	8.71E-12	9.46E-12
Batch 2	3.43E-12	3.03E-11	9.45E-12	1.00E-11	1.30E-11	1.28E-11	1.44E-11
Batch 3	4.71E-12	9.31E-12	5.16E-12	6.68E-12	1.02E-11	1.33E-11	9.14E-12
Batch 3	1.81E-12	1.13E-11	4.64E-12	4.72E-12	5.82E-12	9.34E-12	1.40E-11
Batch 3	3.26E-12	2.82E-11	9.83E-12	9.83E-12	1.28E-11	1.60E-11	2.08E-11

The two-way ANOVA analysis was performed to determine whether the mean of ATP content per cell in the different batches (rows) and days (columns) was the same (Table 5.7). The results show that within batches and the interaction between batches and days present p-values higher than 0.05 and F-values lower than F_{critical} -value. This indicates that there are no significant differences between batches and no significant interaction between the groups. On the other hand, daily intracellular ATP content present a p-value lower than the significance level and the F-value is higher than the F_{critical} -value (Table 5.7).

Table 5.7: Results of two-way ANOVA.

Source of Variation	SS	df	MS	F	P-value	F crit
Batches	9.03E-24	2	4.52E-24	0.20	0.82	3.22
Days	7.91E-22	6	1.32E-22	5.72	0.00	2.32
Interaction	1.41E-22	12	1.18E-23	0.51	0.90	1.99
Within	9.68E-22	42	2.30E-23			
Total	1.91E-21	62				

Data in Appendix IX.

ANOVA results indicate that there are significant differences in the intracellular ATP content between days (p-value<0.05), but it does not provide information about which days

present significant differences. Therefore, a t-test analysis was performed for each batch in all days' combinations to find the stage of the culture that present significant differences in intracellular ATP content (results of t-test in Appendix X).

Experiments Batches 1 and 2 do not present significant differences between days. Experiment Batch 3 presents just one p-value lower than the confidence level (0.05) and the absolute value of the t-value greater than the critical values for the analysis considering time 0 and 120 hours. These results indicate that the concentration of ATP per viable cell can be considered constant between batches and throughout the culture and the value is $9.22 \pm 5.02 \times 10^{-12}$ [mmol ATP/Cell] (Figure 5.21).

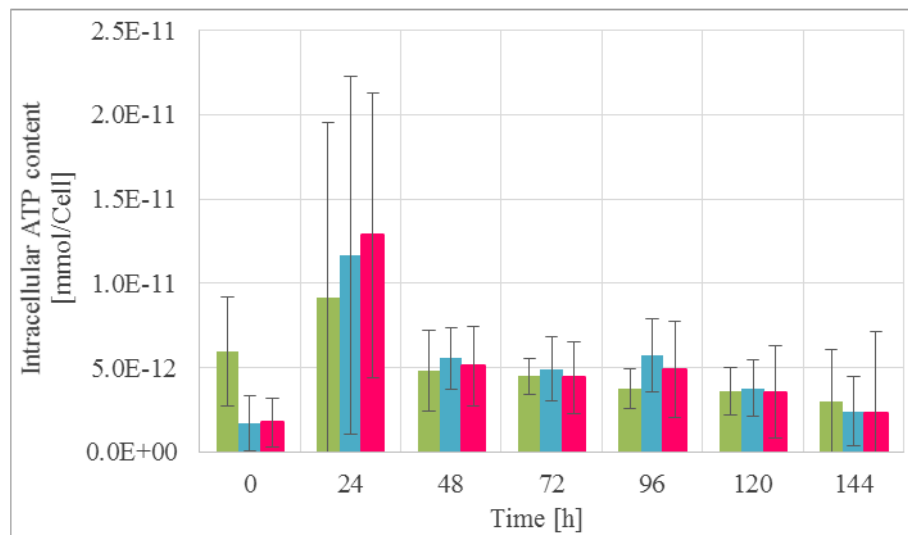


Figure 5.21: Experimental results for intracellular ATP content [mmol ATP/Cell]. (Green) Batch 1, (Blue) Batch 2, (Pink) Batch 3

In Figure 5.21 the standard deviation bars indicate high variability on the data set. This variability was attributed to the serum content of the cultures, which introduces high variability due to its undefined composition. Since this research work is focused on the intracellular ATP balance, it was a required to adapt the cells to serum-free conditions with the aim of achieving less variability and more reproducible results. The medium selection, adaptation protocol and adaptation assessment are described in Appendix VI.

5.6.6 Conclusions

To the best of our knowledge, the Energy Model is the first dynamic model that predicts the intracellular ATP content for GS-NS0 cells in batch cultures in DMEM medium supplemented with 10 % FBS. The model is based on mass balances, modified Monod's kinetics and stoichiometric equations. The formulation of the growth kinetic equations was based on the literature available and the metabolic shifts of arginine, proline, tyrosine, phenylalanine and alanine were successfully incorporated.

The model analysis for the identification of significant model parameters through GSA presented clear and conclusive results. The list of 11 significant parameters were biologically related to the studied output variable proving the impact of the development of mathematical models based on first principles. The GSA not only revealed the biological meaning of the mathematical analysis, it also demonstrated that it captures the changes of the parameters significance over time, reflecting the dynamic of batch cultures, which pass through different stages, allowing us to observe which parameters are more relevant in each stage.

One experiment was designed and performed for the estimation of the subset of significant parameters (Batch 2). Subsequently, the model was validated through the comparison of experimental and simulation results, under the same initial conditions (Batch 3). Finally, the model was used to perform predictions under low and high initial cell densities, the half and twice the recommended value (Batch 1 and Batch 4), respectively. The validation and prediction results prove the ability of the model to describe growth kinetics, glucose and amino acids metabolism, metabolic shifts, mAbs secretion and ATP production and consumption. The model accurately predicts the number of viable, apoptotic and dead cells, and the concentration of ATP, glucose, 15 amino acids and lactate.

The statistical analysis of the results indicates that the intracellular ATP content in viable cells is constant between batches and throughout the culture and its value is $9.22 \pm 5.02 \times 10^{-12}$ [mmol ATP/Cell].

CHAPTER 6: Energy model for GS-NS0 cells in serum-free medium

6 Energy model for GS-NS0 cells in serum-free medium

In chapter 5, the Energy Model was developed for batch cultures of GS-NS0 cells producing the cB72.3 monoclonal antibody in modified DMEM medium supplemented with 10% FBS. The model was based on growth kinetics, nutrient metabolism, mAbs secretion and the production/consumption of ATP. The full range of model parameters was subjected to GSA to identify the significant parameters that were then estimated from a specifically-designed batch experiment and finally, the validity of the model was tested against a set of four independent batch experiments, each of them under different initial conditions. The validated model successfully predicts the number of viable, apoptotic and dead cells, and the concentrations of ATP, glucose, 15 amino acids and lactate. The successful coupling of growth kinetics equations and stoichiometric balances and the *in vitro/in silico* approach enabled us to develop the first dynamic model that predicts the intracellular ATP content in mammalian cell cultures. In chapter 6, the adaptation of the cells to grow in three different chemically-defined/serum-free media was presented. The CD-Hybridoma medium was selected for the next stages, based on its remarkable results compared to the other tested medium.

In the present chapter, the Energy Model is modified by the incorporation of four sets of equations related to serine, glycine, cysteine and tryptophan metabolism to complete the total amino acids profile. The adapted GS-NS0 cells are used for the model validation and the model predictive capabilities testing.

6.1 Introduction

For the optimisation of mAbs production, it is necessary to implement another operation platform, fed-batch culture. The most common approach to implement a fed-batch culture is the design of a supplemental medium followed by the design of a feeding strategy (Xie & Wang, 1993a; Zhang et al., 2004, Spens & Häggström, 2007; Shibuya et al., 2008).

The strategies for the development of a supplemental medium vary from iterative trial-and-error processes to stoichiometric models (Spens & Häggström, 2007; Xie & Wang, 1996). The last one is the most successful technique and it has been widely applied by research groups (Xie & Wang, 1993a; Zhou et al., 1997a-1997b; Sauer et al., 2000; Burky et al., 2006; Li et al., 2009). Currently, mathematical models and computational tools are used to assist these processes, allowing the *in-silico* simulation of cultures under different medium compositions and feeding schedules, reducing the number of experiments to be performed (Dhir et al., 2002; Kontoravdi et al., 2010; Kiparissides et al., 2015).

The components of a supplemental medium include carbon sources, amino acids, vitamins, nucleotides, inorganic salts, buffers and serum. Regarding the mammalian cellular composition, which consists of 1.57% DNA, 5.13 %RNA, 5.58% carbohydrates, 10.30% lipids and 71.37% proteins, in average (Zupke & Stephanopoulos, 1994, Bonarius et al., 1995; Altamirano et al., 2001), the amino acids medium composition has an important influence in cells proliferation. Therefore, the first step in the supplemental medium design should be the estimation of the specific amino acids amount required in fed-batch cultures.

The Energy Model, in chapter 5, successfully describes the profile of fifteen amino acids. Therefore, it is necessary to incorporate four mass balances to predict the profile of the remaining amino acids to ensure coverage of the full set of nineteen amino acids present in the culture medium.

6.2 Aims and objectives

The aim of this chapter is to modify the Energy Model to predict the uptake of the total set of amino acids present in the culture medium. The objectives to achieve this aim were:

- 1) **Model modification:** The Energy Model developed in chapter 5 was modified including mass balances for the amino acids serine, glycine, cysteine and tryptophan.
- 2) **Model analysis and parameter estimation:** The modified Energy Model was subjected to GSA to identify the significant model parameters, which were subsequently re-estimated.
- 3) **Model validation and prediction capability testing:** The model was validated through the comparison of experimental results and simulations predictions under the identical initial conditions. Finally, the model prediction capability was tested under a wide range of scenarios comparing the experimental results to model predictions.
- 4) **Model validation in bioreactor operation:** The model was used to predict the behaviour of GS-NS0 cells cultured in the bioreactor Bioflo-110. The execution of GS-NS0 cultures in the bioreactor is essential for the implementation of fed-batch cultures.

6.3 Methodology

6.3.1 Model modifications: Amino acids mass balances

The original Energy Model equations were not modified, the only modification was the incorporation of four mass balances for the amino acids: serine, glycine, cysteine and tryptophan. The new equations are described below.

6.3.1.1 Serine and glycine

Glycine and cysteine are derived from serine, and in the experimental results from cultures in DMEM medium supplemented with 10% FBS, it was possible to identify glycine accumulation and cysteine decrease. Therefore, in the mass balance for serine, uptake for glycine formation was included.

Equation 6.1: Mass balances for serine and glycine.

$$\frac{d(V[SER])}{dt} = -VX_v(Q_{x,ser} + Q_{mab,ser} + Q_{gly,ser})$$

$$\frac{d(V[GLY])}{dt} = -VX_v(Q_{x,gly} + Q_{mab,gly} - Q_{gly,ser})$$

In the mass balance equations, SER and GLY are serine and glycine concentrations [mM], respectively. The specific uptake of both amino acids has been separated in three different terms: specific consumption rate for proliferation ($Q_{x,ser}$ and $Q_{x,gly}$), specific consumption rate for production ($Q_{mab,ser}$ and $Q_{mab,gly}$) and specific consumption rate of serine for glycine production ($Q_{gly,ser}$), which is also the specific production rate of glycine from serine.

The specific consumption rate for proliferation depends on the growth rate and the yield of biomass per amino acid ($Y_{x,ser}$ [Cell/mmol] and $Y_{x,gly}$ [Cell/mmol]).

Equation 6.2: Serine and glycine specific consumption rates of proliferation.

$$Q_{x,ser} = \frac{\mu}{Y_{x,ser}}$$

$$Q_{x,gly} = \frac{\mu}{Y_{x,gly}}$$

The specific consumption rate for production depends on the specific production rate ($q_{mab,x}$ [mg/Cell h]) and the product composition ($M_{mab,ser}$ [mmol/mg] and $M_{mab,gly}$ [mmol/mg]).

Equation 6.3: Serine and glycine specific consumption rates for production.

$$Q_{mab,ser} = q_{mab,x}M_{mab,ser}$$

$$Q_{mab,gly} = q_{mab,x}M_{mab,gly}$$

Finally, the specific consumption rate of serine for glycine production is represented by $Q_{gly,ser}$, which is composed of one term related to the growth rate:

Equation 6.4: Serine and glycine specific consumption rates for production.

$$Q_{gly,ser} = \frac{\mu}{Y_{gly,ser}}$$

6.3.1.2 Cysteine and tryptophan

Cysteine and tryptophan concentrations decline throughout the culture and their specific consumption rate are related to proliferation and production.

Equation 6.5: Mass balances for cysteine and tryptophan.

$$\begin{aligned} \frac{d(V \text{ CYS})}{dt} &= -VX_v(Q_{x,cys} + Q_{mab,cys}) \\ \frac{d(V \text{ TRP})}{dt} &= -VX_v(Q_{x,trp} + Q_{mab,trp}) \end{aligned}$$

Equation 6.6: Cysteine and tryptophan specific consumption rates for proliferation.

$$\begin{aligned} Q_{x,cys} &= \frac{\mu}{Y_{x,cys}} \\ Q_{x,trp} &= \frac{\mu}{Y_{x,trp}} \end{aligned}$$

Equation 6.7: Cysteine and tryptophan specific consumption rates for production.

$$\begin{aligned} Q_{mab,cys} &= q_{mab,x}M_{mab,cys} \\ Q_{mab,trp} &= q_{mab,x}M_{mab,trp} \end{aligned}$$

6.4 Global sensitivity analysis

The modifications to the original model increased the number of differential (29) and algebraic equations (80), and variables (112); nonetheless, only one parameter was included ($Y_{glu,ser}$). For the GSA, the parameters uncertainty was set to $\pm 50\%$ and the sampling interval was 30 minutes. The scenarios were generated using the sobolset function in MATLAB R2015a and the values of the output variables were obtained simulating the scenarios in gPROMS 4.1.1 through the tool go:MATLAB, which makes the connexion between MATLAB and gPROMS. The graphical representation the generated scenarios were is in Figure 6.1.

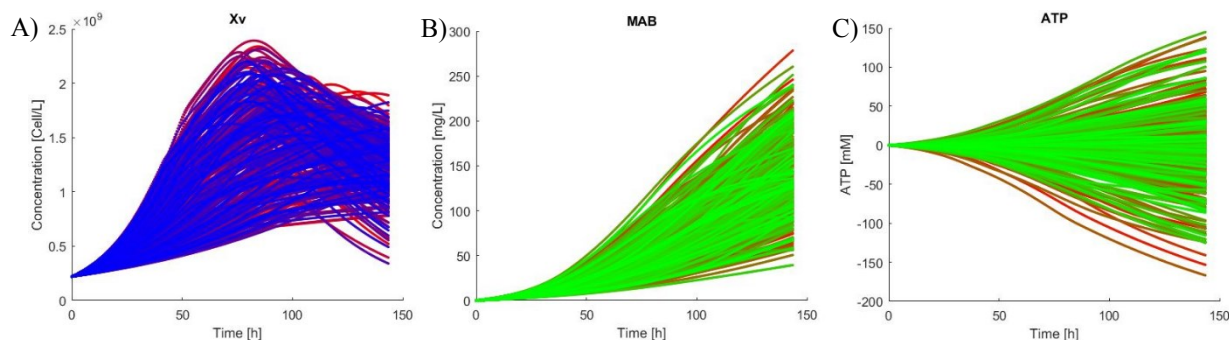


Figure 6.1: Graphical results obtain through gPROMS simulations of the scenarios generated with sobol-set function in MATLAB. A) Viable cells output B) mAbs output C) ATP output.

Based on the scenarios presented in Figure 6.1, a GSA was performed in MATLAB R2015a using the GUI-HDMR toolbox, which calculates the sensitivity index of each parameter. The parameters with sensitivity index values above 0.1 were considered as significant; the graphical results are in Figure 6.2.

The results from GSA presented eight significant parameters, and within them, six are already included in the original Energy Model's list of significant parameters. Moreover, the original and modified models profiles of the sensitivity indices present clear similarities (Figure 5.12 and Figure 6.2), which confirm that modifications incorporated in the Energy Model do not interfere profoundly in its overall structure.

The analysis based on the viable cells concentration as output showed that the number of viable cells is sensitive to the theoretical growth rate ($\mu_{\max}$) and its predominance is permanent throughout the culture. In DMEM medium, the predominance of this parameter was observed during the exponential growth (24-72 hours). This difference could be explained by the fact that the viability of the culture is maintained above 90% for 72 hours and 120 hours in DMEM and CD-Hybridoma medium, respectively. Therefore, the predominance of the significance of $\mu_{\max}$ is related to the viability level. GSA showed the impact of the viability level in the culture, even when the viability is not a variable in the mathematical model.

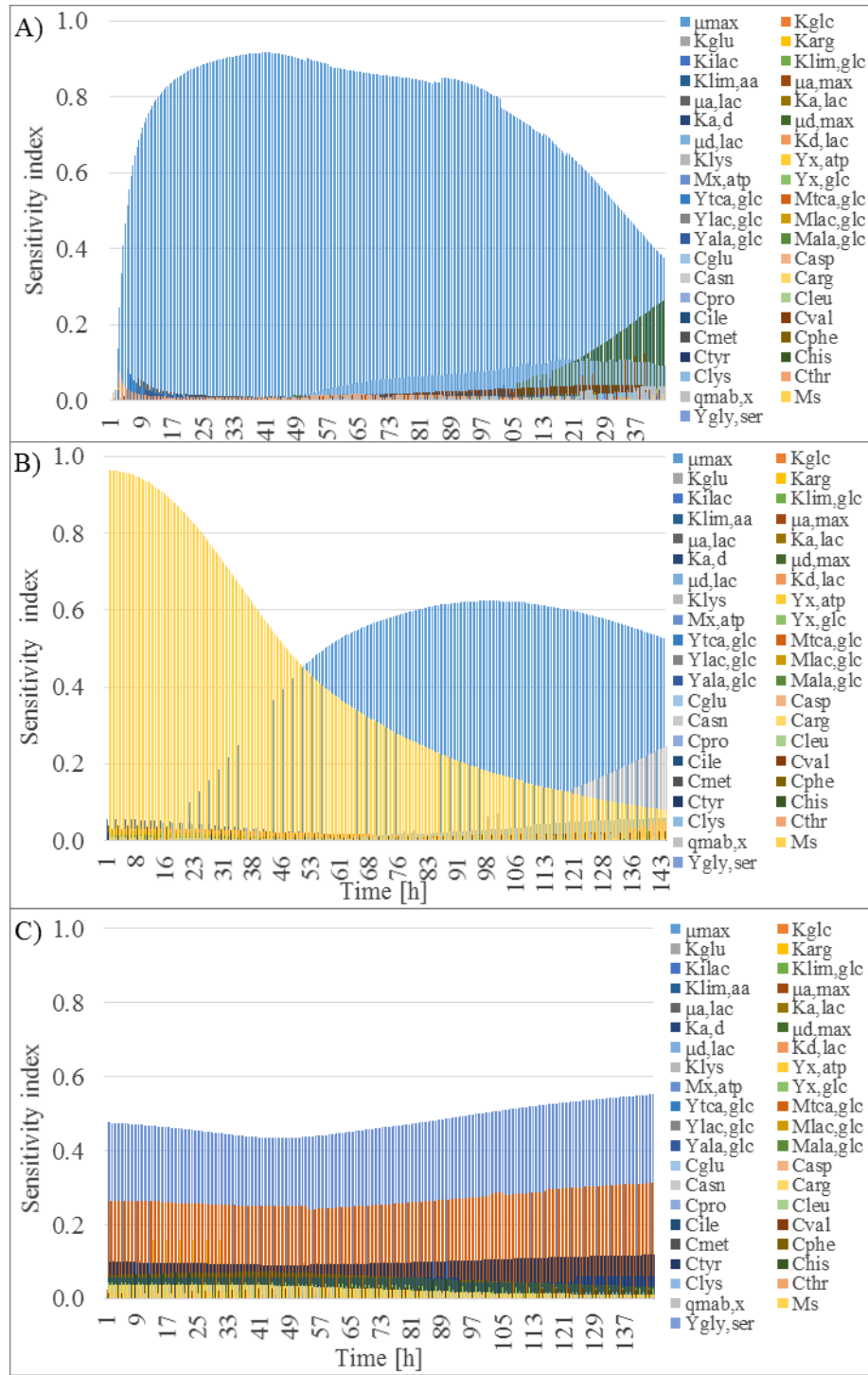


Figure 6.2: Global sensitivity analysis results. Sensitivity index for the parameters assessing the following output variables: A) Viable cells (Xv) B) Monoclonal antibody (MAB) C) Adenosine triphosphate (ATP)

The mAbs concentration is sensitive to exactly the same parameters in DMEM and CD-Hybridoma medium (Figure 5.12 and Figure 6.2): $\mu_{\max}$, specific production rate ($q_{\text{mAb},x}$) and secretion rate (M_s). The sensitivity index of these parameters presents similar profiles in both cases. The secretion rate is predominant at the beginning of the culture because the secretion pathway is the only alternative for mAbs release at early culture stages. Conversely, the specific production rate ($q_{\text{mAb},x}$) takes a significant role at the end of the culture because the mAbs accumulated inside the cells, which depends on the balance between $q_{\text{mAb},x}$ and M_s , is released via cells fragmentation, phenomenon that occurs at late culture stages (Al-Rubeai & Emery, 1990; Simpson et al., 1996).

The last studied output was the ATP concentration. This variable presented a stable GSA profile indicating a consistent sensitivity to the parameters $Y_{x,\text{atp}}$ and $M_{x,\text{atp}}$. The same results were obtained for the original Energy Model.

The relevance of the information collected from GSA applied in a mathematical model based on first principles was discussed in Chapter 5 in relation to the biological meaning of its results, the variation of the parameters' significance over time that capture the different stages in a batch culture. In this chapter, the comparison of GSA applied on the Energy Model for the same cells growing in DMEM 10% FBS and serum-free CD-Hybridoma medium, revealed that GSA captured the main effect of the adaptation, the extension of the culture longevity. This outstanding result proves again the importance of the development of mathematical models based on first principles and the potential of the GSA for model analysis.

6.5 Parameter estimation

The non-significant parameters (sensitivity index below 0.1) were fixed to their initial values and the eight significant parameters were re-estimated using dynamic experimental data (Appendix XI). The parameter estimation was performed in two groups: Group 1: $\mu_{\max}$, $\mu_{a,\text{stv}}$, $\mu_{d,\text{stv}}$, $M_{\text{tca,glc}}$, $q_{\text{mAb},x}$ and M_s and Group 2: $Y_{x,\text{atp}}$ and $M_{x,\text{atp}}$. The method applied for the parameter estimation was the Minimum Likelihood Estimation and both analysis were performed in gPROMS 4.1.0 considering that the constant relative variance of the sensors was equal to 10% (Group 1) and 25% (Group 2). For Group 1, the parameter estimation was performed twice.

The initial values for the first run and second run are presented in Table 6.1 and consisted of 4 and 5 iterations, and took 91 and 375 seconds to be performed, respectively. The t-values showed 95% accuracy for both runs and the final Lack of Fit test showed that the weighted residual value (30.99) was lower than the Chi-squared value (40.11), indicating good fit of the model to the experimental data. For Group 2, the parameter estimation was performed once. The initial values are presented in Table 6.1, consisted of 7 iterations and took 99 seconds to be performed. The t-values showed 95% accuracy and the Lack of Fit test showed that the weighted residual value (3.21) was lower than the Chi-squared value (11.07), indicating also good fit of the model to the experimental data.

Table 6.1: Re-estimation of significant parameters.

Significant parameters	Initial Value 1	Initial Value 2	Final Value	Units
$\mu_{\max}$	0.0170	0.0177	0.0172	h^{-1}
$\mu_{a,\text{stv}}$	7.0e-4	7.7e-4	8.4e-4	h^{-1}
$\mu_{d,\text{stv}}$	1.0e-3	9.8e-4	1.0e-3	
$M_{\text{tca,glc}}$	3.0e-11	3.3e-11	3.6e-11	mmol/Cell h
$q_{m\text{Ab},x}$	1.5e-9	1.35e-9	1.3e-9	mg/Cell h
M_s	9.0e-10	4.5e-10	4.2e-10	mg/Cell h
$Y_{x,\text{atp}}$	6.61e7		6.60e7	mmol/Cell
$M_{x,\text{atp}}$	9.63e-10		1.12e-9	mmol/Cell h

6.6 Results and discussion: Batch cultures in flasks

In this section, the results from the experiments performed for parameter estimation (Batch 2: initial cell density 2×10^8 [Cells/L]), validation (Batch 3: initial cell density 3×10^8 [Cells/L]) and prediction capability testing (Batch 1 and Batch 4, : initial cell density 1×10^8 [Cells/L] and density 4×10^8 [Cells/L]) are presented in the same format used in Chapter 5.

6.6.1 Growth: Viable, apoptotic and death cells

In Figure 6.3 , it is possible to observe that the model predicts the concentrations of (A) viable, (B) total, (C) early apoptotic and (D) dead cells. However, when higher initial cell densities were used, the model did not capture the last sample point for viable and total cells

number. In these cases, the total number of cells abruptly decline during the last hours of the culture and high levels of lysis were observed. To understand this phenomenon, samples from each flask were examined (Figure 6.4 and Figure 6.5).

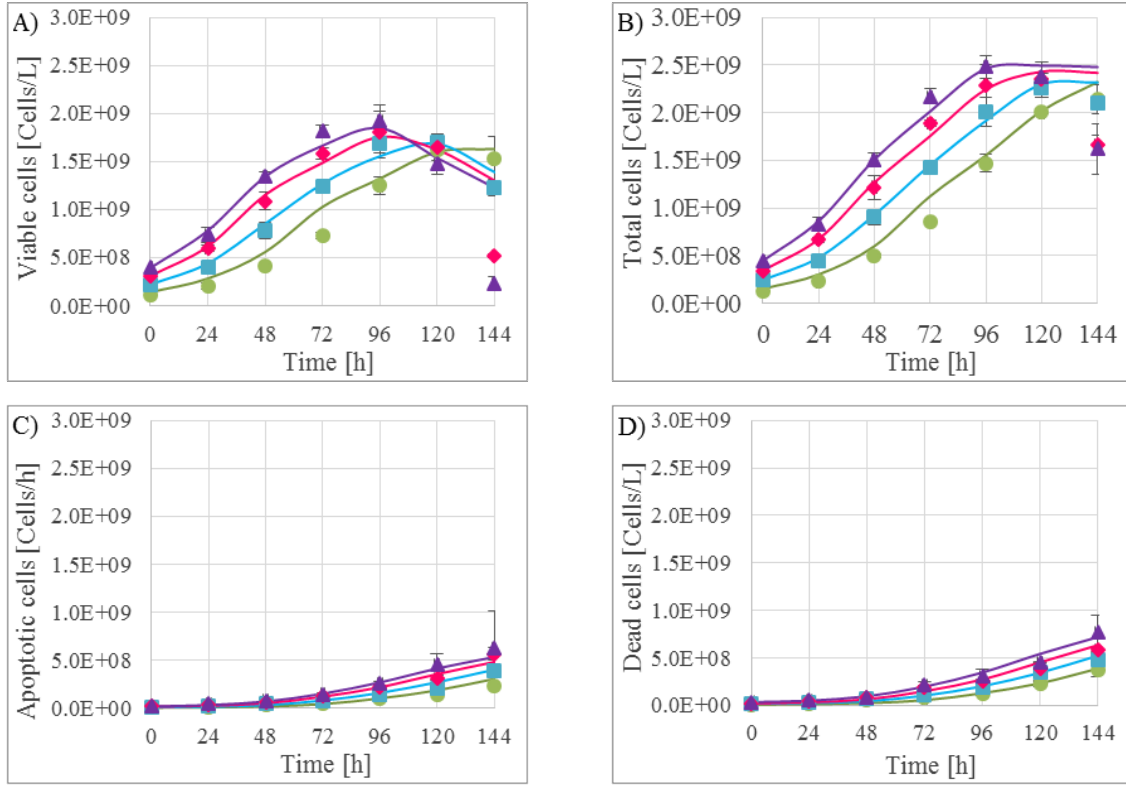


Figure 6.3: Experimental results (dots) and model predictions (lines) for (A) Viable cells, (B) Total cells, (C) Apoptotic cells, and (D) Dead cells. (○-green) Batch 1, (□ - blue) Batch 2, (◇- pink) Batch 3 and (Δ-purple) Batch 4.

In Figure 6.4, pictures of a set of experiments are presented. Cultures with lower initial cell densities, Batch 1 and Batch 2 (Figure 6.4 B and C), did not show any visible unusual feature. The medium was clear and the cell integrity was maintained. On the other hand, Cultures with higher initial cell densities, Batch 3 and Batch 4 (Figure 6.4 D and E), present high aggregation levels and accumulation of cells in the bottom of the flasks in the last day (Batch 4 Figure 6.4 F).

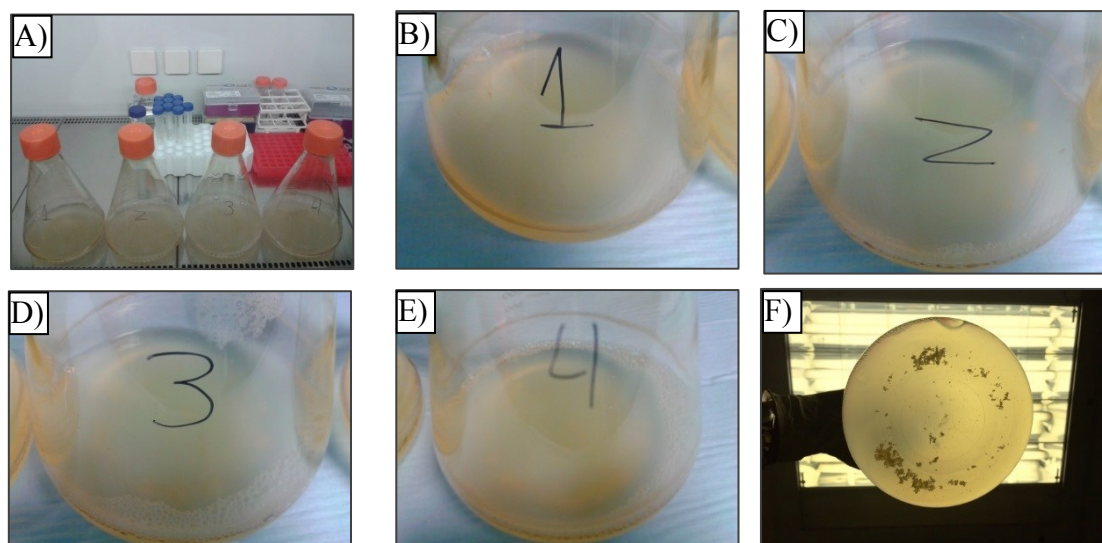


Figure 6.4: Flasks pictures. (A) Set of experiments under different culture conditions (B) Batch 1 (C) Batch 2 (D) Batch 3 (E) Batch 4 (F) Batch 4. Aggregation of cells.

The aggregates were observed on the last day of culture. To analyse the morphological conditions of the cells in the aggregates, samples of the flask Batch 4 were taken and stained with Erythrosine B, which marks cells that have lost membrane integrity. In Figure 6.5, the results from the aggregates staining are presented. Microscopy results showed viable cells being part of the aggregates (green areas in picture A) and the aggregates (B) present higher cell density compared to the supernatant (C). Consequently, when aggregates are formed, the experimental measurement of cell number would provide an incorrect cell concentration. These observations could explain the low number of cells in the experimental results and suggest that the deviation between model predictions and experimental measurements could be imperceptible, if the sample was representative.

These results indicate that the cultures should stop when their viability is 50%, not only because this would ensure the reliability of the sample but also because it would ensure the secretion of a complete and active product (Mukherjee et al., 2007); low levels of proteases that could damage the product (Hill et al., 2001) and low debris accumulation that could interfere in the downstream processing for the purification process. The model allows us to predict when the viability level would reach 50% value from the initial conditions of the

culture. Therefore, the model would allow us to determine how long a culture would last, based on its initial conditions.

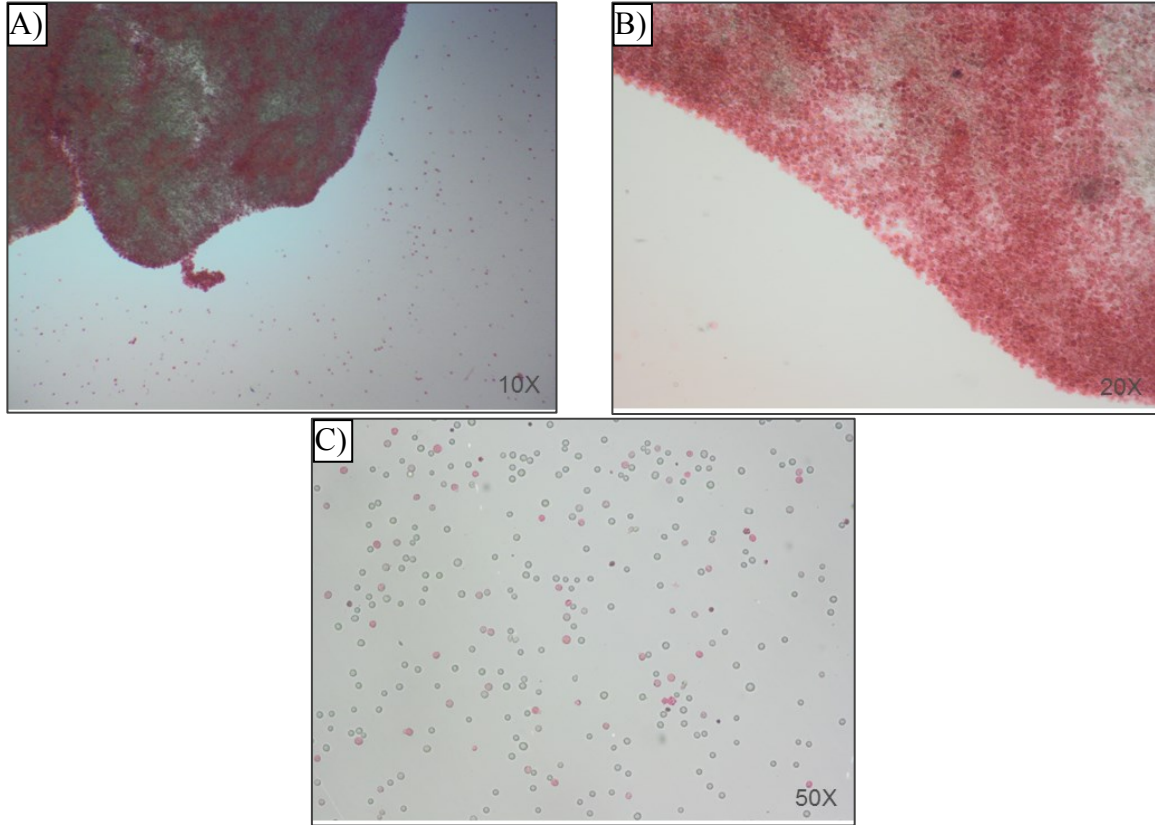


Figure 6.5: Aggregates examination. (A) Magnification of aggregates and supernatant (10X) (B) Magnification of aggregates (20X). (C) Magnification of the supernatant (50X).

6.6.2 Monoclonal antibody production.

In Figure 6.6, the comparison of the experimental results and model prediction is presented, and the grey area in the graphs describes the 95% confidence interval. The model predicted the monoclonal antibody concentration in all cultures and few discrepancies are evident at different time points among batches. Therefore, it is not possible to find an explanation for the discrepancies between the experimental results and model predictions.

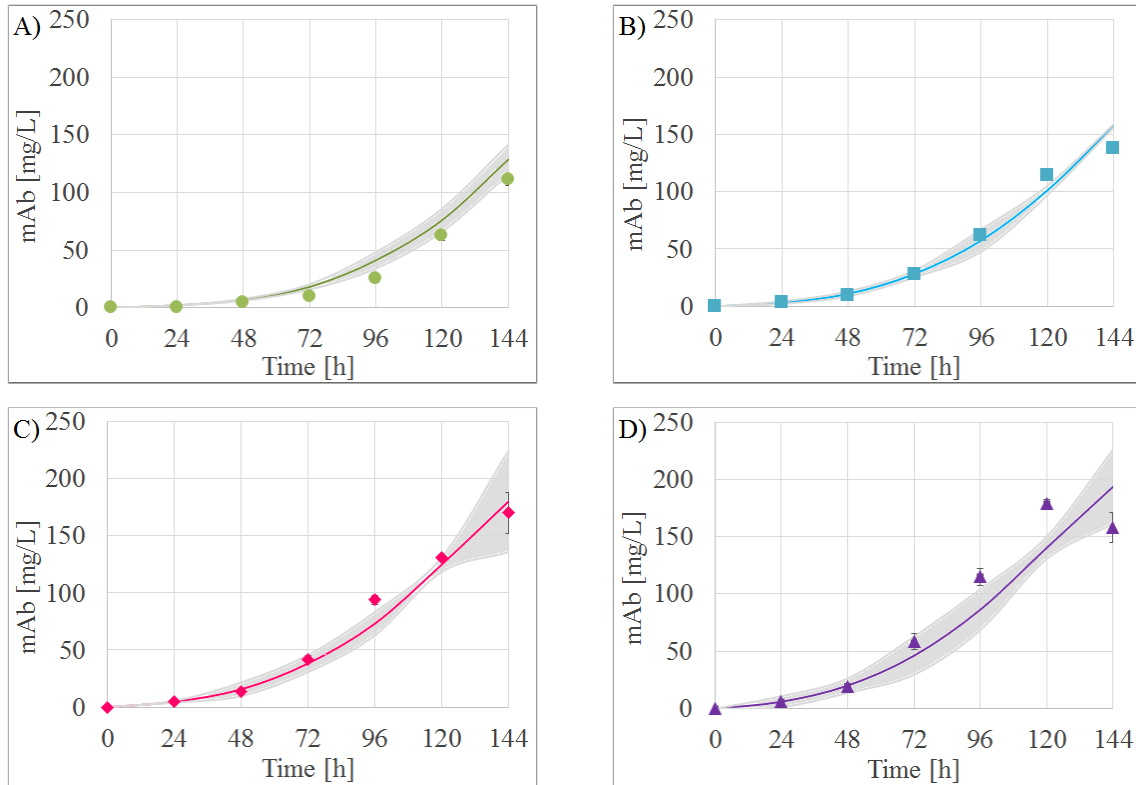


Figure 6.6: Comparison of experimental results (dots) and model predictions (lines) for mAbs concentration. (A) Batch 1, (B) Batch 2, (C) Batch 3 and (D) Batch 4. 95% confidence interval (grey area)

6.6.3 Glucose metabolism and lactate production

In Figure 6.7, the profiles of glucose concentration in cultures (A) with serum and (B) serum-free are compared, and it is possible to observe that the glucose uptake in serum-free cultures was lower in comparison to serum-supplemented cultures. In DMEM medium supplemented with 10% FBS, lactate was accumulated until 72-96 hours, when its concentration reached a plateau (25[mM]) (Figure 5.15). However, after adaptation to serum-free conditions, the cell reached low levels of lactate concentration (5 [mM] in some cases at 48 hours of culture). Therefore, CD-Hybridoma medium supplemented with cholesterol and serum replacement balanced the glucose and amino acids uptake, and cells proliferation did not merely rely on the glycolytic metabolism. In consequence, lactate accumulation was imperceptible. These results show how versatile tumour cells are and how they adjust their metabolism depending on the environmental conditions and nutrients availability. A similar

behaviour has been observed in CHO cultures, where the anaplerotic conversion of pyruvate to oxaloacetate account for 10% of the pyruvate flux with the remaining 90% entering the TCA cycle through acetyl-CoA (Goudar et al., 2010).

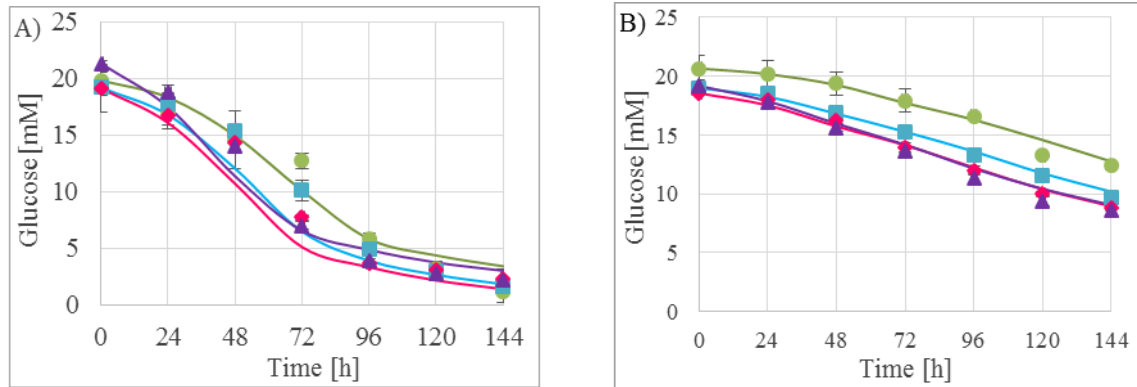


Figure 6.7: Comparison of glucose profile in (A) DMEM 10%FBS medium and (B) CD-Hybridoma medium. Comparison of experimental results (dots) and model predictions (lines) for glucose. (o-green) Batch 1, (□ - blue) Batch 2, (◇ - pink) Batch 3 and (Δ-purple) Batch 4.

6.6.4 Amino acid metabolism

6.6.4.1 Glutamate consumption and metabolic shifts

In Figure 6.8, the experimental results and model predictions for the concentration of amino acids involved in energy production are presented: (A) glutamate, (B) arginine, (C) proline, (D) histidine, (E) tyrosine and (F) phenylalanine. In the figure, it is possible to observe the fit of the model predictions to the experimental results, the similarities between the amino acids concentration profiles trends and the metabolic shifts.

Glutamate is the main source of glutamine for the GS-NS0 system and it is used for the biosynthesis of cell structures, mAbs assembling and energy production, and it is exhausted between 48-72 hours of the culture. Glutamate depletion activates arginine uptake to produce molecules of glutamine and glutamate for biosynthesis. Additionally, phenylalanine, tyrosine, histidine and proline uptake is activated to produce oxaloacetate and energy. In Figure 6.8, these metabolic shifts are evident, the slope of arginine (Figure 6.8 B) and proline (Figure 6.8 C) profiles present a dramatic increase. The uptake coefficient for energy production increase

10 times more for proline ($C_{\text{pro}}=0.5$) than phenylalanine and tyrosine ($C_{\text{phe}}=C_{\text{tyr}}=0.05$), and histidine does not enter into the TCA cycle ($C_{\text{his}}=0$). Therefore, glutamate depletion has low impact in phenylalanine, tyrosine and histidine profiles.

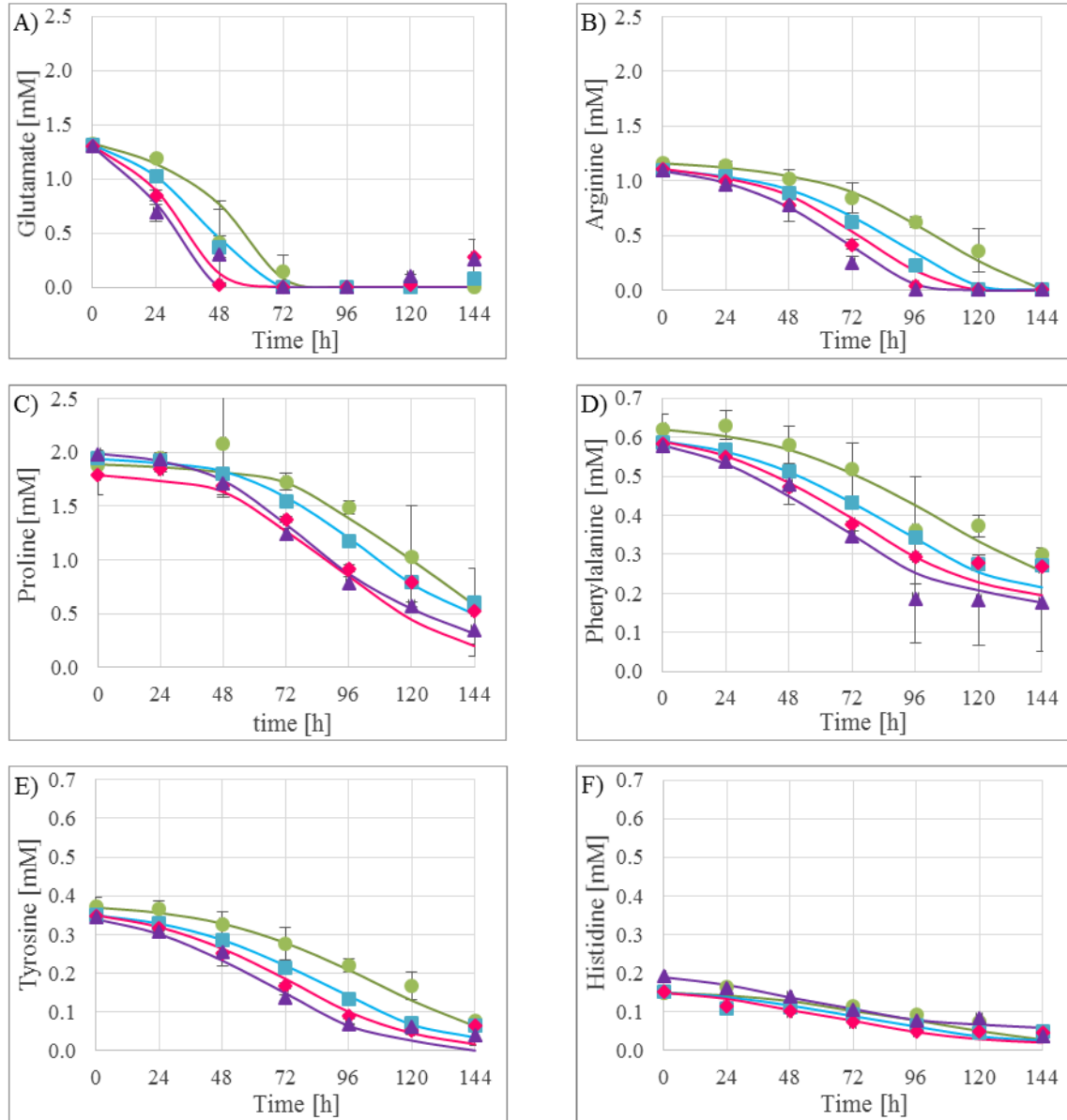


Figure 6.8: Comparison of experimental results (dots) and model predictions (lines) for (A) Glutamate (B) Arginine (C) Proline (D) Histidine (E) Tyrosine (F) Phenylalanine. (○-green) Batch 1, (□-blue) Batch 2, (◇-pink) Batch 3 and (Δ-purple) Batch 4.

In addition to the expected metabolic shifts, alanine concentration profile shifted from accumulation to degradation. This metabolic shift has been included in the model and it was related to glutamate exhaustion. In Figure 6.9, alanine concentration profiles are presented and the fit of the model predictions to the experimental data is clear.

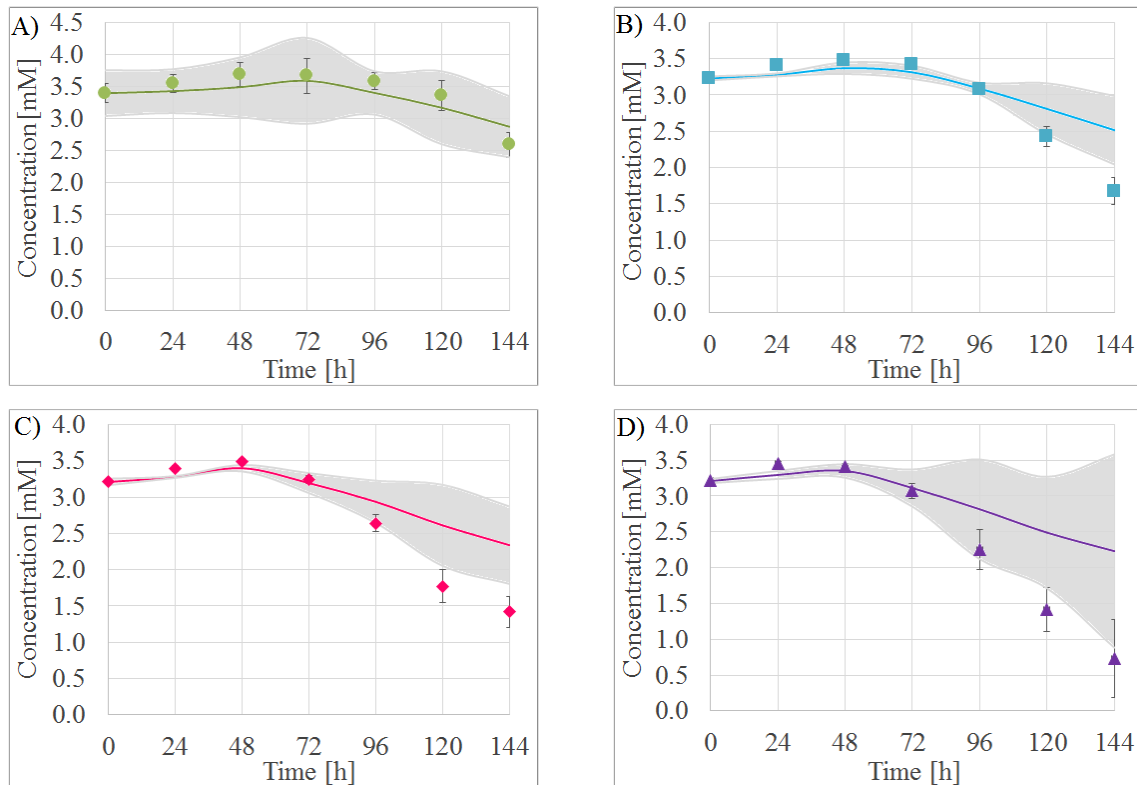


Figure 6.9: Experimental results (dots) and model predictions (lines) for alanine (A) Batch 1, (B) Batch 2, (C) Batch 3 and (D) Batch 4. 95% confidence interval (grey area)

6.6.4.2 Aspartate consumption and metabolic shift

In Figure 6.10, aspartate and asparagine concentration profiles are presented. Aspartate is depleted between 48-96 hours of culture (Figure 6.10 A), following the same trend as glutamate. Here, the model proposes that asparagine is used to supply the exact amount of aspartate required for biosynthesis. This assumption is validated with the comparison between experimental and predictions results (Figure 6.10 B).

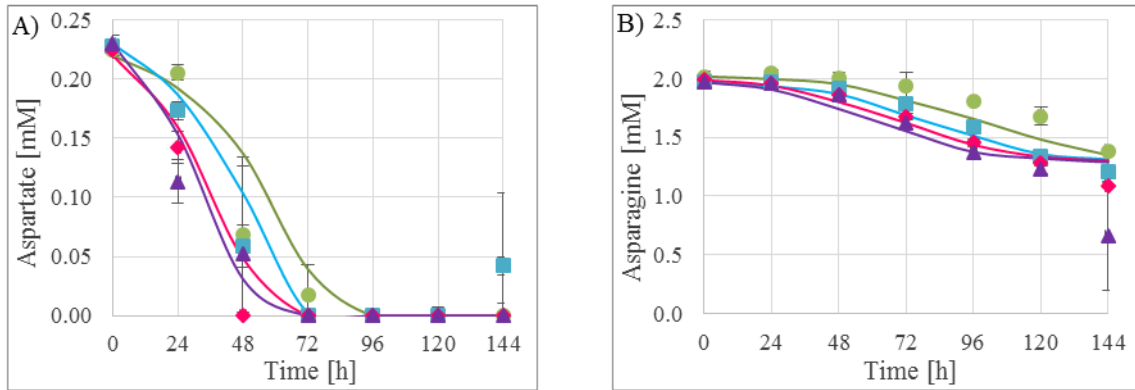


Figure 6.10: Experimental results (dots) and model predictions (lines) for (A) Aspartate and (B) Asparagine. (o-green) Batch 1, (□ - blue) Batch 2, (◇- pink) Batch 3 and (Δ-purple) Batch 4.

6.6.4.3 Essential amino acids exhaustion.

In Figure 6.11, the experimental results and model predictions for the concentration of amino acids involved in energy production are presented: (A) valine, (B) threonine, (C) methionine, (D) lysine, (E) isoleucine and (F) leucine. The depletion of leucine, isoleucine and arginine after 96 hours of the culture (Figure 6.11 A and C, and Figure 6.8 B) induces the activation of apoptosis and necrosis processes, and the viability of the culture decrease to 50% after 24.

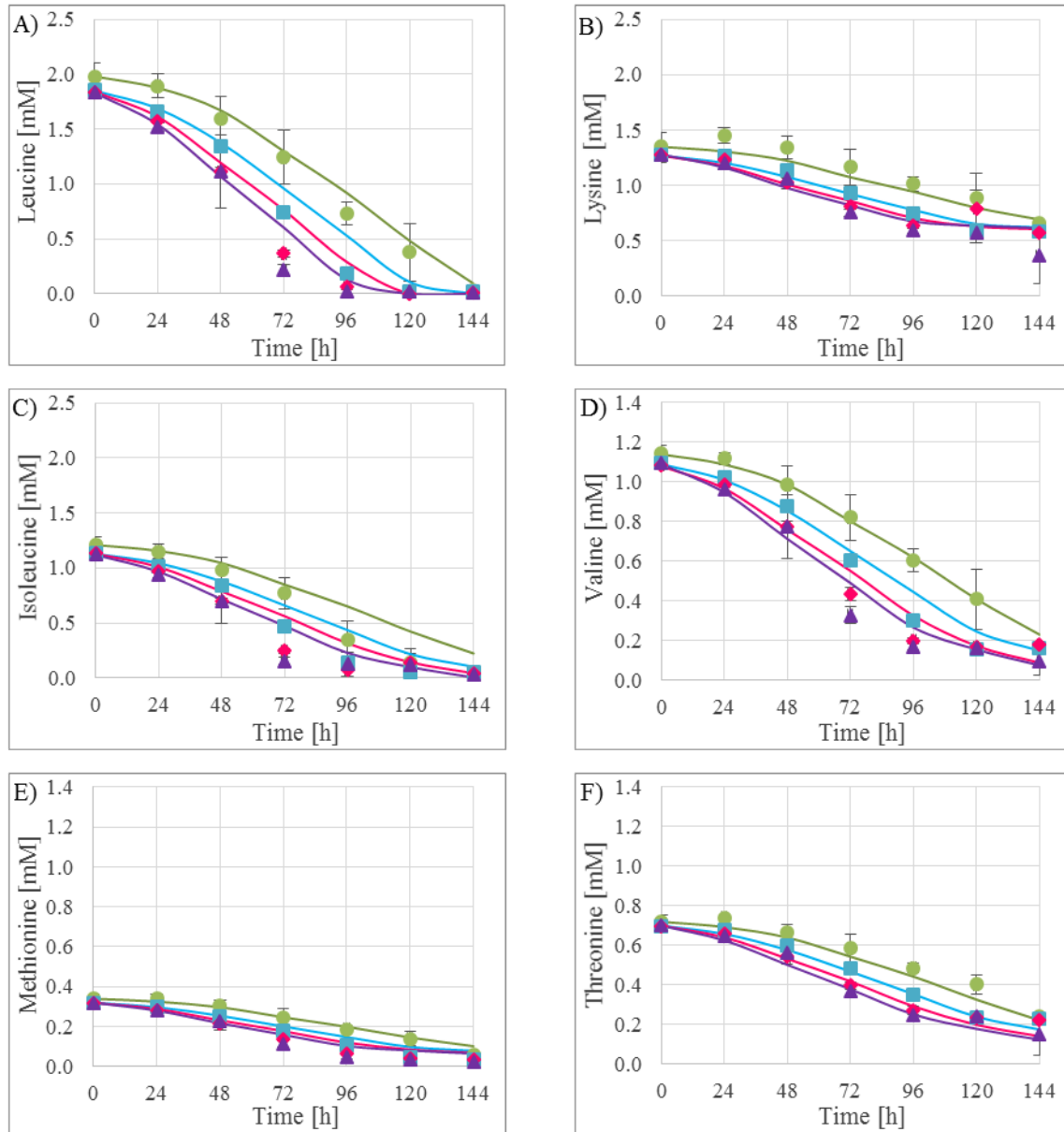


Figure 6.11: Comparison of experimental results (dots) and model predictions (lines) for (A) Leucine, (B) Lysine, (C) Isoleucine, (D) Valine, (E) Methionine and (F) Threonine. (○-green) Batch 1, (□ - blue) Batch 2, (◇- pink) Batch 3 and (Δ-purple) Batch 4.

6.6.5 Serine, glycine, cysteine and tryptophan

In Figure 6.12, the concentration profile of the amino acids incorporated in the model are presented: (A) tryptophan, (B) cysteine, (C) serine and (D) glycine. These amino acids mass balances were incorporated in the Energy Model to predict their uptakes and use this

information for medium design. The model captured the amino acids concentrations trends during the first 72 hours of culture and, after this point, the model underestimated serine concentrations (Figure 6.12 C). The underestimation occurred when the cells stopped producing glycine, therefore, it is concluded that serine mass fraction in the cell composition or mAbs composition has been overestimated. These results suggest that it is necessary to recalculate cellular and product compositions.

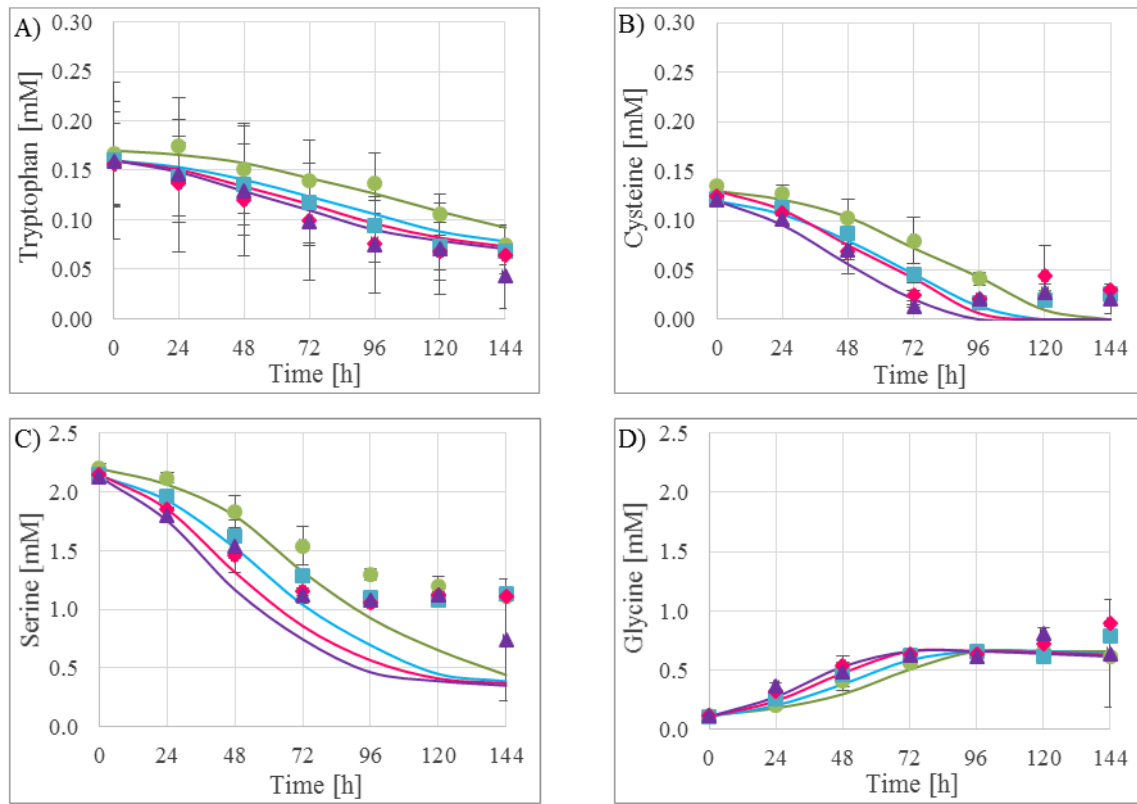


Figure 6.12: Experimental results (dots) and model predictions (lines) for: (A) Tryptophan, (B) Cysteine, (C) Serine and (D) Glycine. (○-green) Batch 1, (□ - blue) Batch 2, (◇- pink) Batch 3 and (Δ-purple) Batch 4.

6.6.6 ATP Balance

In Figure 6.13, it is possible to observe the experimental results (dots), model predictions (solid line) and the 95% interval (grey area) for ATP concentration in the culture medium. These graphical representations allows us to observe the precision of the model predictions.

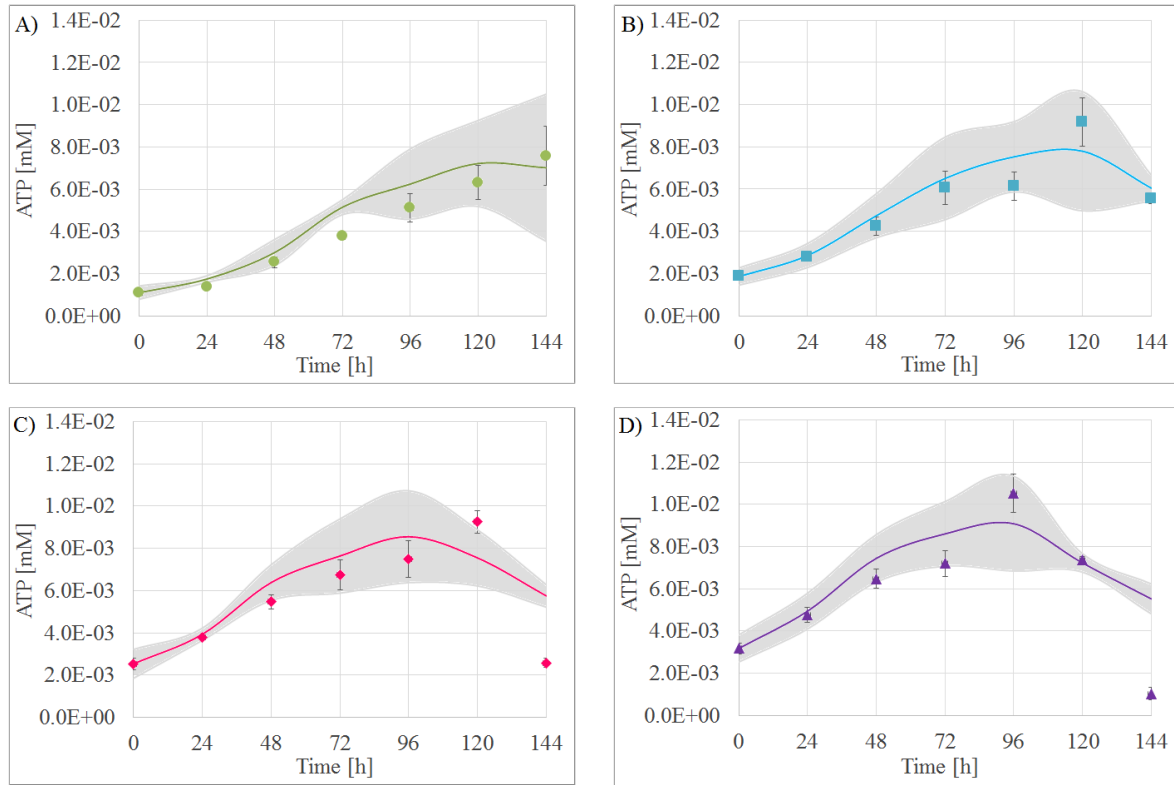


Figure 6.13: Comparison of experimental results (dots) and model predictions (lines) for ATP concentration. (○) Batch 1, (□) Batch 2, (◇) Batch 3 and (Δ) Batch 4.

The ATP mass balance, in the Energy Model, allowed us to estimate the ATP content in cultures with lower initial cell densities, until the end of the culture (Figure 6.13 A and B). The last sample points in the cultures with higher initial cell densities, Batch 3 and Batch 4, show that the model overestimated the ATP concentration; the explanation for these discrepancies is related to the high aggregation level reached at the end of these cultures, which caused the collection of a non-representative samples.

The ATP content per cell was subjected to statistical analysis to determine if its value is constant in viable cells. A two-way ANOVA analysis was performed to determine if there were significant differences within batches, within days and the interaction of these variables, considered initially as independent. The data used for ANOVA analysis are in Table 6.2 and the results in Table 6.3.

Table 6.2: ATP per cell measurements.

Time	Batch 1	Batch 2	Batch 3	Batch 4
[h]	[mmol/Cell]	[mmol/Cell]	[mmol/Cell]	[mmol/Cell]
0	9.69E-12	8.45E-12	8.17E-12	8.08E-12
24	7.06E-12	7.02E-12	6.38E-12	6.55E-12
48	6.17E-12	5.48E-12	5.07E-12	4.83E-12
72	5.19E-12	4.94E-12	4.26E-12	3.97E-12
96	4.08E-12	3.66E-12	4.18E-12	5.52E-12
120	3.89E-12	5.37E-12	5.69E-12	5.01E-12
144	4.96E-12	4.52E-12	4.99E-12	4.61E-12

Table 6.3: Results of ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Batches	1.63E-24	3	5.42E-25	1.19	0.32	2.77
Days	1.68E-22	6	2.80E-23	61.65	7.06E-23	2.27
Interaction	2.22E-23	18	1.23E-24	2.72	0.002	1.79
Within	2.54E-23	56	4.53E-25			
Total	2.17E-22	83				

The results show that within batches there are no significant differences. On the other hand, the analysis within days and between batches and days present p-values lower than the confidence level and F values higher than F-critical, this indicates that there are significant differences within days and an interaction between days and batches. Unfortunately, the test applied does not indicate which day present differences and it was necessary to perform t-test analysis to determine the significance within days.

The t-test analysis was performed for each batch in all days combinations to find the stage of the culture that present a significantly different ATP content.

In Figure 6.14, the experimental results for intracellular ATP content in viable GS-NS0 cells are presented. All experiments present significant differences ($p\text{-value} < 0.05$) between 1 and 24 hours compared to the other stages of the culture. These results indicate that the concentration of ATP per viable cell is not constant throughout the culture time. Moreover, during lag phase, from 0 to 24 hours, the cells presented an intracellular ATP concentration around $7.68 \pm 1.25 \times 10^{-12}$ [mmol ATP/Cell], and during exponential growth, stationary phase and death stage the ATP content dropped to $4.82 \pm 1.09 \times 10^{-12}$ [mmol ATP/Cell].

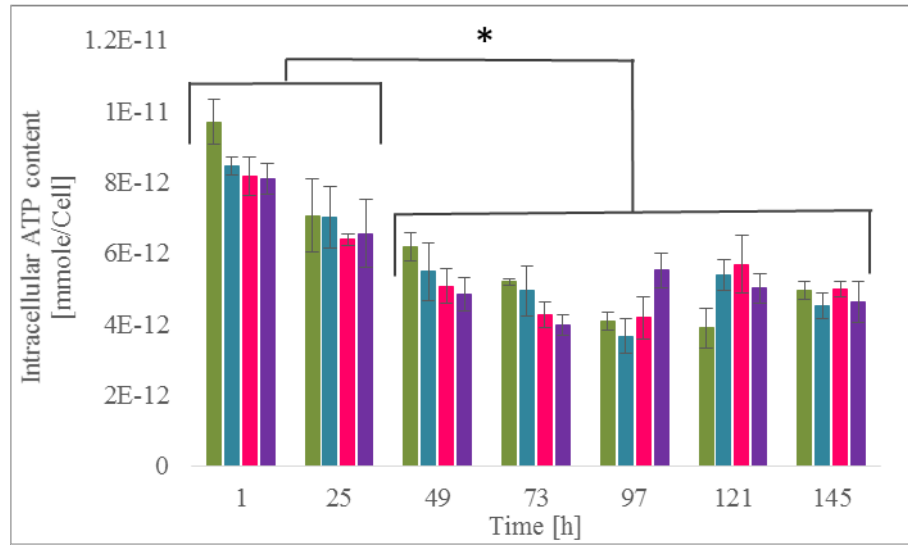


Figure 6.14: Experimental intracellular ATP content [mmol ATP/Cell]. (Green) Batch 1, (Blue) Batch 2, (Pink) Batch 3 and (Purple) Batch 4. (*) $p < 0.05$

6.6.7 Batch cultures in bioreactor Bioflo-110

The implementation of fed-batch cultures in flasks has a wide variety of limitations. Firstly, the temperature inevitably decreases every time a flask is removed from the incubator and placed inside a laminar flow cabinet for sample collection and feeding. This is a perturbation in the system. Secondly, as result of cells by-product release, medium pH is continuously declining, and even when the feeding pH is adjusted to the set-point, the feeding also introduces a perturbation in the system. Taking these two important limitations for fed-batch cultures in flasks, the system was scaled up to bioreactor operation for future fed-batch operation. The bioreactor Bioflo-110 operated under the conditions presented in

Table 6.4.

Table 6.4: Culture conditions in bioreactor Bioflo-110.

Parameter	Set point	Control system
Volume	1.5L	
Working volume	500mL	
Temperature	37°C	Temperature sensor/Water jacket
pH	7.2	pH sensor/CO ₂
O ₂	20%	O ₂ sensor / O ₂ and N ₂ sparger
Agitation	60-80rpm	Tri-blade impeller

Cultures with initial cell densities of $3.10 \pm 0.14 \times 10^8$ [Cells/L] (Flasks) and $3.31 \pm 0.27 \times 10^8$ [Cells/L] (bioreactor) presented the same performance, reaching the same number of viable cells ($1.81 \pm 0.22 \times 10^9$ and $1.98 \pm 0.13 \times 10^9$), total cells ($2.35 \pm 0.18 \times 10^9$ and $2.38 \pm 0.011 \times 10^9$) and product titre (170.11 ± 17.87 [mg/L] and 165.57 ± 3.57 [mg/L]). In addition, the model captured the dynamic of the system in both operation modes and the parameters of the model were not re-estimated. According to the experimental results and the model prediction, the scaling-up of the culture does not have a significant effect in the system. Moreover, it would be possible to implement a feeding strategy in the bioreactor, reducing temperature and pH perturbations.

The experimental results and model predictions are presented in Figure 6.15, Figure 6.16 and Figure 6.17.

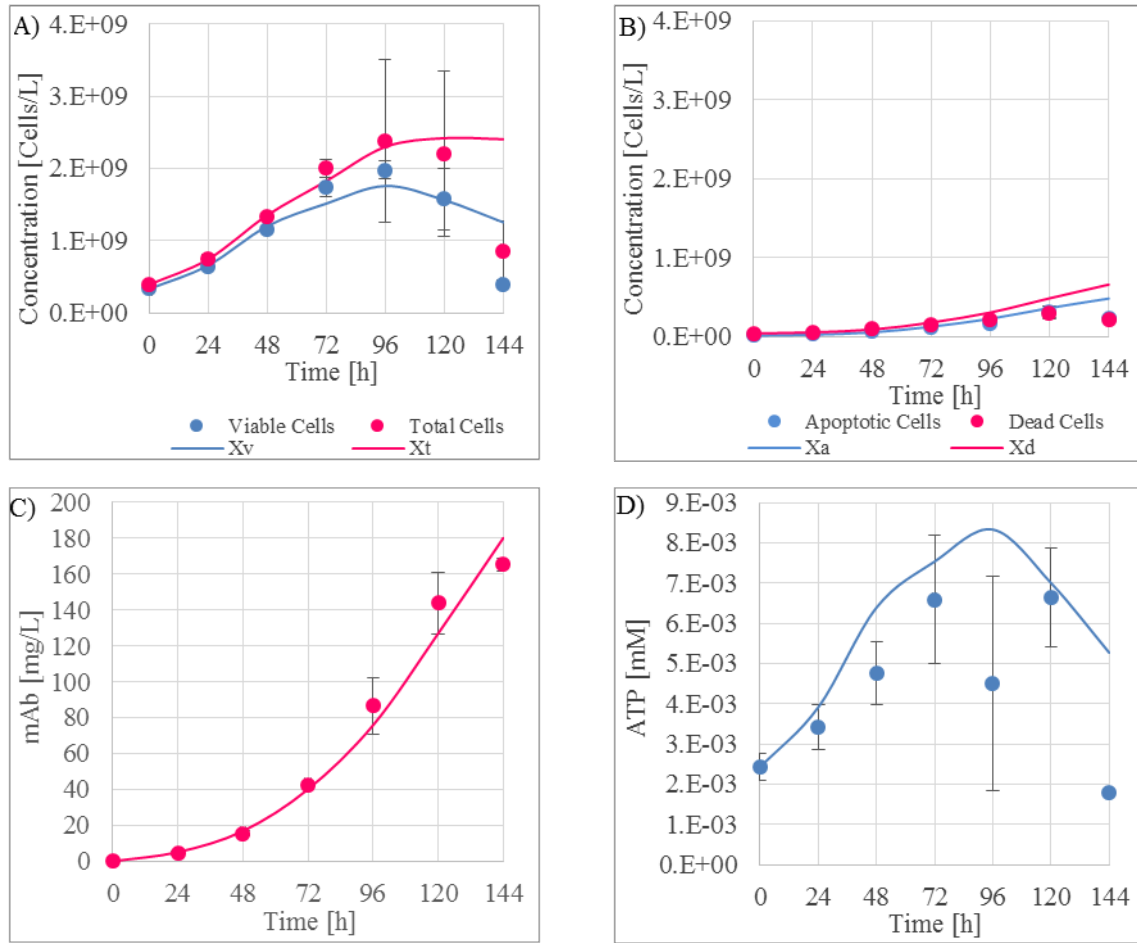


Figure 6.15: Comparison of experimental (dots) and model predictions (solid lines) for cultures in bioreactor Bioflo-110. (A) Viable and total cells (B) Early apoptotic and dead cells (C) Monoclonal antibody concentration (D) ATP content in the culture medium.

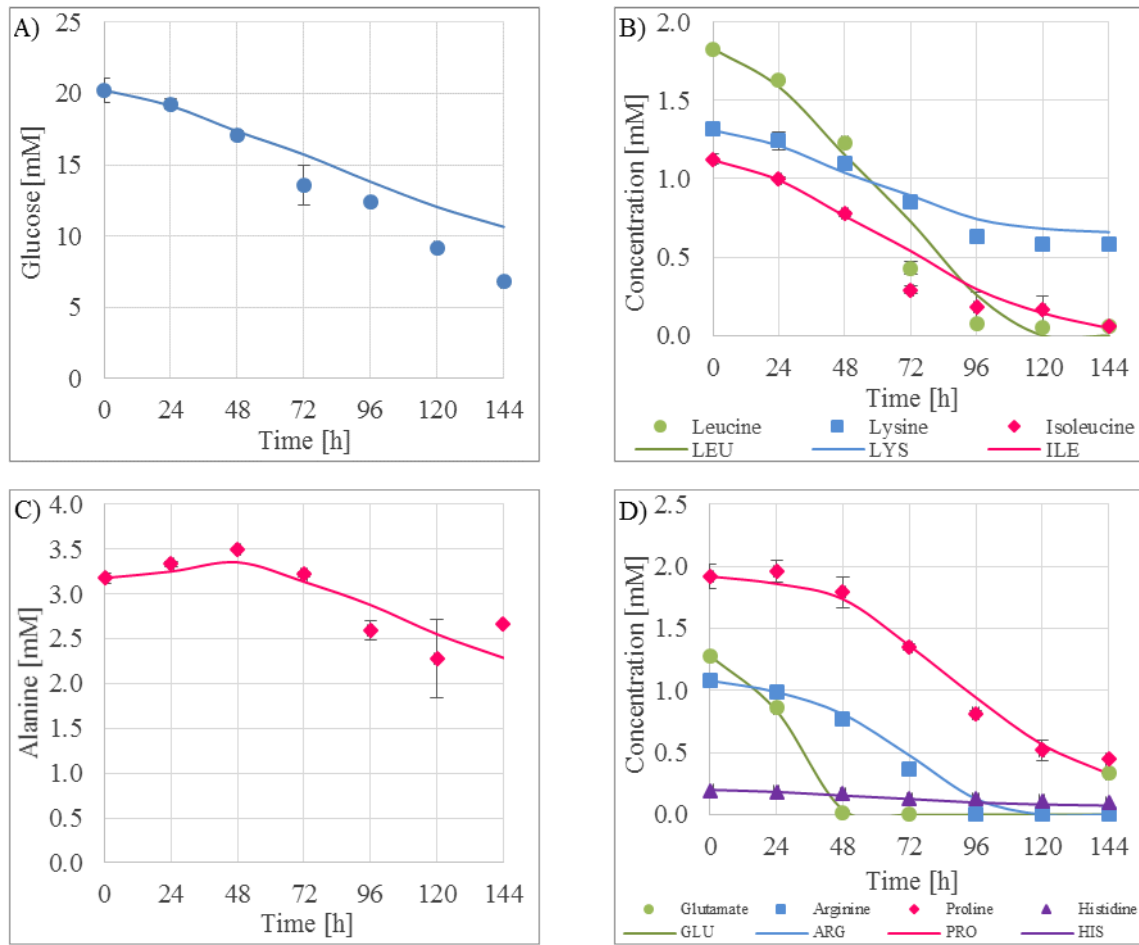


Figure 6.16: Comparison of experimental (dots) and model predictions (solid lines) for cultures in bioreactor Bioflo-110. (A) Glucose concentration (B) Leucine, lysine and isoleucine concentrations (C) Alanine concentration (D) Glutamate, arginine, proline and histidine concentrations.

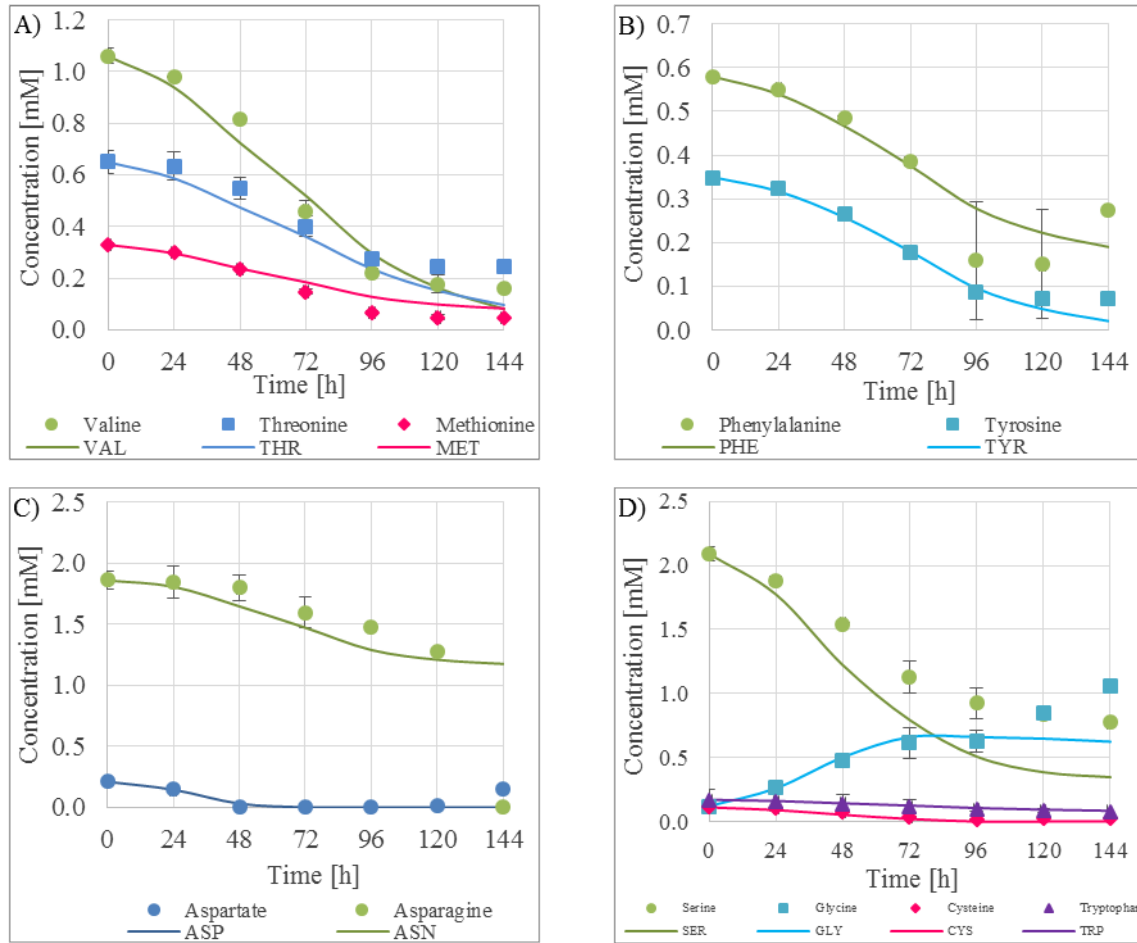


Figure 6.17: Comparison of experimental (dots) and model predictions (solid lines) for cultures in bioreactor Bioflo-110. (A) Valine, threonine and methionine concentrations (B) Phenylalanine and tyrosine concentrations (C) Aspartate and asparagine concentrations (D) Serine, glycine cysteine and tryptophan concentrations.

The results present some discrepancies with the model at the end of the culture. The explanation for these differences is related to the accumulation of cells in some areas of the bioreactor, therefore, the last samples were not representative of the system.

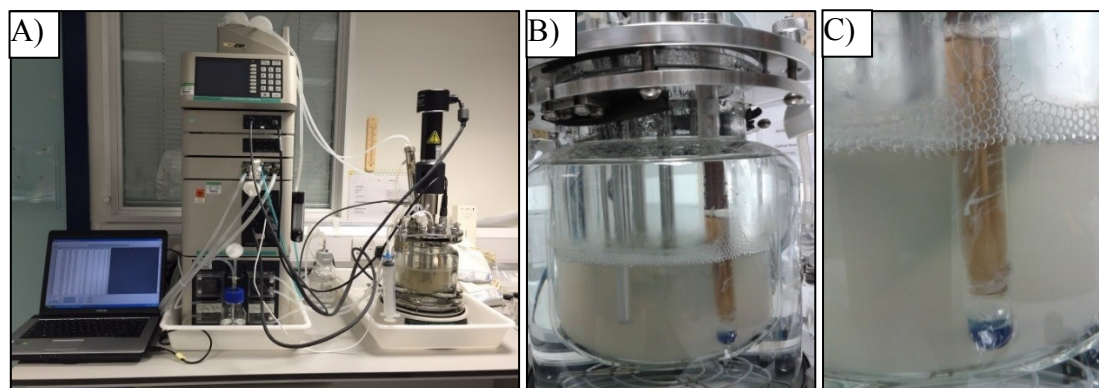


Figure 6.18: (A) Bioreactor Bioflo-110 in operation (Laptop for data collection, control unit and vessel). (B) Last day of culture (vessel) (C) Cells accumulation around the pH probe.

In Table 6.5 is possible to observe that experiments under the same initial conditions but performed in Erlenmeyer flasks or the bioreactor Bioflo 110 did not presented significant differences in the maximum number of viable and total cells, and final product titer. Therefore, the system is scalable to conditions that resemble industrial mAbs production (agitation and aeration configurations, and pH control).

Table 6.5: Comparison of batch culture in Erlenmeyer flasks and Bioflo 110 bioreactor.

Parameter	Flasks	Bioflo 110
Initial cell density	$3.10 \pm 0.14 \times 10^8$ [Cells/L]	$3.31 \pm 0.27 \times 10^8$ [Cells/L]
Maximum viable cell concentration	$1.81 \pm 0.22 \times 10^9$ [Cells/L]	$1.98 \pm 0.13 \times 10^9$ [Cells/L]
Maximum total cell concentration	$2.35 \pm 0.18 \times 10^9$ [Cells/L]	$2.38 \pm 0.011 \times 10^9$ [Cells/L]
Product titre	170.11 ± 17.87 [mg/L]	165.57 ± 3.57 [mg/L]

6.6.8 Conclusions

The Energy Model was successfully modified to predict serine, glycine, cysteine and tryptophan profiles. The model analysis presented the same results for the original and modified Energy Model. The most important result of GSA was the difference in the sensitivity of the viable cells concentration to $\mu_{\max}$, which was predominant during the exponential growth in DMEM medium and permanent throughout the culture in CD-Hybridoma medium, revealing that GSA captured the main effect of the adaptation, the extension of the culture longevity.

This result proved again the importance of the development of mathematical models based on first principles and the potential of the GSA for model analysis.

One experiment was designed and performed for the estimation of the subset of significant parameters (Batch 2). Subsequently, the model was validated through the comparison of experimental and simulations results, under the same initial conditions (Batch 3). Finally, the model was used to perform predictions under low and high initial cell density (Batch 1 and Batch 4). The validation and prediction results prove the capability of the model to describe growth kinetics, glucose and amino acids metabolism, metabolic shifts, mAbs secretion and ATP production and consumption.

The statistical analysis revealed that all experiments present significant differences in the intracellular ATP content, between 1 and 24 hours compared to the other stages of the culture, indicating that the ATP content in viable cells is not constant throughout the culture. The intracellular ATP content was $7.68 \pm 1.25 \times 10^{-12}$ [mmol ATP/Cell] during the lag phase, and $4.82 \pm 1.09 \times 10^{-12}$ [mmol ATP/Cell] after the lag phase and until the end of the culture.

The culture was successfully scaled-up to the bioreactor Bioflo 110, 1.5L capacity with 500mL of working volume. Cultures in flask and the bioreactor under the same initial conditions presented equivalent performance, reaching consistent numbers of viable and total cells, and product titre. In addition, the model captured the dynamic of the system in both operation modes without re-estimation of parameters. According to the experimental results and the model prediction, the scaling-up of the culture does not have a significant effect on the system. Moreover, it would be possible to implement a feeding strategy in the bioreactor, reducing temperature and pH perturbations.

Finally, the successful incorporation of the mass balances for serine, glycine, cysteine and tryptophan in the Energy Model allowed us to predict the number of viable, apoptotic and dead cells; the concentrations of glucose, 19 amino acids and lactate, and the intracellular ATP content in cultures performed in flasks and bioreactor.

CHAPTER 7: Model-based medium design and optimisation of fed-batch cultures

7 Model-based medium design and optimisation of fed-batch cultures.

The modified Energy Model presented in the previous chapter predicts the profile of the entire set of amino acids present in the culture medium for batch cultures in flasks and bioreactors. In this chapter, the model was used to determine the composition of a supplemental medium for fed-batch cultures of GS-NS0 cells, producing cB72.3 mAbs in serum-free CD-Hybridoma medium.

7.1 Introduction

The implementation of a fed-batch culture requires the design of a supplemental medium coupled with a feeding strategy design (Xie & Wang, 1993-1996; Zhang et al., 2004 Altamirano et al., 2004; Spens & Häggström, 2007; Shibuya et al., 2008).

Altamirano et al. implemented two different media, one for the initial batch stage (basal medium) and another for the fed-batch stage (supplemental medium). The basal medium consisted of a commercial medium without amino acids and the added concentration of amino acids was calculated taking into account the expected cell growth rate, the length of the batch stage and the specific consumption rate of each amino acid. The data required to calculate these variables was collected from experiments. The supplemental medium for the fed-batch stage consisted of the same components in the same proportions, but in higher concentrations (alanine, glycine and aspartate were not added) (Altamirano et al., 2004). This approach and the stoichiometric balances for medium design by Xie & Wang were the inspiration for the model-based medium design implemented in the present work (Xie & Wang, 1994a; Xie & Wang, 1994b; Xie & Wang, 1994c; Xie & Wang, 1996a; Xie & Wang, 1996b).

In this work, the Energy Model and computational tools were used for the implementation of model-based medium design and model-based optimisation techniques. *In-silico* simulations allowed us to compare different medium compositions with the designed medium and design a feeding strategy for fed-batch cultures.

7.2 Aims and objectives

The aim of this chapter is the design of a supplemental medium composition and the implementation of a model-based optimisation of a feeding schedule that maximises the mAbs titre. The objectives to achieve this aim were:

- 1) **Supplemental medium design:** for the implementation of fed-batch cultures a supplemental medium is required. In the present work, the supplemental medium was designed using model predictions.
- 2) **Model modifications:** the Energy Model was designed for batch cultures. Therefore, the introduction of an inlet flow term and supplemental medium composition was incorporated to transform the system in a fed-batch culture.
- 3) **Model testing and parameter estimation:** the model was used to design a conventional feeding strategy. The schedule was implemented in flasks and the experimental results were used for parameter estimation.
- 4) **Model-based optimisation and validation:** the model was used to perform independent dynamic optimisation of four output variables, and one of the schedules derived from the optimisations was implemented to validate our approach.

7.3 Methodology

7.3.1 Medium design

The design of the supplemental medium is based on the specific consumption rate of each amino acid and glucose per cell per hour. Altamirano et al. designed a supplemental medium for fed-batch cultures taking into account the expected cell growth rate and the specific consumption rate of each amino acid. Both values were constant and calculated from experimental results from batch experiments (Altamirano et al., 2004).

In this work, the growth rate and the specific consumption rate change throughout the culture; because it is a dynamic system, and the amino acids uptake does not only consider proliferation ($Q_{x,AA}$) but also product assembly ($Q_{mAb,AA}$) and energy production ($Q_{tca,AA}$).

Therefore, the structure of the specific consumption rate includes three separate terms (See description in section 5.3.4).

Equation 7.1: Total specific consumption rate of any amino acids.

$$Q_{AA} = Q_{x,AA} + Q_{mab,AA} + Q_{tca,AA}$$

Equation 7.2: Amino acids specific consumption rate for proliferation.

$$Q_{x,AA} = \frac{\mu}{Y_{x,AA}}$$

Equation 7.3: Amino acids specific consumption rate for product assembly.

$$Q_{mab,AA} = q_{mab,x} M_{mab,AA}$$

Equation 7.4: Amino acids specific consumption rate for energy production.

$$Q_{tca,AA} = C_{AA} \left(\frac{\mu}{Y_{tca,AA}} + M_{tca,AA} \right)$$

The results of the simulation of the amino-acid-specific consumption rate for proliferation are presented in Figure 7.1. The initial conditions for the simulation derive from the CD-Hybridoma culture Batch 2. In Figure 7.1, it is possible to observe the dynamism of the system.

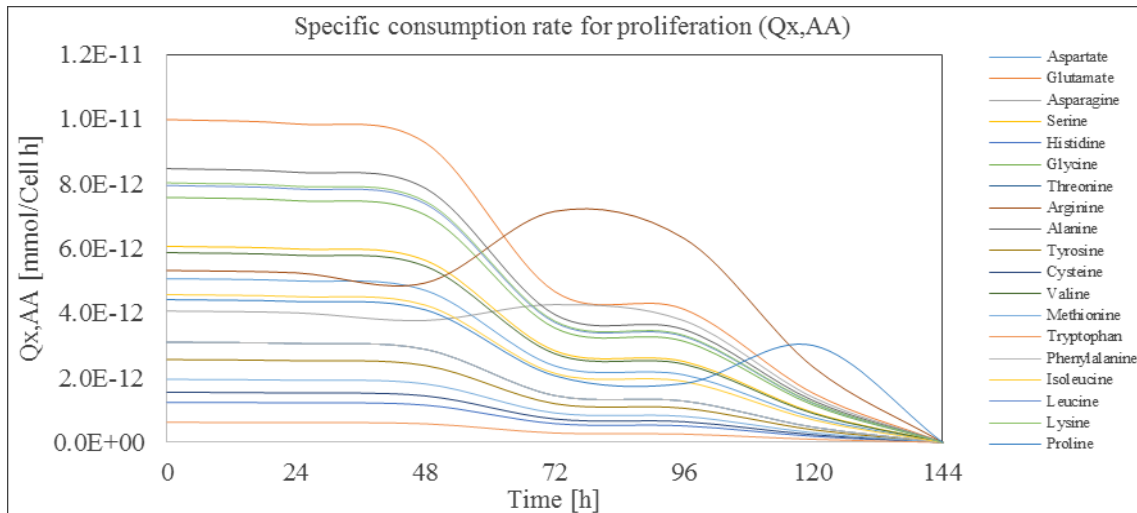


Figure 7.1: Profile of specific rate consumption of amino acids for proliferation.

The uptake for proliferation depends on cell composition and the growth rate. Cell composition has been assumed constant, but the growth rate varies. At the beginning of the culture (0-48 hours), the growth rate is high because of the availability of nutrients, and it drops after glutamate and aspartate depletion (48 hours). Consequently, the profiles of amino acid uptake for proliferation follow the same trends. In Figure 7.1, three amino acids present a substantial shift in their uptake for proliferation: arginine, proline and asparagine. Arginine uptake increases after glutamate depletion because it is used for glutamine and glutamate production and proline is used for the same purpose after arginine depletion. A similar metabolic shift explains the change in asparagine uptake, which is used to produce aspartate after its depletion.

The results for the simulation of amino acids specific consumption rate for mAbs production are presented in Figure 7.2.

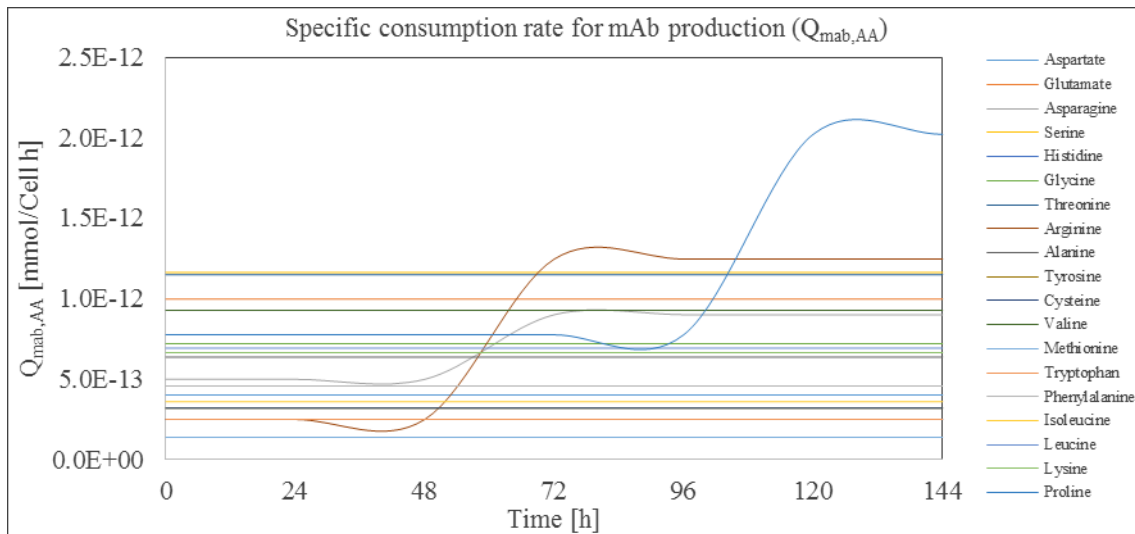


Figure 7.2: Profile of specific rate consumption of amino acids for mAbs production.

The requirements of amino acids for monoclonal antibody production have been considered constant, because the composition of the antibody and the production rate do not change during the culture, but glutamate and aspartate exhaustion induced arginine and asparagine uptake for the production of glutamine/glutamate and aspartate, respectively.

Additionally, arginine exhaustion increases proline uptake for the production of glutamate, glutamine and arginine (Figure 7.2). Without these metabolic shifts to cover the amino acids requirements for biosynthesis, viable cells would not be able to assemble the product because of the absence of the main building blocks of the product structure.

The results for the simulation of amino acids specific consumption rate for energy production are presented in Figure 7.3.

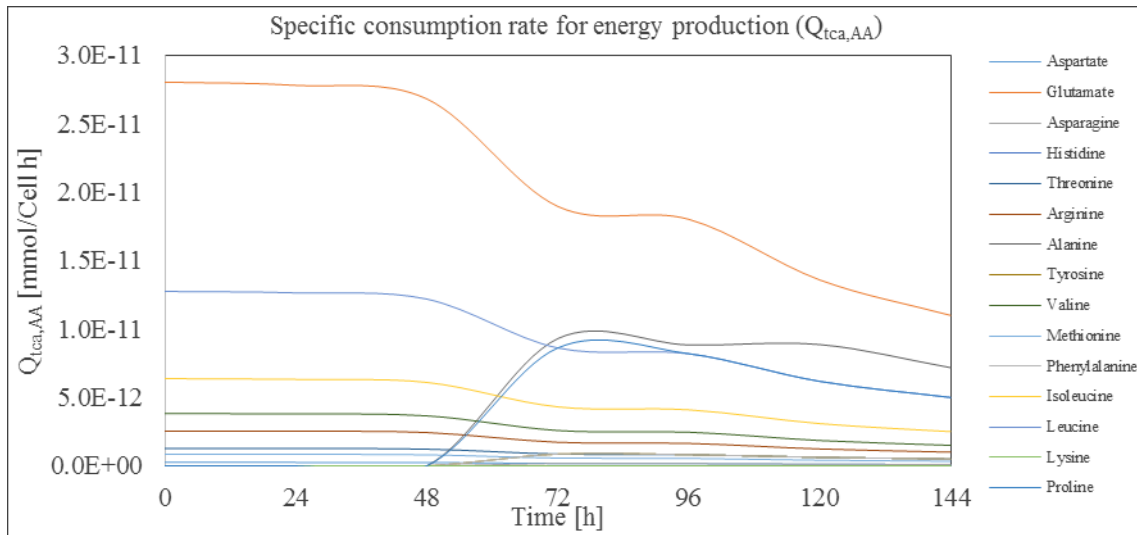


Figure 7.3: Profile of specific rate consumption of amino acids for energy production.

The specific consumption rate for energy production also presents changes, mainly in alanine and proline uptake. Proline was considered the amino acids that replace glutamate energy supply producing oxaloacetate and alanine was considered as the amino acids that replace glutamate energy supply producing acetyl-coA. Finally, these two molecules, oxaloacetate and acetyl-coA, enter into the TCA cycle to produce the first component of the cycle, citrate, increasing the production of energy in reduced glutamate concentrations.

Finally, the total specific consumption rate (Q_{AA}) is the addition of these three fluctuating terms making this variable highly dynamic.

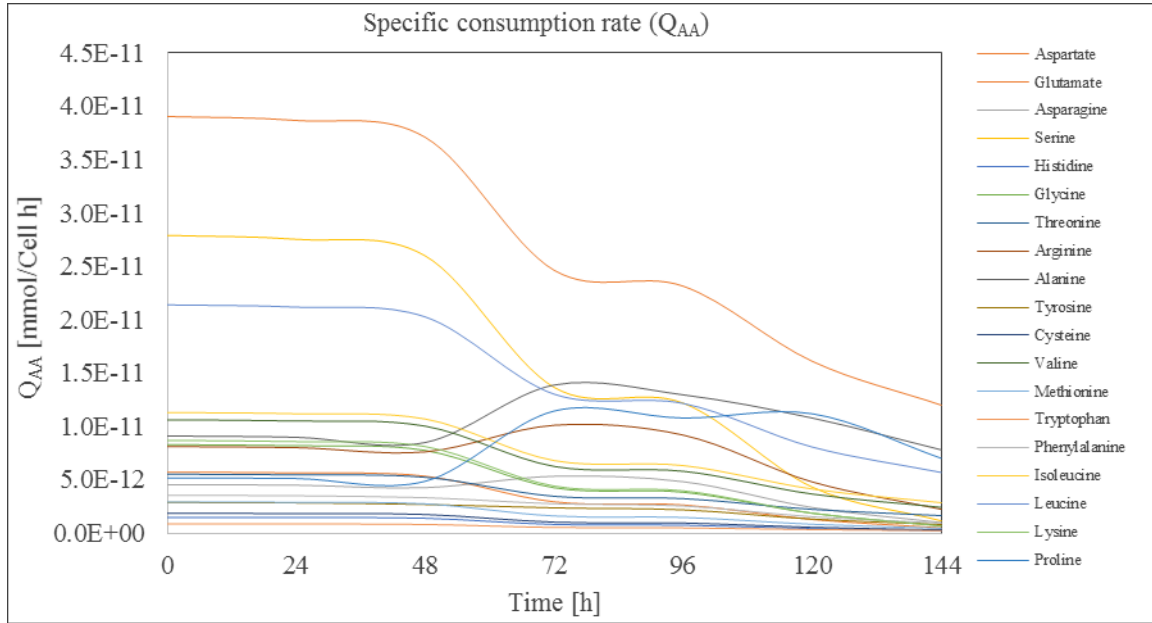


Figure 7.4: Profile of total specific rate consumption of amino acids.

The total specific consumption rate is constant until 48 hours of culture. For this reason, this uptake was considered for the medium design, and the feeding strategy should be applied from this point (48[h]), trying to keep the early exponential growth conditions.

Table 7.1: Specific consumption rate throughout the first 48 hours of culture

Amino acid	Specific consumption rate $Q_{total,AA}$ [mmol/Cell h]	Amino acid	Specific consumption rate $Q_{total,AA}$ [mmol/Cell h]
Aspartate	5.72E-12	Tyrosine	2.88E-12
Glutamate	3.90E-11	Cysteine	1.87E-12
Asparagine	4.56E-12	Valine	1.06E-11
Serine	1.37E-11	Methionine	2.93E-12
Glutamine	-	Tryptophan	8.69E-13
Histidine	1.49E-12	Phenylalanine	3.56E-12
Glycine	-8.30E-12 (Production)	Isoleucine	1.13E-11
Threonine	5.53E-12	Leucine	2.14E-11
Arginine	8.12E-12	Lysine	8.70E-12
Alanine	-9.11E-12 (Production)	Proline	5.19E-12

Table 7.2: Energy Medium composition.

Amino Acid	Concentration [mM]	Amino Acid	Concentration [mM]	Amino acid	Concentration [mM]
Aspartate	8.80	Threonine	8.49	Tryptophan	1.34
Glutamate	60.00	Arginine	12.48	Phenylalanine	5.47
Asparagine	7.01	Alanine	0	Isoleucine	17.38
Serine	20.98	Tyrosine	4.43	Leucine	32.88
Glutamine	0	Cysteine	2.87	Lysine	13.37
Histidine	2.29	Valine	16.33	Proline	7.98
Glycine	0	Methionine	4.50		

7.3.2 Model modifications

The Energy Model had to be modified as it was designed for batch cultures, therefore, terms related to inlet (feeding) and outlet (sampling) were added in some equations (Table 7.3) and the composition of the supplemental medium was also incorporated in the model, as parameters.

Table 7.3: Model modifications.

Balance	Original equation
	Modified equation
Volume	$V = Constant$
	$\frac{dV}{dt} = Fin_{AA} + Fin_{Trace} + Fin_{GLC} - Fout$
Viable cells	$\frac{dVX_v}{dt} = VX_v(\mu - \mu_a - \mu_d)$
	$\frac{dVX_v}{dt} = VX_v(\mu - \mu_a - \mu_d) - X_v F_{out}$
Early apoptotic cells	$\frac{dVX_a}{dt} = VX_v\mu_a - VX_a\mu_{a,d}$
	$\frac{dVX_a}{dt} = VX_v\mu_a - VX_a\mu_{a,d} - X_a F_{out}$
Dead cells	$\frac{dVX_d}{dt} = VX_v\mu_d + VX_a\mu_{a,d} - VX_dK_{lys}$
	$\frac{dVX_d}{dt} = VX_v\mu_d + VX_a\mu_{a,d} - VX_dK_{lys} - X_d F_{out}$
Glucose	$\frac{d(V GLC)}{dt} = -VX_v(Q_{x,glc} + Q_{mab,glc} + Q_{lac,glc} + Q_{tca,glc})$
	$\frac{d(V LAC)}{dt} = Fin_{GLC} GLC_{in} - VX_v(Q_{x,glc} + Q_{mab,glc} + Q_{lac,glc} + Q_{tca,glc}) - FoutGLC$
Lactate	$\frac{d(V[LAC])}{dt} = VX_v 2Q_{lac,glc}$
	$\frac{d(V LAC)}{dt} = VX_v 2Q_{lac,glc} - FoutLAC$
Amino acids	$\frac{d(V AA)}{dt} = -VX_v(Q_{x,AA} + Q_{mab,AA} + Q_{tca,AA})$
	$\frac{d(V AA)}{dt} = Fin_{AA} AA_{in} - VX_v(Q_{x,AA} + Q_{mab,AA} + Q_{tca,AA}) - FoutAA$
Monoclonal antibody	$\frac{d(V MAB)}{dt} = VX_v M_S + VX_v(\mu_a + \mu_d) M_{acc}$
	$\frac{d(V MAB)}{dt} = VX_v M_S + VX_v(\mu_a + \mu_d) M_{acc} - FoutMAB$
ATP	$\frac{d(VATP)}{dt} = VX_v \left(\sum_{i=1}^n \theta_i * Q_{tca,i} \right) - VX_v \left(\frac{\mu}{Y_{x,ATP}} + M_{x,ATP} + M_{mab,ATP} M_x \right)$
	$\frac{d(VATP)}{dt} = VX_v \left(\sum_{i=1}^n \theta_i * Q_{tca,i} \right) - VX_v \left(\frac{\mu}{Y_{x,ATP}} + M_{x,ATP} + M_{mab,ATP} q_{mab,x} \right) - FoutATP$

7.3.3 Parameter estimation

Global sensitivity analysis was not performed for this model because there were no new parameters included. Therefore, the parameter estimation was based on the same list of parameters estimated for the Energy Model for GS-NS0 cells in CD-Hybridoma medium.

The experiment used for parameter estimation was designed to keep the concentration of nutrients in the minimum concentration of glucose 0.5 [mM] and amino acids 0.2 [mM], these concentrations of nutrients induce low lactate production (Gambhir et al., 2003). The system was fed in intervals of 6-6-12 hours (9:00a.m., 3:00p.m. and 9:00 p.m.). The supplemental medium pulses were variable and determined by model predictions with the objective of feeding the exact amount needed to reach the minimum level one hour before the next feeding. The concentrations of amino acids in the supplemental medium were analysed after preparation and the results are in Table 7.4.

Table 7.4: Supplemental medium composition for exploratory experiment 2.

Amino Acid	Concentration [mM]	Amino Acid	Concentration [mM]
Glutamate	61.06	Threonine	10.45
Arginine	17.81	Methionine	4.69
Proline	8.38	Serine	55.33
Histidine	3.07	Cystine	2.51
Aspartate	9.56	Tyrosine	4.09
Asparagine	10.51	Tryptophan	1.06
Isoleucine	17.24	Phenylalanine	5.03
Leucine	30.71	Alanine	0
Lysine	13.90	Glycine	0
Valine	15.88	Glutamine	0

7.3.4 Model-based optimisation.

Model-based dynamic optimisation was performed with the purpose of finding the key performance indicators: viable cell concentration (X_v), the integral of viable cell concentration over time (IVCC) and the integral of monoclonal antibody concentration over time (IMABC) (Koumpouras & Kontoravdi, 2012; Kiparissides et al., 2016).

The dynamic optimisation problem was implemented in gPROMS 4.1.1 using the solver Control Vector Parameterisation Single-Shooting (CVP_SS). The variables to be optimised

were viable cells (X_v), monoclonal antibody concentration (MAB), integral of viable cells (IVCC) and the integral of monoclonal antibody concentration (IMABC).

In exploratory experiments, the effect of flask manipulation (collecting sample and feeding) produce perturbations in the system, pH and temperature, and the cells were less affected when the feeding was done every 12 hours instead of every 6 hours. For this reason, the feeding interval was set to 12 hours and the time horizon was set at 216 hours to extend the culture time by at least 3 days.

There were two control variables, amino acids feeding (Fin_{AA}) and glucose feeding (Fin_{GLC}), the control type was chosen as a piecewise constant (pulse of 1 minute) and the optimisation problem was subjected to one constraint to avoid dilution in the system, with the maximum volume variation being 10%.

The formulation of the optimisation problem and the initial conditions are in Table 7.5 and Table 7.6.

Table 7.5: Optimisation settings.

Settings	Value or equation
Objective functions	Max [X_v] (Fin_{AA} , Fin_{GLC}) Max [MAB] (Fin_{AA} , Fin_{GLC}) Max [IVCC] (Fin_{AA} , Fin_{GLC}) Max [IMABC] (Fin_{AA} , Fin_{GLC})
Objective functions subjected to:	Process model: Energy Model Initial conditions: Table 7.6 Inequality constrains: Interior and end point constrain $0.9 V_0 < V < 1.1 V_0$
Time horizon	216
Duration of the interval	12 hours
Control type	Piecewise constant
Control variables	$u(t)=Fin_{AA} \quad 0 < Fin_{AA} < 0.246 \text{ [L/h]}$ $v(t)=Fin_{GLC} \quad 0 < Fin_{GLC} < 0.12 \text{ [L/h]}$
Inequality constrain	Interior and end point constrain $0.9 V_0 < V < 1.1 V_0$

Table 7.6: Initial conditions.

Variable	Initial value	Units
model_time	0	Hours
V	0.2	L
Xv	2,04e9	Cells/L
Xa	1,27e8	Cells/L
Xd	8,91e7	Cells/L
GLC	19.77	mM
GLU	1.35	mM
ASP	0.15	mM
ASN	2.19	mM
ARG	1.13	mM
LEU	1.85	mM
ILE	1.19	mM
VAL	1.10	mM
MET	0.32	mM
LYS	1.34	mM
PRO	2.00	mM
TYR	0.37	mM
PHE	0.61	mM
ALA	3.21	mM
HIS	0.14	mM
THR	0.69	mM
SER	2.21	mM
GLY	0.12	mM
CYS	0.13	mM
TRP	0.19	mM
MABacc	0	mg/Cell
MAB	0	mg/L
ATP	2.50e3	mM
LAC	0	mM

7.4 Results and discussion

7.4.1 Medium design

The medium was supplemented with amino acids (Table 7.7), glucose (1,000 [mM]), MEM-vitamins solution 100X (Invitrogen, UK), serum replacement 50X (Sigma-Aldrich, UK) and cholesterol 250X (Invitrogen, UK).

Table 7.7: Amino acids supplemental medium composition.

Amino acid	Medium composition [mM]
Aspartate	8.80
Glutamate	60.00
Asparagine	7.01
Serine	20.98
Histidine	2.29
Threonine	8.49
Arginine	12.48
Tyrosine	4.43
Cysteine	2.87
Valine	16.33
Methionine	4.50
Tryptophan	1.34
Phenylalanine	5.47
Isoleucine	17.38
Leucine	32.88
Lysine	13.37
Proline	7.98

Table 7.8: Trace elements solution composition.

Component	Concentration [mM]
FeSO ₄ x 7H ₂ O	0.06
LiCl	9.44
MnSO ₄ x H ₂ O	3E-05
MgCl ₂	4.00
NaH ₂ PO ₄	7.70
ZnSO ₄ x 7H ₂ O	0.12
Na ₂ SeO ₂	0.01
CaCl	4.00
KCl	4.00

7.4.2 Parameter estimation

The set of parameters to be re-estimated was reduced to the last 4 parameters in Table 7.9 because it was observed that the model predicts correctly the number of viable, apoptotic and dead cells and the parameter estimation was more complex for fed-batch cultures than batch cultures. The parameter estimation was performed in two groups: Group 1: $q_{mAb,x}$ and M_s and Group 2: $Y_{x,atp}$ and $M_{x,atp}$. The method applied was the Minimum Likelihood Estimation and both analysis were performed in gPROMS 4.1.0 considering constant relative variance of the sensors equal to 25%. The initial values of the parameters are presented in Table 7.9 and consisted of 1 and 20 iterations and took 34 and 531 seconds to be performed, respectively. The t-values showed 95% accuracy for the parameter in Group 2 and the final Lack of Fit tests showed that the weighted residual values (1.92 and 9.14) were lower than the Chi-squared values (23.68 and 35.17), indicating good fit of the model to the experimental data.

Table 7.9: Parameter estimation results.

Significant parameters	Initial Value	Final Value	Units
μ_{max}	0.0172	0.0172	h^{-1}
$\mu_{a,max}$	8.45e-4	8.45e-4	h^{-1}
$\mu_{d,max}$	1.04e-3	1.04e-3	
$M_{tca,glc}$	3.63e-11	3.63e-11	mmol/Cell h
$q_{mab,x}$	7.50e-10	8.25e-10	mg/Cell h
M_s	4.38e-10	4.40e-10	mg/Cell h
$Y_{x,atp}$	6.61e7	6.605e7	mmol/Cell
$M_{x,atp}$	1.12e-9	1.14e-9	mmol/Cell h

The model accurately predicts viable, total, early apoptotic and dead cells (Figure 7.5), for fed-batch cultures.

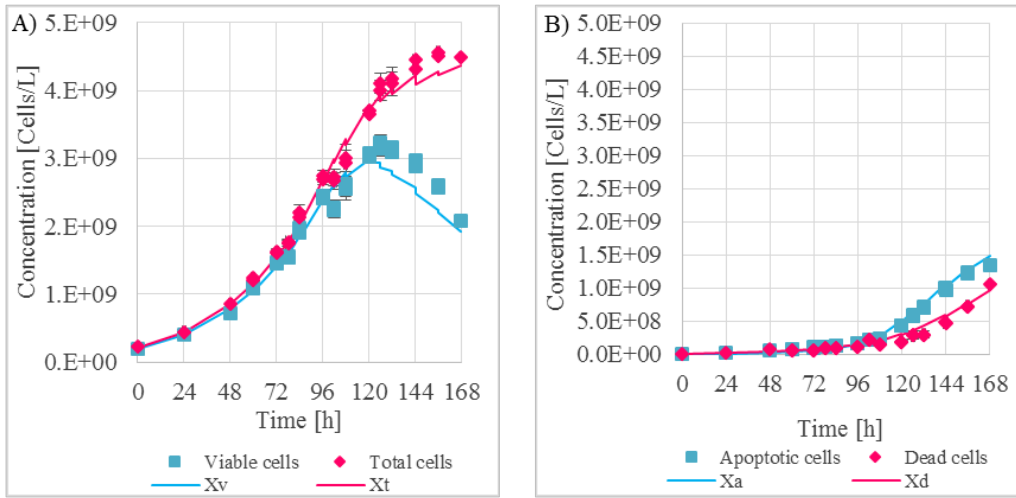


Figure 7.5: Comparison of experimental results (dots) and model predictions (solid lines) for (A) Viable and total cells concentration; and (B) Early apoptotic and dead cells concentration.

The significant parameters for the output variables mAbs and ATP was re-estimated and the model accurately predicts their trends and values. On the other hand, parameters related to glucose and lactate concentrations were not re-estimated, even though the model still predicts the glucose consumption and lactate production with high precision.

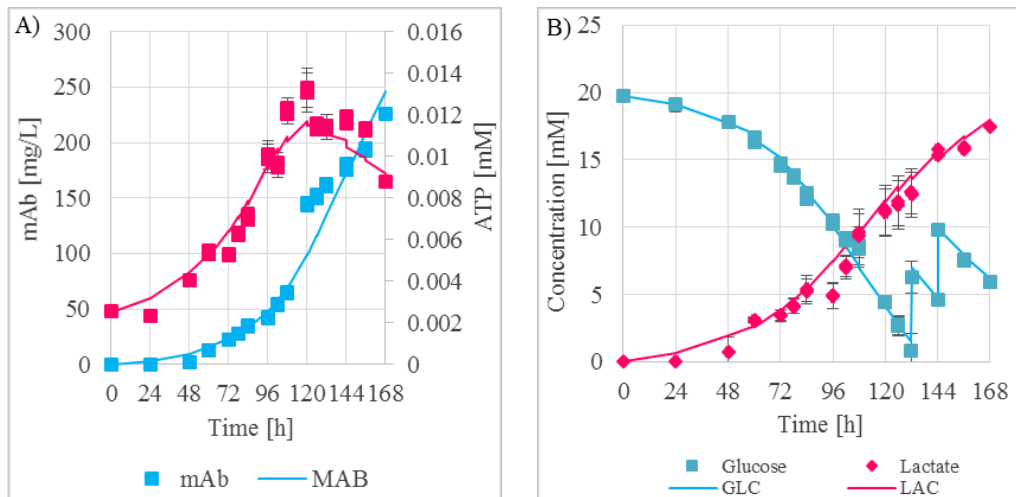


Figure 7.6: Comparison of experimental results (dots) and model predictions (solid lines) for (A) Monoclonal antibody concentration [mg/L] and ATP concentration [mM]; and (B) Glucose and lactate concentration [mM].

Leucine, lysine and isoleucine are precisely estimated. Even when the accumulation trend of alanine profile is captured, alanine concentration is underestimated, a possible explanation could be the underestimation of alanine production from glycolysis.

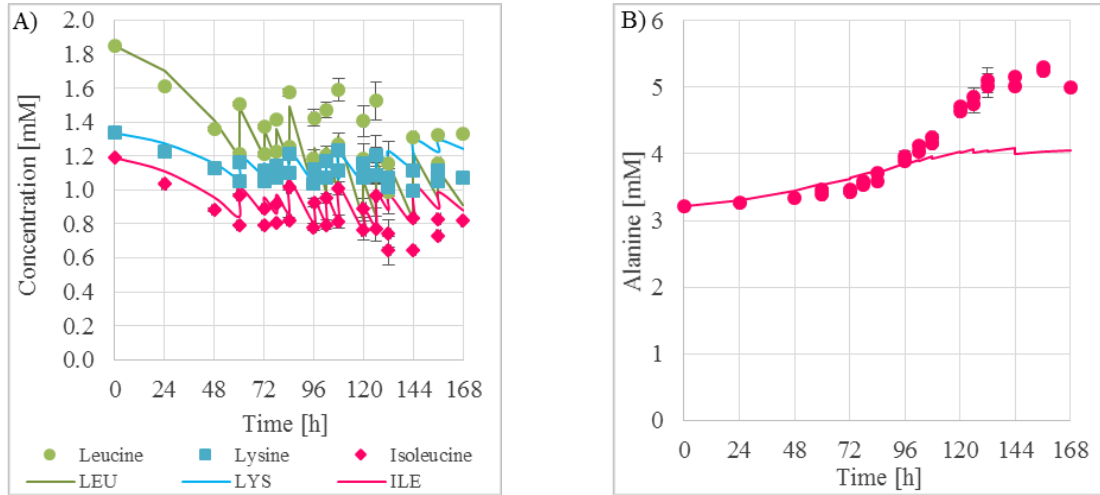


Figure 7.7: Comparison of experimental results (dots) and model predictions (solid lines) for (A) Leucine, lysine and isoleucine concentrations [mM]. (B) Alanine concentration [mM]

Glutamate, arginine, phenylalanine and tyrosine concentrations are slightly overestimated at the end of the culture as consequence an underestimation of the viable cell concentration during the final hours of culture.

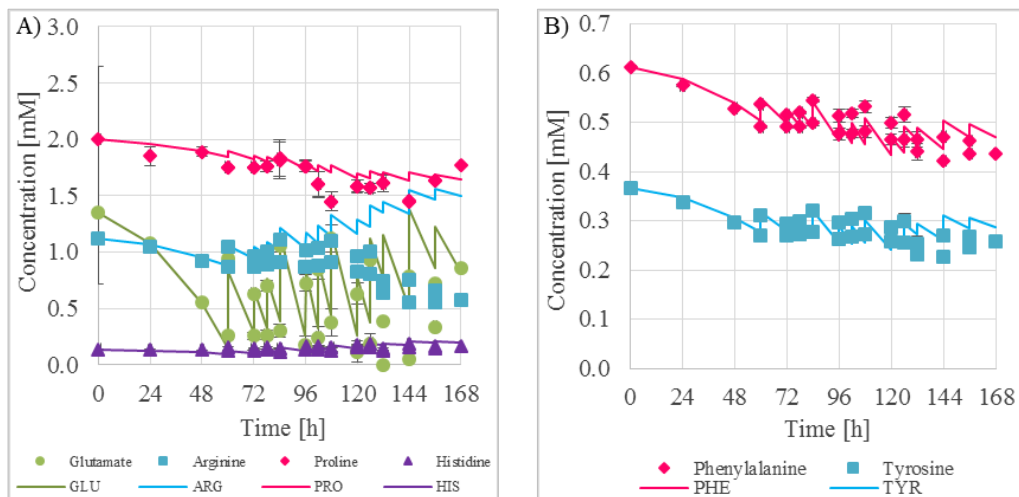


Figure 7.8: Comparison of experimental results (dots) and model predictions (solid lines) for (A) Glutamate, arginine, proline and histidine concentrations [mM] and (B) Phenylalanine and tyrosine concentrations [mM].

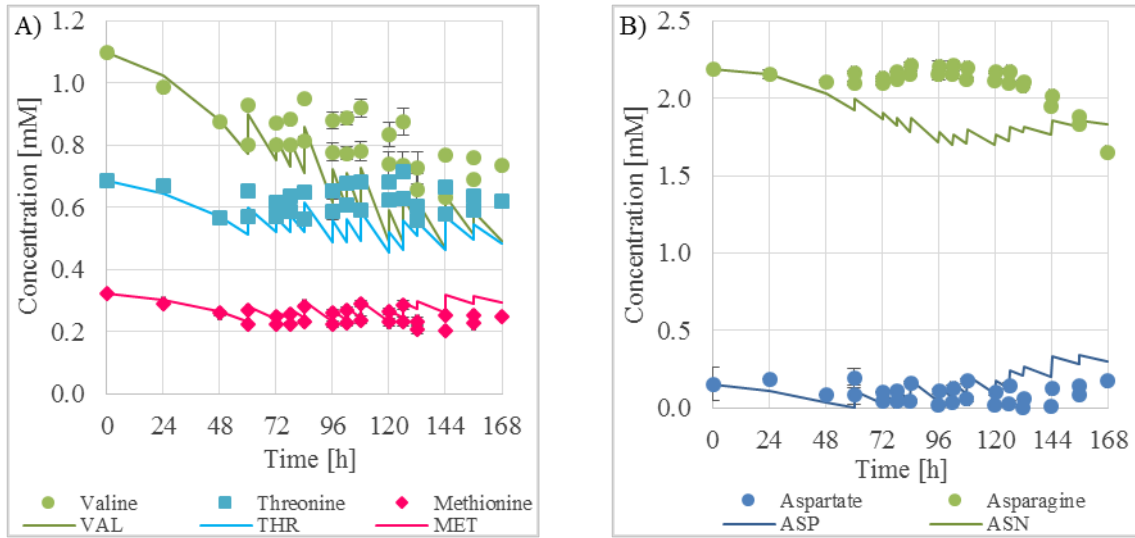


Figure 7.9: Comparison of experimental results (dots) and model predictions (solid lines) for (A) Valine, threonine and methionine concentrations [mM]; and (B) Aspartate and asparagine concentrations [mM].

Serine concentration was underestimated after glycine reached the maximum concentration threshold (0.6[mM]). These results suggest that glycine is produced by other precursors or serine is consumed for other purposes. Cysteine and tryptophan concentrations are under and overestimated by the model, therefore, it is recommended to specifically calculate the cell and product compositions for GS-NS0 cells and cB72.3 mAbs, respectively. However, the medium composition ensures that all the amino acids are present in the culture medium.

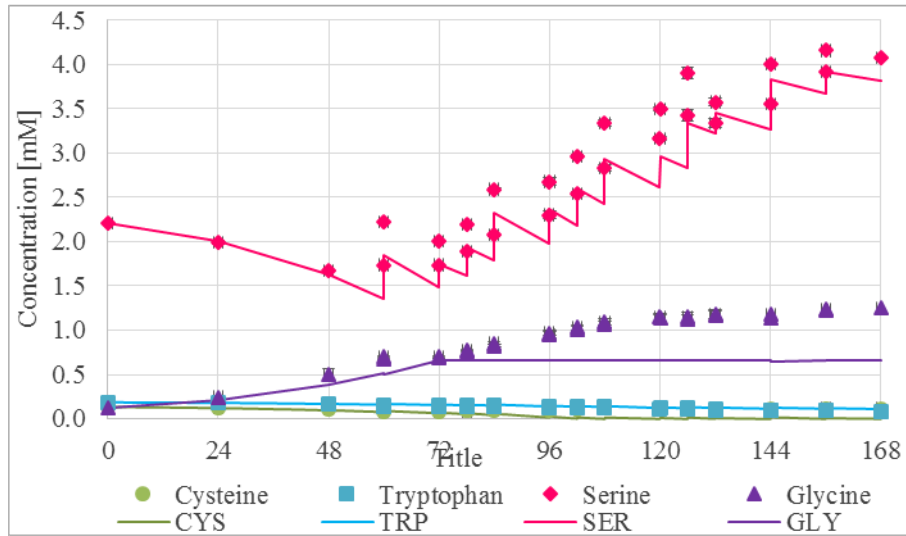


Figure 7.10: Comparison of experimental results (dots) and model predictions (solid lines) for cysteine, tryptophan, serine and glycine concentrations [mM]

After parameter estimation, the modified Energy Model was used to optimise the production of mAbs production.

7.4.3 Model-based optimisation.

The dynamic optimisation problem was implemented in gPROMS 4.1.0 using the solver Control Vector Parameterisation Single-Shooting (CVP_SS). The stepwise parameterization was used to simulate 1 min pulses of amino acids feeding, every 12 hours of culture, and the lower and upper bound for the feeding were 0 and 0,246 [L/h], respectively. The batch cultures duration was 144 hours. Therefore, in order to extend the culture longevity in fed-batch cultures, the time horizon was set at 216 hours (3 days extension). The formulation of the optimisation problem and the initial conditions are in Table 7.5 and Table 7.6.

The dynamic optimisation was performed to maximise the viable cells concentration (X_v) (48 iterations, CPU time: 4 hours and 31 min), monoclonal antibody concentration (MAB) (7 iterations, CPU time: 17min), the integral of the viable cells concentration (IVCC) (85 iterations, CPU time: 4 hours and 11 min), and the integral of the monoclonal antibody concentration (IMABC) (48 iterations, CPU time: 4 hours and 12 min).

The amino acids feeding schedule from the optimisation of Xv, MAB, IVCC and IMABC are presented in Figure 7.11.

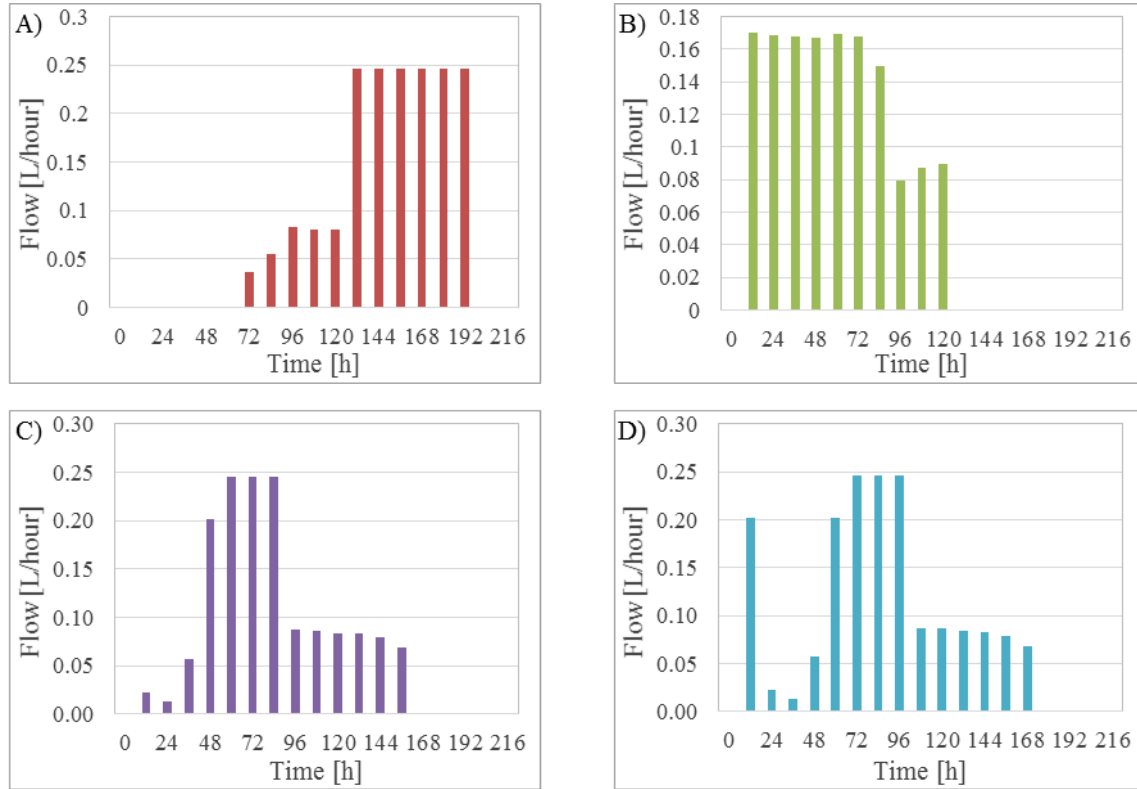


Figure 7.11: Inlet flow of amino acids optimised schedule for (A) Viable cells concentration (B) Monoclonal antibody concentration (C) Integral of the viable cells concentration (D) Integral of the monoclonal antibody concentration.

The schedule obtained from the optimisation of the viable cells concentration present a different profile compared to the other three schedules that present notorious similarities, higher feeding before 96 hours of culture. However, the selection of the feeding schedule depends on the production levels reach using each feeding strategy and the quality of the final product. The assessment of the schedules was based on simulations of a fed-batch culture under the initial conditions of the fed-batch culture presented in the previous section. The results of these simulations are presented in Figure 7.12, where it is possible to observe that while the concentration of viable cells is higher at the end of the culture under the schedule derived from

the optimisation of the viable cells concentration (X_v), the concentration of dead cells is lower and the viability is higher. These results indicate that the cultures under the optimise conditions MAB, IVCC and IMABC should be finished by 168 hours of culture, when the viability is under 50% because the accumulation of debris would difficult the downstream processing for the product purification. Finishing the cultures when they reach 50% viability means that the final mAbs concentration would be 400[mg/L], 340[mg/L], 254[mg/L] and 238 [mg/L] applying the schedules derived from the optimisation of X_v , MAB, IVCC and IMABC, respectively. Therefore, the most productive culture would be the fed-batch culture that operates under the schedule from X_v optimisation.

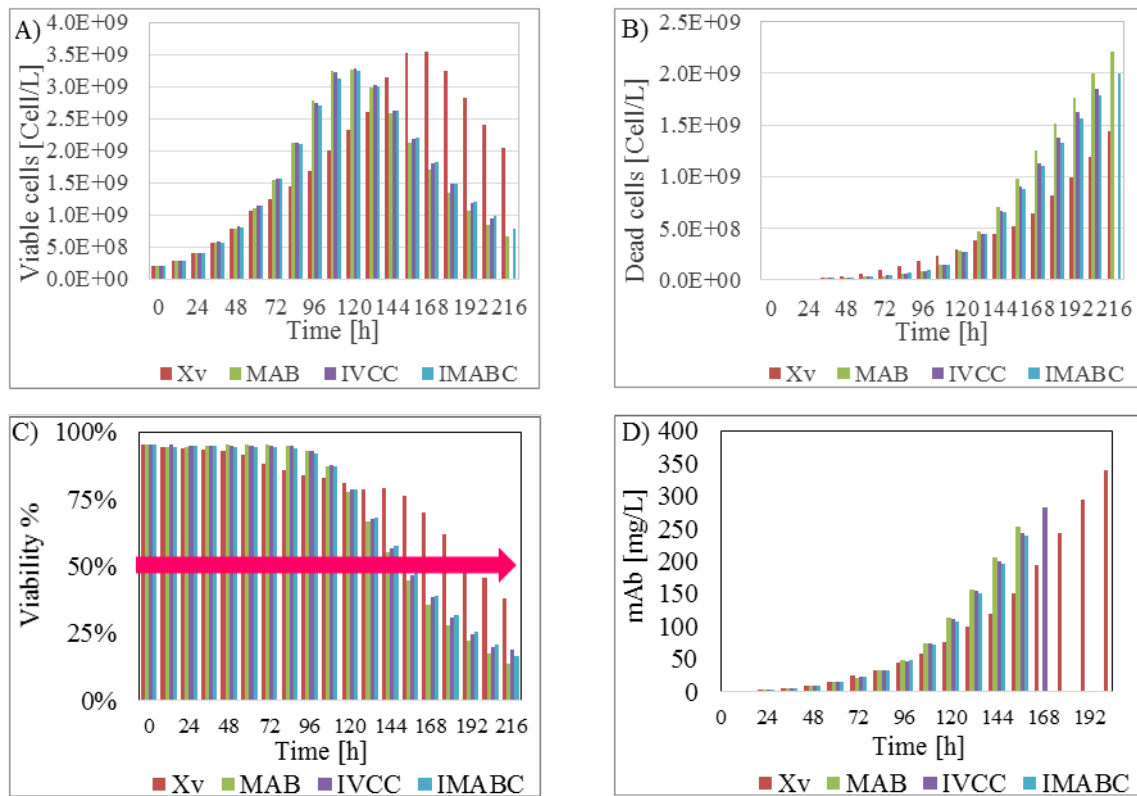


Figure 7.12: Results of simulation under different optimised feeding schedules (X_v , MAB, IVCC and IMABC) for (A) Concentration of viable cells (B) Concentration of dead cells (C) Viability (D) concentration of monoclonal antibody.

Viable cells secrete a complete and active product. On the other hand, when the death processes start, glycosylations have been found to be incomplete (Gu et al., 1997). In the optimisation of MAB, IVCC and IMABC the concentration of dead cells was higher and the increase in mAbs concentration was a consequence of the unspecific release of possibly incomplete product due to the Golgi apparatus fragmentation, apoptosis blebbing and loss of cells membrane integrity throughout the death stage (Mukherjee et al., 2007). Additionally, the release of proteases could damage the product structure (Hill et al., 2001). Therefore, in these three cases, the quality of the product could be compromised.

Finally, the schedule from the optimisation of the concentration of viable cells was used for the optimised fed-batch culture to obtain high mAbs concentrations without compromising product quality.

The validation results of the model-base dynamic optimisation are presented in the following sections.

7.4.3.1 Optimisation results for cell growth

In Figure 7.13, it is possible to observe that the concentration of total cells and dead is accurately predicted by the model; and the number of viable cells is underestimated due to the overestimation of early apoptotic cells concentration. The explanation for these discrepancies is based on the overestimation of lactate formation (Figure 7.14).

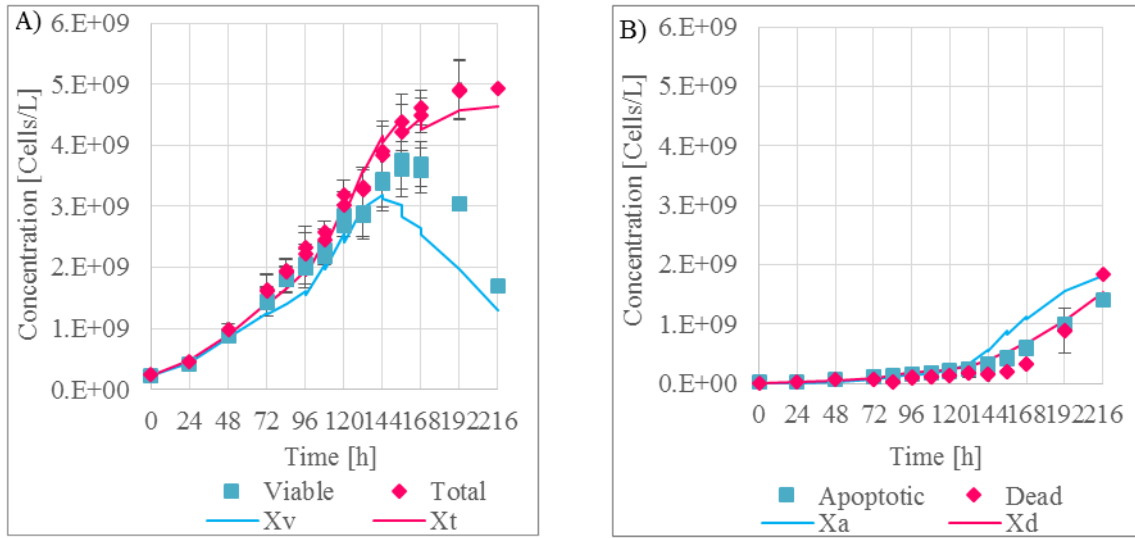


Figure 7.13: Comparison of experimental results and model predictions for (A) Total and viable cells concentrations; and (B) Early apoptotic and dead cells concentrations.

7.4.3.2 Optimisation results for glucose consumption and lactate formation

The grey area in Figure 7.14 indicates the 95% confidence interval for the model predictions for lactate concentration. These area indicates that lactate concentration was slightly overestimated at the beginning and at the end of the culture. The effect of lactate at the beginning of the culture is imperceptible due to its low concentration. However, after 168 hours of culture, the effect of lactate concentration has a significant effect on the viable and early apoptotic cells concentration, which has been under and overestimated, respectively (Figure 7.13).

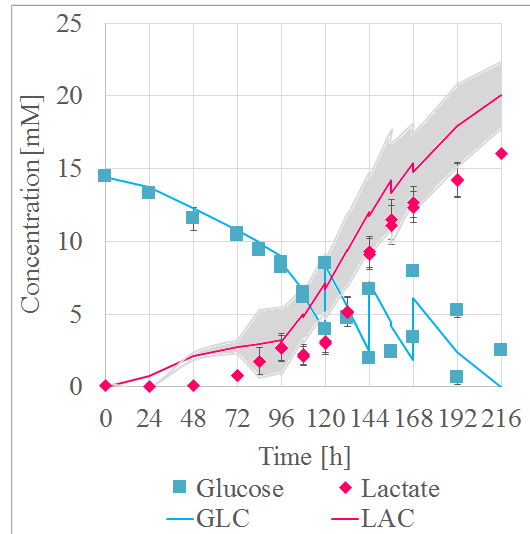


Figure 7.14: Comparison of experimental results and model predictions for glucose and lactate concentrations.

7.4.3.3 Optimisation results for glutamate, arginine, proline and histidine profiles.

The profiles of glutamate concentration and the profile amino acids that are its possible precursors are presented in Figure 7.15. It is evident that the model accurately predicts the amino acid profiles from 0 to 168 hours of culture. After 168 hours the model predicts higher concentrations of amino acids due to the underestimation of viable cells, which is affected by the overestimation of lactate concentration. Arginine profile presents serious differences between experimental results and model predictions. In the previous fed-batch experiment, used for parameter estimation in fed-batch cultures, the model also predicted arginine accumulation (Figure 7.8). Regarding the results obtained after parameter estimation and the present results, it is recommended to improve the calculation of the parameter C_{arg} , which value is not significant for the output variables analysed by GSA (viable cells, mAbs or ATP concentrations), but this parameter is relevant for the model-based medium design calculations to avoid nutrients accumulation. However, the medium composition ensures that all the amino acids are present in the culture medium.

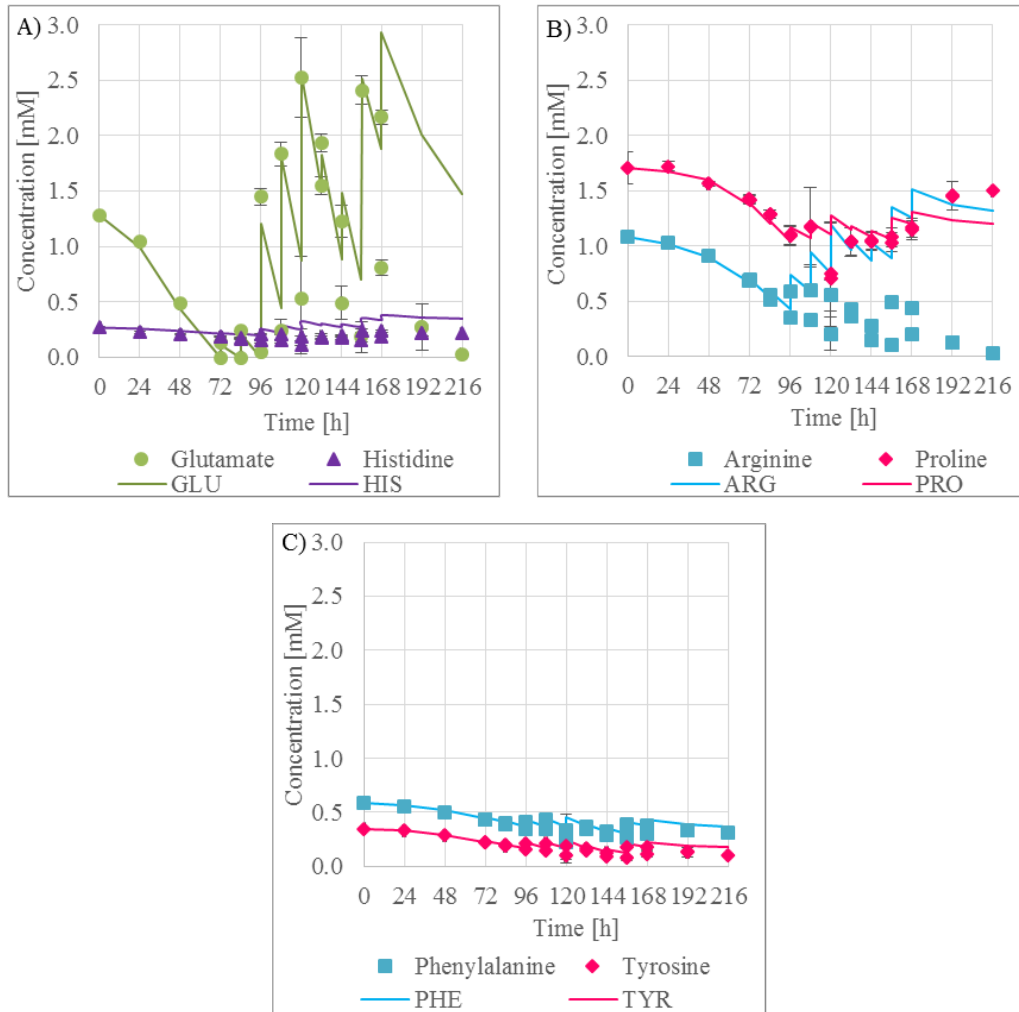


Figure 7.15: Comparison of experimental results and model predictions for (A) Glutamate and histidine, (B) Arginine and proline; and (C) Phenylalanine and tyrosine concentrations.

7.4.3.4 Optimisation results for aspartate and asparagine profiles.

Aspartate presents the same trend as glutamate, histidine, arginine phenylalanine and tyrosine, the model prediction slightly overestimates its concentration due to the overestimation of lactate concentration which has a “domino effect” that affects the viable cell concentration prediction, which is underestimated. Therefore, the uptake of amino acids would be reduced and the amino acid concentrations would be overestimated. On the other hand, asparagine concentration presents an unpredictable behaviour. In the previous fed-batch experiment, used

for parameter estimation, asparagine was accumulated (Figure 7.9) and in this case, asparagine is continuously consumed, the model under and overestimated asparagine concentration, respectively. This erratic behaviour suggests that once asparagine degradation is activated its consumption would be active until the end of the culture.

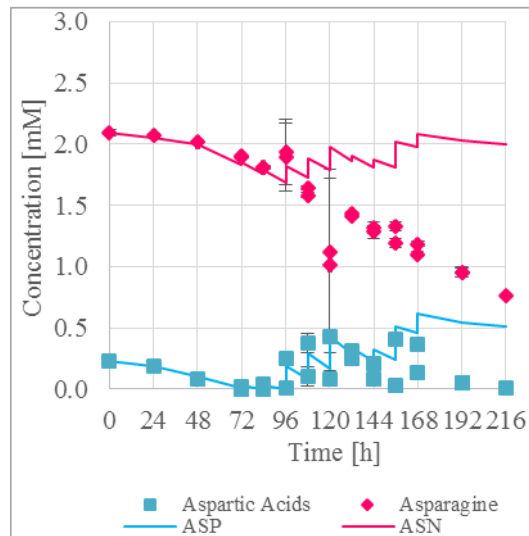


Figure 7.16: Comparison of experimental results and model predictions for aspartate and arginine concentrations.

7.4.3.5 Optimisation results for other energy amino acids

Methionine and threonine profile are accurately predicted, but valine, leucine, lysine and isoleucine present a higher uptake between 72 and 120 hours of culture. These differences have a “domino effect” in the estimation of the amino acids concentrations after this period. Regarding the results obtained after parameter estimation and the present results, it is recommended to estimate the parameters C_{val} , C_{leu} , C_{lys} and C_{iso} . These parameters are relevant for the model-based medium design calculations. Even when none of these amino acids concentrations is zero during the fed-batch process, it is important to precisely estimate their uptake to avoid depletion or accumulation of nutrients.

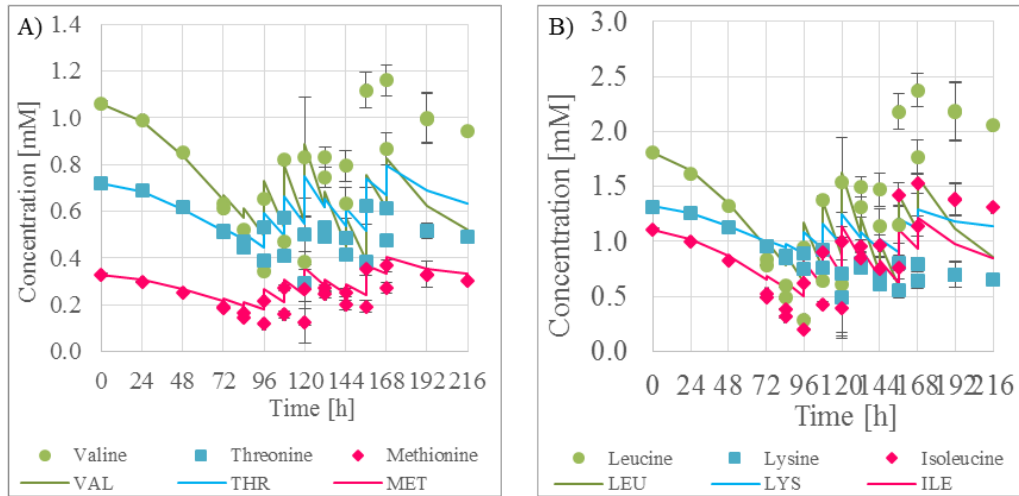


Figure 7.17: Comparison of experimental results and model predictions for (A) valine, threonine and methionine concentrations and (B) leucine, lysine and isoleucine concentrations.

7.4.3.6 Optimisation results for serine, glycine, cysteine and tryptophan.

Serine concentration has been overestimated after glycine reached the maximum concentration threshold (0.6[mM]), therefore, it is recommended to set a new threshold for glycine. Cysteine and tryptophan concentrations are under and overestimated by the model, therefore, it is recommended to specifically calculate the composition of GS-NS0 cells and cB72.3 mAbs, to improve the accuracy of the predictions.

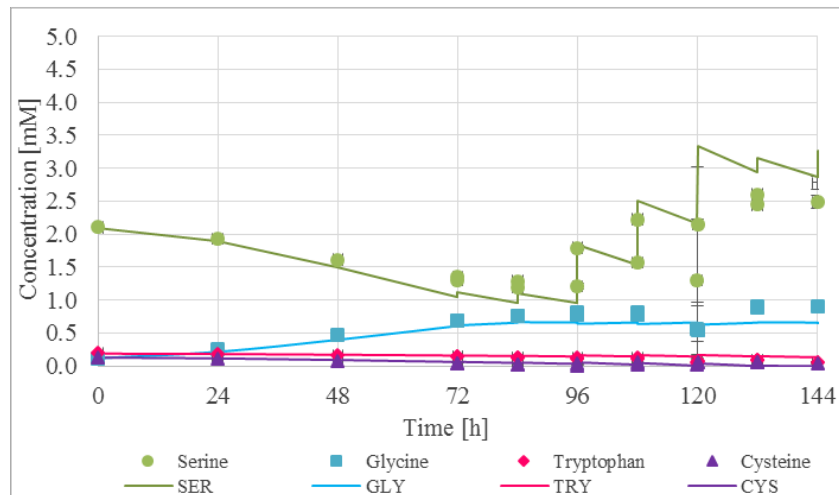


Figure 7.18: Comparison of experimental results and model predictions for serine, glycine, tryptophan and cysteine concentrations.

7.4.3.7 Amino acids profile analysis.

The Energy Medium performance was assessed based on the comparison of the amino acids profiles throughout a fed-batch culture under the optimised feeding schedule, performing simulations of the same feeding strategy using the Energy Medium and two different medium compositions, which were normalised to make the three medium comparable. The first medium selected was CD-Hybridoma medium base on the common practice of using concentrated basal medium as a supplemental medium in fed-batch cultures (Dhir et al., 2000). The second medium selected was RITM02, which was particularly designed for NS0 cells producing a monoclonal antibody (Spens and Haggstrom; 2007).

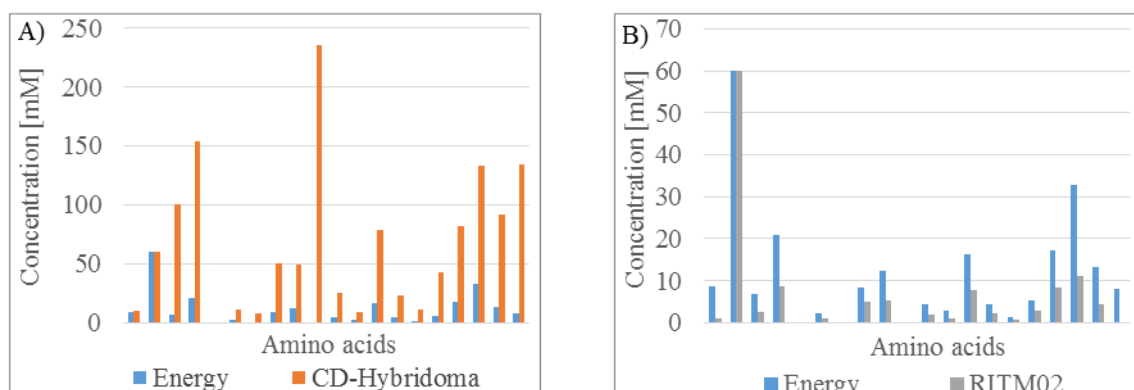


Figure 7.19: Medium composition comparison (A) Energy versus CD-Hybridoma. (B) Energy versus RITM02.

In Figure 7.19 A, it is possible to observe the different proportion and higher amino acids concentration in CD-Hybridoma medium compared to the Energy Medium. On the other hand, in Figure 7.19 B, RITM02 presents similar proportions and lower concentration of amino acids compared to the Energy Medium.

The Energy Medium presented amino acids concentrations between 0.01 [mM] (Cysteine) and 5.61 [mM] (Alanine). None of the amino acids was exhausted throughout the culture.

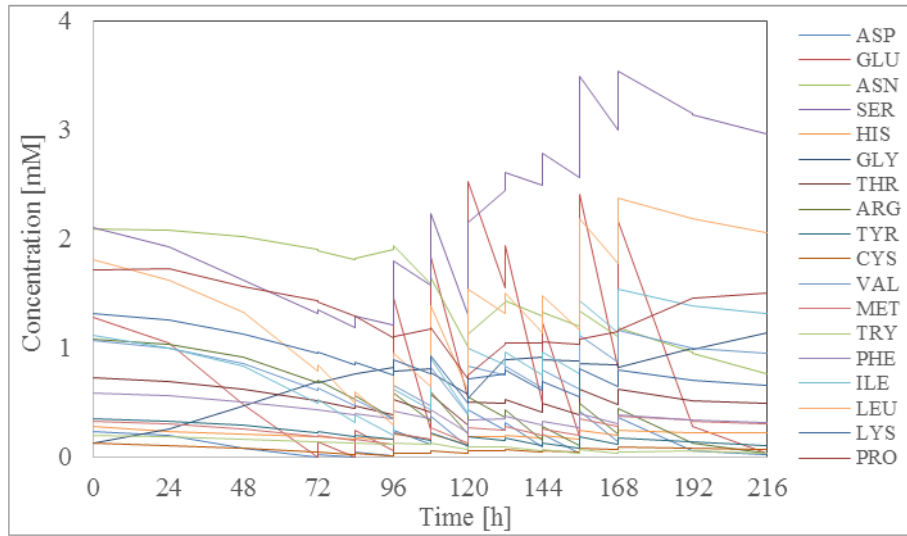


Figure 7.20: Simulation results for amino acids concentration profile using Energy Medium as supplemental medium.

CD-Hybridoma medium presented concentration between 0.06 [mM] (Cysteine) and 34.69 [mM] (Alanine). None of the amino acids was exhausted throughout the culture, but all the amino acids presented high accumulation levels. Therefore, it is not recommended to feed the system with a solution of concentrated basal medium, which would cause toxic levels of nutrients, waste of nutrients and increase in osmolality.

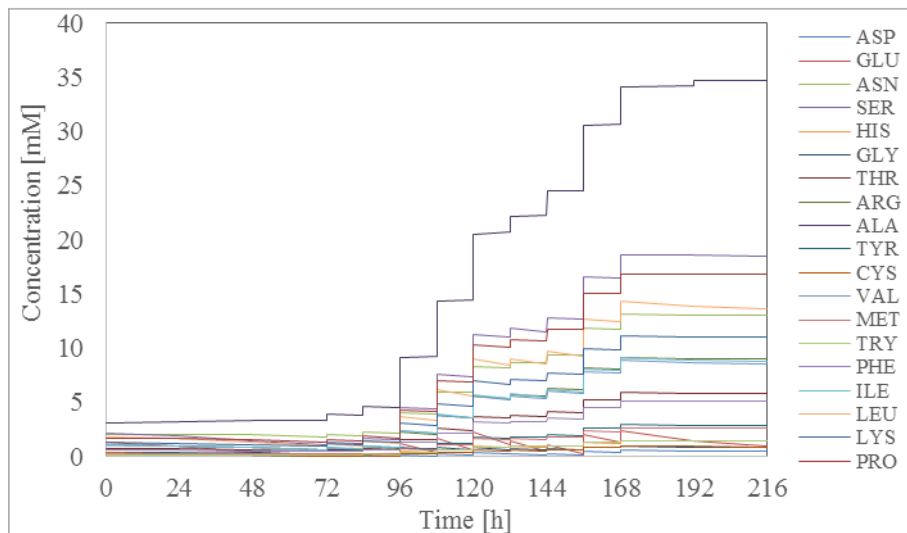


Figure 7.21: Simulation results for amino acids concentration profile using CD-Hybridoma medium as supplemental medium.

RITM02 is a medium developed for NS0 cells producing a monoclonal antibody. Therefore, it was expected that it would present a similar performance compared to the Energy Medium.

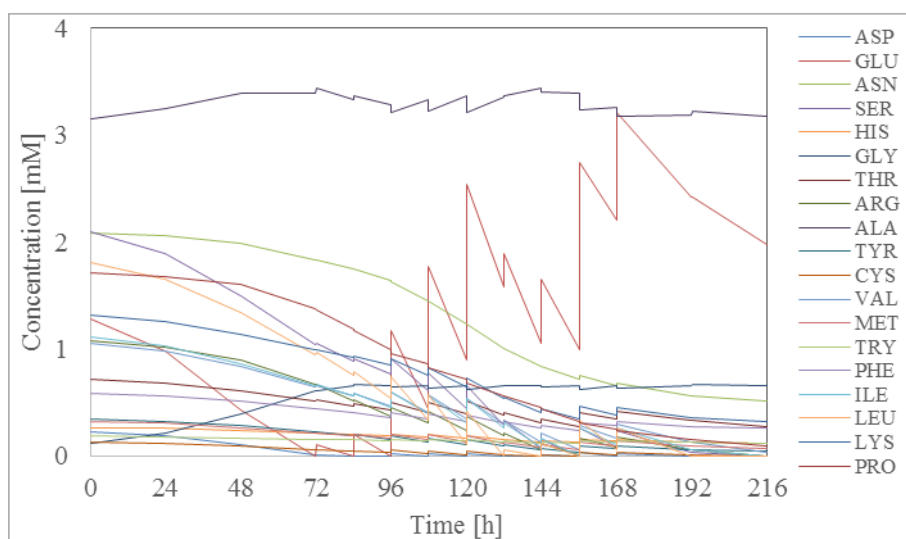


Figure 7.22: Simulation results for amino acids concentration profile using RITM02 medium as supplemental medium.

RITM02 presented concentrations between 0.0 [mM] (serine, arginine, cysteine, isoleucine and leucine) and 3.44 [mM] (Alanine). The profile of amino acids presents a very tight range of concentrations (no waste or excessive osmolality increase), but four essential amino acids were exhausted throughout the culture. The exhaustion of essential amino acids activates apoptosis and necrosis and also restricts the energy availability. Therefore, it is not recommended to use RITM02 as a supplemental medium in fed-batch cultures for the optimisation of the production of cB72.3 mAbs in GS-NS0 cells.

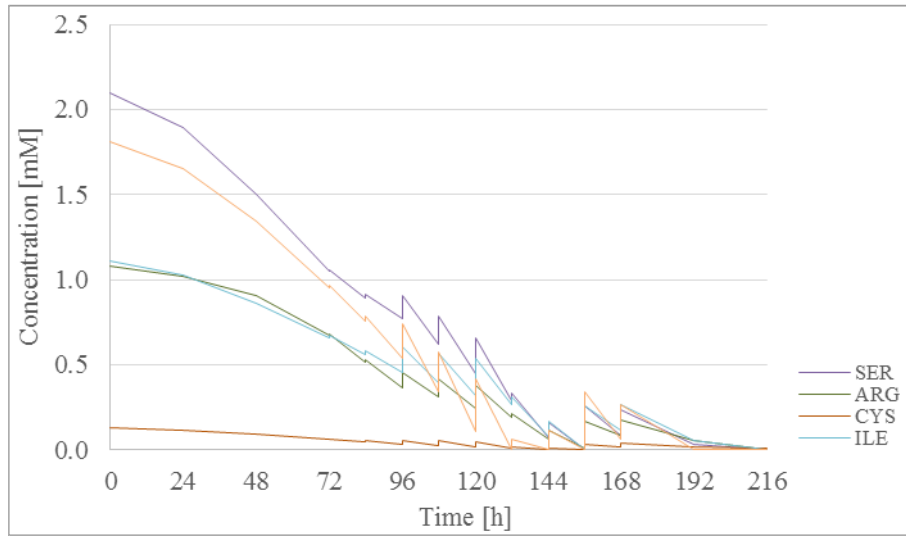


Figure 7.23: Exhaustion of essential amino acids using RITM02 as a supplemental medium.

The simulation of a fed-batch culture in of GS-NS0 cells producing cB72.3 monoclonal antibody revealed that the tailor-made supplemental medium Energy Medium, covers all the requirements of the cells for proliferation, maintenance and production that other medium available in the market would not be able to provide.

7.4.3.8 Product and ATP concentrations

The experimental results for monoclonal antibody concentration have presented a discontinuity in the middle of the culture that is not captured by the model, in this case, the discontinuity is observed between 120-144 hours (Figure 7.24). This phenomenon has been explained by the influence of amino acids metabolic shifts in the mAbs assembly. When the amino acids glutamate and aspartate are exhausted in the culture medium, they are supplied by the degradation of arginine and asparagine, respectively. Once these metabolic shifts occur, the mAbs assembly is re-started.

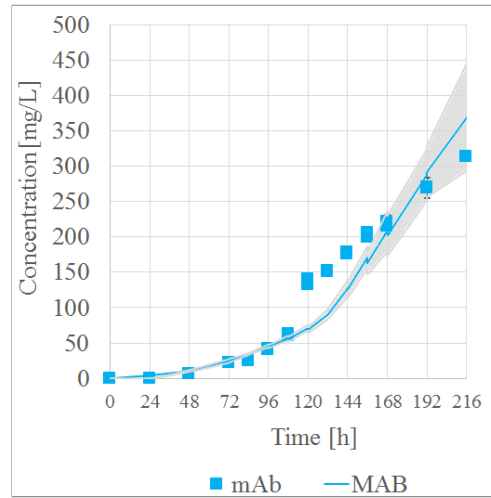


Figure 7.24: Comparison of experimental results (dots) and model predictions (solid line) for mAbs concentration. 95% confidence interval (grey area)

In Figure 7.25, the mAbs concentration profiles in different production platforms (batch and fed-batch) and medium compositions (DMEM 10% FBS and serum-free CD-Hybridoma) are presented. The results show how the extension of the culture longevity allows us to produce in one fed-batch culture the same amount as two consecutive batches, reducing the preparation time for the production run (seed and inoculum train) and sterilization of equipment; the physical equipment capacity for the downstream processes (the product is concentrated in fed-batch culture) and medium costs (it is necessary the half of the initial culture medium volume).

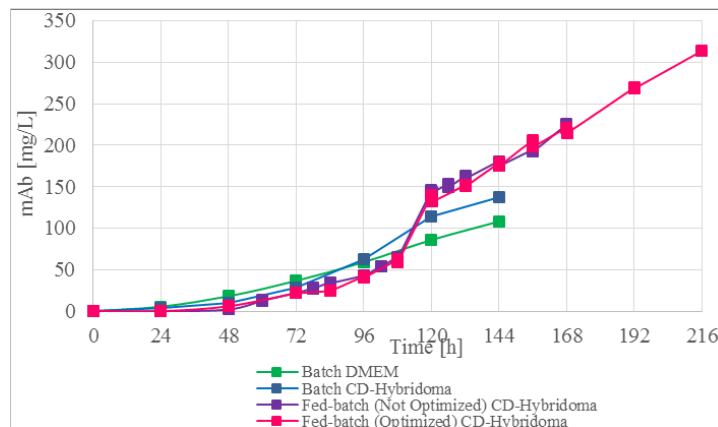


Figure 7.25: mAbs concentration over time in different production platforms (batch and fed-batch) and medium compositions (DMEM% FBS and serum-free CD-Hybridoma).

In Figure 7.26 is presented a comparison between the final monoclonal antibody concentration in batch cultures in modified DMEM medium & 10% FBS and serum-free CD-Hybridoma medium; and fed-batch cultures in serum-free CD-Hybridoma medium without and with optimisation of the feeding strategy. The results show that the optimised culture increased the production by 1.9 and 1.3-fold compared to the batch production mode in DEMEM medium & 10% FBS and serum-free CD-Hybridoma medium, respectively.

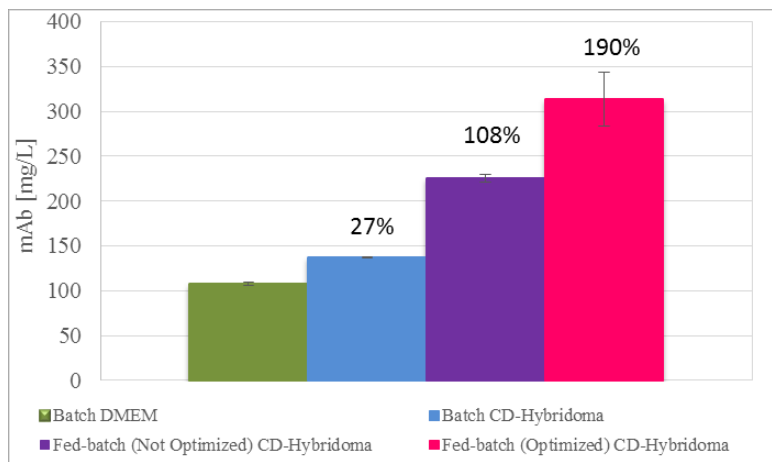


Figure 7.26: Production increase over cultures.

The ATP concentration is an extremely sensitive variable. The only time when the model did not capture the ATP content was between 48-96 hours when glutamate concentration was low. Therefore, these results are clearly related to the metabolic shifts that occur inside the cells to supply glutamate. The over and underestimation of some amino acids involved in energy production has been discussed in this chapter and the re-estimation of some parameters related to the energy amino acids uptake has been suggested. The GSA indicated that the uptake of these amino acids does not have a significant impact on the ATP balance. These results are confirmed by the ATP balance, which predictions present and excellent fit to the experimental results (Figure 7.27).

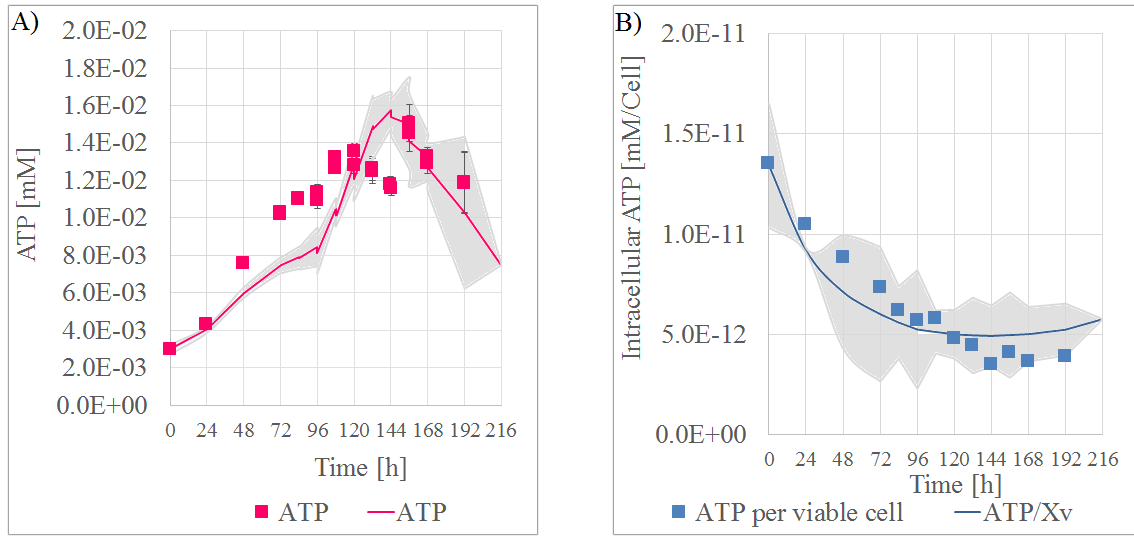


Figure 7.27: Comparison of experimental results (dots) and model predictions (solid line) for (A) ATP concentration and (B) intracellular ATP content. 95% confidence interval (grey area).

The average concentration of intracellular ATP content per viable cell is $4.95 \pm 1.20 \times 10^{-12}$ [mmol ATP/Cell] (Figure 7.18). In batch cultures of GS-NS0 cells in CD-Hybridoma medium the intracellular ATP content, from 0 to 24 hours (lag phase), was $7.68 \pm 1.25 \times 10^{-12}$ [mmol ATP/Cell], and during exponential growth, stationary phase and death stage the intracellular ATP content dropped to $4.82 \pm 1.09 \times 10^{-12}$ [mmol ATP/Cell]. From these results and the level of intracellular ATP registered during the optimised culture, it is possible to conclude that the model-based medium design and the feeding strategy applied kept the intracellular ATP content at optimal levels.

7.5 Conclusions

The technique applied for the development of a supplemental medium for fed-batch cultures was based on the total specific amino acids consumption rate, which was calculated based on the predictive model. The supplemental medium considered that the culture should maintain the concentration of nutrients observed in the initial feeding point.

The Energy Model was modified by introducing terms related to glucose and amino acids inlet. The modified model and the designed supplemental medium were tested through one fed-batch experiment, which was performed based on model predictions and with the aim of

keeping the concentration of glutamate above 0,2 [mM]. The experiment was successful; it extended the longevity of the culture for one day, increased the mAbs titre by 64% and none of the amino acids present in the medium was exhausted throughout the culture time. The results from this experiment were used to re-estimate the significant parameters of the model under fed-batch operation.

The modified model, after parameter re-estimation, was used for the design of a feeding schedule applying model-based dynamic optimisation of the viable cells concentration and using the composition of the supplemental medium previously design. The validation of the model and the supplemental medium and optimisation strategy testing were exceptionally successful, the strategy extended the longevity of the culture for three days, increased the mAbs titre by 190%, none of the amino acids present in the medium was exhausted throughout the culture time and the concentration of amino acids was maintained in a tight range above zero (0,01-5.61 [mM])

CHAPTER 8: Conclusions and future directions

8 Conclusions and final directions

8.1 Conclusions

The Energy Model is the first dynamic model that predicts the intracellular ATP content in mammalian cells.

Metabolic shifts have been extensively documented in metabolic flux analysis research. However, they have been poorly explained or included in mathematical models. Metabolic shifts have been observed in batch, fed-batch and continuous cultures (Gambhir et al., 2003; Niklas et al., 2011b; Dean & Reddy, 2013). Therefore, it is critical to include the metabolic adaptation of mammalian cells to establish a precise description of the biological system. Another relevant aspect of metabolic shifts is their role in the energy homoeostasis, which is essential for cell proliferation and production of heterologous proteins. The incorporation of metabolic shifts in GS-NS0 cultures allowed us to predict changes in the essential amino acids uptake. These amino acids were used as precursors for the biosynthesis of exhausted nutrients and also to produce energy. Consequently, the model had the capability to predict the number of viable, apoptotic and dead cells; the concentration of glucose, 15 amino acids and lactate; and the most importantly it allowed us to calculate the intracellular ATP content in mammalian cells.

The adaptation of GS-NS0 cells from modified DMEM medium supplemented with 10% FBS to serum-free conditions was successfully performed in three different media. All the adapted cultures recovered after cryopreservation and reached high cell densities. Unfortunately, Ex-Cell medium and HyClone medium presented low production levels, reaching 7% and 33% of the mAbs concentration found in batch cultures in DMEM medium. Therefore, the medium selected was CD-Hybridoma, which presented a remarkable performance. The viability of the culture was above 90% for 120 hours in 144-hour cultures, the number of cells increased by 1.5-fold and the production was increased by 20% compared with the conventional culture.

After serum-free adaptation, the Energy Model was modified to predict serine, glycine, cysteine and tryptophan profiles with the aim of predicting the entire set of amino acids present in the culture medium and use the model for medium design. GSA was performed and its results showed that the output variables were sensitive to the same parameters compared to the original Energy Model, indicating that the modifications did not significantly affect the studied output variables. The results comparing the sensitivity of the viable cells concentration to the maximum theoretical growth rate revealed that GSA captured the main effect of the adaptation, the extension of the culture longevity, and additionally it captured the change of the parameters' significance over time, i.e., the analysis captures the culture phases (lag, exponential, stationary and decline). These outstanding results proved the importance of the development of mathematical models based on first principles and the potential of GSA for model analysis.

The modified model for serum-free conditions predicted the same variables as the original model, in addition to serine, glycine, cysteine and tryptophan profiles.

The statistical analysis of the intracellular ATP content revealed that all experiments present significant differences, between 0 and 24 hours compared to the other stages of the culture, indicating that the ATP content in viable cells is not constant throughout the culture time. However, the ATP content was the same after the lag phase in DMEM medium supplemented with 10% FBS and serum-free CD-Hybridoma medium. Therefore, the optimal intracellular ATP content is above $4.82 \pm 1.09 \times 10^{-12}$ [mmol ATP/Cell].

The culture was successfully scaled-up from flasks to the Bioflo 110 bioreactor. Cultures in flasks and the bioreactor, under the identical initial conditions, presented the same performance, reaching the same number of viable cells, total cells and product titre. In addition, the model captured the dynamic of the system in both operation modes, without parameters re-estimation. According to the experimental results and the model prediction, the scaling-up of the culture does not have a significant effect on the system. Moreover, it would be possible to implement a feeding strategy in the bioreactor, reducing temperature and pH perturbations.

For fed-batch culture implementation, the Energy Model was modified including terms related to glucose inlet (FinGLC) and amino acids inlet (FinAA), transforming the batch system in a fed-batch culture. The modified model and the designed supplemental medium were tested

through one fed-batch experiment, which was performed based on model predictions and with the aim of keeping the concentration of glutamate above 0.2 [mM]. The experiment was effective, it extended the longevity of the culture by one day, increased the mAbs titre by 64% and none of the amino acids present in the medium was exhausted throughout the culture time. The results from this experiment were used to re-estimate the significant parameters of the model under fed-batch operation.

The modified model, after parameter estimation, was used for the design of a fed-batch feeding schedule applying model-based dynamic optimisation of the viable cells concentration and using the composition of the supplemental medium previously design. The implementation of the model-based feeding strategy was exceptionally successful, it extended the longevity of the culture by three days, increased the mAbs titre by 190%, none of the amino acids present in the medium was exhausted throughout the culture time and the concentration of amino acids was maintained in a tight range above zero (0,01-5.61 [mM]).

The Energy Model is the first dynamic model that predict the intracellular ATP content in mammalian cells and its capabilities have been tested in cultures of GS-NS0 cells in two culture medium, modified DMEM supplemented with 10 % FBS and serum-free CD-Hybridoma, and in two operations modes, batch and fed-batch. The applications of the model are: model predictions, model-based medium design, fed-batch dynamic optimisation and design of control strategies.

The Energy Model could be combined with the cell cycle (Garcia, 2015) and apoptosis (Usaku, 2016) models developed by our research group. The integrated model would broaden the understanding of the expression system introducing the heterogeneity of the culture (Cell cycle model), the expression of anti-apoptotic genes (Apoptosis model) and energy requirements that should be fulfilled (Energy model). The modelling of these key characteristics of the biological system would allow us to control the culture conditions that promote cell arrest in the most productive cell cycle stage, reduce apoptosis to preserve the culture viability and maintain optimal intracellular levels of energy required for proliferation, maintenance and production. Linking these models would allow us to increase culture yield,

extend culture longevity, promote efficient nutrients consumption, reduce toxic metabolites formation and increase the culture productivity.

The collaboration work presented in this chapter proved the possibility of the design of a controller based on the Energy Model, which could be designed applying the PAROC framework (Pistikopoulos et al., 2015). The controller should be validated *in-silico* as that significantly minimises labour cost and time, as well as the risk of sub-optimal process control, while it ensures that the process operates under optimal conditions. Finally, the physical controller should be built for the experimental validation of the system.

In the present work, two frameworks were successfully applied “Model development framework for biological systems” (Kiparissides et al., 2011) and the “Parametric Optimisation and Control framework” (Pistikopoulos et al., 2015). Additionally, a new framework and its associated users platform is in development, the “Energy Medium Design Platform”, which uses the Energy Model for the development of a tailored medium composition for specific product/expression system combinations. The medium formulation would reduce the medium cost, reduce waste to improve downstream processing and maximise the production. This integrated model-based platform presents a cost-effective and efficient approach for media development and upstream optimisation of a wide range of bio-products.

8.2 Future directions

The framework developed by Kiparissides, Kountinas, Kontoravdi, Mantalaris and Pistikoupoulos (Kiparissides et al., 2011) that combines experimental results, mathematical modelling and computational tools for the optimisation and control of biological systems has been implemented for a wide range of applications: culture predictions, model-based supplemental medium design, model-based optimisation of fed-batch cultures and development of control strategies. The Energy Model developed for the prediction of the intracellular ATP content in GS-NS0 cells producing cB72.3 mAbs successfully described the behaviour of cultures performed in two different medium compositions: modified DMEM medium supplemented with 10% FBS and serum-free CD-Hybridoma medium; and two different operation modes, batch and fed-batch. The successful implementation of the Energy Model revealed its flexibility to modifications and easy parameter estimation. Therefore, it could be applied in the production of a wide range of biopharmaceutical products that produce different recombinant proteins and use different expression systems. In the present chapter future research directions are proposed based on the developed Energy Model.

8.2.1 Cell cycle, apoptosis and energy model

The Energy Model considers cell death in a superficial but effective manner. However, apoptosis is an active process where energy is involved. Therefore, this phenomenon should be precisely described in the mathematical model. Another factor to be considered in future research is the effect of cell cycle on the productivity of the culture.

Combining the three existing models developed in our research group: cell cycle (Garcia, 2015), apoptosis (Usaku, 2015) and the present work, would broaden the understanding of the expression system to select the conditions that promote cell arrest in the most productive cell cycle stage, reduce apoptosis and supply the exact amount of nutrients for proliferation, maintenance, mAbs assembling and energy production. The final goals should be to induce efficient nutrients consumption, reduce toxic metabolites accumulation, extend the culture time and increase the productivity.

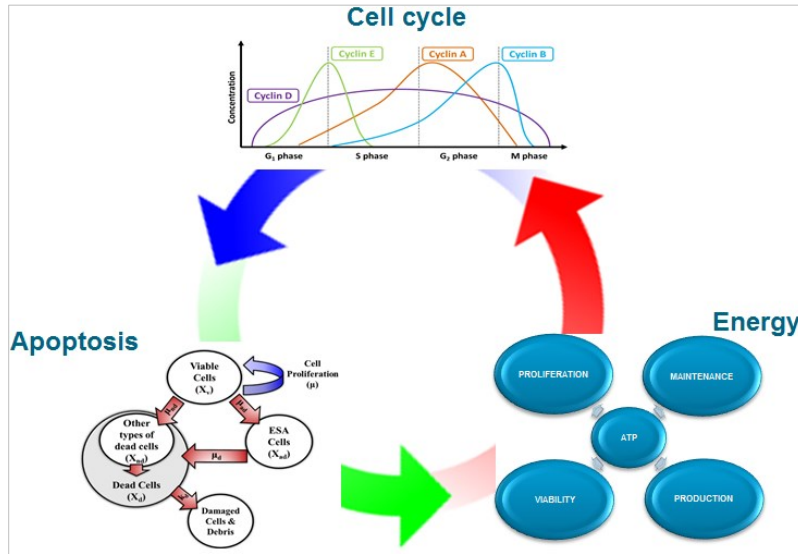


Figure 8.1: Combined Cell cycle-Apoptosis-Energy Model.

8.3 Model-based control strategies

The control of biomanufacturing processes is a challenging task that requires on-line measurements. The currently available electrochemical and optical sensors enable upstream conditions monitoring of pH, temperature and DO online measurements. However, for the control of the product titre and quality it is necessary to measure cell density, major nutrients concentration, toxic metabolites level and product attributes such as activity, aggregation, glycosylation and impurities concentration. The gap between monitoring technologies and the development of model-based control techniques have led us to the development of advanced computational tools, which can provide cost-free simulations and promote the design of advanced optimisation strategies and control policies that could ensure optimal operation. This work is focused on the design and testing of a dynamic process model and an advanced controller for the maximisation of the productivity of cB72.3 mAbs in GS-NS0 in modified DMEM medium 10% FBS cultures.

The development of a control strategy based on the Parametric Optimisation and Control framework (PAROC framework) is proposed in Appendix VII (Pistikopoulos et al., 2015). This work was accomplished in collaboration with two other Ph.D. students, Richard Oberdieck,

who developed the approximate model and Maria Papathanassiou, who developed the controller. The aim was the development and *in-silico* testing of a model-based controller for GS-NS0 cultures in DMEM medium 10% FBS, in order to maintain culture variables within desired limits. A simplified model that captures the dynamic of GS-NS0 cultures in DMEM medium 10% FBS was developed and the PAROC platform was used for the multi-parametric controller design (framework, software and toolboxes). Finally, the controller was validated against model predictions. The proposed future project should follow the same steps presented in Appendix VII for the controller design and *in-silico* validation, and aim to develop a controller based on the Energy Model, build the physical controller or chip and implement the experimental validation of the system.

8.3.1 Model-based medium design framework

In the present work, two frameworks were successfully applied “Model development framework for biological systems” (Kiparissides et al., 2011) and the “Parametric Optimisation and Control framework” (Pistikoupulos et al., 2015). Additionally, a new framework and its user’s platform is in development. The following steps describe the framework for model-based medium design:

8.3.1.1 Data collection:

Some parameters have been fixed in the Energy Model, product and cell composition. However, these parameters depend on the specific product and host. Other parameters related to the specific product/host are the specific production rate, intracellular ATP content and amino acids profiles. These parameters can be obtained from one simple batch experiment.

8.3.1.2 Model modifications

Once the data is collected the product composition, production rate, cell composition and nutrients preferences must be incorporated in the Energy Model.

8.3.1.3 Parameter estimation

The significant parameters in the Energy Model must be re-estimated if the GS-NS0 system is used. If the cells line is different, all the parameters must be re-estimated. The data required for parameter estimation are viable, early apoptotic and dead cells concentrations, glucose and lactate concentrations, amino acids profiles and intracellular ATP content.

8.3.1.4 Supplemental medium design

The supplemental medium composition is designed based on the proportion of the maximum total specific consumption rate of the amino acids before any metabolic shift is detected, i.e., before any nutrient is exhausted.

8.3.1.5 Model validation and medium testing

The model must be validated by a model-based design of a simple fed-batch feeding strategy. Subsequently, the simulation and experimental results must be compared and any difference must be adjusted (parameter estimation).

8.3.1.6 Final media composition

The final medium composition is obtained after the model validation against a fed-batch experiment. If the model required any extra parameter estimation, the results from the fed-batch experiment can be used to perform new parameter estimations until the model presents highly precise predictions.

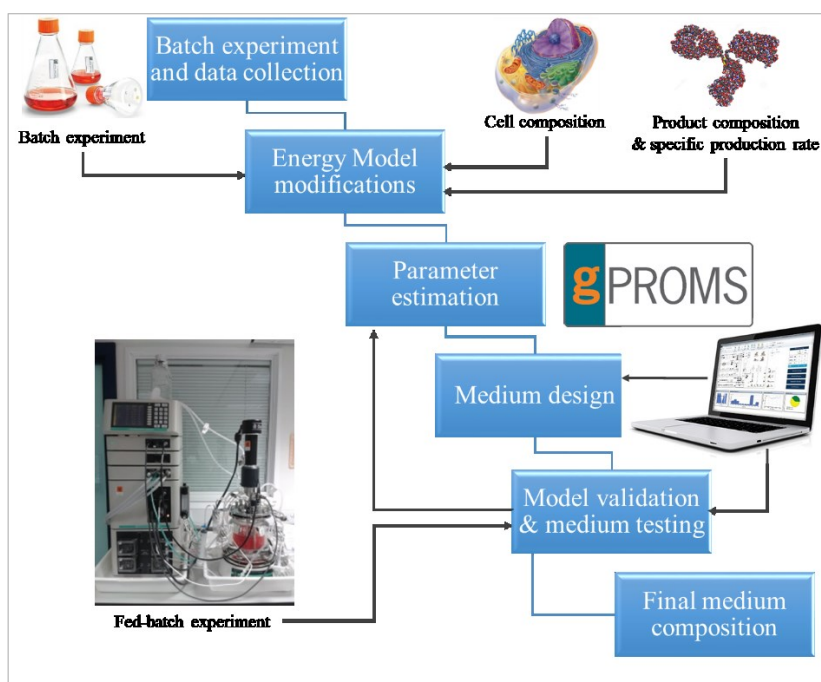


Figure 8.2: Model based medium design framework.

The construction of a database that contains different cells and product composition would allow us to build a medium design platform where the users would be asked to introduce a few parameters to determine the tailored medium for the specific product and expression system. An idea of how the media development user interface should look like is presented in Figure 8.3.

Medium Development Platform

Complete the form and press the blue button

Select cell type

Select specific organism

Select product

Insert production rate of your clone

Calculate Medium Composition

Medium composition

Amino acids	Vitamins	Inorganic salts	Others

Figure 8.3: Interphase design for medium development platform.

The cell type databases should include mammalian, bacteria and yeast and an Energy Model designed for each specific cell type and specific organism should be performed for the specific expression system. The product composition should be included in the databases or, if is not included, the user should provide it. Finally, the production rate of the clone, which is a very specific parameter that depends on the culture conditions implemented by the user, would be the last parameter required.

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Appendices

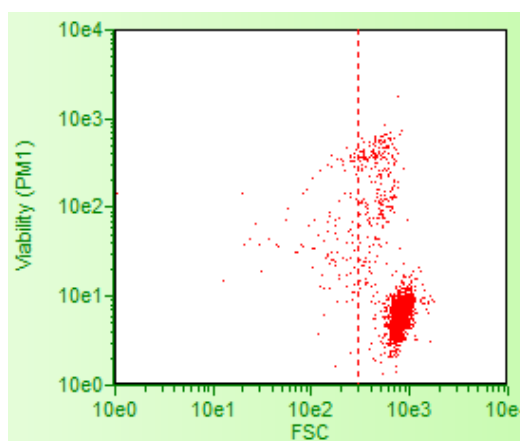
Appendices

I. ViaCount assay

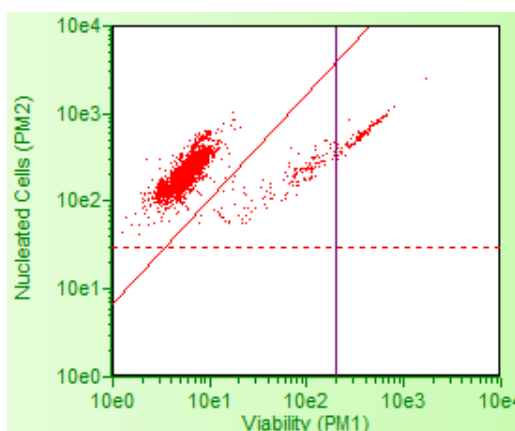
The results from Guava ViaCount application are calculated automatically and are plotted in two dot plots. The first one, Forward Scatter (FSC) vs Viability (PM1), was used to gate the cells from the debris; live cells appear at the bottom of this plot. The second plot, Viability (PM1) vs Nucleated Cells (PM2), was used to gate the viable, apoptotic and dead cells, on the left, centre and right of the plot, respectively.

The software displays the following results:

- Total cells in original sample
- Number of viable cells per mL
- Number of apoptotic cells per mL
- Number of dead cells per mL
- Percent viability
- Dilution factor (input value)



CytoSoft software results. Forward scatter (FSC) vs Viability (PM1)



CytoSoft software results. Viability (PM1) vs Nucleated Cells (PM2)

	Cells/mL	% of Total	Cells in Original Sample
Viable	7.88e05	91.51%	7.88e05
Apoptotic	3.78e04	4.39%	3.78e04
Dead	3.53e04	4.10%	3.53e04
Total	8.61e05	100.0 %	8.61e05
<i>Dilution Factor</i>	20	<i>Original volume</i>	1 mL

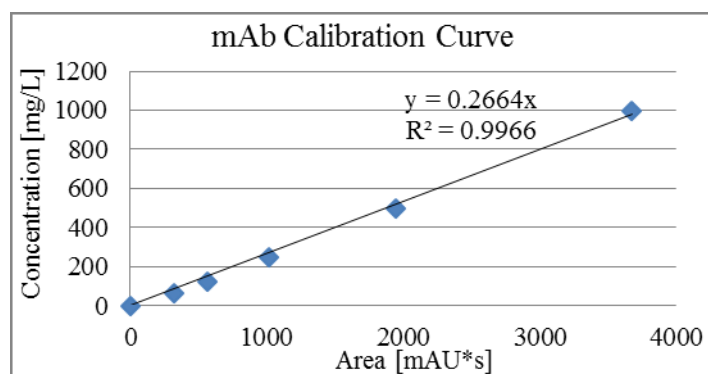
CytoSoft software results.

II. Construction of mAbs standard curve.

The calibration curves were constructed by diluting a mAbs standard 1,000[mg/L] (Lonza Biologics, UK)

Example of calibration curve data.

Concentration	Area
[mg/L]	[mAU×s]
1000	3672.55
500	1949.00
250	1013.40
125	564.59
62.5	321.85
0	0.00



Calibration curve construction.

III. Example of amino acids calibration curve.

The calibration curves were constructed by diluting an amino acids standard solution (AA standard 1 nmol/ μ L, Agilent Technologies, UK). The dilutions in the table below **Error! Reference source not found.** were analysed using the same procedure as that for the culture samples (Section 4.6).

Standard dilution and concentrations

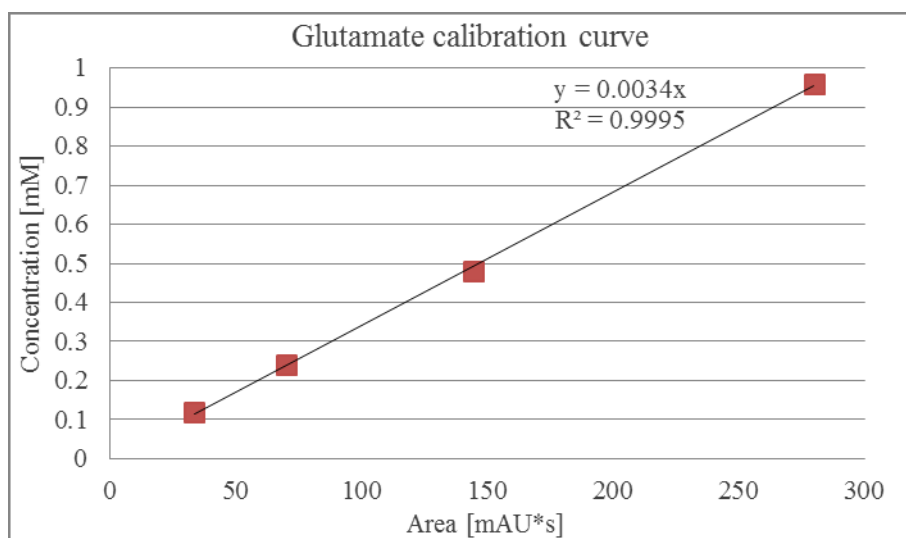
Amino acids	Concentration	Concentration	Concentration	Concentration
	[mM]	[mM]	[mM]	[mM]
Aspartic Acids	0.96	0.48	0.24	0.12
Glutamate	0.96	0.48	0.24	0.12
Asparagine	0.96	0.48	0.24	0.12
Serine	0.96	0.48	0.24	0.12
Glutamine	0.96	0.48	0.24	0.12
Histidine	0.96	0.48	0.24	0.12
Glycine	0.96	0.48	0.24	0.12
Threonine	0.96	0.48	0.24	0.12
Arginine	0.96	0.48	0.24	0.12
Alanine	0.96	0.48	0.24	0.12
Tyrosine	0.96	0.48	0.24	0.12
Cysteine	0.48	0.24	0.12	0.06
Valine	0.96	0.48	0.24	0.12
Methionine	0.96	0.48	0.24	0.12
Tryptophan	0.96	0.48	0.24	0.12
Phenylalanine	0.96	0.48	0.24	0.12
Isoleucine	0.96	0.48	0.24	0.12
Leucine	0.96	0.48	0.24	0.12
Lysine	0.96	0.48	0.24	0.12
Proline	0.96	0.48	0.24	0.12

The analysis was performed in triplicates. The chromatograms obtained using the software OpenLAB Chromatography Data System C.01.04 (Agilent Technologies) and the peak areas were collected from the software reports.

Results collected from OpenLAB Chromatography Data System reports.

Amino acids	Area	Area	Area	Area
	[mAU*s]	[mAU*s]	[mAU*s]	[mAU*s]
Aspartic Acids	261.95	144.76	71.76	35.01
Glutamate	279.93	144.58	70.40	33.69
Asparagine	317.85	165.04	81.32	39.64
Serine	304.74	153.82	75.01	36.29
Glutamine	309.36	156.10	75.92	36.68
Histidine	259.39	130.85	64.41	31.69
Glycine	316.55	156.48	77.18	39.02
Threonine	295.82	154.75	77.13	38.44
Arginine	315.23	158.23	77.35	37.77
Alanine	309.63	158.05	77.63	37.95
Tyrosine	290.58	149.23	73.18	35.74
Cysteine	248.34	129.16	63.33	30.87
Valine	301.27	160.74	81.59	43.02
Methionine	315.22	160.51	79.76	39.72
Tryptophan	292.57	148.56	73.18	35.99
Phenylalanine	292.15	149.96	73.82	36.34
Isoleucine	294.93	153.77	75.92	37.38
Leucine	310.26	157.95	77.66	38.18
Lysine	525.42	247.37	117.07	54.90
Proline	202.29	120.00	55.10	38.78

The area (horizontal axis) and concentration (vertical axis) in the table presents an example of glutamate calibration curve. Linear regression was applied to the data to relate the concentration of amino acid standards with the areas.



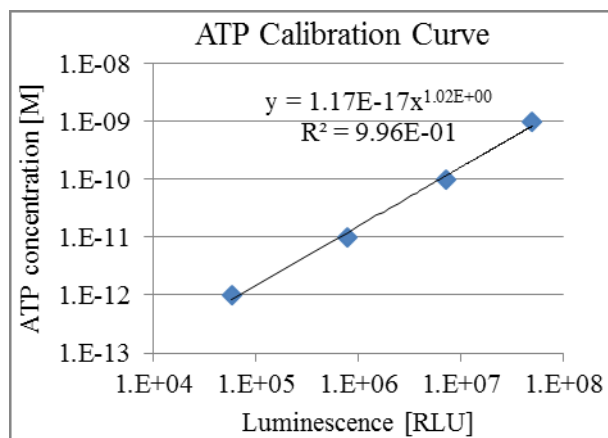
Example of amino acid calibration curve construction.

IV. ATP calibration curve.

The calibration curves were constructed by diluting an ATP standard solution in fresh medium at room temperature (ATP standard 10^{-7} [M], Promega, UK).

Example of calibration curve data.

Sample	Blank	ATP 10^{-9} [M]	ATP 10^{-10} [M]	ATP 10^{-11} [M]	ATP 10^{-12} [M]
1	2.91E+04	5.04E+07	7.15E+06	7.72E+05	8.08E+04
2	2.81E+04	5.05E+07	7.15E+06	7.75E+05	8.09E+04
3	2.84E+04	5.05E+07	7.19E+06	7.81E+05	8.16E+04
Replicate 1	1.70E+04	5.10E+07	7.24E+06	8.42E+05	8.20E+04
Replicate 2	1.64E+04	5.11E+07	7.90E+06	8.50E+05	8.27E+04
Replicate 3	1.64E+04	5.10E+07	7.28E+06	8.53E+05	8.28E+04
Replicate 1	2.73E+04	4.92E+07	7.00E+06	7.65E+05	7.99E+04
Replicate 2	2.66E+04	4.93E+07	7.04E+06	7.73E+05	8.09E+04
Replicate 3	2.66E+04	4.92E+07	7.01E+06	7.81E+05	7.98E+04
Replicate 1	1.63E+04	4.97E+07	7.16E+06	8.32E+05	8.12E+04
Replicate 2	1.61E+04	4.96E+07	7.28E+06	8.25E+05	8.09E+04
Replicate 3	2.26E+04	5.01E+07	7.20E+06	7.82E+05	5.87E+04
Average-Blank average	2.26E+04	5.01E+07	7.20E+06	7.82E+05	5.87E+04



ATP calibration curve construction.

V. Dry cell weight calculation

The cells were cultivated for 48 hours (3 Flasks, 200mL working volume). Three samples were collected from each flask to count viable, early apoptotic and dead cells. Sample tubes previously weight were used to take samples of 15, 25 and 50mL. The samples were centrifuged for 5 minutes at 800rpm and the supernatant was discarded and the cells in the tubes dried at 60°C (Bonarius *et al.*, 1996).

Dry cell weight results.

Cell density	Volume	Weight	DCW
[Cell/L]	[L]	[g]	[g/Cell]
1.27E+09	0.015	0.0069	3.62E-10
1.27E+09	0.015	0.0072	3.78E-10
8.90E+08	0.025	0.0137	6.16E-10
8.86E+08	0.025	0.0118	5.33E-10
1.00E+09	0.05	0.0229	4.58E-10
1.00E+09	0.05	0.0191	3.82E-10
DCW			4.55E-10

VI. Adaptation of GS-NS0 cells to serum-free conditions.

The trend towards chemically-defined media, completely free of animal-derived components is driven by the biopharma awareness of potentially infectious adventitious agents into the production processes and the final products (Merten, 1999). Accordingly, European regulations indicate that non-animal compounds should be used for the production of therapeutic drugs and urge manufacturers to always justify the use of materials of animal origin (Zhang and Robinson, 2005).

The adaptation of GS-NS0 cells from DMEM medium supplemented with 10% FBS to serum-free conditions is presented in this section. In the adaptation, three different chemically-defined culture medium were tested Ex-Cell, HyClone and CD-Hybridoma. The adaptation schedule, culture conditions and medium composition are described in detail and the results are presented to justify the selection of the final chemically-defined medium to be used in the serum-free cultures implementation.

The objectives to achieve the serum free adaptation were:

- 1) **Selection of serum-free culture media:** Selection of suitable media for adaptation of GS-NS0 cells to serum-free conditions and identification of the special requirements of the cells.
- 2) **Design of adaptation protocol:** Design and implementation of adaptation experiments.
- 3) **Assessment and medium selection:** Evaluation of the adapted cells performance through cryopreservation recovery, viability assays and production measurements; and selection of the serum-free culture medium that presented the best performance for the development of a cell bank for future experiments.

1) Medium selection

The cell bank used to perform the experiments was provided by Lonza Biologics and the medium used was DMEM modified medium described in Section 4.1 . Previous attempts to adapt this clone to serum-free conditions were focussed on a progressive reduction of serum concentration every three or four passages, reaching a high number of passages and reducing the concentration of serum to 2.5% only. For this reason, the cells were adapted to grow in 2% FBS and subsequently adapted to serum replacement.

The approach in the present work was the adaptation to three different chemically media available in the market, Ex-Cell® (Raja et al., 2011), HyClone™ (Raja et al., 2011) and CD-Hybridoma® (Gorfien et al., 2000) and supplement them with specific substances that could increase the adaptation probabilities in a reduced number of passages. The details and descriptions of the market media selected are in the table below.

Chemically-defined medium descriptions.

Medium	Company	Supplier product description
Ex-Cell®	Sigma-Aldrich	Animal-component free, protein-free, chemically-defined, serum-free medium developed for the long-term growth of NS0-related cells in suspension culture. NS0 hybridoma suspension cultures can be subcultured directly into EX-CELL NS0 from serum-supplemented or serum-free media. Suspension cultures in EX-CELL NS0 have been carried for more than 50 passages with no loss of growth or viability.
HyClone™	GE Healthcare	Protein-free and contains no animal-derived components. This regulatory-friendly medium is developed through the HyClone Metabolic Pathway Design process to increase the process yields for the manufacture of antibodies and antibody fragments for therapeutic use in a variety of engineered hybridoma and myeloma cell lines. It increases process yields in the manufacturing of mAbs using a variety of NS0 cell clones with GE Healthcare HyClone CDM4NS0 media designed to require no cholesterol or GS-supplement.
CD-Hybridoma®	Gibco	Animal origin-free, chemically-defined, protein-free medium specifically developed for the ability to support the growth of hybridomas for antibody production. Cholesterol-dependent cultures such as NS0 require the addition of 250X Cholesterol Lipid Concentrate.

Special cell's requirements**Lipids: Cholesterol**

Cholesterol is essential for cells proliferation as it is one of the building blocks for cellular structures such as the cell membrane. Unfortunately, NS0 cells are cholesterol auxotroph due to their deficiency of 17- β -hydroxysteroid dehydrogenase type7 (Seth et al., 2006). Therefore, cholesterol has to be supplemented for cell's survival. Fortunately, synthetic cholesterol has been developed to overcome the inconveniences of having medium components from animal origin. The product selected for cholesterol supply was 250X Cholesterol Lipid Concentrate (12531018, Invitrogen, UK)

Proteins: Albumin

Serum contains a wide variety of proteins that serve as carriers and protective agents for other molecules such as albumin, a protein that binds and protects vitamins, fatty acids and ions, thus these molecules became soluble, stable and are available for cell consumption. In particular, albumin avoids the precipitation of cholesterol vesicles in NS0 cultures, which are cholesterol-dependent cell lines (Keen & Steward, 1995). The product selected for albumin supply was 50X Serum Replacement (S0638, Sigma-Aldrich).

Hormones: Insulin

Insulin regulates cellular uptake, utilisation, and storage of glucose, amino acids and fatty acids and inhibits the breakdown of glycogen, protein and fat. In studies of NS0 cell adaptation to protein-free medium, it was observed that insulin increased the total number of cells and viability of the culture when serum is also supplemented and cells die in absence of serum (De la Luz et al., 2008). Additionally, Chang adapted hybridoma cells to grow in serum-free condition and supplemented the medium with a combination of physiological levels of insulin and transferrin obtaining excellent adaptation results (Chang, 1980). These results indicate that insulin is favourable but not the sufficient to adapt the cells directly to serum-free conditions. The product selected for insulin supply was 50X Serum Replacement (S0638, Sigma-Aldrich), which also contains transferrin.

2) Design of adaptation protocol

The adaptation process started taking one cryovial from the working bank, containing cryopreserved cells in DMEM medium & 10% FBS and DMSO. The cryovial was removed from the liquid nitrogen storage tank and thawed at 37°C in a water bath for 5min. The content of the cryovial was transferred to a 15ml Falcon tube (Corning, UK), combined with 10mL of pre-warmed fresh medium, gently mixed and centrifuged at 800rpm for 5min (Rotina 46R centrifuge, Hettich Zentrifugen, DE). The supernatant was discarded and the cell pellet resuspended in 10mL of fresh medium and inoculated at 2×10^5 cells/mL in a 125mL Erlenmeyer flask (Corning) with 30mL working volume. Cells from cultures in mid-exponential growth were passaged every 2 days at 2×10^5 cells/mL inoculation cell density, three times prior experiments.

In the first passage, the cells were centrifuged at 800rpm for 5min (Rotina 46R centrifuge, Hettich Zentrifugen, DE), the medium was discarded and the cells re-suspended in pre-warmed mixture of the modified DMEM medium & 10% FBS and the chemically-defined medium in the evaluation.

Chemically-defined medium compositions (1L).

Volume	Ex-Cell®	HyClone™	CD-Hybridoma®
	[L]	[L]	[L]
Medium	958.5	979.5	958.5
Serum Replacement 50X	0.50	0.50	0.50
Cholesterol 250X	1.00	0.00	1
GS-Supplement 50X	20	0	20
Penicillin/Streptomycin 100X	10	10	10
MSX solution (Final concentration 25[μM])	10	10	10

*Each medium was prepared following the supplier recommendations.

Passages schedule

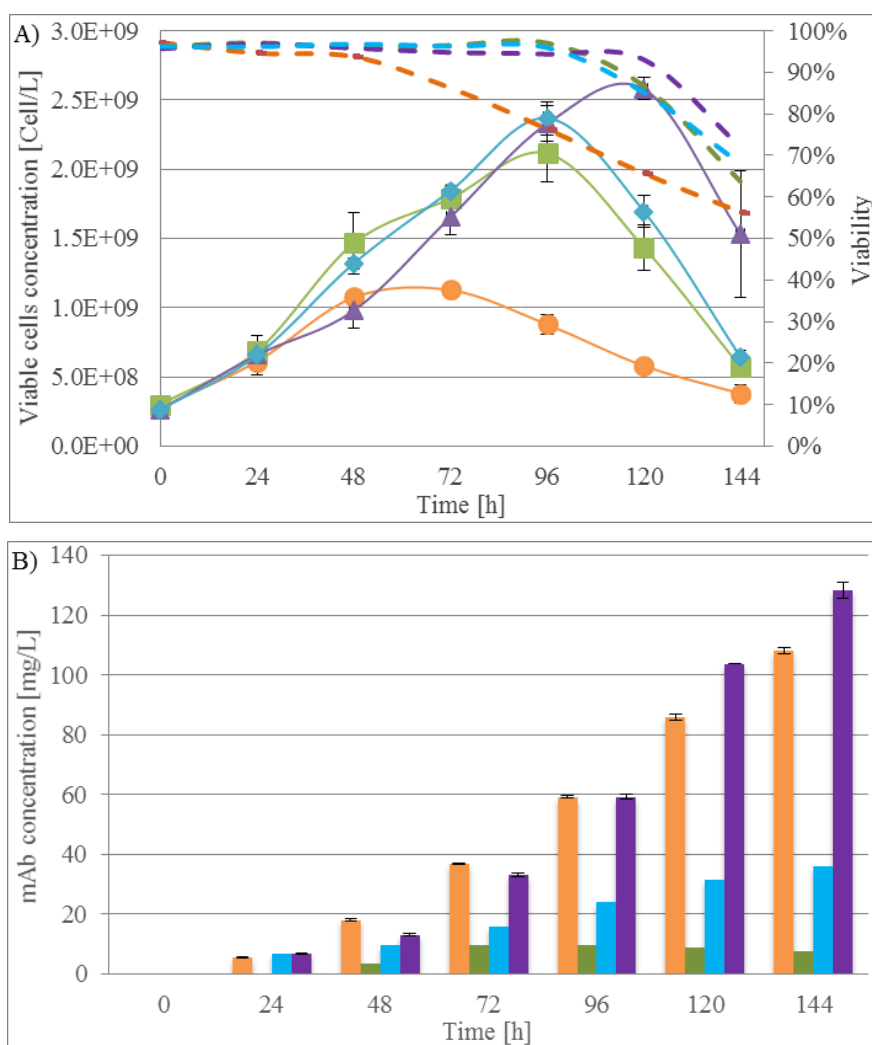
Passage	Volume	Percentage of Modified DMEM medium & 10% FBS	Percentage of Chemically-defined medium & 2% FBS
Initial culture	30 [mL]	100%	0%
Passage 1	50 [mL]	100%	0%
Passage 2	50 [mL]	100%	0%
Passage 3	50 [mL]	75%	25%
Passage 4	50 [mL]	75%	25%
Passage 5	50 [mL]	75%	25%
Passage 6	50 [mL]	50%	50%
Passage 7	50 [mL]	50%	50%
Passage 8	50 [mL]	50%	50%
Passage 9	50 [mL]	25%	75%
Passage 10	50 [mL]	25%	75%
Passage 11	50 [mL]	25%	75%
Bank (Passage 12)	200[mL]	25%	75%
Passage 12	50 [mL]	0%	100%
Passage 13	50 [mL]	0%	100%
Passage 14	50 [mL]	0%	100%
Bank (Passage 15)	200[mL]	0%	100%
Passage 15	50 [mL]	0%	100% Chemically-defined medium & 10% Serum replacement
Passage 16	50 [mL]	0%	100% Chemically-defined medium & 10% Serum replacement
Passage 17	50 [mL]	0%	100% Chemically-defined medium & 10% Serum replacement
Bank (Passage 18)	200[mL]	0%	100% Chemically-defined medium & 10% Serum replacement

3) Assessment and medium selection

The criteria applied for medium selection were based on viable cells concentration, viability and mAbs accumulation levels achieved in each system. These levels should be equal or higher than the levels reached in modified DMEM supplemented with 10% FBS cultures, under the same culture conditions: initial cell density (2×10^9 [Cells/L]), volume (200 [mL]), agitation (130 rpm), CO₂ concentration (5%), initial pH (7.2) and temperature (37°C).

In the figure below (A), the concentration of viable cells and viability of the cultures are presented. The culture in modified DMEM medium supplemented with 10% FBS presents a

maximum concentration of cells, 1.2×10^9 [Cells/L], between 48-72 hours of cultures, whilst the serum-free cultures in Excell, HyClone and Cd-Hybridoma media reached concentrations 1-fold higher, around 2.5×10^9 [Cells/L], between 96-120 hours of culture. The viability in serum-free cultures was maintained over 90% for 2 days more in serum-free cultures compared to FBS supplemented cultures. In the figure below (B), it is possible to observe that the only serum-free culture that reached a similar mAbs titre to the FBS supplemented medium, at the end of the culture, was the chemically-defined CD-Hybridoma medium.



Experimental results for (A) Viable cells (solid lines) and viability (dotted lines) and (B) mAbs concentration (bars). (Orange) DMEM medium, (Green) Ex-Cell medium (Blue) HyClone medium and (Purple) CD-Hybridoma medium.

The serum replaced components were cholesterol, albumin, insulin and transferrin. Cholesterol was replaced with a lipids solution and the three other components were replaced with a serum replacement solution. The selection and supply of the replaced serum components was successful according to the reached cells concentrations in each adapted culture.

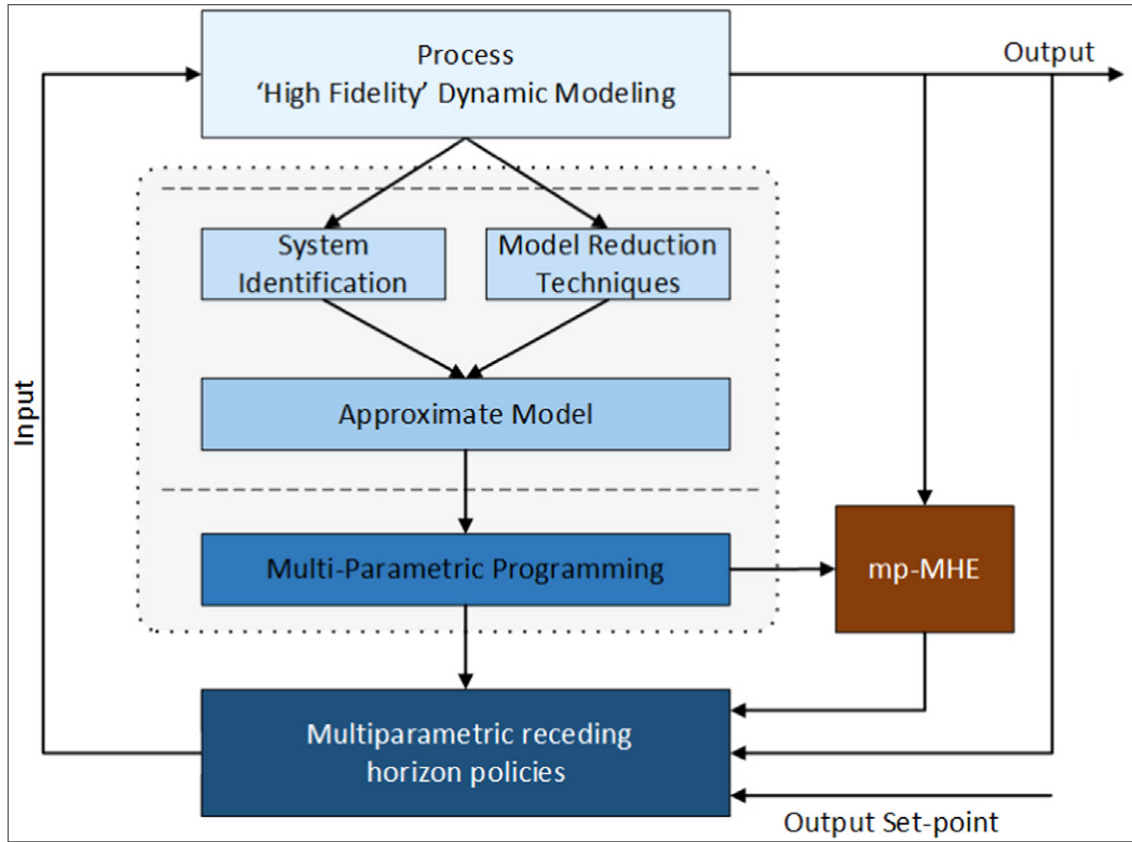
Serum-free adaptation was successfully performed in three different tested media: ExCell, HyClone and CD-Hybridoma, supplemented with cholesterol and serum replacement. The experimental design allowed us to adapt the cell line to serum-free conditions in 5 weeks, without important viability decrease throughout the adaptation process (above 80%).

All the adapted cultures recovered after cryopreservation and reached high cell densities. Unfortunately, Ex-Cell medium and HyClone medium presented low production levels, reaching 7% and 33% of the mAbs concentration found in batch cultures in modified DMEM medium supplemented with 10% FBS. Therefore, the medium selected was CD-Hybridoma, which presented a remarkable performance. The viability of the culture was above 90% for 120 hours in 144 hours long cultures, the number of cells increased by 1.5-fold and the production was increased by 20% compared with the conventional culture. Therefore, CD-Hybridoma bank was selected to be used in future experiments.

VII. Model-based control strategies

Controller design: PAROC framework.

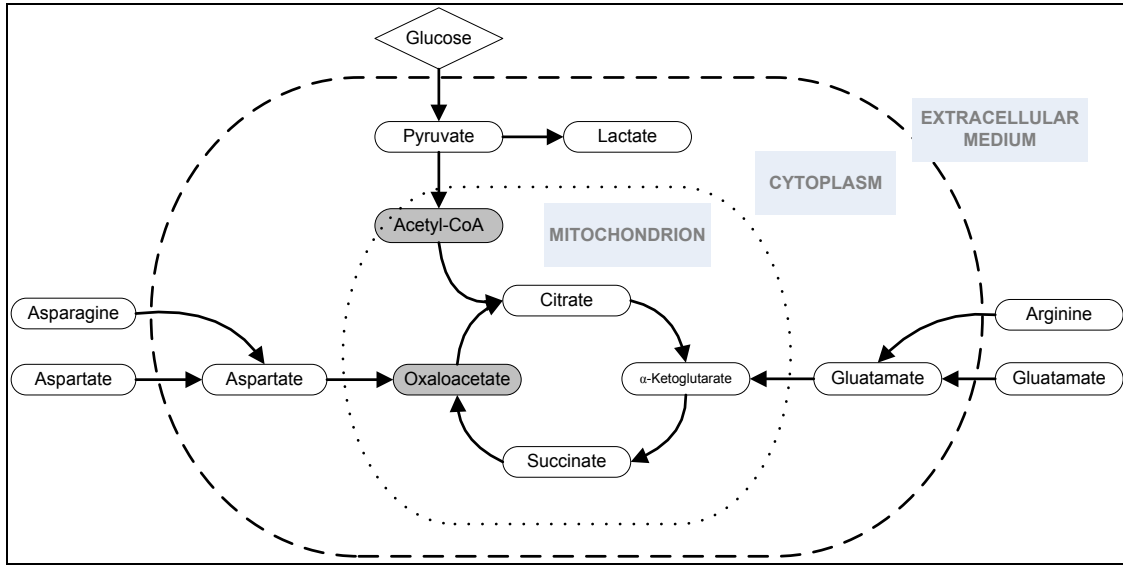
The design of the controller for the maximisation of mAbs production follows the PAROC framework/software platform, which is a generic framework designed for the development of advanced controllers based on multiparametric techniques. The framework comprises four main steps: (1) development of a high-fidelity dynamic model, model analysis and validation (2) model approximation, including system identification and model reduction (3) controller design and (4) *in-silico* controller testing.



PAROC Framework (Pistikopoulos et al., 2015).

Model development

The model was designed for GS-NS0 cells in DMEM medium 10% FBS, in a 1L bioreactor with 200 mL working volume. The metabolic network used for the model development was reduced, to moderate the model complexity, considering the most significant nutrients: glucose, aspartate, arginine, asparagine and glutamate.



Reduced metabolic network.

The proposed model describes a simplified metabolic network of GS-NS0 cells, based on four key nutrients and glucose. The model equations considered in this work are described below.

Material balance around the culture vessel.

$$\frac{dV}{dt} = F_{IN} - F_{OUT}$$

Mass balance for viable cells.

$$\frac{dVX_v}{dt} = VX_v(\mu - \mu_d) - F_{OUT}X_v$$

Mass balance for dead cells.

$$\frac{dVX_d}{dt} = VX_v\mu_d - F_{OUT}X_d$$

Cell growth depends on the availability of a wide variety of nutrients, in particular carbohydrates and amino acids. Glucose is the most predominant carbohydrate consumed by mammalian cells and it follows different metabolic pathways: (1) the pentose phosphate pathway (PPP) to produce DNA precursors, (2) the anaerobic pathway or glycolysis to produce energy and (3) the aerobic pathway where glucose is completely oxidised through the tricarboxylic acid (TCA) cycle that also incorporates amino acids to produce energy. Amino

acids are considered as the building blocks for the production mAbs and proteins that correspond to 70% of the cell composition. In the case of GS-NS0 cells, glutamate is considered as the main source of glutamine used for biosynthesis of cellular structures and product. However, it has been reported that glutamate is not only used as a biosynthesis precursor but also as one of the main energy sources in mammalian cells, contributing more than 50% of the produced ATP (Reitzer et al., 1979; Brand, 1985; Fitzpatrick et al., 1993; Newsholme et al., 2003). Cells require glutamate also for glutamine production, proliferation, mAbs assembly and energy production. The depletion of glutamate and aspartate are followed by decreased growth rate and two metabolic shifts where arginine and asparagine uptakes are increased. Metabolic shifts have been extensively reported in NS0 and CHO cultures but their mechanism still remains an open challenge (Khoo & Al-Rubeai, 2009; Ma et al., 2009; Dorka et al., 2009; Wilkens et al., 2011; Luo et al., 2011; Mulukutla et al., 2012; Kyriakopoulos et al. 2013).

Specific growth rate.

$$\mu = \mu_{max} \left(\frac{GLC}{K_{glc} + GLC} \right) \left(\frac{GLU}{K_{glu} + GLU} + \frac{ARG}{K_{arg} + ARG} \right) \left(\frac{ASP}{K_{asp} + ASP} + \frac{ASN}{K_{asn} + ASN} \right)$$

Specific death rate.

$$\mu_d = \mu_{d,max} \left(\frac{K_{glc,lim}}{K_{glc,lim} + GLC} + \frac{K_{glu,lim}}{K_{glu,lim} + GLU} + \frac{K_{asp,lim}}{K_{asp,lim} + ASP} + \frac{K_{arg,lim}}{K_{arg,lim} + ARG} + \frac{K_{asn,lim}}{K_{asn,lim} + ASN} \right)$$

The specific glucose consumption for biomass production ($Q_{x,glc}$) is described by biomass yield from glucose ($Y_{x,glc}$), that is modelled as a function of the growth rate (μ) and considers the consumption of glucose for biosynthesis of multiple structural components of the cell, including non-essential amino acids, lipids, nucleotides and DNA. Furthermore, $Q_{glyc,glc}$ corresponds to the specific consumption rate of glucose per cell for the production of energy through glycolysis, while Q_{MET} depicts the effect of glutamate concentration in glucose uptake for the production of energy through glycolysis (Kiparissides et al., 2015). The specific consumption of glucose per cell to produce energy through TCA cycle is described by $Q_{tca,glc}$. Moreover, the specific consumption of amino acids is linked to both biomass ($Q_{x,glu}$) and energy

($Q_{tca,asp}$) production. The balance for the mAbs is described by a non-growth associated/maintenance term that ($M_{mAbs,x}$) that describes the constant production of mAbs by viable cells.

Mass balance for glucose.

$$\frac{d(V\ GLC)}{dt} = -VX_v(Q_{x,glc} + Q_{lac,glc} + Q_{tca,glc}) + F_{IN}GLC_{IN} - F_{OUT}GLC$$

$$Q_{x,glc} = \frac{\mu}{Y_{x,glc}}$$

$$Q_{lac,glc} = (1 - Q_{MET}) \left(\frac{\mu}{Y_{lac,glc}} + M_{lac,glc} \right)$$

$$Q_{MET} = \frac{K_{MET}}{K_{MET} + GLU}$$

$$Q_{tca,glc} = \frac{\mu}{Y_{tca,glc}} + M_{tca,glc}$$

Mass balance for lactate.

$$\frac{d(V\ LAC)}{dt} = VX_v(2 * Q_{lac,glc}) - F_{OUT}LAC$$

Mass balance for glutamate.

$$\frac{d(V\ GLU)}{dt} = VX_v(Q_{glu,arg} - Q_{x,glu} - Q_{tca,glu}) + F_{IN}GLU_{IN} - F_{OUT}GLU$$

$$Q_{glu,arg} = \begin{cases} 0 & GLU > 0 \\ C_{arg} * Q_{tca,glu} & GLU = 0 \end{cases}$$

$$Q_{x,glu} = \frac{\mu}{Y_{x,glu}}$$

$$Q_{tca,glu} = \frac{\mu}{Y_{tca,glu}} + M_{tca,glu}$$

Mass balance for arginine.

$$\frac{d(V\ ARG)}{dt} = -VX_v(Q_{x,arg} + Q_{glu,arg}) + F_{IN}ARG_{IN} - F_{OUT}ARG$$

$$Q_{x,arg} = \frac{\mu}{Y_{x,arg}}$$

Mass balance for aspartate.

$$\frac{d(V\ ASP)}{dt} = VX_v(Q_{asp,asn} - Q_{x,asp} - Q_{tca,asp}) + F_{IN}ASP_{IN} - F_{OUT}ASP$$

$$Q_{asp,asn} = \begin{cases} 0 & ASP > 0 \\ C_{asn} Q_{tca,asp} & ASP = 0 \end{cases}$$

$$Q_{x,asp} = \frac{\mu}{Y_{x,asp}}$$

$$Q_{tca,asp} = C_{asp} * Q_{tca,glu}$$

Mass balance for asparagine.

$$\frac{d(V ASN)}{dt} = -VX_v(Q_{asp,asn} + Q_{x,asn}) + F_{IN}ASN_{IN} - F_{OUT}ASN$$

$$Q_{x,asn} = \frac{\mu}{Y_{x,asn}}$$

Mass balance for product.

$$\frac{d(V MAB)}{dt} = VX_v q_{mAb,x}$$

The suggested model assumes that arginine and asparagine are used to supply glutamate and aspartate, respectively, as well as for cell proliferation and survival. Cell growth rate depends on the availability of nutrients in the culture, e.g., the depletion of glutamate and/or aspartate leads to the decrease in the growth rate, while cell growth is diminished with the exhaustion of either glutamate/arginine or aspartate/asparagine. In addition, cell starvation (exhaustion of nutrients) produces cellular stress and activates apoptosis pathways and subsequently cell death. The independent effect of each nutrient has been depicted as an additive effect.

The model consists of 21 differential and algebraic equations, 24 variables and 25 parameters. The model was simulated in gPROMS® v4.1.1. Following the framework presented by Kiparissides, Kountinas, Kontoravdi, Mantalaris and Pistikoupoulos (Kiparissides et al., 2011), the model was subjected to sensitivity analysis for the identification of the significant parameter set.

The GSA was performed for 25 parameters using a sampling time of 2 hours and a parameter variation of $\pm 50\%$. The GSA results indicate 6 significant parameters that were re-

estimated using data obtained from a tailor-made experiment. Parameter estimation was performed within gPROMS® 4.1.1.

Results of the GSA procedure and the final parameter values.

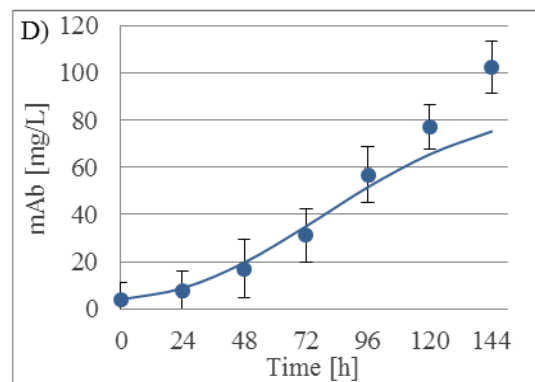
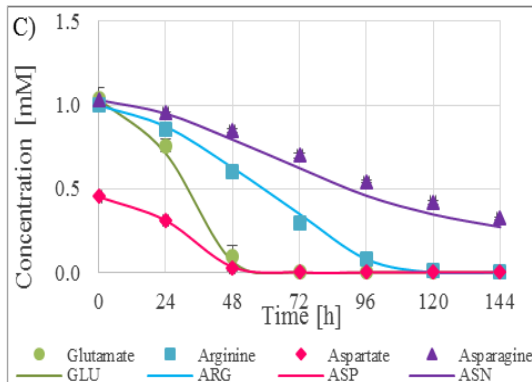
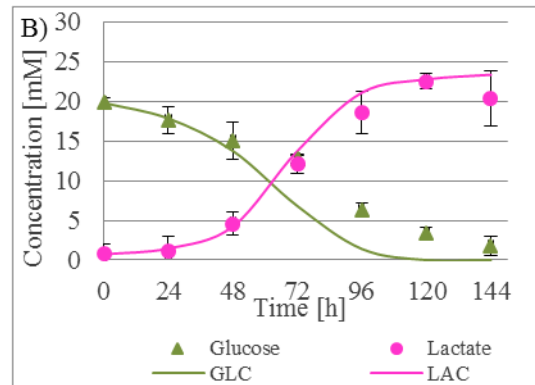
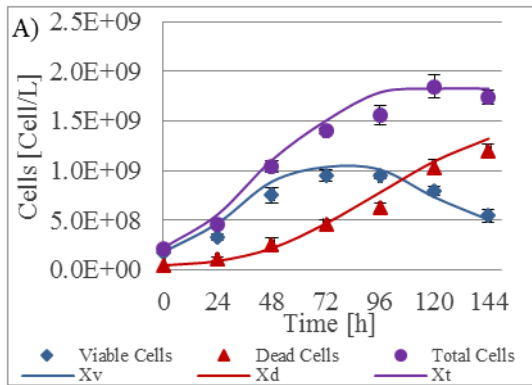
(*) corresponds to the significant parameters that were re-estimated using experimental data

Parameter	Value	Units
$\mu_{\max}$ (*)	0.023	h^{-1}
K_{met}	0.09	mM
K_{glc}	3.50	mM
K_{glu}	0.50	mM
K_{asp} (*)	0.47	mM
K_{arg}	0.001	mM
K_{asn}	0.001	mM
$\mu_{\text{d,max}}$ (*)	0.003	mM
$K_{\text{lim,glc}}$	3.5	mM
$K_{\text{lim,glu}}$	0.5	mM
$K_{\text{lim,asp}}$	0.5	mM
$K_{\text{lim,arg}}$	0.5	mM
$K_{\text{lim,asn}}$	0.5	mM
$Y_{\text{x,glc}}$	1.0E+09	Cells/mM
$Y_{\text{glyc,glc}}$	9.0E+07	Cells/mM
$M_{\text{glyc,glc}}$	2.0E-11	Cells/mM h
$Y_{\text{tca,glc}}$	3.0E+08	Cells/mM
$M_{\text{tca,glc}}$	2.0E-11	Cells/mM h
$Y_{\text{tca,lac}}$	2.0E+07	Cells/mM h
$M_{\text{tca,lac}}$	2.0E-11	Cells/mM h
$Y_{\text{x,glu}}$	9.3E+09	Cells/mM
$Y_{\text{tca,glu}}$	4.0E+09	Cells/mM
$M_{\text{tca,glu}}$ (*)	2.5E-11	Cells/mM h
$\text{Coeff}_{\text{asp}}$	0.6	-
$\text{Coeff}_{\text{asn}}$	0.2	-
$\text{Coeff}_{\text{arg}}$ (*)	0.34	-
$M_{\text{mAb,x}}$ (*)	6.6E-10	mg/Cell h

The final model was validated against the experimental results from “Control Experiment” on the table below. The experimental results and model predictions are in the figure below.

Experimental validation of model for the development of control strategies.

Time	Viable Cells	Dead Cells	Total Cells	Glucose	Lactate	Glutamate	Arginine	Aspartate	Asparagine	mAb
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mg/L]
0	1.78E+08	4.41E+07	2.14E+08	19.83	0.73	1.04	1.00	0.45	1.03	4.06
24	3.29E+08	1.03E+08	4.51E+08	17.64	1.06	0.76	0.86	0.31	0.95	7.52
48	7.52E+08	2.51E+08	1.04E+09	15.03	4.58	0.09	0.60	0.03	0.85	17.12
72	9.49E+08	4.60E+08	1.40E+09	12.77	12.10	0.00	0.30	0.00	0.70	31.23
96	9.45E+08	6.27E+08	1.56E+09	6.32	18.63	0.00	0.08	0.00	0.54	56.83
120	7.96E+08	1.03E+09	1.85E+09	3.43	22.55	0.00	0.01	0.00	0.42	77.19
144	5.42E+08	1.19E+09	1.74E+09	1.75	20.41	0.00	0.01	0.00	0.32	102.36



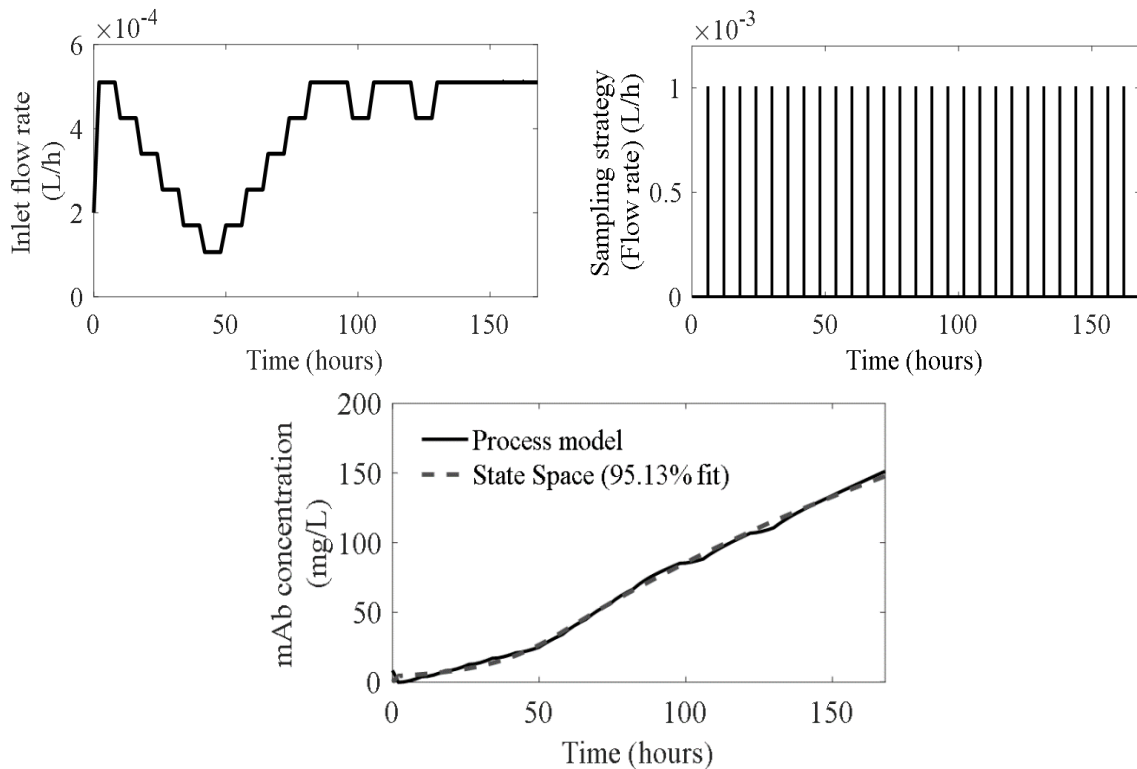
Model validation: comparison of simulation results and experimental data: The presented model provided a satisfactory representation of the examined system.

Model approximation (Richard Oberdieck)

First-principle models describe the dynamic of biological systems through a complex set of equations, making control studies challenging to perform. Therefore, the design of an approximate model is required for the formulation of the control problem. The process model was re-formulated to a state space using identification techniques (*ident* toolbox, MATLAB®)

to obtain a linear representation of the system that fits the dynamics of the original process model.

The model was re-formulated to a state space considering the inlet flow rate of the supplemental medium as the input and mAbs concentration as the output. In addition, the sampling was introduced as a measured disturbance for the design of the state space model (1 mL of sample collected every 6 hours). Moreover, standard practice indicates that the culture volume should not vary more than $\pm 10\%$ from its initial value, considering this constraint as an additional system state, an output with a predefined set-point.



(A) Random flow rate (Input). (B) Sampling (Disturbance). (C) mAbs concentration (Output): comparison between model prediction (solid line) and state space (dotted line).

Controller design & ‘closed-loop’ validation (Maria Papathanassiou)

After the model approximation, a state-space model is obtained and used for the formulation and solution of the multi-parametric problem, where the optimisation problem is solved offline using the POP toolbox in MATLAB ®. Later, the controllers are validated *in-*

silico, in a “closed-loop” fashion against the process model, where their capabilities and/or limitations are assessed. The validation/testing was performed using the tool gO:MATLAB that allows seamless data exchange between MATLAB® (control actions) and gPROMS® (process model).

The multi-parametric problem was formulated and solved based on the state space model described in the previous section. The problem parameters are in the table below.

State space parameters.

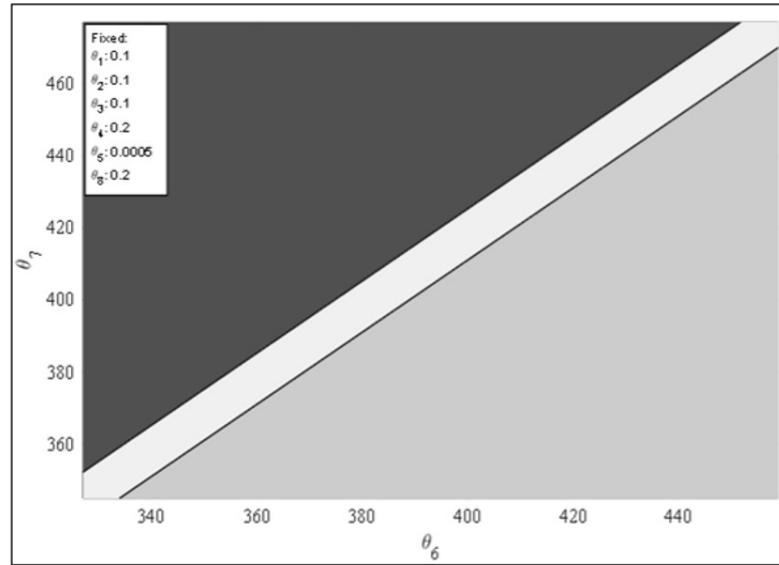
Parameter	Description
$\theta_1, \dots, \theta_4$	System states ($x_1, \dots, x_4$)
θ_5	Disturbance (Sampling)
θ_6	Output (mAbs concentration)
θ_7	Set-point (mAbs)
θ_8	Set-point (Volume)

The control problem comprises 8 parameters and it was solved using POP toolbox in MATLAB®.

Tuning parameters.

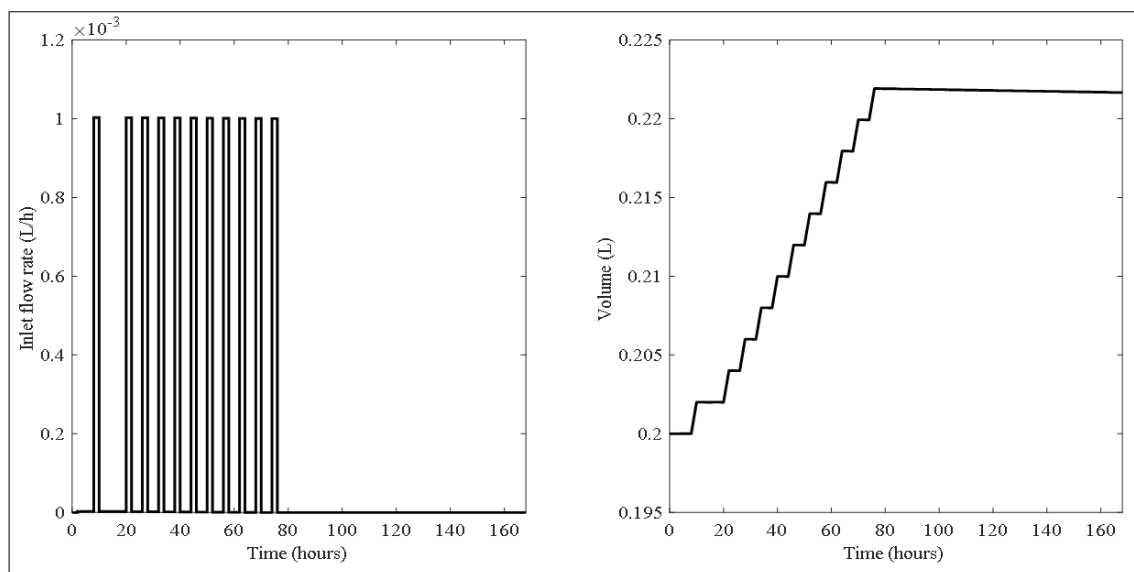
Parameter	Value	Description
Output horizon	4	The controller predicts 4 hours ahead
Control horizon	2	The controller takes an action every 2 hours.

The results provided a map of the critical regions that correspond to affine function evaluations of the problem parameters and result into the optimal control actions.



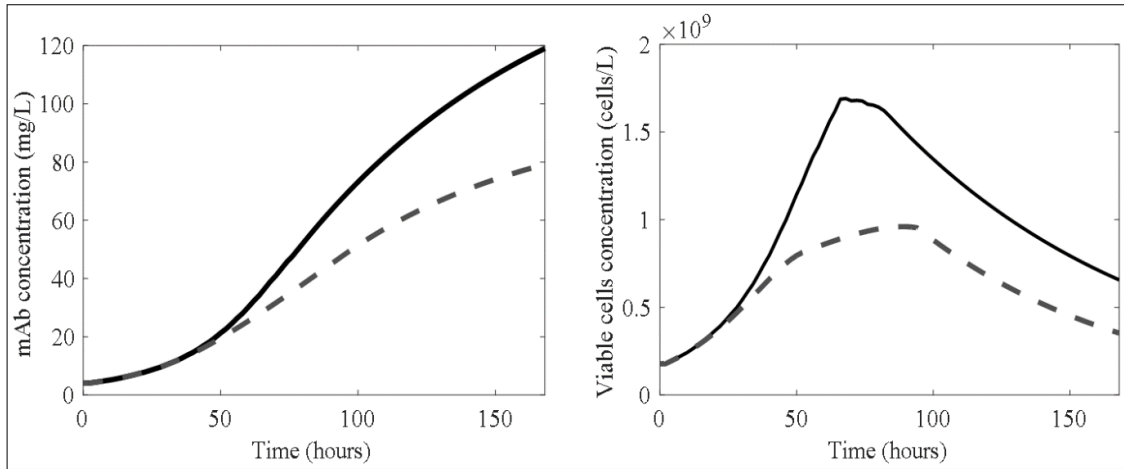
Critical regions for parameters θ_6 (Output) and θ_7 (Set-point).

Following its design, the controller was validated *in-silico* against the process model. The model was simulated for 168 hours (7 days), under periodic sampling (1mL every 6 hours) and the behaviour of the controller was assessed. The results suggest fed-batch feeding, that would minimise the excess of nutrients in the culture and promote the effective consumption of glucose, reducing the formation of toxic metabolites, such as lactate. In a similar manner, the controller intensifies the feeding between 20-76 hours of culture, where the culture is undergoing its exponential phase and maximum cell growth is achieved.



Controller results: (A) Inlet flow rate (control output). (B) Evolution of culture volume under the suggested feeding strategy.

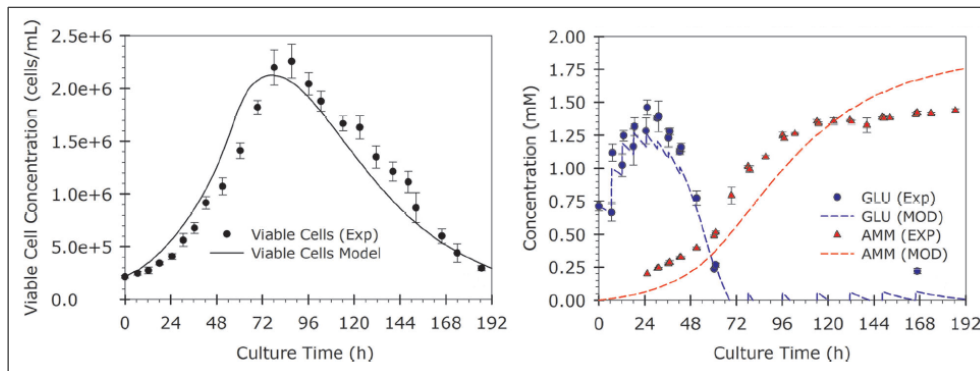
The suggested strategy for nutrient supply not only increases the number of viable cells, but also prolongs the culture longevity, leading to increased productivity. The controller indicates that feeding should be provided until the 76 hours of culture, when arginine is exhausted (essential amino acids and not provided in the feeding), and apoptosis and cell death are triggered and irreversible. Any medium provided past this point would increase process waste and would not improve culture productivity. Furthermore, these results provide a valid indication of the optimal time point that the culture should be terminated in order to minimise product degradation and therefore to improve the performance of the downstream purification.



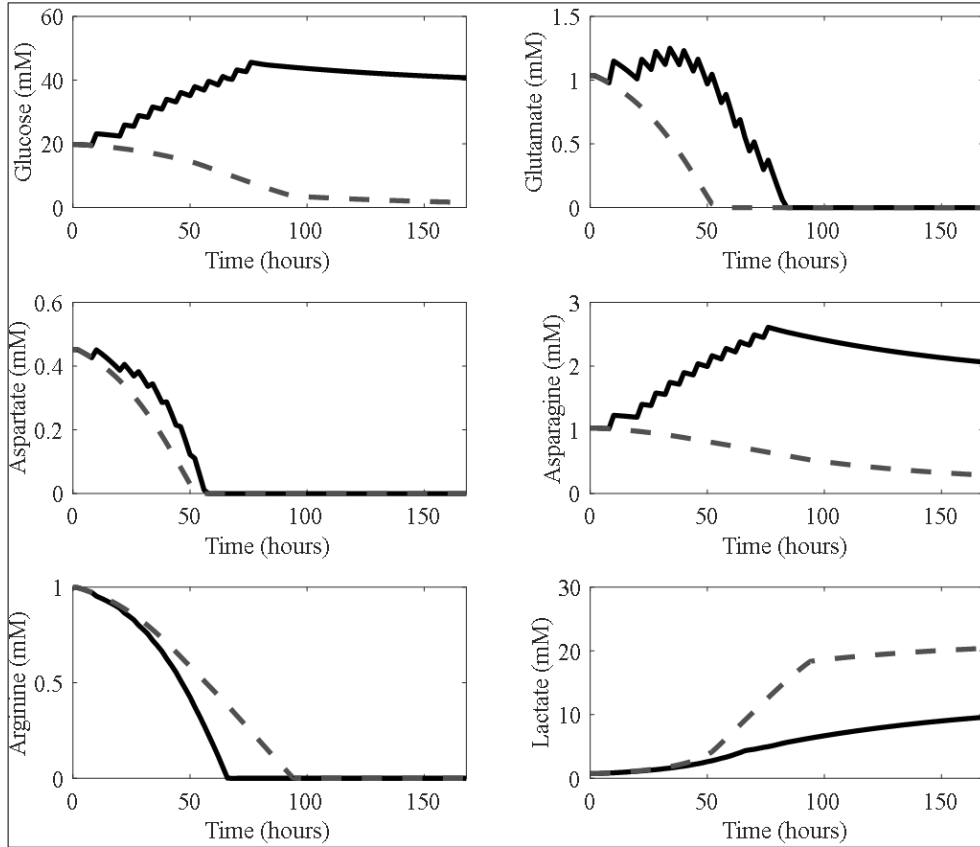
Comparison between the fed-batch (solid line) and the batch (dotted line) system for: (A) mAbs concentration and (B) viable cells, over 168 hours of culture.

In addition, the controller efficiently manages to maintain the working volume within the permitted range ($\pm 10\%$ from the starting value of 0.2 L). The volume of feeding required in order to maintain the system in exponential growth, the accumulation of glucose and asparagine, and the limitation of glutamate and arginine revealed the opportunity to use the proposed controller for medium development, in order to simultaneously optimise the feeding composition and feeding schedule.

Similar results have been obtained through model-based optimisation of fed-batch cultures (Kiparissides et al., 2015).



Model-based optimisation results (A) Experimental (dots) and simulation (solid line) results for viable cells concentration. (B) Experimental (dots) and simulation (solid line) results for glutamate (blue) and ammonia (red) (Kiparissides et al., 2015)



Comparison between the fed-batch (solid line) and the batch (dotted line) system for the concentrations of: (A) glucose, (B) glutamate, (C) aspartate, (D) asparagine, (E) arginine and (F) lactate, over 168 hours of culture.

Conclusions

The design and testing of a model-based controller for GS-NS0 cultures in DMEM medium 10% FBS were successful. The high fidelity model accurately represented the system and the model approximation, tested *in-silico* against model predictions, presented a satisfactory fitting.

The designed controller considers a tight constraint on the culture volume, as well as a periodic sampling (disturbance). The controller managed to efficiently increase the final product concentration under fed-batch feeding.

The presented results demonstrate how powerful computational tools can provide *in-silico* experimentation that significantly minimises labour costs and time, as well as the risk of infeasible and/or sub-optimal process design, while ensuring that the process operates under optimal conditions.

The proposed future project should aim to develop a controller based on the Energy Model, build the physical controller or chip and implement the experimental validation of the system.

VIII. Experimental results: GS-NS0 Batch in flasks, modified DMEM medium &10% FBS.

Exploratory experiment: Batch culture.

Time	Viable Cells	Apoptotic Cells	Dead Cells	Total Cells	ATP	mAb	Lactate	Glucose	Aspartate	Glutamate	Asparagine	Glutamine	Histidine	Threonine	Arginine	Alanine	Tyrosine	Valine	Methionine	Phenylalanine	Isoleucine	Leucine	Lysine	Proline
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
0	2.80E+08	2.89E+07	8.36E+06	3.17E+08	1.89E-03	0.00	0.00	21.20	0.43	1.02	0.94	0.02	0.10	1.02	0.98	0.44	0.55	1.32	0.26	0.58	1.28	1.14	1.08	0.66
24	5.90E+08	8.67E+07	1.25E+07	6.89E+08	6.30E-03	2.56	3.57	20.00	0.24	0.65	0.91	0.04	0.06	0.99	0.82	0.62	0.54	1.20	0.23	0.57	1.13	0.97	1.05	0.63
48	1.21E+09	3.25E+08	3.87E+07	1.57E+09	7.60E-03	9.99	9.30	16.87	0.00	0.00	0.74	0.01	0.04	0.85	0.46	0.68	0.48	0.93	0.17	0.49	0.84	0.65	0.85	0.48
72	1.23E+09	4.48E+08	8.30E+07	1.76E+09	7.94E-03	24.84	20.67	9.83	0.00	0.00	0.59	0.01	0.05	0.88	0.17	0.52	0.42	0.77	0.15	0.46	0.63	0.42	0.81	0.31
96	1.08E+09	5.98E+08	2.94E+08	1.97E+09	7.50E-03	52.51	26.90	4.97	0.00	0.00	0.47	0.03	0.09	0.90	0.04	0.46	0.42	0.67	0.13	0.46	0.44	0.28	0.80	0.16
120	6.01E+08	5.37E+08	5.18E+08	1.66E+09	6.00E-03	75.85	26.20	3.23	0.00	0.00	0.37	0.04	0.09	0.90	0.00	0.42	0.39	0.61	0.13	0.45	0.35	0.20	0.80	0.13
144	2.44E+08	3.57E+08	7.51E+08	1.35E+09	4.00E-03	106.81	24.23	2.83	0.00	0.00	0.28	0.05	0.09	0.87	0.00	0.39	0.41	0.56	0.12	0.44	0.30	0.16	0.78	0.13

Time	Viable Cells	Apoptotic Cells	Dead Cells	Total Cells	ATP	mAb	Lactate	Glucose	Aspartate	Glutamate	Asparagine	Glutamine	Histidine	Threonine	Arginine	Alanine	Tyrosine	Valine	Methionine	Phenylalanine	Isoleucine	Leucine	Lysine	Proline
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mg/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
0	1.70E+08	2.30E+07	2.11E+07	2.14E+08	9.59E-04	0.00	0.00	19.83	0.45	1.04	1.03	0.03	0.35	1.06	1.00	0.44	0.60	1.38	0.28	0.56	1.25	1.15	1.12	0.66
24	3.48E+08	5.46E+07	4.83E+07	4.51E+08	5.68E-03	2.56	0.00	17.13	0.31	0.76	0.95	0.06	0.34	1.06	0.86	0.56	0.58	1.26	0.26	0.53	1.10	1.01	1.07	0.63
48	7.91E+08	1.21E+08	1.30E+08	1.04E+09	7.13E-03	9.99	3.87	14.57	0.03	0.09	0.85	0.04	0.31	0.97	0.60	0.67	0.53	1.09	0.21	0.48	0.96	0.82	0.94	0.48
72	9.36E+08	2.20E+08	2.41E+08	1.40E+09	6.67E-03	24.84	11.90	12.73	0.00	0.00	0.70	0.02	0.29	0.99	0.30	0.59	0.48	0.91	0.18	0.45	0.73	0.56	0.86	0.31
96	9.36E+08	4.02E+08	2.25E+08	1.56E+09	8.73E-03	52.51	18.03	5.83	0.00	0.00	0.54	0.02	0.28	0.98	0.08	0.48	0.46	0.76	0.15	0.42	0.53	0.36	0.79	0.16
120	8.13E+08	5.70E+08	4.62E+08	1.85E+09	7.53E-03	75.85	22.27	3.20	0.00	0.00	0.42	0.03	0.28	0.97	0.01	0.43	0.44	0.68	0.14	0.41	0.39	0.25	0.78	0.13
144	5.45E+08	5.28E+08	6.66E+08	1.74E+09	6.18E-03	106.81	19.03	1.23	0.00	0.00	0.32	0.04	0.27	0.96	0.01	0.38	0.43	0.62	0.13	0.41	0.32	0.19	0.77	0.13

Batch 1: Low initial cell density. Experimental results used to test the prediction capability of the model.

Batch 2: Average results. Experimental results used for parameter estimations.

Time	Viable Cells	Apoptotic Cells	Dead Cells	Total Cells	ATP	mAb	Lactate	Glucose	Aspartate	Glutamate	Asparagine	Glutamine	Histidine	Threonine	Arginine	Alanine	Tyrosine	Valine	Methionine	Phenylalanine	Isoleucine	Leucine	Lysine	Proline
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mg/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
0	2.76E+08	3.46E+07	4.66E+07	3.57E+08	9.45E-04	0.00	0.00	19.20	0.45	1.04	1.03	0.02	0.35	1.15	1.02	0.44	0.60	1.38	0.28	0.56	1.24	1.16	1.14	0.67
24	5.98E+08	9.65E+07	7.99E+07	7.74E+08	8.60E-03	5.44	3.93	17.80	0.23	0.60	0.93	0.05	0.33	1.09	0.79	0.59	0.55	1.21	0.24	0.52	1.06	0.95	1.03	0.62
48	1.10E+09	1.65E+08	1.64E+08	1.43E+09	7.86E-03	18.06	7.27	15.40	0.00	0.00	0.78	0.02	0.29	0.98	0.46	0.65	0.50	0.99	0.19	0.45	0.83	0.68	0.88	0.56
72	1.10E+09	3.83E+08	2.25E+08	1.71E+09	8.38E-03	36.67	16.73	10.13	0.00	0.00	0.61	0.02	0.28	0.96	0.17	0.50	0.31	0.81	0.16	0.42	0.62	0.45	0.81	0.47
96	9.93E+08	4.80E+08	2.51E+08	1.72E+09	1.00E-02	59.23	22.40	4.90	0.00	0.00	0.47	0.03	0.27	0.97	0.04	0.43	0.44	0.70	0.14	0.41	0.42	0.28	0.78	0.26
120	6.90E+08	6.04E+08	7.17E+08	2.01E+09	7.55E-03	85.87	22.67	3.00	0.00	0.00	0.36	0.04	0.27	0.96	0.01	0.38	0.43	0.63	0.13	0.41	0.33	0.20	0.76	0.10
144	4.28E+08	6.23E+08	1.01E+09	2.07E+09	5.04E-03	108.15	21.90	1.63	0.00	0.00	0.27	0.05	0.27	0.95	0.00	0.36	0.41	0.59	0.13	0.40	0.28	0.16	0.76	0.05

Batch 3: Experimental results used for the model validation.

Time	Viable Cells	Apoptotic Cells	Dead Cells	Total Cells	ATP	mAb	Lactate	Glucose	Aspartate	Glutamate	Asparagine	Glutamine	Histidine	Threonine	Arginine	Alanine	Tyrosine	Valine	Methionine	Phenylalanine	Isoleucine	Leucine	Lysine	Proline
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mg/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
0	3.49E+08	4.06E+07	2.67E+07	4.17E+08	1.16E-03	0.00	0.00	19.13	0.45	1.03	1.03	0.02	0.35	1.16	1.02	0.45	0.60	1.38	0.28	0.56	1.24	1.15	1.13	0.66
24	6.38E+08	1.00E+08	5.77E+07	7.96E+08	1.04E-02	7.96	3.37	16.80	0.16	0.45	0.91	0.04	0.32	1.08	0.73	0.61	0.55	1.17	0.23	0.50	1.02	0.91	0.99	0.61
48	1.25E+09	2.50E+08	1.04E+08	1.60E+09	8.24E-03	24.09	9.93	14.37	0.03	0.02	0.74	0.15	0.29	0.99	0.36	0.59	0.49	0.92	0.18	0.44	0.75	0.60	0.85	0.57
72	1.24E+09	4.29E+08	4.11E+08	2.08E+09	8.76E-03	44.27	19.97	7.73	0.00	0.00	0.56	0.02	0.28	0.95	0.11	0.46	0.39	0.76	0.15	0.41	0.53	0.37	0.78	0.47
96	1.00E+09	6.08E+08	3.28E+08	1.94E+09	9.47E-03	70.38	23.57	3.73	0.00	0.00	0.39	0.04	0.27	0.97	0.01	0.39	0.44	0.64	0.13	0.41	0.34	0.21	0.77	0.45
120	5.45E+08	6.07E+08	7.81E+08	1.93E+09	6.90E-03	97.86	24.37	3.07	0.00	0.00	0.32	0.04	0.27	0.95	0.00	0.36	0.42	0.44	0.10	0.40	0.30	0.17	0.75	0.05
144	2.79E+08	5.78E+08	9.01E+08	1.76E+09	4.11E-03	118.06	21.93	2.37	0.00	0.00	0.24	0.05	0.27	0.93	0.00	0.38	0.41	0.57	0.12	0.40	0.27	0.15	0.75	0.05

Batch 4: High initial cell density. Experimental results used to test the prediction capability of the model.

Time	Viable Cells	Apoptotic Cells	Dead Cells	Total Cells	ATP	mAb	Lactate	Glucose	Aspartate	Glutamate	Asparagine	Glutamine	Histidine	Threonine	Arginine	Alanine	Tyrosine	Valine	Methionine	Phenylalanine	Isoleucine	Leucine	Lysine	Proline
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mg/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
0	4.43E+08	6.66E+07	1.10E+08	6.19E+08	2.99E-03	0.00	0.00	21.33	0.45	1.04	1.04	0.02	0.36	1.10	1.03	0.44	0.61	1.39	0.28	0.56	1.25	1.15	1.14	0.68
24	7.84E+08	1.30E+08	2.92E+08	1.21E+09	9.54E-03	10.52	5.83	18.80	0.11	0.32	0.88	0.05	0.11	0.84	0.65	0.60	0.54	1.13	0.22	0.49	1.01	0.87	0.93	0.61
48	1.35E+09	1.15E+08	4.31E+08	1.89E+09	7.13E-03	30.17	13.70	14.03	0.00	0.00	0.68	0.03	0.10	0.78	0.33	0.55	0.48	0.88	0.17	0.43	0.75	0.56	0.81	0.51
72	1.23E+09	4.22E+08	6.25E+08	2.27E+09	7.06E-03	51.96	22.83	7.03	0.00	0.00	0.51	0.03	0.11	0.81	0.11	0.45	0.45	0.74	0.15	0.41	0.53	0.35	0.75	0.42
96	9.81E+08	5.72E+08	6.72E+08	2.22E+09	6.66E-03	79.20	24.63	3.90	0.00	0.00	0.39	0.05	0.11	0.86	0.03	0.42	0.44	0.67	0.14	0.41	0.42	0.25	0.75	0.28
120	4.71E+08	5.96E+08	1.19E+09	2.25E+09	6.27E-03	107.57	25.17	2.83	0.00	0.00	0.26	0.04	0.11	0.81	0.00	0.35	0.42	0.58	0.12	0.39	0.31	0.16	0.71	0.05
144	3.19E+08	5.52E+08	1.27E+09	2.14E+09	2.46E-03	120.94	23.50	2.20	0.00	0.00	0.19	0.04	0.11	0.80	0.00	0.44	0.40	0.57	0.12	0.39	0.29	0.15	0.70	0.05

IX. Statistical analysis (ANOVA Tow-ways) for intracellular ATP content.

Data

Intracellular ATP content per viable cells.

	0 [h]	24 [h]	48 [h]	72 [h]	96 [h]	120 [h]	144 [h]
Batch 1	[mmol ATP/Cell]	[mmol ATP/Cell]	[mmol ATP/Cell]	[mmol ATP/Cell]	[mmol ATP/Cell]	[mmol ATP/Cell]	[mmol ATP/Cell]
Replicate 1	4.30E-12	5.86E-12	3.72E-12	3.65E-12	3.85E-12	6.27E-12	8.87E-12
Replicate 2	8.28E-12	1.30E-11	7.94E-12	8.30E-12	8.66E-12	1.27E-11	1.92E-11
Replicate 3	7.62E-12	1.33E-11	7.04E-12	7.41E-12	8.40E-12	1.08E-11	2.25E-11
Batch 2							
Replicate 1	5.05E-12	7.90E-12	4.98E-12	7.28E-12	9.09E-12	1.13E-11	1.12E-11
Replicate 2	1.81E-12	7.69E-12	7.05E-12	5.43E-12	7.90E-12	8.71E-12	9.46E-12
Replicate 3	3.43E-12	3.03E-11	9.45E-12	1.00E-11	1.30E-11	1.28E-11	1.44E-11
Batch 3							
Replicate 1	4.71E-12	9.31E-12	5.16E-12	6.68E-12	1.02E-11	1.33E-11	9.14E-12
Replicate 2	1.81E-12	1.13E-11	4.64E-12	4.72E-12	5.82E-12	9.34E-12	1.40E-11
Replicate 3	3.26E-12	2.82E-11	9.83E-12	9.83E-12	1.28E-11	1.60E-11	2.08E-11

X. T-test analysis results for ATP content in viable cells

DMEM Batch 1

0/24	0	24	0/48	0	48	0/72	0	72	0/96	0	96	0/120	0	120	0/144	0	144	24/48	24	48
Mean	6.7E-12	1.1E-11	Mean	6.7E-12	6.2E-12	Mean	6.7E-12	6.5E-12	Mean	6.7E-12	7.0E-12	Mean	6.7E-12	9.9E-12	Mean	6.7E-12	1.7E-11	Mean	1.1E-11	6.2E-12
Variance	4.5E-24	1.8E-23	Variance	4.5E-24	4.9E-24	Variance	4.5E-24	6.1E-24	Variance	4.5E-24	7.3E-24	Variance	4.5E-24	1.1E-23	Variance	4.5E-24	5.1E-23	Variance	1.8E-23	4.9E-24
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	3		df	4		df	4		df	4		df	3		df	2		df	3	
t Stat	-1.46		t Stat	0.28		t Stat	0.15		t Stat	-0.12		t Stat	-1.40		t Stat	-2.36		t Stat	1.63	
P(T<=t) one-tail	0.12		P(T<=t) one-tail	0.40		P(T<=t) one-tail	0.44		P(T<=t) one-tail	0.46		P(T<=t) one-tail	0.13		P(T<=t) one-tail	0.07		P(T<=t) one-tail	0.10	
t Critical one-tail	2.35		t Critical one-tail	2.13		t Critical one-tail	2.13		t Critical one-tail	2.13		t Critical one-tail	2.35		t Critical one-tail	2.92		t Critical one-tail	2.35	
P(T<=t) two-tail	0.24		P(T<=t) two-tail	0.79		P(T<=t) two-tail	0.89		P(T<=t) two-tail	0.91		P(T<=t) two-tail	0.26		P(T<=t) two-tail	0.14		P(T<=t) two-tail	0.20	
t Critical two-tail	3.18		t Critical two-tail	2.78		t Critical two-tail	2.78		t Critical two-tail	2.78		t Critical two-tail	3.18		t Critical two-tail	4.30		t Critical two-tail	3.18	
24/72	24	72	24/96	24	96		24	120	24/144	24	144	48/72	48	72	48/96	48	96	48/120	48	120
Mean	1.1E-11	6.5E-12	Mean	1.1E-11	7.0E-12	Mean	1.1E-11	9.9E-12	Mean	1.1E-11	1.7E-11	Mean	6.2E-12	6.5E-12	Mean	6.2E-12	7.0E-12	Mean	6.2E-12	9.9E-12
Variance	1.8E-23	6.1E-24	Variance	1.8E-23	7.3E-24	Variance	1.8E-23	1.1E-23	Variance	1.8E-23	5.1E-23	Variance	4.9E-24	6.1E-24	Variance	4.9E-24	7.3E-24	Variance	4.9E-24	1.1E-23
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	3		df	3		df	4		df	3		df	4		df	4		df	4	
t Stat	1.51		t Stat	1.29		t Stat	0.26		t Stat	-1.30		t Stat	-0.11		t Stat	-0.36		t Stat	-1.60	
P(T<=t) one-tail	0.11		P(T<=t) one-tail	0.14		P(T<=t) one-tail	0.41		P(T<=t) one-tail	0.14		P(T<=t) one-tail	0.46		P(T<=t) one-tail	0.37		P(T<=t) one-tail	0.09	
t Critical one-tail	2.35		t Critical one-tail	2.35		t Critical one-tail	2.13		t Critical one-tail	2.35		t Critical one-tail	2.13		t Critical one-tail	2.13		t Critical one-tail	2.13	
P(T<=t) two-tail	0.23		P(T<=t) two-tail	0.29		P(T<=t) two-tail	0.81		P(T<=t) two-tail	0.29		P(T<=t) two-tail	0.91		P(T<=t) two-tail	0.73		P(T<=t) two-tail	0.18	
t Critical two-tail	3.18		t Critical two-tail	3.18		t Critical two-tail	2.78		t Critical two-tail	3.18		t Critical two-tail	2.78		t Critical two-tail	2.78		t Critical two-tail	2.78	
48/144	48	144	72/96	72	96	72/120	72	120	72/144	72	144	96/120	96	120	96/144	96	144	120/144	120	144
Mean	6.2E-12	1.7E-11	Mean	6.5E-12	7.0E-12	Mean	6.5E-12	9.9E-12	Mean	6.5E-12	1.7E-11	Mean	7.0E-12	9.9E-12	Mean	7.0E-12	1.7E-11	Mean	9.9E-12	1.7E-11
Variance	4.9E-24	5.1E-23	Variance	6E-24	7E-24	Variance	6.1E-24	1.1E-23	Variance	6.1E-24	5.1E-23	Variance	7.3E-24	1.1E-23	Variance	7.3E-24	5.1E-23	Variance	1.1E-23	5.1E-23
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	2		df	4.00		df	4		df	2		df	4		df	3		df	3	
t Stat	-2.47		t Stat	-0.24		t Stat	-1.46		t Stat	-2.3966		t Stat	-1.20		t Stat	-2.25		t Stat	-1.54	
P(T<=t) one-tail	0.07		P(T<=t) one-tail	0.41		P(T<=t) one-tail	0.11		P(T<=t) one-tail	0.0694		P(T<=t) one-tail	0.15		P(T<=t) one-tail	0.05		P(T<=t) one-tail	0.11	
t Critical one-tail	2.92		t Critical one-tail	2.13		t Critical one-tail	2.13		t Critical one-tail	2.92		t Critical one-tail	2.13		t Critical one-tail	2.35		t Critical one-tail	2.35	
P(T<=t) two-tail	0.13		P(T<=t) two-tail	0.82		P(T<=t) two-tail	0.22		P(T<=t) two-tail	0.1388		P(T<=t) two-tail	0.30		P(T<=t) two-tail	0.11		P(T<=t) two-tail	0.22	
t Critical two-tail	4.30		t Critical two-tail	2.7764		t Critical two-tail	2.78		t Critical two-tail	4.3027		t Critical two-tail	2.78		t Critical two-tail	3.18		t Critical two-tail	3.18	

DMEM Batch 2

0/24	0	24	0/48	0	48	0/72	0	72	0/96	0	96	0/120	0	120	0/144	0	144	24/48	24	48
Mean	3.4E-12	1.5E-11	Mean	3.4E-12	7.2E-12	Mean	3.4E-12	7.6E-12	Mean	3.4E-12	1E-11	Mean	3.4E-12	1.1E-11	Mean	3.4E-12	1.2E-11	Mean	1.5E-11	7.2E-12
Variance	5.2E-24	1.7E-22	Variance	5.2E-24	5E-24	Variance	5.2E-24	5.3E-24	Variance	5.2E-24	7.2E-24	Variance	5.2E-24	4.2E-24	Variance	5.2E-24	6.2E-24	Variance	1.7E-22	5E-24
Observations	2	3	Observations	2	3	Observations	2	3	Observations	2	3	Observations	2	3	Observations	2	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	2		df	2		df	2		df	3		df	2		df	2		df	2	
t Stat	-1.5457		t Stat	-1.8005		t Stat	-1.9748		t Stat	-2.932		t Stat	-3.7269		t Stat	-3.8015		t Stat	1.06887	
P(T<=t) one-tail	0.13111		P(T<=t) one-tail	0.1068		P(T<=t) one-tail	0.09349		P(T<=t) one-tail	0.03045		P(T<=t) one-tail	0.03253		P(T<=t) one-tail	0.03138		P(T<=t) one-tail	0.19852	
t Critical one-tail	2.91999		t Critical one-tail	2.91999		t Critical one-tail	2.91999		t Critical one-tail	2.35336		t Critical one-tail	2.91999		t Critical one-tail	2.91999		t Critical one-tail	2.91999	
P(T<=t) two-tail	0.26221		P(T<=t) two-tail	0.21359		P(T<=t) two-tail	0.18698		P(T<=t) two-tail	0.0609		P(T<=t) two-tail	0.06505		P(T<=t) two-tail	0.06275		P(T<=t) two-tail	0.39704	
t Critical two-tail	4.30265		t Critical two-tail	4.30265		t Critical two-tail	4.30265		t Critical two-tail	3.18245		t Critical two-tail	4.30265		t Critical two-tail	4.30265		t Critical two-tail	4.30265	
24/72	24	72	24/96	24	96	24/120	24	120	24/144	24	144	48/72	48	72	48/96	48	96	48/120	48	120
Mean	1.5E-11	7.6E-12	Mean	1.5E-11	1E-11	Mean	1.5E-11	1.1E-11	Mean	1.5E-11	1.2E-11	Mean	7.2E-12	7.6E-12	Mean	7.2E-12	1E-11	Mean	7.2E-12	1.1E-11
Variance	1.7E-22	5.3E-24	Variance	1.7E-22	7.2E-24	Variance	1.7E-22	4.2E-24	Variance	1.7E-22	6.2E-24	Variance	5E-24	5.3E-24	Variance	5E-24	7.2E-24	Variance	5E-24	4.2E-24
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	2		df	2		df	2		df	2		df	4		df	4		df	4	
t Stat	1.01476		t Stat	0.69071		t Stat	0.57712		t Stat	0.4759		t Stat	-0.2192		t Stat	-1.4105		t Stat	-2.141	
P(T<=t) one-tail	0.2085		P(T<=t) one-tail	0.28057		P(T<=t) one-tail	0.31108		P(T<=t) one-tail	0.34053		P(T<=t) one-tail	0.41861		P(T<=t) one-tail	0.1156		P(T<=t) one-tail	0.04949	
t Critical one-tail	2.91999		t Critical one-tail	2.91999		t Critical one-tail	2.91999		t Critical one-tail	2.91999		t Critical one-tail	2.13185		t Critical one-tail	2.13185		t Critical one-tail	2.13185	
P(T<=t) two-tail	0.41701		P(T<=t) two-tail	0.56114		P(T<=t) two-tail	0.62216		P(T<=t) two-tail	0.68106		P(T<=t) two-tail	0.83722		P(T<=t) two-tail	0.23121		P(T<=t) two-tail	0.09898	
t Critical two-tail	4.30265		t Critical two-tail	4.30265		t Critical two-tail	4.30265		t Critical two-tail	4.30265		t Critical two-tail	2.77645		t Critical two-tail	2.77645		t Critical two-tail	2.77645	
48/144	48	144	72/96	72	96	72/120	72	120	72/144	72	144	96/120	96	120	96/144	96	144	120/144	120	144
Mean	7.6E-12	1E-11	Mean	7.6E-12	1E-11	Mean	7.6E-12	1.1E-11	Mean	7.6E-12	1.2E-11	Mean	1E-11	1.1E-11	Mean	1E-11	1.2E-11	Mean	1.1E-11	1.2E-11
Variance	5.3E-24	7.2E-24	Variance	5.3E-24	7.2E-24	Variance	5.3E-24	4.2E-24	Variance	5.3E-24	6.2E-24	Variance	7.2E-24	4.2E-24	Variance	7.2E-24	6.2E-24	Variance	4.2E-24	6.2E-24
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	4		df	4		df	4		df	4		df	4		df	4		df	4	
t Stat	-1.1957		t Stat	-1.1957		t Stat	-1.881		t Stat	-2.0945		t Stat	-0.4649		t Stat	-0.7834		t Stat	-0.4015	
P(T<=t) one-tail	0.14893		P(T<=t) one-tail	0.14893		P(T<=t) one-tail	0.06657		P(T<=t) one-tail	0.05215		P(T<=t) one-tail	0.33308		P(T<=t) one-tail	0.2386		P(T<=t) one-tail	0.35429	
t Critical one-tail	2.13185		t Critical one-tail	2.13185		t Critical one-tail	2.13185		t Critical one-tail	2.13185		t Critical one-tail	2.13185		t Critical one-tail	2.13185		t Critical one-tail	2.13185	
P(T<=t) two-tail	0.29786		P(T<=t) two-tail	0.29786		P(T<=t) two-tail	0.13313		P(T<=t) two-tail	0.1043		P(T<=t) two-tail	0.66616		P(T<=t) two-tail	0.4772		P(T<=t) two-tail	0.70858	
t Critical two-tail	2.77645		t Critical two-tail	2.77645		t Critical two-tail	2.77645		t Critical two-tail	2.77645		t Critical two-tail	2.77645		t Critical two-tail	2.77645		t Critical two-tail	2.77645	

DMEM Batch 3

0/24			0/48			0/72			0/96			0/120			0/144			24/48		
0	24		0	48		0	72		0	96		0	120		0	144		24	48	
Mean	3.3E-12	1.6E-11	Mean	3.3E-12	6.5E-12	Mean	3.3E-12	7.1E-12	Mean	3.3E-12	9.6E-12	Mean	3.3E-12	1.3E-11	Mean	3.3E-12	1.5E-11	Mean	1.6E-11	6.5E-12
Variance	4.2E-24	1.1E-22	Variance	4.2E-24	8.2E-24	Variance	4.2E-24	6.6E-24	Variance	4.2E-24	1.2E-23	Variance	4.2E-24	1.1E-23	Variance	4.2E-24	3.4E-23	Variance	1.1E-22	8.2E-24
Observations	2	3	Observations	2	3	Observations	2	3	Observations	2	3	Observations	2	3	Observations	2	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	2		df	3		df	3		df	3		df	3		df	3		df	2	
t Stat	-2.1118		t Stat	-1.4965		t Stat	-1.8378		t Stat	-2.5345		t Stat	-3.9808		t Stat	-3.0869		t Stat	1.56545	
P(T<=t) one-tail	0.08455		P(T<=t) one-tail	0.11572		P(T<=t) one-tail	0.0817		P(T<=t) one-tail	0.04254		P(T<=t) one-tail	0.01418		P(T<=t) one-tail	0.02692		P(T<=t) one-tail	0.12898	
t Critical one-tail	2.91999		t Critical one-tail	2.35336		t Critical one-tail	2.35336		t Critical one-tail	2.35336		t Critical one-tail	2.35336		t Critical one-tail	2.35336		t Critical one-tail	2.91999	
P(T<=t) two-tail	0.16911		P(T<=t) two-tail	0.23144		P(T<=t) two-tail	0.16339		P(T<=t) two-tail	0.08509		P(T<=t) two-tail	0.02836		P(T<=t) two-tail	0.05384		P(T<=t) two-tail	0.25796	
t Critical two-tail	4.30265		t Critical two-tail	3.18245		t Critical two-tail	3.18245		t Critical two-tail	3.18245		t Critical two-tail	3.18245		t Critical two-tail	3.18245		t Critical two-tail	4.30265	
24/72			24/96			24/120			24/144			48/72			48/96			48/120		
24	72		24	96		24	120		24	144		48	72		48	96		48	120	
Mean	1.6E-11	7.1E-12	Mean	1.6E-11	9.6E-12	Mean	1.6E-11	1.3E-11	Mean	1.6E-11	1.5E-11	Mean	6.5E-12	7.1E-12	Mean	6.5E-12	9.6E-12	Mean	6.5E-12	1.3E-11
Variance	1.1E-22	6.6E-24	Variance	1.1E-22	1.2E-23	Variance	1.1E-22	1.1E-23	Variance	1.1E-22	3.4E-23	Variance	8.2E-24	6.6E-24	Variance	8.2E-24	1.2E-23	Variance	8.2E-24	1.1E-23
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	2		df	2		df	2		df	3		df	4		df	4		df	4	
t Stat	1.48961		t Stat	1.05444		t Stat	0.53796		t Stat	0.23546		t Stat	-0.2395		t Stat	-1.1646		t Stat	-2.4934	
P(T<=t) one-tail	0.13739		P(T<=t) one-tail	0.20113		P(T<=t) one-tail	0.32223		P(T<=t) one-tail	0.4145		P(T<=t) one-tail	0.41124		P(T<=t) one-tail	0.15446		P(T<=t) one-tail	0.03362	
t Critical one-tail	2.91999		t Critical one-tail	2.91999		t Critical one-tail	2.91999		t Critical one-tail	2.35336		t Critical one-tail	2.13185		t Critical one-tail	2.13185		t Critical one-tail	2.13185	
P(T<=t) two-tail	0.27478		P(T<=t) two-tail	0.40226		P(T<=t) two-tail	0.64446		P(T<=t) two-tail	0.82901		P(T<=t) two-tail	0.82248		P(T<=t) two-tail	0.30892		P(T<=t) two-tail	0.06724	
t Critical two-tail	4.30265		t Critical two-tail	4.30265		t Critical two-tail	4.30265		t Critical two-tail	3.18245		t Critical two-tail	2.77645		t Critical two-tail	2.77645		t Critical two-tail	2.77645	
48/144			72/96			72/120			72/144			96/120			96/144			120/144		
48	144		72	96		72	120		72	144		96	120		96	144		120	144	
Mean	6.5E-12	1.5E-11	Mean	7.1E-12	9.6E-12	Mean	7.1E-12	1.3E-11	Mean	7.1E-12	1.5E-11	Mean	9.6E-12	1.3E-11	Mean	9.6E-12	1.5E-11	Mean	1.3E-11	1.5E-11
Variance	8.2E-24	3.4E-23	Variance	6.6E-24	1.2E-23	Variance	6.6E-24	1.1E-23	Variance	6.6E-24	3.4E-23	Variance	1.2E-23	1.1E-23	Variance	1.2E-23	3.4E-23	Variance	1.1E-23	3.4E-23
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	3		df	4		df	4		df	3		df	4		df	3		df	3	
t Stat	-2.1479		t Stat	-0.9992		t Stat	-2.3794		t Stat	-2.0436		t Stat	-1.1691		t Stat	-1.2758		t Stat	-0.4519	
P(T<=t) one-tail	0.06047		P(T<=t) one-tail	0.18712		P(T<=t) one-tail	0.03802		P(T<=t) one-tail	0.06679		P(T<=t) one-tail	0.15365		P(T<=t) one-tail	0.14592		P(T<=t) one-tail	0.34101	
t Critical one-tail	2.35336		t Critical one-tail	2.13185		t Critical one-tail	2.13185		t Critical one-tail	2.35336		t Critical one-tail	2.13185		t Critical one-tail	2.35336		t Critical one-tail	2.35336	
P(T<=t) two-tail	0.12095		P(T<=t) two-tail	0.37424		P(T<=t) two-tail	0.07604		P(T<=t) two-tail	0.13358		P(T<=t) two-tail	0.30729		P(T<=t) two-tail	0.29185		P(T<=t) two-tail	0.68203	
t Critical two-tail	3.18245		t Critical two-tail	2.77645		t Critical two-tail	2.77645		t Critical two-tail	3.18245		t Critical two-tail	2.77645		t Critical two-tail	3.18245		t Critical two-tail	3.18245	

XI. Experimental results: GS-NS0 Batch in flasks, serum-free CD-Hybridoma.

Batch 1: Low initial cell density. Experimental results used to test the prediction capability of the model.

Time	Viable Cells	Apoptotic Cells	Dead Cells	Total Cells	ATP	mAb	Lactate	Glucose	Aspartate	Glutamate	Asparagine	Serine	Glutamine	Histidine	Glycine	Threonine	Arginine	Alanine	Tyrosine	Cysteine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine	Leucine	Lysine	Proline
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mg/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
0	1.14E+08	5.32E+06	7.98E+06	1.28E+08	1.11E-03	0.00	0.00	20.67	0.22	1.33	2.02	2.20	0.03	0.15	0.12	0.72	1.16	3.40	0.37	0.13	1.14	0.34	0.17	0.62	1.21	1.98	1.35	1.89
24	2.01E+08	1.22E+07	1.53E+07	2.29E+08	1.39E-03	0.00	0.00	20.20	0.21	1.19	2.05	2.12	0.08	0.17	0.20	0.74	1.14	3.55	0.37	0.13	1.12	0.34	0.17	0.63	1.15	1.89	1.45	1.95
48	4.11E+08	2.97E+07	5.40E+07	4.95E+08	2.54E-03	4.19	0.00	19.40	0.07	0.41	2.00	1.83	0.08	0.12	0.41	0.67	1.02	3.68	0.33	0.10	0.98	0.30	0.15	0.58	0.98	1.59	1.34	2.09
72	7.26E+08	5.44E+07	7.69E+07	8.57E+08	3.77E-03	9.84	0.00	17.93	0.02	0.14	1.94	1.54	0.07	0.11	0.56	0.58	0.84	3.67	0.28	0.08	0.82	0.25	0.14	0.52	0.77	1.24	1.17	1.73
96	1.25E+09	1.00E+08	1.24E+08	1.47E+09	5.11E-03	25.10	0.00	16.63	0.00	0.00	1.81	1.30	0.06	0.09	0.66	0.49	0.62	3.59	0.22	0.04	0.60	0.19	0.14	0.36	0.35	0.73	1.01	1.49
120	1.63E+09	1.47E+08	2.30E+08	2.00E+09	6.31E-03	62.50	0.00	13.27	0.00	0.00	1.68	1.20	0.05	0.07	0.65	0.40	0.36	3.36	0.17	0.02	0.41	0.14	0.11	0.37	0.14	0.38	0.88	1.03
144	1.52E+09	2.38E+08	3.79E+08	2.14E+09	7.58E-03	111.45	0.00	12.40	0.00	0.00	1.39	1.11	0.05	0.05	0.62	0.24	0.00	2.59	0.08	0.02	0.17	0.06	0.07	0.30	0.05	0.03	0.66	0.56

Batch 2: Experimental results used for parameter estimations.

Time	Viable Cells	Apoptotic Cells	Dead Cells	Total Cells	ATP	mAb	Lactate	Glucose	Aspartate	Glutamate	Asparagine	Serine	Glutamine	Histidine	Glycine	Threonine	Arginine	Alanine	Tyrosine	Cysteine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine	Leucine	Lysine	Proline
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mg/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
0	2.20E+08	9.97E+06	1.64E+07	2.47E+08	1.86E-03	0.00	0.00	19.00	0.23	1.32	1.97	2.14	0.04	0.15	0.11	0.70	1.10	3.23	0.35	0.12	1.09	0.32	0.16	0.59	1.13	1.85	1.27	1.94
24	4.02E+08	2.11E+07	2.55E+07	4.49E+08	2.80E-03	3.42	0.00	18.57	0.17	1.02	1.97	1.96	0.08	0.11	0.26	0.68	1.04	3.40	0.33	0.11	1.02	0.30	0.14	0.57	1.02	1.66	1.27	1.93
48	7.84E+08	4.42E+07	7.50E+07	9.03E+08	4.25E-03	9.81	0.00	16.93	0.06	0.37	1.91	1.62	0.07	0.11	0.46	0.60	0.89	3.47	0.28	0.09	0.88	0.25	0.14	0.51	0.83	1.34	1.14	1.80
72	1.23E+09	7.93E+07	1.13E+08	1.43E+09	6.07E-03	27.70	0.00	15.23	0.00	0.00	1.79	1.29	0.06	0.09	0.63	0.48	0.62	3.42	0.21	0.05	0.60	0.18	0.12	0.43	0.47	0.74	0.93	1.54
96	1.69E+09	1.38E+08	1.85E+08	2.01E+09	6.14E-03	62.16	0.00	13.33	0.00	0.00	1.59	1.10	0.04	0.07	0.66	0.35	0.22	3.08	0.13	0.02	0.30	0.11	0.09	0.34	0.14	0.18	0.75	1.17
120	1.70E+09	2.11E+08	3.46E+08	2.26E+09	9.18E-03	114.05	0.00	11.50	0.00	0.01	1.34	1.08	0.04	0.05	0.62	0.24	0.00	2.43	0.07	0.02	0.16	0.05	0.07	0.27	0.05	0.02	0.59	0.80
144	1.23E+09	3.86E+08	4.81E+08	2.10E+09	5.54E-03	137.31	0.00	9.67	0.04	0.08	1.20	1.13	0.15	0.05	0.78	0.23	0.00	1.68	0.07	0.03	0.16	0.04	0.07	0.27	0.05	0.02	0.58	0.60

Batch 3: Experimental results used for the model validation.

Time	Viable Cells	Apoptotic Cells	Dead Cells	Total Cells	ATP	mAb	Lactate	Glucose	Aspartate	Glutamate	Asparagine	Serine	Glutamine	Histidine	Glycine	Threonine	Arginine	Alanine	Tyrosine	Cysteine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine	Leucine	Lysine	Proline
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mg/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
0	3.10E+08	1.63E+07	2.00E+07	3.46E+08	2.54E-03	0.00	0.00	18.57	0.22	1.31	1.99	2.15	0.04	0.15	0.11	0.70	1.11	3.21	0.35	0.13	1.08	0.32	0.16	0.58	1.13	1.84	1.27	1.79
24	5.94E+08	3.15E+07	4.41E+07	6.69E+08	3.78E-03	5.22	0.00	17.97	0.14	0.85	1.96	1.85	0.08	0.11	0.32	0.66	0.99	3.40	0.32	0.11	0.99	0.28	0.14	0.55	0.97	1.57	1.23	1.84
48	1.08E+09	5.79E+07	7.81E+07	1.22E+09	5.46E-03	14.01	0.00	16.27	0.00	0.02	1.86	1.46	0.06	0.10	0.54	0.54	0.77	3.49	0.25	0.07	0.77	0.21	0.12	0.47	0.70	1.11	1.03	1.69
72	1.58E+09	1.27E+08	1.82E+08	1.89E+09	6.73E-03	42.11	0.00	13.97	0.00	0.00	1.68	1.15	0.05	0.07	0.63	0.40	0.42	3.24	0.17	0.02	0.43	0.14	0.10	0.38	0.25	0.37	0.81	1.37
96	1.81E+09	2.25E+08	2.57E+08	2.29E+09	7.49E-03	94.10	0.00	11.97	0.00	0.00	1.45	1.06	0.04	0.05	0.64	0.27	0.03	2.64	0.09	0.02	0.20	0.07	0.08	0.29	0.07	0.06	0.64	0.92
120	1.65E+09	3.15E+08	3.85E+08	2.35E+09	9.26E-03	130.87	0.00	10.03	0.00	0.03	1.28	1.12	0.14	0.05	0.72	0.23	0.00	1.77	0.05	0.04	0.17	0.04	0.07	0.28	0.14	0.00	0.80	0.80
144	5.15E+08	5.62E+08	5.83E+08	1.66E+09	2.57E-03	170.11	0.00	8.83	0.00	0.28	1.09	1.11	0.11	0.05	0.89	0.22	0.00	1.42	0.06	0.03	0.18	0.03	0.06	0.27	0.05	0.01	0.57	0.53

Batch 4: High initial cell density. Experimental results used to test the prediction capability of the model.

Time	Viable Cells	Apoptotic Cells	Dead Cells	Total Cells	ATP	mAb	Lactate	Glucose	Aspartate	Glutamate	Asparagine	Serine	Glutamine	Histidine	Glycine	Threonine	Arginine	Alanine	Tyrosine	Cysteine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine	Leucine	Lysine	Proline
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mg/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
0	3.94E+08	1.97E+07	3.38E+07	4.47E+08	3.18E-03	0.00	0.00	19.20	0.23	1.30	1.97	2.13	0.04	0.19	0.11	0.70	1.09	3.21	0.34	0.12	1.09	0.32	0.16	0.58	1.12	1.83	1.28	1.99
24	7.38E+08	4.26E+07	5.21E+07	8.32E+08	4.77E-03	6.13	0.00	17.83	0.11	0.69	1.96	1.80	0.07	0.16	0.36	0.65	0.97	3.45	0.31	0.10	0.96	0.28	0.15	0.54	0.93	1.52	1.21	1.94
48	1.34E+09	8.00E+07	8.23E+07	1.51E+09	6.47E-03	19.49	0.00	15.60	0.05	0.30	1.86	1.54	0.06	0.14	0.49	0.56	0.78	3.41	0.26	0.07	0.77	0.23	0.13	0.48	0.69	1.11	1.06	1.71
72	1.81E+09	1.47E+08	2.10E+08	2.17E+09	7.20E-03	58.52	0.00	13.63	0.00	0.00	1.62	1.12	0.04	0.11	0.63	0.37	0.25	3.07	0.14	0.01	0.33	0.11	0.10	0.35	0.15	0.21	0.76	1.24
96	1.92E+09	2.56E+08	3.07E+08	2.48E+09	1.05E-02	114.81	0.00	11.33	0.00	0.00	1.37	1.07	0.06	0.08	0.62	0.25	0.00	2.25	0.07	0.02	0.17	0.05	0.07	0.19	0.12	0.02	0.59	0.78
120	1.48E+09	4.50E+08	4.51E+08	2.38E+09	7.35E-03	178.78	0.00	9.37	0.00	0.10	1.22	1.12	0.18	0.08	0.80	0.24	0.01	1.42	0.06	0.03	0.16	0.04	0.07	0.18	0.12	0.02	0.57	0.57
144	2.27E+08	6.26E+08	7.70E+08	1.62E+09	1.04E-03	157.79	0.00	8.63	0.00	0.26	0.66	0.74	0.06	0.04	0.64	0.15	0.00	0.73	0.04	0.02	0.09	0.02	0.04	0.18	0.03	0.01	0.37	0.34

XII. Bioreactor: Experimental results used to test the prediction capability of the model.

Time	Viable Cells	Apoptotic Cells	Dead Cells	Total Cells	ATP	mAb	Lactate	Glucose	Aspartate	Glutamate	Asparagine	Serine	Glutamine	Histidine	Glycine	Threonine	Arginine	Alanine	Tyrosine	Cysteine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine	Leucine	Lysine	Proline
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mg/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
0	3.31E+08	1.75E+07	4.47E+07	3.94E+08	2.43E-03	0.00	0.00	20.25	0.21	1.28	1.86	2.09	0.03	0.20	0.12	0.65	1.08	3.18	0.35	0.11	1.06	0.33	0.17	0.58	1.12	1.83	1.31	1.92
24	6.49E+08	4.28E+07	5.85E+07	7.50E+08	3.43E-03	4.65	0.00	19.25	0.15	0.86	1.85	1.88	0.08	0.18	0.26	0.63	0.99	3.34	0.32	0.11	0.98	0.30	0.16	0.55	1.00	1.63	1.24	1.96
48	1.16E+09	7.19E+07	9.78E+07	1.33E+09	4.76E-03	15.50	0.00	17.10	0.00	0.01	1.80	1.54	0.07	0.17	0.47	0.55	0.77	3.49	0.27	0.08	0.82	0.24	0.14	0.49	0.78	1.23	1.10	1.79
72	1.74E+09	1.17E+08	1.49E+08	2.00E+09	6.60E-03	42.77	0.00	13.60	0.00	0.00	1.59	1.13	0.05	0.13	0.61	0.40	0.37	3.22	0.18	0.03	0.46	0.15	0.12	0.39	0.29	0.43	0.86	1.35
96	1.98E+09	1.75E+08	2.25E+08	2.38E+09	4.51E-03	86.66	0.00	12.40	0.00	0.00	1.48	0.93	0.04	0.12	0.63	0.27	0.00	2.60	0.09	0.01	0.22	0.07	0.10	0.16	0.18	0.07	0.63	0.82
120	1.58E+09	3.15E+08	3.06E+08	2.20E+09	6.64E-03	143.97	0.00	9.20	0.01	0.04	1.28	0.84	0.13	0.11	0.85	0.25	0.00	2.28	0.07	0.03	0.18	0.05	0.08	0.15	0.16	0.05	0.59	0.52
144	4.00E+08	2.40E+08	2.20E+08	8.60E+08	1.80E-03	165.57	0.00	6.80	0.15	0.33	0.00	0.77	0.05	0.09	1.06	0.24	0.00	2.67	0.07	0.03	0.16	0.05	0.08	0.28	0.06	0.06	0.58	0.44

XIII. T-test analysis results for ATP content in viable cells

CD-Hybridoma: Batch 1

0/24	0	24	0/48	0	48	0/72	0	72	0/96	0	96	0/120	0	120	0/144	0	144	24//48	24	48
Mean	9.7E-12	7.1E-12	Mean	9.7E-12	6.2E-12	Mean	9.7E-12	72	Mean	9.7E-12	4.1E-12	Mean	9.7E-12	3.9E-12	Mean	9.7E-12	5E-12	Mean	7.1E-12	6.2E-12
Variance	5.8E-25	1.6E-24	Variance	5.8E-25	2.3E-25	Variance	5.8E-25	5.2E-12	Variance	5.8E-25	9.1E-26	Variance	5.8E-25	4.7E-25	Variance	5.8E-25	9.8E-26	Variance	1.6E-24	2.3E-25
Observations	3	3	Observations	3	3	Observations	3	1.1E-26	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	3	Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	3		df	3		df	2		df	3		df	4		df	3		df	3	
t Stat	3.11243		t Stat	6.76506		t Stat	10.1293		t Stat	11.837		t Stat	9.78089		t Stat	9.9365		t Stat	1.14939	
P(T<=t) one-tail	0.02639		P(T<=t) one-tail	0.0033		P(T<=t) one-tail	0.0048		P(T<=t) one-tail	0.00065		P(T<=t) one-tail	0.00031		P(T<=t) one-tail	0.00108		P(T<=t) one-tail	0.16686	
t Critical one-tail	2.35336		t Critical one-tail	2.35336		t Critical one-tail	2.91999		t Critical one-tail	2.35336		t Critical one-tail	2.13185		t Critical one-tail	2.35336		t Critical one-tail	2.35336	
P(T<=t) two-tail	0.05278		P(T<=t) two-tail	0.0066		P(T<=t) two-tail	0.00961		P(T<=t) two-tail	0.0013		P(T<=t) two-tail	0.00061		P(T<=t) two-tail	0.00217		P(T<=t) two-tail	0.33372	
t Critical two-tail	3.18245		t Critical two-tail	3.18245		t Critical two-tail	4.30265		t Critical two-tail	3.18245		t Critical two-tail	2.77645		t Critical two-tail	3.18245		t Critical two-tail	3.18245	
24/72	24	72	24/96	24	96	24/120	24	120	24/144	24	144	48/72	48	72	48/96	48	96	48/120	48	120
Mean	7.1E-12	5.2E-12	Mean	7.1E-12	4.1E-12	Mean	7.1E-12	3.9E-12	Mean	7.1E-12	5E-12	Mean	6.2E-12	5.2E-12	Mean	6.2E-12	4.1E-12	Mean	6.2E-12	3.9E-12
Variance	1.6E-24	1.1E-26	Variance	1.6E-24	9.1E-26	Variance	1.6E-24	4.7E-25	Variance	1.6E-24	9.8E-26	Variance	2.3E-25	1.1E-26	Variance	2.3E-25	9.1E-26	Variance	2.3E-25	4.7E-25
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	2		df	2		df	3		df	2		df	2		df	3		df	4	
t Stat	2.57267		t Stat	3.99227		t Stat	3.83442		t Stat	2.81126		t Stat	3.42189		t Stat	6.32491		t Stat	4.68473	
P(T<=t) one-tail	0.06184		P(T<=t) one-tail	0.0287		P(T<=t) one-tail	0.01563		P(T<=t) one-tail	0.05333		P(T<=t) one-tail	0.03791		P(T<=t) one-tail	0.00399		P(T<=t) one-tail	0.00471	
t Critical one-tail	2.91999		t Critical one-tail	2.91999		t Critical one-tail	2.35336		t Critical one-tail	2.91999		t Critical one-tail	2.91999		t Critical one-tail	2.35336		t Critical one-tail	2.13185	
P(T<=t) two-tail	0.12368		P(T<=t) two-tail	0.05739		P(T<=t) two-tail	0.03127		P(T<=t) two-tail	0.10667		P(T<=t) two-tail	0.07582		P(T<=t) two-tail	0.00799		P(T<=t) two-tail	0.00941	
t Critical two-tail	4.30265		t Critical two-tail	4.30265		t Critical two-tail	3.18245		t Critical two-tail	4.30265		t Critical two-tail	4.30265		t Critical two-tail	3.18245		t Critical two-tail	2.77645	
48/144	48	144	72/96	72	96	72/120	72	120	72/144	72	144	96/120	96	120	96/144	96	144	120/144	120	144
Mean	6.2E-12	5E-12	Mean	5.2E-12	4.1E-12	Mean	5.2E-12	3.9E-12	Mean	5.2E-12	5E-12	Mean	4.1E-12	3.9E-12	Mean	4.1E-12	5E-12	Mean	3.9E-12	5E-12
Variance	2.3E-25	9.8E-26	Variance	1.1E-26	9.1E-26	Variance	1.1E-26	4.7E-25	Variance	1.1E-26	9.8E-26	Variance	9.1E-26	4.7E-25	Variance	9.1E-26	9.8E-26	Variance	4.7E-25	9.8E-26
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	3		df	2		df	2		df	2		df	3		df	4		df	3	
t Stat	3.62458		t Stat	5.99046		t Stat	3.2291		t Stat	1.19978		t Stat	0.44684		t Stat	-3.48054		t Stat	-2.44694	
P(T<=t) one-tail	0.01807		P(T<=t) one-tail	0.01338		P(T<=t) one-tail	0.042		P(T<=t) one-tail	0.17654		P(T<=t) one-tail	0.34264		P(T<=t) one-tail	0.01267		P(T<=t) one-tail	0.04596	
t Critical one-tail	2.35336		t Critical one-tail	2.91999		t Critical one-tail	2.91999		t Critical one-tail	2.91999		t Critical one-tail	2.35336		t Critical one-tail	2.13185		t Critical one-tail	2.35336	
P(T<=t) two-tail	0.03613		P(T<=t) two-tail	0.02675		P(T<=t) two-tail	0.084		P(T<=t) two-tail	0.35307		P(T<=t) two-tail	0.68528		P(T<=t) two-tail	0.02534		P(T<=t) two-tail	0.09193	
t Critical two-tail	3.18245		t Critical two-tail	4.30265		t Critical two-tail	4.30265		t Critical two-tail	4.30265		t Critical two-tail	3.18245		t Critical two-tail	2.77645		t Critical two-tail	3.18245	

CD-Hybridoma: Batch 2

0/24	0	24	0/48	0	48	0/72	0	72	0/96	0	96	0/120	0	120	0/144	0	144	24//48	24	48
Mean	8.45E-12	7.02E-12	Mean	8.45E-12	5.48E-12	Mean	8.45E-12	4.94E-12	Mean	8.45E-12	3.66E-12	Mean	8.45E-12	5.37E-12	Mean	8.45E-12	4.52E-12	Mean	7.02E-12	5.48E-12
Variance	9.17E-26	1.1E-24	Variance	9.17E-26	1.01E-24	Variance	9.17E-26	7.33E-25	Variance	9.17E-26	3.76E-25	Variance	9.17E-26	2.84E-25	Variance	9.17E-26	1.9E-25	Variance	1.1E-24	1.01E-24
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	2		df	2		df	2		df	3		df	3		df	4		df	4	
t Stat	2.266723		t Stat	4.893195		t Stat	6.687353		t Stat	12.10519		t Stat	8.697487		t Stat	12.80384		t Stat	1.826466	
P(T<=t) one-tail	0.075792		P(T<=t) one-tail	0.019659		P(T<=t) one-tail	0.010819		P(T<=t) one-tail	0.000607		P(T<=t) one-tail	0.001599		P(T<=t) one-tail	0.000107		P(T<=t) one-tail	0.070904	
t Critical one-tail	2.919986		t Critical one-tail	2.919986		t Critical one-tail	2.919986		t Critical one-tail	2.353363		t Critical one-tail	2.353363		t Critical one-tail	2.131847		t Critical one-tail	2.131847	
P(T<=t) two-tail	0.151583		P(T<=t) two-tail	0.039318		P(T<=t) two-tail	0.021638		P(T<=t) two-tail	0.001213		P(T<=t) two-tail	0.003199		P(T<=t) two-tail	0.000214		P(T<=t) two-tail	0.141808	
t Critical two-tail	4.302653		t Critical two-tail	4.302653		t Critical two-tail	4.302653		t Critical two-tail	3.182446		t Critical two-tail	3.182446		t Critical two-tail	2.776445		t Critical two-tail	2.776445	
24/72	24	72	24/96	24	96	24/120	24	120	24/144	24	144	48/72	48	72	48/96	48	96	48/120	48	120
Mean	7.02E-12	4.94E-12	Mean	7.02E-12	3.66E-12	Mean	7.02E-12	5.37E-12	Mean	7.02E-12	4.52E-12	Mean	5.48E-12	4.94E-12	Mean	5.48E-12	3.66E-12	Mean	5.48E-12	5.37E-12
Variance	1.1E-24	7.33E-25	Variance	1.1E-24	3.76E-25	Variance	1.1E-24	2.84E-25	Variance	1.1E-24	1.9E-25	Variance	1.01E-24	7.33E-25	Variance	1.01E-24	3.76E-25	Variance	1.01E-24	2.84E-25
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	4		df	3		df	3		df	3		df	4		df	3		df	3	
t Stat	2.656294		t Stat	4.775391		t Stat	2.424626		t Stat	3.801837		t Stat	0.717372		t Stat	2.681546		t Stat	0.177782	
P(T<=t) one-tail	0.028304		P(T<=t) one-tail	0.008725		P(T<=t) one-tail	0.04689		P(T<=t) one-tail	0.015982		P(T<=t) one-tail	0.256403		P(T<=t) one-tail	0.037475		P(T<=t) one-tail	0.43511	
t Critical one-tail	2.131847		t Critical one-tail	2.353363		t Critical one-tail	2.353363		t Critical one-tail	2.353363		t Critical one-tail	2.131847		t Critical one-tail	2.353363		t Critical one-tail	2.353363	
P(T<=t) two-tail	0.056609		P(T<=t) two-tail	0.01745		P(T<=t) two-tail	0.09378		P(T<=t) two-tail	0.031965		P(T<=t) two-tail	0.512807		P(T<=t) two-tail	0.07495		P(T<=t) two-tail	0.870221	
t Critical two-tail	2.776445		t Critical two-tail	3.182446		t Critical two-tail	3.182446		t Critical two-tail	3.182446		t Critical two-tail	2.776445		t Critical two-tail	3.182446		t Critical two-tail	3.182446	
48/144	48	144	72/96	72	96	72/120	72	120	72/144	72	144	96/120	96	120	96/144	96	144	120/144	120	144
Mean	5.48E-12	4.52E-12	Mean	4.94E-12	3.66E-12	Mean	4.94E-12	5.37E-12	Mean	4.94E-12	4.52E-12	Mean	3.66E-12	5.37E-12	Mean	3.66E-12	4.52E-12	Mean	5.37E-12	4.52E-12
Variance	1.01E-24	1.9E-25	Variance	7.33E-25	3.76E-25	Variance	7.33E-25	2.84E-25	Variance	7.33E-25	1.9E-25	Variance	3.76E-25	2.84E-25	Variance	3.76E-25	1.9E-25	Variance	2.84E-25	1.9E-25
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	3		df	4		df	3		df	3		df	4		df	4		df	4	
t Stat	1.525614		t Stat	2.095231		t Stat	-0.73797		t Stat	0.752011		t Stat	-3.63238		t Stat	-1.97234		t Stat	2.130715	
P(T<=t) one-tail	0.112262		P(T<=t) one-tail	0.052107		P(T<=t) one-tail	0.257009		P(T<=t) one-tail	0.253334		P(T<=t) one-tail	0.011057		P(T<=t) one-tail	0.059924		P(T<=t) one-tail	0.050064	
t Critical one-tail	2.353363		t Critical one-tail	2.131847		t Critical one-tail	2.353363		t Critical one-tail	2.353363		t Critical one-tail	2.131847		t Critical one-tail	2.131847		t Critical one-tail	2.131847	
P(T<=t) two-tail	0.224525		P(T<=t) two-tail	0.104213		P(T<=t) two-tail	0.514018		P(T<=t) two-tail	0.506667		P(T<=t) two-tail	0.022113		P(T<=t) two-tail	0.119848		P(T<=t) two-tail	0.100127	
t Critical two-tail	3.182446		t Critical two-tail	2.776445		t Critical two-tail	3.182446		t Critical two-tail	3.182446		t Critical two-tail	2.776445		t Critical two-tail	2.776445		t Critical two-tail	2.776445	

CD-Hybridoma: Batch 3

0/24	0	24	0/48	0	48	0/72	0	72	0/96	0	96	0/120	0	120	0/144	0	144	24//48	24	48
Mean	8.17E-12	6.38E-12	Mean	8.17E-12	5.07E-12	Mean	8.17E-12	4.26E-12	Mean	8.17E-12	4.18E-12	Mean	8.17E-12	5.69E-12	Mean	8.17E-12	4.99E-12	Mean	6.38E-12	5.07E-12
Variance	4.42E-25	4.68E-26	Variance	4.42E-25	3.65E-25	Variance	4.42E-25	1.91E-25	Variance	4.42E-25	5.35E-25	Variance	4.42E-25	9.65E-25	Variance	4.42E-25	7.64E-26	Variance	4.68E-26	3.65E-25
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	2		df	4		df	3		df	4		df	4		df	3		df	3	
t Stat	4.447089		t Stat	5.976495		t Stat	8.529198		t Stat	7.001805		t Stat	3.628146		t Stat	7.668799		t Stat	3.521879	
P(T<=t) one-tail	0.023513		P(T<=t) one-tail	0.001969		P(T<=t) one-tail	0.001693		P(T<=t) one-tail	0.001095		P(T<=t) one-tail	0.011098		P(T<=t) one-tail	0.002303		P(T<=t) one-tail	0.019432	
t Critical one-tail	2.919986		t Critical one-tail	2.131847		t Critical one-tail	2.353363		t Critical one-tail	2.131847		t Critical one-tail	2.131847		t Critical one-tail	2.353363		t Critical one-tail	2.353363	
P(T<=t) two-tail	0.047027		P(T<=t) two-tail	0.003939		P(T<=t) two-tail	0.003386		P(T<=t) two-tail	0.00219		P(T<=t) two-tail	0.022196		P(T<=t) two-tail	0.004606		P(T<=t) two-tail	0.038865	
t Critical two-tail	4.302653		t Critical two-tail	2.776445		t Critical two-tail	3.182446		t Critical two-tail	2.776445		t Critical two-tail	2.776445		t Critical two-tail	3.182446		t Critical two-tail	3.182446	
24/72	24	72	24/96	24	96	24/120	24	120	24/144	24	144	48/72	48	72	48/96	48	96	48/120	48	120
Mean	6.38E-12	4.26E-12	Mean	6.38E-12	4.18E-12	Mean	6.38E-12	5.69E-12	Mean	6.38E-12	4.99E-12	Mean	5.07E-12	4.26E-12	Mean	5.07E-12	4.18E-12	Mean	5.07E-12	5.69E-12
Variance	4.68E-26	1.91E-25	Variance	4.68E-26	5.35E-25	Variance	4.68E-26	9.65E-25	Variance	4.68E-26	7.64E-26	Variance	3.65E-25	1.91E-25	Variance	3.65E-25	5.35E-25	Variance	3.65E-25	9.65E-25
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	3		df	2		df	2		df	4		df	4		df	4		df	3	
t Stat	7.53773		t Stat	4.997304		t Stat	1.188027		t Stat	6.87171		t Stat	1.899212		t Stat	1.634236		t Stat	-0.92415	
P(T<=t) one-tail	0.00242		P(T<=t) one-tail	0.018894		P(T<=t) one-tail	0.17839		P(T<=t) one-tail	0.001175		P(T<=t) one-tail	0.065179		P(T<=t) one-tail	0.088774		P(T<=t) one-tail	0.211785	
t Critical one-tail	2.353363		t Critical one-tail	2.919986		t Critical one-tail	2.919986		t Critical one-tail	2.131847		t Critical one-tail	2.131847		t Critical one-tail	2.131847		t Critical one-tail	2.353363	
P(T<=t) two-tail	0.004841		P(T<=t) two-tail	0.037788		P(T<=t) two-tail	0.35678		P(T<=t) two-tail	0.002349		P(T<=t) two-tail	0.130357		P(T<=t) two-tail	0.177548		P(T<=t) two-tail	0.42357	
t Critical two-tail	3.182446		t Critical two-tail	4.302653		t Critical two-tail	4.302653		t Critical two-tail	2.776445		t Critical two-tail	2.776445		t Critical two-tail	2.776445		t Critical two-tail	3.182446	
48/144	48	144	72/96	72	96	72/120	72	120	72/144	72	144	96/120	96	120	96/144	96	144	120/144	120	144
Mean	5.07E-12	4.99E-12	Mean	4.26E-12	4.18E-12	Mean	4.26E-12	5.69E-12	Mean	4.26E-12	4.99E-12	Mean	4.18E-12	5.69E-12	Mean	4.18E-12	4.99E-12	Mean	5.69E-12	4.99E-12
Variance	3.65E-25	7.64E-26	Variance	1.91E-25	5.35E-25	Variance	1.91E-25	9.65E-25	Variance	1.91E-25	7.64E-26	Variance	5.35E-25	9.65E-25	Variance	5.35E-25	7.64E-26	Variance	9.65E-25	7.64E-26
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	3		df	3		df	3		df	3		df	4		df	3		df	2	
t Stat	0.227426		t Stat	0.156844		t Stat	-2.309		t Stat	-2.44631		t Stat	-2.13644		t Stat	-1.78978		t Stat	1.192623	
P(T<=t) one-tail	0.417355		P(T<=t) one-tail	0.442664		P(T<=t) one-tail	0.052063		P(T<=t) one-tail	0.045991		P(T<=t) one-tail	0.049743		P(T<=t) one-tail	0.085713		P(T<=t) one-tail	0.177662	
t Critical one-tail	2.353363		t Critical one-tail	2.353363		t Critical one-tail	2.353363		t Critical one-tail	2.353363		t Critical one-tail	2.131847		t Critical one-tail	2.353363		t Critical one-tail	2.919986	
P(T<=t) two-tail	0.83471		P(T<=t) two-tail	0.885329		P(T<=t) two-tail	0.104126		P(T<=t) two-tail	0.091981		P(T<=t) two-tail	0.099485		P(T<=t) two-tail	0.171426		P(T<=t) two-tail	0.355325	
t Critical two-tail	3.182446		t Critical two-tail	3.182446		t Critical two-tail	3.182446		t Critical two-tail	3.182446		t Critical two-tail	2.776445		t Critical two-tail	3.182446		t Critical two-tail	4.302653	

CD-Hybridoma: Batch 4

0/24			0/48			0/72			0/96			0/120			0/144			24//48		
0	24		0	48		0	72		0	96		0	120		0	144		24	48	
Mean	8.08E-12	6.55E-12	Mean	8.08E-12	4.83E-12	Mean	8.08E-12	3.97E-12	Mean	8.08E-12	5.52E-12	Mean	8.08E-12	5.01E-12	Mean	8.08E-12	4.61E-12	Mean	6.55E-12	4.83E-12
Variance	2.87E-25	1.38E-24	Variance	2.87E-25	3.27E-25	Variance	2.87E-25	1.38E-25	Variance	2.87E-25	3.54E-25	Variance	2.87E-25	2.52E-25	Variance	2.87E-25	4.94E-25	Variance	1.38E-24	3.27E-25
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	3		df	4		df	4		df	4		df	4		df	4		df	3	
t Stat	2.056094		t Stat	7.188024		t Stat	10.93401		t Stat	5.55411		t Stat	7.255096		t Stat	6.8025		t Stat	2.28295	
P(T<=t) one-tail	0.065995		P(T<=t) one-tail	0.000992		P(T<=t) one-tail	0.000199		P(T<=t) one-tail	0.002572		P(T<=t) one-tail	0.000958		P(T<=t) one-tail	0.00122		P(T<=t) one-tail	0.053323	
t Critical one-tail	2.353363		t Critical one-tail	2.131847		t Critical one-tail	2.131847		t Critical one-tail	2.131847		t Critical one-tail	2.131847		t Critical one-tail	2.131847		t Critical one-tail	2.353363	
P(T<=t) two-tail	0.13199		P(T<=t) two-tail	0.001985		P(T<=t) two-tail	0.000397		P(T<=t) two-tail	0.005143		P(T<=t) two-tail	0.001916		P(T<=t) two-tail	0.00244		P(T<=t) two-tail	0.106645	
t Critical two-tail	3.182446		t Critical two-tail	2.776445		t Critical two-tail	2.776445		t Critical two-tail	2.776445		t Critical two-tail	2.776445		t Critical two-tail	2.776445		t Critical two-tail	3.182446	
24/72			24/96			24/120			24/144			48/72			48/96			48/120		
24	72		24	96		24	120		24	144		48	72		48	96		48	120	
Mean	6.55E-12	3.97E-12	Mean	6.55E-12	5.52E-12	Mean	6.55E-12	5.01E-12	Mean	6.55E-12	4.61E-12	Mean	4.83E-12	3.97E-12	Mean	4.83E-12	5.52E-12	Mean	4.83E-12	5.01E-12
Variance	1.38E-24	1.38E-25	Variance	1.38E-24	3.54E-25	Variance	1.38E-24	2.52E-25	Variance	1.38E-24	4.94E-25	Variance	3.27E-25	1.38E-25	Variance	3.27E-25	3.54E-25	Variance	3.27E-25	2.52E-25
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	2		df	3		df	3		df	3		df	3		df	4		df	4	
t Stat	3.634853		t Stat	1.363373		t Stat	2.094374		t Stat	2.455209		t Stat	2.190787		t Stat	-1.43801		t Stat	-0.40383	
P(T<=t) one-tail	0.034026		P(T<=t) one-tail	0.133032		P(T<=t) one-tail	0.063623		P(T<=t) one-tail	0.045628		P(T<=t) one-tail	0.058084		P(T<=t) one-tail	0.111906		P(T<=t) one-tail	0.353498	
t Critical one-tail	2.919986		t Critical one-tail	2.353363		t Critical one-tail	2.353363		t Critical one-tail	2.353363		t Critical one-tail	2.353363		t Critical one-tail	2.131847		t Critical one-tail	2.131847	
P(T<=t) two-tail	0.068052		P(T<=t) two-tail	0.266063		P(T<=t) two-tail	0.127245		P(T<=t) two-tail	0.091255		P(T<=t) two-tail	0.116169		P(T<=t) two-tail	0.223813		P(T<=t) two-tail	0.706996	
t Critical two-tail	4.302653		t Critical two-tail	3.182446		t Critical two-tail	3.182446		t Critical two-tail	3.182446		t Critical two-tail	3.182446		t Critical two-tail	2.776445		t Critical two-tail	2.776445	
48/144			72/96			72/120			72/144			96/120			96/144			120/144		
48	144		72	96		72	120		72	144		96	120		96	144		120	144	
Mean	4.83E-12	4.61E-12	Mean	3.97E-12	5.52E-12	Mean	3.97E-12	5.01E-12	Mean	3.97E-12	4.61E-12	Mean	5.52E-12	5.01E-12	Mean	5.52E-12	4.61E-12	Mean	5.01E-12	4.61E-12
Variance	3.27E-25	4.94E-25	Variance	1.38E-25	3.54E-25	Variance	1.38E-25	2.52E-25	Variance	1.38E-25	4.94E-25	Variance	3.54E-25	2.52E-25	Variance	3.54E-25	4.94E-25	Variance	2.52E-25	4.94E-25
Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3	Observations	3	3
Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0		Hypothesized Mean	0	
df	4		df	3		df	4		df	3		df	4		df	4		df	4	
t Stat	0.417195		t Stat	-3.8227		t Stat	-2.88536		t Stat	-1.40391		t Stat	1.129951		t Stat	1.699428		t Stat	0.793581	
P(T<=t) one-tail	0.348977		P(T<=t) one-tail	0.015758		P(T<=t) one-tail	0.022386		P(T<=t) one-tail	0.127479		P(T<=t) one-tail	0.160832		P(T<=t) one-tail	0.082233		P(T<=t) one-tail	0.235929	
t Critical one-tail	2.131847		t Critical one-tail	2.353363		t Critical one-tail	2.131847		t Critical one-tail	2.353363		t Critical one-tail	2.131847		t Critical one-tail	2.131847		t Critical one-tail	2.131847	
P(T<=t) two-tail	0.697954		P(T<=t) two-tail	0.031516		P(T<=t) two-tail	0.044771		P(T<=t) two-tail	0.254958		P(T<=t) two-tail	0.321664		P(T<=t) two-tail	0.164465		P(T<=t) two-tail	0.471858	
t Critical two-tail	2.776445		t Critical two-tail	3.182446		t Critical two-tail	2.776445		t Critical two-tail	3.182446		t Critical two-tail	2.776445		t Critical two-tail	2.776445		t Critical two-tail	2.776445	

XIV. Experimental results: GS-NS0 Fed-batch in flasks, serum-free CD-Hybridoma. Optimisation

Experimental results for fed-batch culture used for parameter estimation.

Time	Viable cells	Apoptotic cells	Dead cells	Total cells	ATP	mAb	Lactate	Glucose	Aspartate	Glutamate	Asparagine	Serine	Glutamine	Histidine	Glycine	Threonine	Arginine	Alanine	Tyrosine	Cysteine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine	Leucine	Lysine	Proline
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mg/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
0	2.04E+08	1.26E+07	8.91E+06	2.25E+08	2.55E-03	0.00	0.00	19.77	0.15	1.35	2.19	2.21	0.04	0.14	0.12	0.69	1.13	3.21	0.37	0.13	1.10	0.32	0.19	0.61	1.19	1.85	1.34	2.00
24	4.05E+08	2.36E+07	1.92E+07	4.48E+08	2.34E-03	0.00	0.00	19.10	0.19	1.08	2.15	1.99	0.09	0.14	0.24	0.67	1.04	3.26	0.34	0.12	0.99	0.29	0.18	0.58	1.03	1.61	1.23	1.85
48	7.27E+08	6.43E+07	7.01E+07	8.61E+08	4.05E-03	2.05	0.77	17.83	0.09	0.56	2.11	1.67	0.08	0.13	0.50	0.57	0.92	3.34	0.30	0.11	0.88	0.26	0.16	0.53	0.88	1.36	1.13	1.89
60	1.10E+09	8.40E+07	5.72E+07	1.24E+09	5.45E-03	13.12	3.13	16.70	0.09	0.26	2.09	1.74	0.07	0.13	0.70	0.57	0.87	3.46	0.27	0.09	0.80	0.22	0.14	0.49	0.79	1.21	1.05	1.75
60.0167	1.08E+09	8.21E+07	5.59E+07	1.22E+09	5.32E-03	12.82	3.06	16.32	0.19	0.94	2.17	2.22	0.07	0.16	0.68	0.65	1.05	3.39	0.31	0.10	0.93	0.27	0.15	0.54	0.97	1.51	1.16	1.75
72	1.46E+09	1.03E+08	6.01E+07	1.63E+09	5.29E-03	22.08	3.47	14.73	0.04	0.26	2.09	1.74	0.07	0.13	0.70	0.57	0.87	3.46	0.27	0.09	0.80	0.22	0.14	0.49	0.79	1.21	1.05	1.75
72.0167	1.45E+09	1.02E+08	5.93E+07	1.61E+09	5.22E-03	21.81	3.42	14.55	0.10	0.63	2.13	2.00	0.07	0.15	0.69	0.61	0.97	3.42	0.29	0.09	0.87	0.25	0.15	0.52	0.89	1.37	1.11	1.75
78	1.56E+09	1.18E+08	1.01E+08	1.78E+09	6.30E-03	27.70	4.17	13.83	0.04	0.27	2.12	1.89	0.07	0.13	0.77	0.59	0.89	3.59	0.27	0.09	0.80	0.23	0.14	0.49	0.81	1.22	1.08	1.76
78.0167	1.53E+09	1.17E+08	9.93E+07	1.75E+09	6.21E-03	27.30	4.11	13.63	0.11	0.70	2.17	2.19	0.07	0.15	0.76	0.64	1.00	3.54	0.30	0.10	0.88	0.26	0.15	0.52	0.92	1.41	1.15	1.76
84	1.98E+09	1.29E+08	9.66E+07	2.20E+09	7.20E-03	34.85	5.43	12.50	0.04	0.30	2.15	2.07	0.07	0.11	0.85	0.56	0.91	3.71	0.28	0.10	0.81	0.23	0.14	0.50	0.82	1.25	1.10	1.84
84.0167	1.91E+09	1.25E+08	9.34E+07	2.13E+09	6.96E-03	33.69	5.25	12.08	0.16	1.06	2.22	2.59	0.07	0.15	0.82	0.65	1.11	3.59	0.32	0.11	0.95	0.28	0.15	0.55	1.01	1.57	1.21	1.81
96	2.45E+09	1.69E+08	1.18E+08	2.74E+09	1.01E-02	42.65	4.93	10.47	0.02	0.18	2.15	2.30	0.06	0.14	0.97	0.59	0.87	3.97	0.26	0.10	0.78	0.22	0.13	0.48	0.78	1.19	1.04	1.77
96.0167	2.41E+09	1.66E+08	1.15E+08	2.69E+09	9.89E-03	41.88	4.84	10.28	0.11	0.73	2.21	2.68	0.06	0.16	0.95	0.65	1.02	3.90	0.30	0.11	0.88	0.26	0.14	0.51	0.92	1.43	1.12	1.76
102	2.28E+09	2.26E+08	2.19E+08	2.72E+09	9.67E-03	54.51	7.13	9.17	0.04	0.24	2.15	2.54	0.06	0.14	1.03	0.61	0.88	4.12	0.27	0.11	0.77	0.23	0.13	0.48	0.79	1.21	1.07	1.60
102.0167	2.23E+09	2.21E+08	2.14E+08	2.67E+09	9.48E-03	53.42	6.99	8.98	0.13	0.85	2.21	2.96	0.06	0.17	1.01	0.68	1.04	4.03	0.30	0.12	0.89	0.27	0.14	0.52	0.95	1.47	1.17	1.60
108	2.62E+09	2.34E+08	1.48E+08	3.01E+09	1.23E-02	65.48	9.60	8.63	0.06	0.37	2.12	2.83	0.07	0.12	1.09	0.59	0.91	4.26	0.27	0.12	0.78	0.24	0.12	0.48	0.81	1.27	1.11	1.45
108.0167	2.56E+09	2.28E+08	1.45E+08	2.93E+09	1.20E-02	63.86	9.36	8.42	0.17	1.12	2.20	3.34	0.06	0.15	1.06	0.68	1.11	4.15	0.32	0.13	0.92	0.29	0.13	0.53	1.01	1.59	1.23	1.45
120	3.08E+09	4.38E+08	1.88E+08	3.71E+09	1.33E-02	145.45	11.27	4.47	0.02	0.12	2.12	3.16	0.07	0.15	1.15	0.62	0.83	4.71	0.26	0.11	0.74	0.23	0.11	0.47	0.76	1.18	1.08	1.59
120.0167	3.03E+09	4.31E+08	1.85E+08	3.65E+09	1.31E-02	143.00	11.08	4.39	0.10	0.63	2.17	3.50	0.07	0.18	1.13	0.68	0.96	4.63	0.29	0.12	0.84	0.27	0.12	0.50	0.89	1.40	1.16	1.59
126	3.23E+09	5.89E+08	2.90E+08	4.11E+09	1.16E-02	153.14	11.93	2.73	0.03	0.19	2.10	3.42	0.08	0.16	1.15	0.63	0.80	4.86	0.26	0.12	0.74	0.23	0.11	0.47	0.77	1.20	1.09	1.57
126.0167	3.15E+09	5.75E+08	2.83E+08	4.01E+09	1.13E-02	149.39	11.64	2.67	0.14	0.93	2.17	3.91	0.07	0.19	1.12	0.72	1.00	4.74	0.30	0.13	0.88	0.28	0.12	0.52	0.96	1.53	1.21	1.57
132	3.16E+09	7.19E+08	2.97E+08	4.17E+09	1.15E-02	162.92	12.63	0.87	0.00	0.00	2.08	3.34	0.07	0.12	1.18	0.56	0.64	5.11	0.23	0.10	0.66	0.21	0.10	0.44	0.64	0.99	1.02	1.62
132.0167	3.10E+09	7.06E+08	2.92E+08	4.10E+09	1.13E-02	160.05	12.41	6.27	0.06	0.39	2.11	3.57	0.06	0.14	1.16	0.60	0.74	5.02	0.25	0.11	0.73	0.23	0.10	0.46	0.74	1.16	1.07	1.61
144	2.97E+09	1.00E+09	4.81E+08	4.45E+09	1.19E-02	180.58	15.80	4.57	0.01	0.05	1.95	3.55	0.11	0.15	1.18	0.58	0.56	5.16	0.23	0.10	0.63	0.20	0.09	0.42	0.64	1.00	1.00	1.46
144.0167	2.88E+09	9.75E+08	4.67E+08	4.32E+09	1.16E-02	175.36	15.34	9.78	0.12	0.78	2.02	4.01	0.11	0.19	1.14	0.66	0.76	5.01	0.27	0.11	0.77	0.25	0.10	0.47	0.83	1.31	1.11	1.45
156	2.61E+09	1.24E+09	7.24E+08	4.57E+09	1.14E-02	194.72	15.97	7.57	0.08	0.34	1.84	3.92	0.20	0.15	1.24	0.59	0.55	5.31	0.25	0.11	0.69	0.23	0.09	0.44	0.73	1.16	1.05	1.64
156.0167	2.57E+09	1.22E+09	7.15E+08	4.51E+09	1.12E-02	192.27	15.77	7.47	0.14	0.72	1.88	4.16	0.19	0.16	1.23	0.64	0.65	5.24	0.27	0.11	0.76	0.25	0.09	0.46	0.83	1.32	1.11	1.64
168	2.09E+09	1.35E+09	1.05E+09	4.49E+09	8.78E-03	225.29	17.47	5.97	0.17	0.86	1.65	4.08	0.27	0.17	1.25	0.62	0.57	5.00	0.26	0.12	0.73	0.25	0.09	0.44	0.82	1.33	1.07	1.77

Validation of feeding strategy for fed-batch cultures.

Time	Viable	Apoptotic	Dead	Total	ATP	mAb	Lactate	Glucose	Aspartate	Glutamate	Asparagine	Serine	Glutamine	Histidine	Glycine	Threonine	Arginine	Alanine	Tyrosine	Cysteine	Valine	Methionine	Tryptophan	Phenylalanine	Isoleucine	Leucine	Lysine	Proline
[h]	[Cells/L]	[Cells/L]	[Cells/L]	[Cells/L]	[mM]	[mg/L]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]	[mM]
0	221666666.7	1.37E+07	7.47E+06	2.43E+08	2.98E-03	0.00	0.07	14.45	0.23	1.28	2.09	2.10	0.04	0.27	0.12	0.72	1.08	3.15	0.35	0.13	1.06	0.33	0.19	0.59	1.11	1.81	1.32	1.71
24	408000000	2.64E+07	1.79E+07	4.52E+08	4.28E-03	0.00	0.00	13.30	0.19	1.04	2.07	1.93	0.08	0.23	0.25	0.69	1.03	3.28	0.33	0.10	0.99	0.30	0.18	0.56	1.00	1.62	1.26	1.72
48	864333333.3	6.31E+07	6.15E+07	9.89E+08	7.54E-03	5.99	0.07	11.55	0.08	0.48	2.02	1.61	0.08	0.21	0.47	0.62	0.91	3.34	0.29	0.08	0.85	0.25	0.16	0.50	0.82	1.32	1.13	1.56
72	1443333333	1.13E+08	7.22E+07	1.63E+09	1.03E-02	21.86	0.78	10.51	0.00	0.00	1.91	1.31	0.06	0.18	0.69	0.51	0.68	3.39	0.22	0.04	0.61	0.18	0.14	0.43	0.49	0.79	0.95	1.43
72.0167	1425931003	1.11E+08	7.13E+07	1.61E+09	1.02E-02	21.59	0.77	10.38	0.02	0.13	1.89	1.35	0.05	0.19	0.68	0.52	0.70	3.35	0.23	0.04	0.63	0.19	0.14	0.43	0.53	0.84	0.96	1.42
84	1806666667	1.20E+08	2.07E+07	1.95E+09	1.10E-02	24.28	1.78	9.44	0.00	0.00	1.81	1.19	0.05	0.16	0.76	0.44	0.52	3.30	0.19	0.02	0.47	0.15	0.12	0.38	0.31	0.49	0.84	1.29
84.0167	1794597770	1.19E+08	2.06E+07	1.93E+09	1.10E-02	24.11	1.76	9.37	0.04	0.24	1.82	1.29	0.05	0.17	0.76	0.47	0.56	3.27	0.20	0.03	0.52	0.16	0.13	0.40	0.39	0.60	0.87	1.29
96	2060000000	1.58E+08	1.01E+08	2.32E+09	1.14E-02	42.01	2.74	8.55	0.01	0.05	1.90	1.21	0.04	0.16	0.81	0.39	0.35	3.22	0.15	0.01	0.34	0.12	0.11	0.34	0.20	0.29	0.75	1.09
96.0167	1980208347	1.52E+08	9.72E+07	2.23E+09	1.09E-02	40.38	2.63	8.22	0.25	1.45	1.94	1.79	0.04	0.21	0.78	0.53	0.58	3.09	0.21	0.03	0.65	0.22	0.12	0.41	0.62	0.95	0.89	1.10
108	2283333333	1.77E+08	1.16E+08	2.58E+09	1.32E-02	61.99	2.22	6.46	0.11	0.24	1.59	1.57	0.04	0.15	0.81	0.41	0.33	3.26	0.15	0.03	0.47	0.16	0.11	0.35	0.43	0.64	0.76	1.17
108.0167	2181706814	1.69E+08	1.11E+08	2.46E+09	1.26E-02	59.24	2.12	6.17	0.38	1.83	1.64	2.23	0.04	0.21	0.77	0.57	0.60	3.11	0.22	0.05	0.82	0.27	0.13	0.43	0.90	1.38	0.92	1.18
120	2843333333	2.11E+08	1.38E+08	3.19E+09	1.35E-02	139.61	3.15	3.94	0.09	0.53	1.01	1.30	0.03	0.11	0.57	0.29	0.21	2.30	0.10	0.03	0.38	0.12	0.06	0.22	0.40	0.61	0.49	0.71
120.0167	2689045707	2.00E+08	1.30E+08	3.02E+09	1.28E-02	132.03	2.97	8.47	0.43	2.52	1.12	2.15	0.03	0.19	0.54	0.50	0.55	2.18	0.19	0.06	0.83	0.27	0.08	0.33	1.00	1.54	0.71	0.75
132	2890000000	2.46E+08	1.75E+08	3.31E+09	1.26E-02	151.88	5.18	4.77	0.25	1.55	1.42	2.45	0.05	0.18	0.90	0.49	0.36	3.72	0.15	0.06	0.75	0.24	0.09	0.35	0.85	1.32	0.76	1.04
132.0167	2860123397	2.44E+08	1.74E+08	3.28E+09	1.25E-02	150.31	5.13	4.72	0.31	1.93	1.43	2.60	0.05	0.19	0.89	0.53	0.43	3.68	0.17	0.06	0.83	0.27	0.09	0.37	0.96	1.49	0.80	1.04
144	3446666667	3.15E+08	1.46E+08	3.91E+09	1.18E-02	178.55	9.29	1.96	0.08	0.49	1.28	2.49	0.07	0.17	0.91	0.41	0.15	4.38	0.10	0.05	0.63	0.20	0.05	0.29	0.75	1.14	0.61	1.04
144.0167	3380129710	3.09E+08	1.43E+08	3.83E+09	1.16E-02	175.10	9.11	6.66	0.21	1.23	1.32	2.78	0.07	0.20	0.89	0.49	0.28	4.30	0.13	0.06	0.79	0.25	0.06	0.32	0.96	1.47	0.69	1.05
156	3750000000	4.30E+08	2.00E+08	4.38E+09	1.51E-02	205.82	11.51	2.46	0.03	0.18	1.19	2.56	0.07	0.16	0.88	0.39	0.10	4.64	0.08	0.04	0.62	0.19	0.03	0.26	0.77	1.16	0.55	1.03
156.0167	3616395127	4.14E+08	1.93E+08	4.22E+09	1.45E-02	198.48	11.10	2.37	0.41	2.41	1.33	3.48	0.07	0.24	0.85	0.62	0.49	4.47	0.18	0.08	1.12	0.35	0.06	0.39	1.42	2.18	0.80	1.08
168	3686666667	6.01E+08	3.29E+08	4.62E+09	1.33E-02	221.00	12.69	3.42	0.13	0.81	1.10	2.99	0.15	0.19	0.85	0.47	0.21	4.72	0.11	0.06	0.87	0.27	0.03	0.30	1.14	1.77	0.64	1.14
168.0167	3584229282	5.84E+08	3.20E+08	4.49E+09	1.29E-02	214.86	12.34	7.90	0.36	2.17	1.18	3.53	0.15	0.24	0.82	0.62	0.44	4.59	0.17	0.08	1.16	0.37	0.05	0.38	1.53	2.38	0.79	1.16
192	3046666667	9.88E+08	8.87E+08	4.92E+09	1.19E-02	269.56	14.25	0.63	0.05	0.28	0.96	3.15	0.55	0.22	0.99	0.52	0.13	5.35	0.13	0.07	1.00	0.33	0.05	0.34	1.39	2.19	0.70	1.46
192.0167	3034003645	9.84E+08	8.84E+08	4.90E+09	1.19E-02	268.44	14.19	5.24	0.05	0.27	0.95	3.14	0.55	0.22	0.99	0.52	0.13	5.33	0.13	0.07	1.00	0.33	0.05	0.34	1.38	2.18	0.70	1.45
216	1700000000	1.40E+09	1.83E+09	4.93E+09		313.87	16.06	2.48	0.01	0.03	0.76	2.96	0.61	0.22	1.14	0.49	0.03	5.61	0.10	0.06	0.94	0.30	0.04	0.31	1.31	2.06	0.65	1.50