Universidade do Minho
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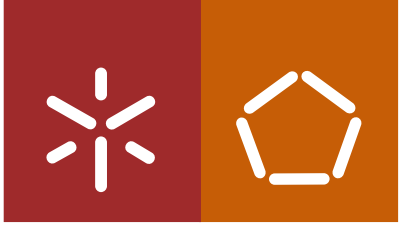

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Development of chemically defined medium for biopharmaceuticals production using mammalian cell lines guided by metabolic modelling tools and metabolomics measurements

Doctoral Thesis
Doctoral Program in Bioengineering

Work developed under supervision of
Professor Isabel Cristina A. Pereira da Rocha

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ABSTRACT

Development of chemically defined medium for biopharmaceuticals production using mammalian cell lines guided through metabolic modelling tools and metabolomics measurements

Systems biology and metabolic engineering tools hold a tremendous promise in improving biomanufacturing attributes. The emergence of omics tools and computational modeling potentiated the development of new approaches to optimize several expression platforms, in particular mammalian cell lines of which Chinese hamster ovary (CHO) cells, the most used platform for recombinant proteins production. This optimization envisions not only growth parameters of CHO, but also the final product titers.

In this context, a CHO genome scale metabolic model (iCHO1766) and flux balance analysis (FBA) were used to study metabolic mechanisms in response to variations in environmental constraints (e.g., amino acids levels) aiming at optimizing cell culture medium formulations. Hence, iCHO1766, combined with an in-house developed algorithm (OptiModels) was first used to determine the minimal medium formulation able to sustain growth of both naïve and recombinant CHO cells lines. Subsequently, based on the prediction results, α-ketoglutarate (AKG) was determined as a potential media supplement and its effect on culture was investigated experimentally. Further, spent media analyses were performed to understand the influence of AKG on CHO metabolism and media formulation was optimized based on balancing the levels of non-essential amino acids together with supplementing AKG and ammonium.

As a result of adding AKG to the media, growth parameters were improved, and ammonia accumulation during the process was reduced. In addition, recombinant protein titers were increased by 1.9-fold. Following, specific productivities were improved when rebalancing nutrient levels in the media, together with supplementing AKG, leading to more efficient metabolic features of CHO.

In conclusion, *in silico*-based approaches for medium optimization are powerful tools for predicting the metabolic interconnexions within a cell and hold great potential in improving media design and bioprocess optimization.

Key words: CHO cells, GSMM, media optimization, α-ketoglutarate (AKG).

RESUMO

Desenvolvimento de meio definido para produção de biofármacos usando células de mamíferos guiado por modelos metabólicos e metabolómica

As ferramentas de biologia de sistemas e engenharia metabólica constituem uma grande promessa na melhoria do desempenho da bio-manufactura. As ferramentas "ómicas" e bioinformáticas potencializaram o desenvolvimento de novas abordagens para otimizar os parâmetros de crescimento e o rendimento do produto final em diversas plataformas de expressão, em particular linhas de células de mamíferos, sendo as células de ovário de Hamster Chinês ("CHO") uma das linhas celulares mais utilizadas para a produção de proteínas recombinantes.

Neste contexto, o modelo metabólico à escala do genoma (GSMM) de células CHO iCHO1766 foi utilizado com o objetivo de estudar o comportamento metabólico das células em resposta a variações nas restrições ambientais, por exemplo, níveis de aminoácidos, visando a otimização da formulação do meio de cultura para células CHO. Para estudar essa influência, o modelo, combinado com um algoritmo desenvolvido internamente, foi usado para determinar a formulação de meio mínima para sustentar o crescimento de CHO não recombinantes, bem como de células recombinantes. Portanto, com base nos resultados da previsão, a suplementação de diferentes níveis de α-cetoglutarato (AKG) à composição do meio padrão foi estudada experimentalmente, e foi realizada uma análise do meio resultante para avaliar os efeitos de AKG sobre o metabolismo de CHO. Por fim, a formulação do meio de cultura foi otimizada com base no equilíbrio dos níveis de aminoácidos não essenciais em conjunto com a suplementação de AKG e amónio. A suplementação com diferentes níveis de AKG permitiu melhorar os parâmetros de crescimento e reduzir a acumulação de amónia. Foi ainda observado um aumento nas concentrações e produtividades específicas das células produtoras, sendo que esta foi melhorada em 1,9 vezes. Por conseguinte, ao utilizar a formulação de meio otimizado, observou-se um aumento da produtividade específica das células, bem como características metabólicas mais eficientes. As abordagens *in silico* para otimização de meio são, assim, ferramentas poderosas para prever a interconexão metabólica na célula e possuem um grande potencial para melhorar o desenho e otimização do meio de culturas.

Palavras-chave: células CHO, GSMM, otimização do meio de cultura, α-cetoglutarato (AKG).

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LIST OF ABBREVIATIONS AND ACRONYMS

1,3BP6	1,3 bisphosphoglycerate
2PG	3-pPhosphoglyceric acid
3PG	2-pPhosphoglyceric acid
AA	Amino acids
ACA	Anti-clumping agent
AcCoA	AcetylCoA
AKG	α-ketoglutarate
ALA	Alanine
ALAAT	Alanine aminotransferase
AlaGln	l-alanyl-l-glutamine
ALT	Alanine transaminase
AMP	Adenosine monophosphate
ASP	Aspartate
ATP	Adenosine Triphosphate
bp	Base pairs
BPCY	Biomass product coupled yield
CCD	Cumulative cell density
cdkis	Cyclin-dependent kinase inhibitors
CDM	Chemically defined media
CHO	Chinese hamster ovary
CIT	Citrate
CO2	Carbon dioxide
CRISPR	Clustered regularly interspaced short palindromic repeats
DHAP	Dihydroxyacetone phosphate
DHFR	Dihydrofolate reductase
DoE	Design of experiment
DSP	Downstream processing
E. coli	Escherichia coli
EAAs	Essential amino acids
FBA	Flux balance analysis

FDA	Food and drug administration
FMOC	Fluorenylmethyloxycarbonyl
FRAMED	Framework for metabolic engineering and design
FRU6P	Fructose-6-pPhosphate
FVA	Flux variability analysis
GCL3P	Glucose-3-pPhosphate
GDH	Glutamate dehydrogenase
GLC	Glucose
GLC6P	Glucose-6-pPhosphate
GLN	Glutamine
GLU	Glutamate
GOI	Gene of interest
GPR	Genes proteins and reactions
GS	Glutamine synthase
GSH	Glutathione
GSMM	Genome scale metabolic model
HCP	Host cell proteins
HER-2	Human epidermal growth factor receptor 2
IgG	Immunoglobulin G
IVCD	Integral viable cell density
LAC	Lactate
lncRNAs	Long non-coding ribonucleic acid
LP	Linear programming
LTM	Logic transformation of model
mAbs	Monoclonal antibodies
MAL	Malate
Mbp	Mega base pairs
MeOH	Methanol
MILP	Mixed integer linear programming
mM	Millimolar
MSX	Methionine sulfoximine
mTOR	Mechanistic target of rapamycin

MTX	Methotrexate
NAD	Adenine dinucleotide
NEAAs	Non-essential amino acids
OAA	Oxaloacetate
ODEs	Ordinary differential equations
OPA	O-phthalaldehyde
P5P	Pyridoxal-5'-phosphate
PEP	Phosphoenolpyruvate
pFBA	Parsimonious flux balance analysis
PPP	Pentose phosphate pathway
PTMs	Post translational modifications
PYR	Pyruvate
R&D	Research and development
rcf	Relative centrifugal force
rpm	Rotation per minute
SDH	Serine ammonia lyase
siRNAs	Small interfering ribonucleic acids
SNPs	Single nucleotide polymorphisms
TCA	Tricarboxylic acid
tPA	Tissue plasminogen activator
USP	Upstream processing
VCD	Viable cell density
VCV	Viable cell volume
µm	Micrometer

SCIENTIFIC OUTPUT

According to the 2nd paragraph of the article 8 of the Portuguese Decree-Law no. 388/70, the scientific outputs of this thesis are listed below.

The results presented in this thesis have been partially published elsewhere.

Peer reviewed journal articles:

Hamdi, A.; Széliová, D.; Ruckerbauer, D.E.; Rocha, I.; Borth, N.; Zanghellini, J. Key Challenges in Designing CHO Chassis Platforms. Processes **2020**, 8, 643. https://doi.org/10.3390/pr8060643

Oral presentation in conferences:

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Posters in conferences:

Hamdi, A.; Santos, S.; Rocha, I; Zanghellini, J. *In silico*-based approaches towards optimization of CHO cell culture medium. European Summit of Industrial Biotechnology, Graz, Austria, 2019.

Hamdi, A.; Santos, S.; Baumann, M.; Borth, N.; Zanghellini, J, Rocha, I. Towards improvement of cho cells culture medium using *in silico*-based approaches. Cell culture engineering, Tucson, Arizona, U.S, 2020 (Postponed conference).

Peer reviewed journal articles in preparation:

Hamdi A., Santos S., Borth, N., Széliová, D., Zanghellini, J., Rocha I.; *In silico*-based approach for medium optimization of CHO cells (To be submitted in 2021).

CHAPTER 1

Motivation and outline of the thesis

Biopharmaceuticals or biologics are large molecules derived from living organisms that, in the correct structure, can be very effective for preventing or treating a wide range of conditions such as infectious diseases and cancer. Examples of such biologics are vaccines, recombinant proteins and growth factors [1]. Along the years, there has been a continuous demand for developing complex biopharmaceuticals and the corresponding manufacturing processes, due to their therapeutic potential and global need in case of epidemics to control potential outbreaks that can be induced by continuously mutating pathogens (e.g., Influenza viruses or SARS-CoV) [2]. Indeed, the increased adoption of bio-based products holds a tremendous promise in improving current prevention and therapeutic procedures, especially in the field of vaccinology, gene therapy and cancer treatment [3].

Long time prior to the emergence of recombinant DNA technology, biotherapeutics were isolated from animals (e.g., Insulin being isolated from cows and pigs) or produced in animal tissues [4,5]. Following the revolutionary discovery of DNA recombination, biologics shifted to be produced in microbes, *in vitro*. The insertion of the insulin gene into a bacterial genome was an important stepping stone that drove the large scale manufacturing of human-insulin precursors in alternative producers already in the late 1970s, with the major production cell factories being *Escherichia coli* (*E.coli*) and *Saccharomyces cerevisiae* [6,7]. These efforts resulted in the approval of the first biopharmaceutical product by the regulatory bodies in the early 80s, Humulin® produced by DNA recombination technology using bacterial cells [8,9]. Due to its rapid growth to high cell densities in cheap and simple media formulations and the ease with which it can be genetically manipulated, *E. coli* remains a prime production host in the biopharmaceutical and biotechnological industries [10–13].

Bacterial systems are not the only hosts that have been used for insulin production. Other cell factories such as yeast systems have been explored as a result of advancement in genetic engineering [14,15]. For peptides/proteins that require the formation of disulfide bonds, including insulin as the best-known example, several yeast species have been explored and now have well established platform technologies available [16]. However, for the production of high value biotherapeutics that require human-like glycosylation or other types of complex post-translational modifications (PTMs), complex organisms, such as mammalian cells are required [17,18].

As a result of these breakthrough technologies over the last 25 years, the pharmaceutical industry invested a great deal of resources into research and development (R&D) [19], aiming at generating groundbreaking complex biologics using heterologous expression in mammalian platforms, mainly the Chinese hamster ovary cells (CHO) due to its potential in producing high-quality biopharmaceuticals [20,21].

Since then, innovation in the biopharmaceutical field triggered the development of various novel compounds that demonstrated great therapeutic potential towards the treatment of both existing and emerging diseases [22]. The spectrum of produced biologics broadened along the years and the focus shifted towards even more sophisticated bio-based therapies, that are unable to be produced by microbial systems. Ergo, mammalian cell factories became one of the most important systems for the manufacturing of complex biopharmaceuticals such as monoclonal antibodies and recombinant therapeutic proteins. Since the introduction of human tissue plasminogen activator (tPA) to the market, the first bio-based therapy generated by mammalian cells, the biopharmaceutical industry continues to generate thumping profits overtime [23].

Currently, 316 biopharmaceuticals are on the market [24]. In fact, about 51% of the total produced biotherapeutic proteins are generated using mammalian cell lines, including 95% of the total produced therapeutic monoclonal antibodies (mAbs) and 83% of the total recombinant blood factors [25].

Emphasizing on monoclonal antibodies-based therapies, the number of commercialized treatments sextupled between 2012 and 2018 [26,27]. A total of 13 mAbs-based drugs were approved in 2018 along with 5 other potential therapies undergoing clinical trials. As an example, Humira® is the most selling drug in the United States, with a profit close to 18 billion dollars in 2017. The latter is a tumor necrosis factor (TNF)-inhibiting and anti-inflammatory drug used for the treatment of many conditions (e.g. plaque psoriasis, rheumatoid arthritis and Crohn's disease) [28]. As a matter of fact, in 2018, the food and drug administration (FDA) approved 59 novel treatments, especially for cancer and infectious diseases. This is considered a 20 years record of approved biopharmaceuticals after 1996, where FDA authorized 53 novel treatments for various conditions [29,30]. As a consequence of this trend, the market size of the biopharmaceutical sector has greatly expanded overtime, with a total of US$ 228 billion in global sales in 2016 [31]. Simultaneously, the global bioprocess technology market is also expected to achieve 71 billion dollars by 2022 [32]. This indicates the market value of the total material needed for producing biopharmaceuticals (e.g., bioreactors, raw materials, chemicals, *etc.*).

Breakthroughs in the biopharmaceutical R&D technologies allowed the large-scale production of various bio-based therapies and made the manufacturing of these products easier and more profitable.

Parameters such as process yield and productivity are fundamental for the economic value and profitability of bioprocesses. Due to the importance of biotherapeutics and the large competition between manufacturers, several attempts have been made to "modernize" the process optimization strategies throughout designing innovative engineering approaches, relying for instance on using different omics data sets to boost process titer, product quality and to decrease the production cost of biopharmaceuticals.

In the post-genomic era, it became easier to study the genome of several species. Relying on modern sequencing techniques, depicting the genetic information of several organisms became faster and less expensive. Lately, sequencing became affordable and optimized to high-throughput [33] and is evolving to be the base for studying specific traits of industrially important cell lines. Due to the easiness of sequencing, it became more straightforward to think about combining genomics data with several other omics datasets for instance, transcriptomics, proteomics, fluxomics and especially metabolomics, referred to as high-dimensional biology [34]. The analysis of these data represents the core of computational systems biology, enabling understanding and optimizing cellular machineries, by guiding, simultaneously, the modification of its genetic information and the rewiring of its metabolic flux distribution towards expressing phenotypes of interest.

Along with this progress, there is still a need for improving production pipelines and room for further studying the cell at "omics" levels. In this era of big data, mathematical modeling has the potential to integrate multi-omics data sets into a single model and to correlate the observed changes in one dataset to observed changes in another [35]. Systems biology, which uses mathematical modeling to integrate current knowledge in a holistic manner, is a promising approach to optimize the time and resources required in biopharmaceutical production and to improve the industrial phenotypes of interest.

While mathematical modeling in the context of systems biology is leading to many great advances in bioprocessing, it is still not fully translated to industry due to lack of expertise in the field [36].

1.1. Context and motivation

While optimizing heterologous protein expression of important biopharmaceuticals, it is necessary to focus on studying the cellular metabolism in order to determine the metabolic bottlenecks of the cell and to optimize its machinery. The latter is controlled by multiple genes and interconnected metabolic pathways.

Cell culture medium contains the most important components for CHO cell lines growth, as well as the main fuel for production of recombinant proteins. Several efforts have been performed to optimize the nutrient levels in these formulations and customize them to the cell's need. This approach can help improving growth parameters of the cells as well as boosting the production of high titers of recombinant proteins, improving also its glycosylation patterns [37,38].

Glucose, amino acids and vitamins are the most important nutrients of cell culture medium. These metabolites are the main providers of carbon, nitrogen and other elements, crucial for proteins synthesis and essential for various biochemical reactions in the cell. Therefore, several media formulations have already been tested by various manufacturers and research groups to evaluate their production potential, especially by employing CHO cells [39]. These formulations have been improved over the years (e.g., the current use of protein-free chemically defined media) but are nowhere close to optimal.

In the past, time-consuming, laborious and relatively inaccurate methodologies have been used for media optimization (e.g., strategies based on varying one factor at a time) [40]. However, modern approaches based on the use of high-throughput strategies together with deterministic and mechanistic modeling approaches, for instance relying on the use of genome scale reconstructs and various omics data, improved the robustness of medium design and provided a solid base to overcome the use of the classical optimization methodologies [41,42]. Refining cell culture media formulation to the metabolic requirements of the cells can solve several bioprocessing problems, for instance, decreasing by-products levels during production. These improvements can be made by assessing metabolic requirements through studying, for instance, nutrient uptake rates and transporters capacity. Tools such as constraint-based modeling are very important to study the metabolic behavior of the cells in answer to variation in environmental conditions, *in silico*. In general, using *in silico*-based strategies and genome-scale metabolic models for studying CHO metabolism is a powerful approach to optimize growth parameters of CHO. These models not only provide a holistic overview about CHO metabolic network, but also can define robust strategies for predicting the cellular phenotypic changes in response to environmental adjustments [43], which in long term will reduce R&D time and costs.

Subsequently, developing a refined formulation of cell culture media based on CHO cells specific needs, based on studying the effects of different nutrients such as amino acids on the metabolism of CHO cells is a groundbreaking ambition that will subdue many biomanufacturing problems such as low cell growth, low cell densities, low product yields and the accumulation of cell culture by-products.

1.2. Research aims

The present PhD thesis focuses on optimizing the growth parameters of 3 different CHO strains (producer and nonproducer strains) as well as their production capabilities of an Immunoglobulin G (Trastuzumab) antibody (in case of producer cells). To achieve this target, we recurred to employ a combination of robust *in silico*-based approaches that have the potential to underline the metabolic bottlenecks of CHO and to design an optimized cell culture medium formulation. Respectively, we used a genome-scale metabolic model of CHO cells to predict the effect of balancing amino acids levels in the medium and its influence on cell growth and productivity of recombinant proteins. Subsequently, we validated the *in silico* results experimentally by studying the effect of supplementation of AKG (predicted as a potential additive to the cell culture medium) as an alternative source for both glutamine and glutamate. This experimental study aimed at understanding the different metabolic fluxes of CHO involved when the cells adapt to the new culture environment, underlying its production capacities under these constraints.

In detail, the use of *in silico* approaches allowed a faster prediction of not only the metabolic behavior of the cells in response to the variation of amino acids levels in the medium, but also possible additives that can be supplemented to the culture. The process was characterized from the point of view of monitoring the specific growth rate values, the maximal cell densities, the specific productivities of the cells, the final titer of Trastuzumab, as well as the levels of toxic by-products (e.g., lactate and ammonia). Predictions were based on the use of CHO genome scale metabolic model published by Hefzi et al., 2016 [44] together with Optflux, relying on the use of constraint-based approaches, for instance parsimonious flux balance analysis (pFBA). In addition, following the experimental validation and to better understand the metabolic differences among the tested conditions, metabolomics studies were performed to highlight the flow of amino acids and other metabolites in different experimental conditions by determining the uptake and secretion rates of key amino acids. The optimization results were compared to a positive control, where CHO cells were grown in standard culture conditions using CD CHO® cell culture media.

The thesis aims therefore at:

- Using the universal genome scale metabolic model of CHO (iCHO1766) [44] combined with an in-house evolutionary algorithm to:
    - Determine a possible minimum medium formulation that promotes CHO growth.

  - Test different media candidates *in silico* (through modifying the original model environmental constraints) to portray CHO metabolic flux distributions under these conditions.
  - Determine the growth rate together with the consumption/production rate values of extracellular metabolites based on pFBA.
  - Determine the influence of medium optimization on by-products secretion (e.g., ammonium).
- Test the optimal *in silico* results experimentally by:
  - Studying the influence of supplementing different concentrations of AKG to the cell culture medium, on 3 different CHO strains. Growth, viability, cell characteristics, maximal cell density, production rate of Trastuzumab and secretion of by-products were assessed.
  - Varying the levels of non-essential amino acids in the culture and studying the effect of these changes on the metabolic network, with and without supplementation of AKG.
  - Studying the effect of supplementing ammonium to the cultures as a possible nitrogen source together with balancing the amino acids levels in the medium.

1.3. Outline of the thesis

The research performed for this thesis is contained in six chapters. The first chapter comprises a general introduction about the importance of bio-based therapies and a brief highlight about economic aspects of the biopharmaceutical industry and its evolution overtime. Furthermore, an introduction and contextualization of the problems addressed in this thesis are presented. Chapters 2 to 5 contain a description of the state of the art, the experiments performed, and the results obtained. Chapter 6 encompasses the final research conclusions and recommends future approaches in the field.

The three chapters exploring the research aims are organized as follows:

- In chapter 2, a comprehensive review on the state of the art of the extensive areas of expertise addressed in this thesis is performed. An overview about the importance of mammalian cell lines, mainly CHO, is provided. In addition, a comparison of CHO production platforms to other cell factories is highlighted, focusing on the complexity of CHO compared with other production platforms (e.g., bacteria, yeast). Modern engineering strategies, as well as various topics addressing optimization efforts towards improving mammalian cell factories are also described.

Chapter 2 was partially adapted from the following publication: *Key Challenges in Designing CHO Chassis Platforms*. Hamdi et al., 2020.

- In chapter 3, the determination of minimal medium composition for CHO, based on the use of the corresponding universal genome scale metabolic model, iCHO1766, is described. We highlight the use of a python-based evolutionary algorithm developed at the center of biological engineering, University of Minho and its potential in determining the optimal minimal set of reactions that constitute the medium formulation for CHO when applying specific constraints. Further, evaluating potential additives that can support a specific objective function, for instance, maximizing either growth or maximizing both growth and production of IgG is described. Further, Optflux is the main framework used to study the effect of balancing amino acids levels in the medium from an *in silico* standpoint.
- In chapter 4, an experimental validation based on the aforementioned *in silico* results is highlighted. The effect of supplementing different AKG concentrations on CHO metabolism through evaluating the different growth parameters and production of Trastuzumab is studied. In addition, the exchange of amino acids under the different studied experimental conditions is explored.
- In chapter 5, the effect of balancing the non-essential amino acids levels on growth and productivity and metabolism of CHO producer cells is examined. Strategies such as evaluating the supplementation of ammonium when balancing amino acids in the medium is also studied experimentally.
- Chapter 6 summarizes the main research conclusions and discusses future perspectives.

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CHAPTER 2

State of the art

Partially adapted from:

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2.1. Mammalian cell factories

Biopharmaceuticals are mainly produced using heterologous expression in recombinant cells or microorganisms [1]. Mammalian cell factories are successful platforms for the production of recombinant proteins, especially monoclonal antibodies (mAbs), where Chinese hamster ovary (CHO) are predominant hosts [2]. This success is not only linked to their production capacity, but also to their history of safety, regarding the low susceptibility to viral infections [3]. These cells hold various unique features, namely, their adaptation capacity to high density suspension cultures, their easy scale-up and also effortless compliance to serum-free medium conditions, the most preferred formulations for biomanufacturing nowadays [4]. Alongside, one of the main features of mammalian platforms is linked to their ability to perform complex human-like Post-Translational Modifications (PTMs) [5]. Among these, glycosylation represents one of the most important attributes [6,7] and the most common structurally diversified modification in secreted proteins [8]. Correct glycosylation is required to sustain optimal pharmacokinetic and pharmacodynamic properties of biopharmaceuticals, since it affects the efficacy and *in vivo* turnover rate of therapeutics, and prevents immune responses triggered by non-human glycans [9,10].

2.2. CHO cells lines: Pioneering the production of recombinant proteins

Within mammalian platforms, CHO cell factories dominate the production of recombinant proteins in today's biopharmaceutical industry. CHO cells, as indicated by their name, were derived from the ovary of the Chinese hamster. They are in fact mainly of epithelial phenotype [11]. Originally, CHO cells were established in the late 1950s by Theodore T. Puck [12]. Since then, the family of CHO cells has expanded, giving rise to various new lineages such as CHO-K1, CHO-S, CHO-GS, CHO-DG44, *etc*.

These lineages were developed to fulfill specific industrial requirements, such as suspension culture or specific gene deficiencies that enabled selection, and are the result of genetic modifications via chemical and radiation mutagenesis [13], targeted gene knockouts and adaptation to new culture conditions [14,15].

Thereupon, due to their comparatively simple handling, CHO cell lines have proven to be crucial for the industrial manufacturing of recombinant proteins [16]. However, despite several decades of research and process design, the productivity remains low compared to the theoretical maximum productivity predicted *in silico* by a genome-scale metabolic model of CHO [17]. Hence, along with the increasing demand for biopharmaceutical products, there is a growing need to optimize CHO's production yield and to fast track the development of newly optimized production cell lines, in order to satisfy the large demands for complex biotherapeutics nowadays.

In view of the many different aspects of mammalian platforms, several engineering approaches have been developed to address, typically individually, the many challenges encountered during cell line development and manufacturing of highly complex biotherapeutics. Medium optimization and high-throughput screening for good producers were previously described [18,19]. Alternative optimization strategies based on modular design, synthetic biology and systems metabolic engineering, hold also tremendous promise to study the metabolic network of the cells and further improve productivity, yield, product quality and to reduce the time and cost of cell line development. Nonetheless, applying such rational engineering tools to mammalian cells is more difficult compared to other platforms, due to the complexity of the system. Many important aspects need to be considered, namely, the large genome, sophisticated regulatory, signaling, and metabolic networks, genome instability and epigenetic regulation [20].

2.3. CHO compared to other industrially relevant platforms

From a genetic perspective, mammalian cells are considered more complex than microbial systems as their genome is by far larger than that of *E. coli* and *S. cerevisiae*. The assembly of the sequenced CHO-K1 genome comprises 2.45 Gb with 24,383 predicted genes [3], while microbial cells used in the biotechnological industry have a smaller genome size by one to two orders of magnitude. A comparison of *E. coli*, *S. cerevisiae* and CHO platforms is summarized in Table 1. Even though having a larger genome does not necessarily relate to the cell's morphological complexity, it can be an indication of the intricacy of its proteome, fluxome, transcriptome and metabolome and, in particular, of its regulatory

capacities. Even from the viewpoint of the proteins that are encoded in these genomes, the proteins constituting prokaryotic cells are considered less complex than those of eukaryotic cells. The latter idea was claimed by different researchers such as Zhang et al and Wang et al [21,22] stating that the organism's protein structural complexity (e.g., length) can directly affect the growth performance of cells. It was demonstrated that, when optimizing for growth, a higher growth rate was observed for cell types containing smaller proteins. This is due to the cell tendency to increase its mass-normalized kinetic efficiencies during growth [23].

Table 2.1 Comparison of the three major platforms for biopharmaceuticals production.

Characteristic	*E. coli*	*S. cerevisiae*	CHO	References
Genome size (Mbp)	4.6	12.1	2450	[3], [24], [25]
Cell size (μm)	<1	3-5	12-24	[26–28]
Cell volume (μm^3)	0.3-3	30-100	900-7200	[27–29]
Doubling time (h)	Fast (0.5-4)	Fast (1.5-6)	Slow (18-48)	[30–33]
N-Linked Glycosylation	No	High mannose	Complex	[34]
Gene length (bp)	~1000	~1000	~1300/18000 †	[29,35]
Promoter length (bp)	~100	~1000	~10^4-10^5 *	[29,36]
Number of protein coding genes	~4300	~5300-5400	~24000	[35,37,38]
Proteins per cell	~10^6	~10^8	~10^{10}	[29,33]
Cell culture medium complexity	Low	Low	High	[34]
Cost of cell culture medium	Low	Low	High	[34]

†Coding/transcript; * HeLa cell line

In addition, cultivating mammalian cells is considered more demanding compared to microbial organisms, especially when focusing on their bioprocessing requirements. Due to the lack of a cell wall, there is significantly higher shear sensitivity and the cell culture medium must contain a higher number of essential nutrients compared to microbial systems. Bacteria (e.g., *E. coli*) and yeast (e.g., *S. cerevisiae*) can grow in a simple medium containing solely basic elements (e.g., glucose and salts) and usually only in specific cases a few amino acids (AA) or vitamins are added. In contrast, mammalian cells require a larger and more complex set of nutrients, including amino acids, organic acids, vitamins,

cofactors, carbohydrates and salts. This complexity of the growth medium reveals the strict nutritional demand of mammalian cells.

A major difference between microbial and mammalian cells is the fact that the genome of the latter actually encodes many different types of cells and developmental stages, namely more than 100 different types of tissues that are part of a mammalian body. To ensure correct expression of the required genes at the necessary level in each of these different tissue types, a much more complex regulatory network is required that includes highly sophisticated mechanisms such as epigenetics and chromatin remodeling that simply are not necessary for microbial cells and therefore are not present or are only at immature levels of development [39]. Apart from these chromatin state and epigenetic mechanisms, other regulatory factors are abundant in mammalian cells, such as microRNAs or long-non-coding RNAs (lncRNAs), which are transcribed in large numbers [40,41].

Over the last years, scientists are moving forward to employ optimization strategies that have been successfully used to study simpler organisms to other less explored systems. For that, synthetic biology, systems biology and metabolic engineering have been employed towards this goal. The use of these tools is facilitated by the availability of the genetic information [42] of different organisms.

While many of these tools are already widely applied in the field of recombinant protein production or strain engineering in microbial research, up to the level of design of chassis strains, its application to mammalian production hosts is still fragmentary and lagging far behind.

2.4. Cell culture media and its importance in bioprocessing

Cell culture media is a complex mixture of nutrients, energy sources and trace elements, essential for the growth and maintenance of the cells *ex-vivo* (figure 1). This concept was first described in the groundbreaking work of Eagle Dulbecco and Freeman, in the early fifties, stating that amino acids combined with other nutrients such as vitamins, are the core base of cell line cultivation, especially in adherent mode [43].

Nowadays due to regulatory guidelines, using animal derived serum poses various problems from bioprocessing standpoint (lot-to-lot variation), safety (contamination with viruses, mycoplasma, prions, *etc.*) and also from ethical point of view [44–46]. To overcome the use of serum in bioproduction, hydrolysates were employed in various processes to improve growth and productivity of the cells by developing cell culture media containing animal free components. Both plant and yeast hydrolysates

were tested with mammalian cells, especially using CHO cell lines [47,48]. However, hydrolysates also contain undefined concentrations of components, which can impair the consistency of both upstream processing (USP) and also downstream processing (DSP) [49].

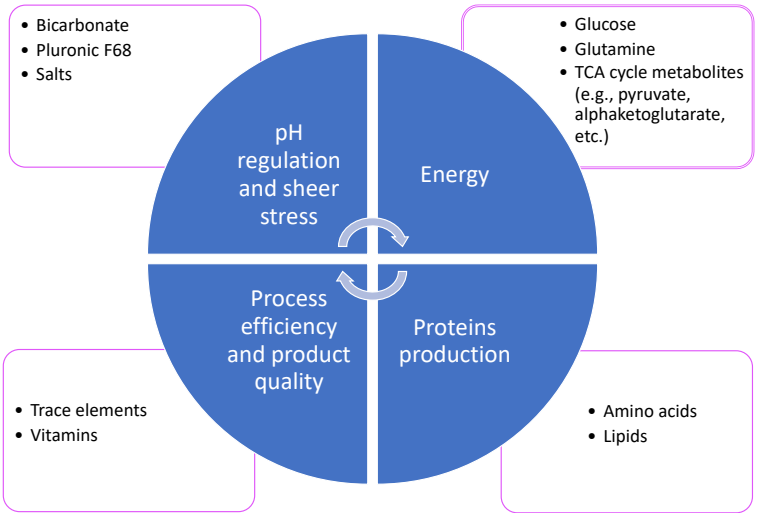

Figure 2.1 Mammalian cell culture media components.

Currently, industry is trying to veer the attention towards the use of chemically defined media (CDM) deprived from animal components to design solid processes with consistent product titers among batches, together with producing potent and clinically safe biopharmaceuticals. In culture, additives such as Pluronic F68 is very useful for CDM, since it can reduce the shear generated by the hydrodynamic motion during mixing [50].

2.5. Highlighting CHO metabolism

For growth, CHO cells require different nutrients (Nitrogen and carbon sources), energy carrying molecules (Adenosine Triphosphate ((ATP)), and other cofactors (e.g., adenine dinucleotide NAD+). These molecules are fundamental for sustaining the basic metabolic functions of mammalian cells [51]. Among the most important nutrients, glucose and glutamine represent a prime energy sources to the cell [52]. They are usually provided *in vitro* throughout the cell culture medium delivering carbon and nitrogen atoms to the cells in order to support its basic mechanisms such as proliferation [53].

In cell culture media, a balance between glucose and glutamine levels is essential, not only for maintaining optimal cell growth but also for sustaining the glycosylation of the produced recombinant proteins [54]. Tight regulation between glucose and glutamine metabolism was previously discussed by

Zeng and Deker [55]. That being said, glycolysis, as part of the central carbon metabolism and glutaminolysis are the major metabolic pathways for mammalian cells [56].

2.5.1. Glycolysis

The central carbon metabolism in mammalian cells is a complex set of biochemical reactions, transforming glucose into different metabolites, generating cell's biomass and various metabolic precursors, essential for various metabolic reactions [57]. This system is composed of three main pathways, including glycolysis, pentose phosphate pathway (PPP) and tricarboxylic acid cycle (TCA cycle) [52]. Glucose is the major player in the central metabolism since it is the main carbon and energy source in mammalian cells. Glycolysis is one of the most important pathways in the cell, where glucose is phosphorylated to glucose-6-phosphate and finally oxidized to 2 molecules of pyruvate, that are channeled into the mitochondria in order to enter the TCA cycle [58] (Figure 2).

2.5.2. Glutaminolysis

Glutamine represents a versatile donor and the main provider of nitrogen to the cells, together with several other amino acids. It is also the main substrate of the glutaminolytic pathway in mammalian cells (Figure 2). Glutamine degradation fuels the TCA cycle, generating cellular energy and boosting the biosynthetic pathways [59,60].

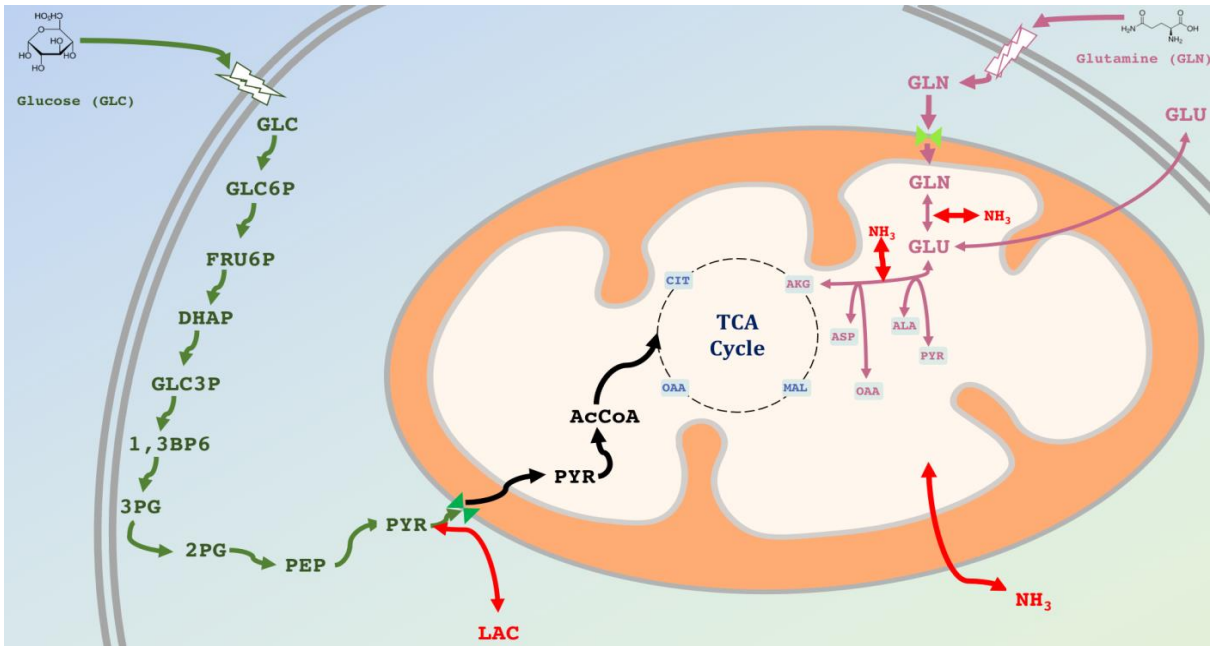

Figure 2.2 Representation of the glycolysis and glutaminolysis in CHO metabolism.

Legend: GLC (Glucose); GLC6P (Glucose-6-Phosphate); FRU6P (Fructose-6-Phosphate); DHAP (Dihydroxyacetone phosphate); GLC3P (Glucose-3-Phosphate); 1,3BP6 (1,3 bisphosphoglycerate); 3PG (3-Phosphoglyceric acid); 2PG (2-Phosphoglyceric acid); PEP (Phosphoenolpyruvate); PYR (Pyruvate); LAC (Lactate); AcCoA (AcetylCoA); CIT (Citrate); AKG (α-ketoglutarate); MAL (Malate); OAA (Oxaloacetate); ASP (Aspartate); ALA (Alanine); GLN (Glutamine); GLU (Glutamate).

On the one hand, glutamine degrades to glutamate via phosphate-dependent glutaminase and then to α-ketoglutarate via enzymatic reactions involving glutamate dehydrogenase (GDH). Following this metabolic path, the carbon backbone of glutamine is oxidized to CO_2 and malate that will be converted to pyruvate molecules [61,62]. On the other hand, the conversion of glutamate to α-ketoglutarate via aspartate/alanine transaminase is usually activated to overcome the overproduction of ammonium by the cells involving either pyruvate or oxaloacetate molecules [63]. The latter mechanism is important to overcome the overproduction of the toxic ammonium during glutaminolysis.

2.5.3. Amino acids and mammalian cell culture

Amino acids are vital for mammalian cells cultivated *in vitro*. They are divided into two categories: essential and non-essential. Essential amino acids (EAAs) cannot be synthesized *de novo* by the cells and have to be provided in the cell culture media. On the other hand, non-essential amino acids (NEAAs) can be synthesized by the cells in order to sustain and support growth. The latter are produced relying on several metabolites such as TCA cycle intermediates or others generated through the glycolytic pathway. A list of different essential and non-essential amino acids for mammalian cell culture are described in Table 2 and the work published by Salazar et al., 2016 [64].

Table 2.2 Essential and non-essential amino acids for mammalian cells. Adapted from [64].

Essential amino acids	Non-essential amino acids
Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine Tryptophan and valine	Alanine, asparagine, aspartate, cysteine, glutamate, glutamine, hydroxyproline, proline, serine and tyrosine

The chemical and biological attributes of these metabolites are crucial for cell growth and they constitute the building blocks for naïve/recombinant proteins synthesis. They are precursors for important metabolic pathways and source of nitrogen and carbon atoms [65]. The catabolism of several

amino acids is performed either via transamination (e.g., glutamate) or direct deamination (e.g., serine and threonine) [66].

Several studies aimed at adjusting the levels of several amino acids in the media due to their importance for obtaining high growth and yield of recombinant proteins simultaneously [67]. The levels are adjusted according to the metabolic requirements of cells [64]. A lack of specific amino acids can starve the cells; however, an excess of other amino acids, for instance lysine can hamper cell growth [68]. In fact, the consumption of amino acids during culture is directly dependent on many factors such as the culture environment or the cell cycle [69,70]. Furthermore, amino acids transporters play a role in sensing the different levels of amino acids outside of the cell. As a consequence, any change in the levels of amino acids in the extracellular environment is able to influence the activity of AA transporters within the cell [71].

Finally, it is important to highlight that the combination of the essential and non-essential amino acids in the cell culture medium is important for triggering various reactions and complexes in the cell, as for example the mechanistic target of rapamycin (mTOR), being mTORC1 one of the most studied pathways [72,73]. The latter is strongly dependent on amino acids presence, especially leucine [74]. It represents the penstock of cellular growth and metabolism [72]. Activating this sophisticated signaling network is the basis for cell proliferation, lipid synthesis, protein synthesis and also mitochondrial metabolism and biogenesis [75].

2.5.4. Metabolic flow path in CHO culture: By-products accumulation

The metabolism of mammalian cells, especially CHO is very complex and far away from being optimized for bioprocessing [20,58,66]. In order to achieve healthy proliferation of the cells and generate high yields of biotherapeutics with correct PTMs, it is important to control the cell culture environmental conditions to limit the secretion of toxic by-products that can hamper cell growth and alter product quality attributes [76]. In the cell, metabolic pathways are interconnected, for instance, glucose and amino acids metabolism. Any variation regarding the consumption rate of one of these metabolites during culture, can influence the metabolic homeostasis and can lead to accumulation of unwanted products.

As a matter of fact, the central carbon metabolism changes depending on the culture phase, cells metabolic need and the availability of nutrients in the cell culture media. In experimental conditions,

glucose and glutamine are quickly consumed by the cells [77]. During growth, CHO cells take up glucose at a very high rates, generating significant amounts of pyruvate. As a consequence of the inefficient metabolism of CHO, a drift to lactate production by the action of lactate dehydrogenase is usually observed, despite sufficient oxygen supply to the cells [66]. This phenomenon is the so called "Warburg effect" [78]. Lactate accumulation can be responsible for decreasing cellular growth rate, halting product formation and dramatically changing the osmolality of the culture medium when its concentration is above toxicity levels [79–81]. In the stationary phase (non-growth phase), on the other hand, most of the carbon consumed by the cells is channeled through the PPP pathway, instead of glycolysis, together with an increased consumption of several key amino acids. As a result, the production rate of lactate from pyruvate decreases significantly and most of the produced pyruvate fuels the TCA cycle, jointly with a metabolic switch based on lactate consumption that might occur [82]. The biological mechanisms underlying the lactate switch phenomena are not yet fully understood. On the other hand, glutamine directly supports mitochondrial oxidative pathways [83], playing a key role in triggering initial growth of CHO cells [84]. When glutamine is lacking in the medium, the glycolytic pathway is upregulated, and the secretion rates of lactate and other key metabolites of the glycolytic pathways are higher comparing to the cultures supplemented with glutamine [84]. In the presence of glutamine in the cell culture medium, glutaminolysis (previously described) is upregulated. Thereupon, ammonia is secreted since it is known to be mainly the product of glutaminolysis and represents one of the major byproducts of amino acids metabolism. The latter can strongly hamper growth, specific productivity and also impact the sialyation of recombinant proteins [66,85]. Ammonia builds-up in the cell cytoplasm, then it diffuses throughout the cell membrane and generates an intense perturbation of the intracellular pH and its electrochemical gradients. Its accumulation during culture with a concentration close to the range of 4-6 mM is proven to be inhibitory for growth [76].

Summarizing, high secretion of lactate and ammonium during mammalian cell cultures is undesirable [54,66]. When accumulated, these two metabolites are considered the main source of metabolic stress of mammalian cells, which comes out with a fast decrease in cell's growth rate, leading to apoptosis [86]. In fact, a major part of carbon and nitrogen consumed by the cells is not completely used to produce neither biomass nor recombinant proteins [87]. According to previously published reports, between 35 % [51] and 70 % [88] of the absorbed glucose is converted to toxic by-products. These facts reflect the ineffective metabolism of CHO.

2.5.5. Accumulation of other potential toxic metabolites

Apart from lactate and ammonia, further studies determined that accumulation of several other metabolites can hamper process attributes and final titers. Subsequently, a number of metabolites (besides glucose and glutamine), were proven to be quickly depleted in culture, for instance, asparagine and serine [89]. Asparagine is highly consumed by the cells and its catabolism involves several pathways such as alanine, aspartate and pyruvate metabolism. In these reactions, asparagine synthase converts asparagine to glutamate and the latter is converted to alanine via alanine aminotransferase (ALAAT). Additionally, alanine can be secreted to the extracellular milieu after being converted from pyruvate via alanine transaminase (ALT) enzyme. Previous studies highlighted that levels of alanine above 3 mM in culture can impair cell growth since high alanine concentrations can block proline uptake [90,91].

On the other hand, serine is directly responsible for the production of formate and glycine. Concentrations of formate between 4-10 mM was proven to be inhibitory for growth [66]. On the other hand, glycine accumulation has a positive effect on CHO culture [81], but its concentration is recommended to not exceed 1mM due to growth inhibitory effects [92]. Previous studies highlighted the interconnexion of metabolic reactions of serine and asparagine even with different metabolic functions [93].

In the culture medium, the starting concentrations of nutrients has to be rigorously controlled. Higher amounts of some amino acids, for instance in the formulation of CHO medium, can negatively impact cell growth. In previous studies, it was mentioned that concentrations above 1 mM of leucine, methionine, phenylalanine, serine, tryptophan, tyrosine and threonine in fed batch cell cultures can interrupt cell growth [66]. Likewise, several by-products of amino acid metabolism (e.g., homocysteine and indolelactate) as well as metabolites derived from lipid metabolism were determined to be detrimental to CHO growth [66], [92]. Moreover, a set of amino acids derivatives can impair growth. Metabolites such as dimethylarginine accumulate in the cell culture medium when arginine is oversupplied [94]. The accumulation of dimethylarginine can promote apoptosis through stimulating the production of intracellular reactive oxygen species [95]. Another example is methylglyoxal which can be generated throughout the metabolism of various amino acids for instance serine, glycine and threonine [96]. Previous reports demonstrated that methylglyoxal is noxious to the cells since lactate is the end product of its metabolism. Following, nucleotide metabolism also play a role in inducing cells apoptosis. The metabolism of some nucleotides such as adenosine monophosphate (AMP) can alter growth and

induce apoptosis, when its concentration is above 2 mM in the cell culture medium [97]. In bioprocesses, overconsuming amino acids by the cells is economically and biologically unfavorable. Depending on the cell line in use, it is important to adjust the levels of amino acids in the culture medium in order to avoid the negative outcomes of their accumulation in culture and to boost cell growth and improve the final process yield. Therefore, the importance of using CDM for production emerges, since the latter can open various windows for media optimization and process improvement. For that, it is necessary to employ additional tools for depicting the metabolic features of CHO, and to engineer it for a better performance.

2.6. Engineering CHO Bioprocess for a better performance

2.6.1. Reducing by-products accumulation

Along the years, several strategies have been used to control the levels of toxic metabolites during culture. However, until now, it has not been fully understood how to regulate lactate consumption/secretion and what are the cellular pathways that directly modulate this phenomenon. In this regard, expressing anti-apoptotic genes was used not only to extend the viability of CHO cells but also to manipulate the central carbon metabolism to efficiently guide pyruvate to mitochondrial oxidation instead of lactate production [98,99]. In addition, downregulating the expression of genes controlling the activity of important kinases (e.g. lactate dehydrogenase) was previously studied using different methodologies, for instance, using small interfering RNAs (siRNAs) vectors that showed a promise in reducing the levels of lactate in the culture without influencing the process productivity [100–103].

Alongside, efforts have been done to control ammonium secretion during the process. Several studies showed that CHO cells can grow independently from glutamine presence [15,104]. Since glutamine is considered as the main source of cellular glutamate, its lack in the cell culture medium could influence cell behavior towards growth. Indeed, the metabolic profiles of glutamine-supplemented and deprived cells were previously studied [33]. At first, glutamine depletion in culture alter the synthesis of important metabolites, for instance, glutathione (GSH) [105]. GSH is composed of cysteine, glutamate and glycine, and plays a very important role in protecting the cells from oxidative stress [106]. Furthermore, previous studies showed that glucose uptake rate, lactate production rate and also the amount of lactate produced per glucose consumed were not varied in case of limited glutamine levels in cell culture medium [15]. Hence, one of the most important features of CHO cultured in glutamine-free

medium, is the increase in the integral viable cell density (IVCD). The latter is considered as one of the main important aspect of product yield [15].

The degradation rate of glutamine in culture is high, outside of cooling conditions, compared to other amino acids, which explains its low stability in the medium. From a bioprocessing standpoint, removing or replacing glutamine by other nutrients in the medium is a rational optimization strategy. More stable metabolites were used to replace glutamine in culture. Di-peptides, for instance, l-alanyl-l-glutamine (AlaGln) were previously used [107]. These metabolites are more stable compared to glutamine, since their degradation rate is lower. In this case, glutamine availability in culture is entirely dependent on peptidases activity [63]. In addition, TCA cycle intermediates (e.g., α-ketoglutarate (AKG)) were also used to replace glutamine [108]. Cells cultured with AKG in the culture medium generated a decrease in growth rate and also a decrease in ammonium levels, but on the other hand, improved titers and specific productivity of the cells [67,86,108].

2.6.2. Increasing productivity and improving process performance

It is already known that CHO cells are auxotrophic for several amino acids and therefore need to take them up from the medium to support growth and recombinant protein production. However, the uptake of these amino acids is quite low, despite the fact that their amount in the medium is high [33], which might be due to insufficient capacity of the transporters. Increasing the transport capacity of essential amino acids or inserting the complete pathways for their synthesis might be favorable for cell growth and protein production. Geoghegan et al identified amino acid transporters that are likely upregulated in producer cell lines and suggested that overexpression of one or more amino acid transporters might improve growth and productivity of the cells [109].

Besides, several studies demonstrated that overexpressing anti-apoptotic genes may improve the productivity of the cells through extending their lifespan [110,111]. It also results in a lower release of proteins from lysed cells, which were shown to make up a considerable amount of the total protein in culture supernatants [112,113]. Here, Fukuda et al recently described a novel approach based on the development of anxa2- and ctsd-knockout CHO cell lines aiming at minimizing the release of host cell proteins (HCPs) into culture supernatants during production of biotherapeutics [114]. Subsequently, Kol et al described a novel model-based approach to predict the effect of decreasing the secretion of HCPs on CHO and its impact on the cell productivity. These predictions helped directing the design of “clean” cells by knocking-out 14 genes (using multiplex CRISPR-Cas9) that were proven to be responsible for

the production of HCPs [115]. The main outcomes of this study were the improvement of recombinant proteins production, in part due to the release of resources, and the reduction of impurities at the end of the culture. More targeted approaches were directed against specific HCPs that are known to pose problems in downstream processing [116]. An excellent summary of the different engineering approaches that have been applied to CHO so far is provided by Fischer et al [117].

In order to tackle different challenges of biopharmaceuticals production, omics technologies emerged as important tools for process optimization. From an omics standpoint, studies were mainly focusing on identifying limiting pathways and genes. together with determining strategies to engineer them either by overexpression or knock-out [118,119]. Most of these addressed the endoplasmic reticulum and the unfolded protein response, as cells producing high amounts of a "foreign" protein tend to have problems in processing and assembling such large cargos. Here, approaches to either overexpress specific helper proteins such as protein disulfide isomerase or to upregulate the entire ER were reported, as shown for example in [120–122].

The success of many of these studies was hampered by the fact that the differential regulation of a single gene is not likely to completely change the behavior or phenotype of a cell line [123]. Very little overlap in specific genes was observed in all of these studies, although frequently similar pathways were identified [124,125]. This led to the search for global engineering approaches where multiple genes and entire pathways could be controlled in a single step. One option that was extensively investigated, was the engineering of microRNAs (miRNAs), which globally regulate post-transcriptional processing of messenger RNAs (mRNAs) and protein translation. With the annotation of miRNAs [126–128], their potential towards enhancing protein productivity by influencing various cellular pathways (e.g., cell cycle, apoptosis, metabolism, protein expression, *etc.*) was taken advantage of [129–133].

2.6.3. Maintaining the balance between growth and productivity

Growth-coupled production is a common design principle employed for the generation of several compounds using microbial cell factories. However, this approach is limited to simple metabolites which can be stoichiometrically coupled to growth, and cannot be applied to protein production, since it is competitive to growth. Hence, a contrasting solution is usually applied for the production of recombinant proteins in CHO cells, where growth and production phases are separated [134]. The switch from high-proliferation to high-production is commonly triggered by a reduction in cultivation temperature [135–137] or by treating the cells with certain chemicals, such as sodium butyrate, which

promotes gene expression and growth suppression [138]. In addition, cell cycle arrest in the G1 phase was achieved by controlling the activity of cyclin-dependent kinase inhibitors (cdkis) and resulted in an increase in the specific productivity of the cells [139,140].

Various approaches have already been employed to control the proliferation of cells during cultivation. So far, mathematical modeling, although of great promise, has not been fully exploited to simulate bioprocesses and design better control of the switch from cell proliferation to increased heterologous protein production. One of the few examples is a study by Klamt et al, who computationally compared the volumetric productivities of two-stage fermentation strategies against the conventional one-stage production system [141].

2.6.4. Ensuring product quality

An additional area of focus, apart from improving growth and final titers, is controlling the PTMs, especially glycosylation. Not only focusing on improving productivity is essential, but also producing high quality complex biopharmaceuticals is a must.

While all mammalian cells are natively able to generate proteins with desired glycosylation patterns, there are some variants which may give rise to immunogenic reactions. This has been observed, for instance, in therapeutics produced in cell lines of mouse origin [142]. Indeed, glycosylation patterns are often heterogeneous and they are heavily influenced by the culture status [2,143]. It has been described that glycosylation patterns within CHO platforms can be modulated by varying the culture conditions (e.g., culture medium supplements) [144]. Identifying the role of the individual glycosylation enzymes as well as the limiting steps of glycosylation would help to engineer CHO cells to consistently produce completely glycosylated proteins with desired glycosylation patterns. In particular, for monoclonal antibodies, the specific glycan structure plays an important role in the immunoactivity of the product [145] which led to the development of production cell lines that lack, for instance, fucosyltransferase FUT8, or that overexpress enzymes to generate more complex glycan structures [146,147]. Coats et al showed that with increased productivity, the quality of N-glycosylation of EpoFc decreases [148]. The next step would be to identify the rate limiting step(s) and overexpress the necessary glycosylation enzymes or pathways for precursor synthesis. Fisher et al described several approaches for modulating post-translational modifications of recombinant proteins by genome editing in CHO [117].

Steps towards developing custom and consistent glycosylation profiles have already been taken. For example, a panel of cell lines expressing custom glycosylation patterns was created with the use of CRISPR/Cas9 technology [149]. In another study, the level of galactosylation was manipulated based on predictions from a kinetic model, leading to a reduction in glycan heterogeneity [150]. While for monoclonal antibodies, with their relatively simple glycosylation pattern, work on detailed control has already been initiated, the field is still open for more complex proteins bearing multiple glycosylation structures with high prevalence of tetra-antennary structure and the need for full terminal sialylation [151].

2.7. Systems biology for studying and improving CHO

The need for developing new methodologies for process optimization drove scientists to combine novel omics technologies with bioinformatics to understand biological systems [152]. Due to the high complexity of cells, mainly mammalian cells, it is mandatory to focus on developing robust models that, combined with omics data, can mathematically describe the metabolism of the cell, allowing the prediction of the effect of different culture conditions, as well as the determination of optimization strategies, which are particularly relevant for industrially valuable platforms such as microbial and mammalian cell factories.

Systems biology and metabolic engineering represent some of the most promising tools for process improvement. These technologies target the amelioration of cellular phenotypes via the manipulation of their biochemical pathways, for instance, through metabolic flux optimization. Optimization goals include increasing cell specific productivity by maintaining the balance between the competing interests of growth and productivity, generating an efficient and targeted metabolism to enhance product quality, and decreasing the level of process by-products [153]. Subsequently, following the success of using metabolic modeling strategies in microbial cells, it is promising to apply these tools to metabolically optimize mammalian cells [154]. Designing rational engineering approaches to enhance CHO-based bioprocesses via modulating cell metabolism is a promising strategy [17].

2.7.1. Omics picture of CHO

The CHO-K1 genome sequence, published in 2011 [3], was an important stepping stone towards the application of systems biology methods to CHO. However, in contrast to microbial systems, the genome of CHO cells often contains various chromosomal abnormalities caused by its genetic instability

[155,156]. Thus, cells belonging to the same lineage within a CHO family can have distinct genetic information and phenotypes. Differences in phenotype can even be observed among cells that normally belong to the same lineage but were grown in different laboratories and under various culture conditions. This can in part be explained by the structural variations in the genome and the accumulated genetic changes such as single nucleotide polymorphisms (SNPs), transgene copy number variations and chromosomal rearrangements [157–160]. Vcelar et al showed that chromosomal rearrangements within the genome of a population are observed during subcloning, adaptation of the cells to a new medium or simply during long-term cultivation [161,162]. On top of this, variations in phenotypes that cannot be explained by genomic diversity and variation alone, are frequently observed, even in subclones of subclones [163]. These facts suggested that the genetic information of the CHO-K1 cells sequenced in 2011 [3] are not representative of all CHO cell lines and subclones, so a reliable and stable reference genome was needed. This was addressed by the generation of a common reference genome of the Chinese hamster *Cricetulus griseus* [5,164], which was further improved by a more complete genome assembly in 2018 [35]. These, along with the genomes of other cell lines sequenced in the meantime [5,159,160] serve as basic datasets for *in silico* studies of CHO via integrative analyses of omics data relying, for example, on the use of genome scale metabolic models (GSMMs) which can support genetic and metabolic engineering studies.

2.7.2. Genome scale metabolic models

Genome scale metabolic models (GSMMs) are novel tools for systems biology that carry information regarding different genes, proteins, and reactions (GPR) being an integral representation of the cellular metabolism. The availability of full genomic sequences, as well as omics data of various organisms enabled researchers to reconstruct cellular metabolism *in silico*, linking different level of information to calculate metabolic fluxes [165]. Indeed, GSMMs are powerful tools in systems biology and can help predicting the changes in metabolic behavior of the studied organisms, not only in response to changes in environmental constraints (e.g., medium formulation) but also to genetic manipulation of the cells (e.g., gene knockouts) [166–168]. These strategies allow to mechanistically link organism's genetic information and phenotype [92, 93] and can be very useful for rational identification of engineering targets [167,171].

GSMMs are emerging as a common practice in metabolic engineering. Therefore, several reports are available nowadays describing, in a detailed manner, the protocol for reconstructing GSMMs

[165,172,173]. This process relies heavily on the information regarding genes, metabolites and enzymes activity of the specific organism being investigated. In fact, following the development of the genome scale model for *H. influenzae* [174], various genome scale reconstructions were made available to the scientific community [175]. Due to the importance of CHO cells in the biopharma industry, scientists joined efforts to develop a CHO genome scale metabolic model [17]. Therefore, researchers are veering the attention towards implementing computational models for cell culture improvement, but still few successful studies were focused on employing CHO GSMM to improve growth and productivity of the cells [176,177]. The universal metabolic model iCHO1766 was built jointly with various research groups and comprises the most complete representation of CHO so far. The metabolic network was reconstructed and associated with over 1700 genes, 2300 metabolites and over 6000 reactions in the *Cricetulus griseus* genome. The model was built based on the information described in the global human metabolic network (Recon 1) [178], knowledge from the updated version Recon 2 [179] and a curated version of the Recon 2 model [180]. Combining these reconstructions, *Cricetulus griseus* homologs were determined. Further details are described in Hefzi et al, 2016 [17].

Accordingly, CHO model represents a powerful tool for untangling the complexity of CHO, investigating bioprocess capabilities and improving cell line development strategies [17], which can be further improved by integrating additional biochemical information to understand cellular processes beyond metabolism [181].

Recently, several efforts were performed to curate GSMMs and to improve its prediction accuracy via modifying it [177]. These efforts generated, for instance, the updated models iCHO2101 [182] and iCHO2291 [183]. Clearly, refining our knowledge about the metabolism of CHO cells allows scientists to continue optimizing the available genome scale metabolic models by complementing genetic and metabolic information of CHO [177].

Prediction tools based on metabolic modeling have only recently started to be applied to the design of engineering strategies in mammalian cells, mainly CHO cells (Table 2). One of the first examples where the GSMM of CHO was applied to an industrial process, is the work performed by Calmels et al, where the genome-scale metabolic model [17] was curated and tailored to a CHO-DG44 producer cell line. They performed corrections, such as modifying 601 reactions (for example silencing of 537 amino acids transporters), which led to an improvement of the growth rate and exometabolome predictions *in silico* [177].

In addition, the secretory pathway was integrated into the GSMM of CHO by Gutierrez et al, to enable predictions of energetic and machinery demands of secreted proteins [184], which might lead to better predictions of engineering targets that aim at improving protein production, as shown for example in the work of Kol et al [115]. On the other hand, many studies based on mapping the intracellular fluxes throughout metabolomics studies can improve cell culture parameters [185].

New perspectives aiming at overcoming the redundancy of the large scale metabolic models and decreasing the computational time are moving towards the reconstruction of minimal metabolic networks that contain the most essential genetic and metabolic information needed for predictions [186].

Table 2.3 Recent studies focusing on the use of GSMMs for CHO bioprocess optimization in the last 4 years.

Publication	Aim of the study	Reference
Calmels et al., 2018	Curation of genome scale metabolic model to construct CHO-DG44 specific model.	[177]
Szeliova et al., 2020	Determination of CHO biomass variations among different strains	[33]
Szeliova et al., 2020 (2)	Experimental measurement errors and its impact on *in silico*-based predictions	[154]
Huang et al., 2020	Improvement of cell's productivity relying on the use of CHO GSMM.	[187]
Gutierrez et al., 2020	Generation of CHO species-specific secretory pathway reconstructions.	[184]
Schinn et al., 2021	Prediction of amino acid concentrations in cultures and predict nutrient feeding strategy.	[188]
Pérez-Fernández et al., 2021	Media optimization for continuous CHO-K1 cultures.	[189]
Szeliova et al., 2021	Inclusion of maintenance energy improves the intracellular flux predictions of CHO	[190]

As a matter of fact, due to the size and complexity of the metabolic models, mainly mammalian cells models, computational support is necessary in order to predict optimal intervention strategies from the combinatorial universe of possible modifications. Computational strain design methods, many of which are based on constraint-based analysis of cellular metabolism [168,191–193] are available towards this end [194] and are continuously being refined. Combined with bioinformatics, GSMMs are very effective

in studying metabolic responses of different stimuli and in determining the metabolic bottlenecks of the cells. In fact, mathematical modeling strategies emerged as a very promising advancement to analyze different biochemical complex networks reflecting a complete picture that draws the different reactions in the studied organism and the specific genes that encode for them [192].

The mathematical representation of these metabolic reactions considering steady state forms a system of linear equations [192]. Tools such as flux balance analysis (FBA), play an important role in solving these equations and help in quantifying the level of contribution of different reactions to a specific target phenotype in response to environmental and genetic changes [195].

2.7.3. Flux Balance Analysis

Flux Balance Analysis (FBA) is a widely used mathematical tool to study metabolic networks and to predict phenotypes [196] which can be easily applied to genome-scale metabolic networks. FBA helps understanding the metabolic distribution inside of a complex network, relying on the use of linear optimization, assuming steady state conditions. FBA targets the maximization of a specific objective function, usually defined as biomass formation [197]. One of the advantages of FBA is to calculate different metabolic flux distributions when varying environmental constraints (e.g., medium formulations) or when performing gene knockouts.

Prediction assuming steady state means to consider that the sum of the rates of formation of all internal metabolites is equal to the sum of their production rates. In general, the modeling approach consists of deducing a stochiometric matrix (S)**.** Within this matrix, the rows and columns represent respectively, the metabolites and the reactions. Assuming the steady state conditions is explained by ($S.v = 0$), where (v) represents the flux vector indicating the specific rates for each reaction, and where every mass balance in the system is represented by a linear equation. This computational approach is considered very useful in systems biology and its prediction accuracy can increase when feeding these models with experimental omics data, for example by means of specifying the flux boundaries. The upper and lower limits of fluxes can be used for every reaction inside of the model where ($v_{lower} \leq v \leq v_{upper}$). From a bioprocessing standpoint, this mathematical tool can be employed for studying metabolic pathways in the cell targeting either maximizing growth or maximizing product formation in industrial cell lines [192]. Inserting experimental data and calculating fluxes taking into consideration the thermodynamic capability of some pathways can improve the prediction accuracy. In addition, other factors such as experimental data quality can imperatively influence the prediction results since the analytical error can

propagate through FBA. Therefore, it is important to establish solid experimental protocols to overcome possible prediction inaccuracies [154].

Another mathematical tool is parsimonious flux balance analysis (pFBA). The latter is a variant of FBA, and assumes the usage of the minimal amount of metabolic fluxes within a metabolic network in order to sustain an objective (e.g., maximal growth), always taking into consideration steady state assumption [177,186,198]. The latter is considered as an improved version of standard FBA, since in 2 prediction steps, this optimization method predicts the most adequate pathways reflecting, in a different mathematical representation, the idea of "maximum biomass per number of fluxes" described by Schuetz et al [198,199]. In a nutshell, this tool minimizes the total sum of fluxes in the network by removing futile loops [198].

2.7.4. Defining the objective function: The biomass function

The biomass function is a very important parameter for *in silico*-based predictions using genome scale metabolic models. When maximizing for growth, the biomass function is usually described as the objective function [197]. In this case, it describes the rate at which the different metabolic components are converted to biomass elements.

The biomass function can be formulated at different levels of detail. Typically, it contains information regarding different components in the cell such as proteins, RNA, DNA and lipids. It can be further detailed by adding different layers of information (e.g., cofactors, vitamins and elements) [200].

The biomass equation is described as following, where C_i represents the coefficient of each biomass component X_i :

$$\sum_{i=1}^{n} C_i X_i \rightarrow Biomass$$

As previously described, different strains/cell lines hold different genetic and metabolic features. It is ideal to build a specific biomass function for each cell type. *In silico* metabolic phenotypes can vary also according to the biomass objective function used for each cell line [201,202]. Subsequently, it is often important to build strain and condition specific biomass functions, given that they can improve the model capabilities to predict metabolic fluxes [33,203].

As an example, it was previously discussed that yeast biomass composition can vary depending on the physiological conditions were it is grown [204]. A similar study concluded the same results for CHO cells [33]. Besides, it was also described that an accurate estimation of cell biomass composition is needed for robust predictions using GSMMs, not only when using microbial models but also for mammalian metabolic models (CHO) [33].

In iCHO1766 [17], two different biomass functions were included in the model. The first biomass equation (R_biomass_cho) is employed in order to predict growth of naïve or (non-recombinant) CHO cells, whereas the second biomass function (R_biomass_cho_producing) is used to predict the growth for recombinant CHO cells targeting the production of monoclonal antibodies. These two different biomass equations were built due to the differences between calculated gross cell composition in non-recombinant CHO cell lines and measured values for IgG-producing hybridoma lines (e.g., the amount of protein fraction constitute more than 70 % of cell dry weight in a producing cell, while it was calculated to be 55% in a non-producing cell). Using a specific biomass function for each cell type can significantly improve prediction accuracy. It also facilitates understanding of the metabolic features of these cell types in different environmental conditions, for instance, media formulations.

In a nutshell, strategies based on *in silico* predictions using cell line specific genome scale models can play a role in decreasing the experimental workload as well as in avoiding needless laboratory costs by defining the most important parameters *in silico* without recurring to test all conditions experimentally [205]. Henceforth, *in silico* approaches hold a tremendous promise in improving bioprocesses relying on the study of cell culture attributes (e.g., cell growth and exchange rates of metabolites) towards improving both final product titer and quality attributes throughout predicting its post-translational modification patterns [206].

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CHAPTER 3

In silico-based approaches for CHO cell culture media optimization

The information presented in this chapter is being prepared for submission to a peer reviewed journal:

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3.1. Introduction

In a cell, nutrients are consumed and channeled through different pathways within its metabolic network. The latter is composed of a myriad of biochemical reactions and metabolic pathways that are catalyzed by several enzymes under the control of specific genes. This complex network is an organized combination of integrated functions of various genetic circuits controlling the machinery of various macromolecules in the cell. The latter represents a primary factor in studying the relationship between genotype and phenotype [1,2]. Improving our knowledge regarding the genotype-phenotype link will allow us to understand how to design a specific phenotype of interest. The different genetic mechanisms in the cell are far away from being simple and multiple genes can be responsible for generating one single protein that can have different functions within the metabolic network [3]. To better study and understand metabolic networks, it is essential to rely on using genome scale metabolic models (GSMMs) together with computational tools to facilitate strain and media optimization. Therefore, the availability of a full reconstruction of cellular metabolism *in silico* through genome-scale metabolic models is essential for the rational identification of metabolic engineering targets [4,5].

In bioprocessing, various modeling strategies emerged to support optimization of various production platforms. Among these modeling strategies, mechanistic dynamic and constraint-based modeling approaches have been described in literature [6]. Mechanistic modeling approaches rely on the use of mathematical equations. However, it is still difficult to employ these tools for studying complex organisms, such as mammalian systems, mostly due to the lack of resources, especially at academia, generating a lack of kinetic data. As an alternative, constraint-based modeling approaches such as FBA,

rely only on stoichiometric information, making it a very useful tool to study metabolic networks at different levels of complexity [8].

Metabolic optimization pipeline using constraint-based modeling approaches is mainly based on rewiring cell's tightly regulated metabolism to fulfill an objective, for instance, improving growth, the production of a compound/recombinant proteins of interest, improve its quality attributes of a specific recombinant protein or decrease process related by-products. FBA boosts the efforts to computationally understand metabolic networks and to optimize cellular capabilities through studying the changes in metabolic fluxes when manipulating different factors, for instance, the environmental constraints. Besides, flux variability analysis (FVA) is a tool that emerged to assess the robustness of genome scale metabolic models, under various conditions. Using a set of constraints in a metabolic network, FVA comes to evaluate the minimal and the maximal rate of specific fluxes based on single or a double linear programming (LP) problems (e.g., a maximization and a following minimization for specific reactions of interest) [9]. Several advantages are behind the use of FVA in constraint-based modelling, include finding alternative optima in a metabolic network, studying suboptimal growth conditions, understanding its metabolic flux distribution and evaluating the metabolic network robustness [10–12]. In the case of CHO cells, using FVA can be very useful to study the system behavior when maximizing for both, cell growth and recombinant protein production.

Recently, various tools were used to engineer mammalian cells, mainly CHO, aiming at increasing their production performance. The universal GSMM of CHO cells, iCHO1766 [13], has been used to predict strategies for improving mAbs production in producer CHO cells, for instance, through media optimization. This tool is very useful to study, *in silico*, how the metabolism changes when changing the growth conditions [5,14]. Several computation tools and constraint-based methodologies have been developed to design novel cell factories and to improve existing strains [15]. In table 1, we can observe different computational frameworks that were developed for metabolic engineering, most of them were used for the study of various microbial cell factories. Several tools can be applied for the optimization of various strains, including CHO cells and can be useful for the cell line development stage of the process. Among the frameworks described in table 1, Optflux was used in this study to optimize CHO cell platforms. The latter is an open-source software that allows, in a user-friendly manner, to perform *in silico* metabolic engineering tasks [16].

Table 3.1 Computational frameworks used for metabolic engineering.

Tool	Year	Prediction	Application (Strains)	References
OptKnock	2003	Possible knockouts that can improve production yield. Maximize the target compound production using Bi-Level linear programming	*E. coli, G. sulfurreducens,* *S. cerevisiae*	[17–19]
OptStrain	2004	Reaction addition/deletion Possible knockouts. Possible insertions	*E. coli, C. acetobutylicum,* *M. extorquens*	[20]
OptReg	2005	Up and downregulation of reactions for strain design	*E. coli*	[21]
OptGene	2008	Optimization of non-linear objective functions Faster predictions compared to OptKnock	*S. cerevisiae*	[22]
RobustKnock	2009	Triple level optimization pipeline The framework targets the maximization of minimal target fluxes of industrial targets based on FBA and FVA Production of chemicals of interest is an obligatory by-product of growth rate maximization	*E. coli*	[18,19]
GDLS	2009	Employs MILP search approach Finding direct gene-deletion targets Low-complexity search of the space of genetic manipulations ignoring Gene-Protein-Reaction relationships	*E. coli*	[23]
Optflux	2010	Bi-level Optimization (OptGene/OptKnock) Multiobjective Optimization	*E. coli, yeast,* mammalian cells	[16]
OptORF	2010	Depict metabolic engineering strategies based on gene deletion/overexpression	*E. coli*	[24]
SimOptstrain	2011	Concurrent gene insertions and knockouts Non-native reaction addition. Bi-level strain design	*E. coli*	[25]
BiMoMa	2011	Knockout predictions based on mixed-integer programming solution techniques GPR association	*E. coli*	[25]
ReacKnock	2013	Predictions using Bi-Level FBA Improved computation time compared to OptKnock	*E. coli*	[26]

MOMAKnock	2013	Relies on MOMA assumption to restrict constraints to steady-state fluxes Identification of robust knockout strategies	*E. coli* core *model*	[27]
OptGeneKnock	2015	Incorporates logic transformation of model (LTM) with a bilevel mixed integer linear programming (MILP)-based knockout method Designing fast genetic intervention strategies	*E. coli*	[28]
APCG	2016	Analysis of production and growth coupling Identification of gene targets for improving production of the desired metabolite	*E. coli*	[29]
IdealKnock	2016	Predict knockout strategies for overproduction of compounds of interest	*Y. lipolytica*	[30]
SelFI	2017	Identification of selection pathways for directed enzyme evolution		
OptPipe	2017	Knockout prediction procedures and rank the suggested mutants according to the expected growth rate, production and a new adaptability measure	*C. glutamicum*	[31]
gcOPT	2018	Prediction of possible strong growth-coupling combinations and intervention strategies	*E. coli*	[18]
OptCouple	2019	Determination of knockouts, insertions and also medium supplements	*E. coli*	[32]
OptRAM	2019	Overexpression, knockdown or knockout of both metabolic genes and transcription factors	*Saccharomyces cerevisiae*	[33]
egKnock	2019	Find gene deletion targets for maximization of minimum target flux of industrial objective in flux variability analysis This tool takes gene-protein-reaction relationships into consideration	*E. coli*	[34]

3.2. Methodology

The universal CHO GSMM, iCHO1766, published by Hefzi et al, 2016 [35] was used in this work. The optimization pipeline is divided into two main parts. The first part is based on developing a new approach aiming at determining the minimal medium formulation using an in-house built evolutionary algorithm (optiModels), while the second part of the *in silico* work is aiming at evaluating cell culture parameters based on using the results obtained in the first part of the study. For that, evaluating the metabolic changes when varying the medium formulation (based on optiModels predictions and literature studies) were also explored, focusing on determining growth rate values together with the different exchange rates of metabolites in the network under various constrains. The optimal results of this study will be used for experimental validation.

3.2.1. Prediction of the minimal number of exchange reactions using optiModels

Aiming at determining an optimal minimal medium formulation for CHO to support growth, a novel evolutionary algorithms tool was used aiming at predicting a combination of the most essential transport reactions within CHO model, to sustain optimal growth values. Identifying this set of optimal candidate reactions will allow us to refine the medium formulation for CHO.

In fact, optiModels is a framework fully implemented in Python language that enables analysis, simulation and optimization of stoichiometric, dynamic and Gecko models [36] (integrated models of metabolism, protein synthesis, and protein secretion), used for single and multi-organism cultures. OptiModels use the theory of natural evolution that aims at finding the best set of solutions to specific biological questions. Two different biomass functions (R_biomass_cho) and (R_biomass_cho_producing) were used in this study. The prediction pipeline starts with a population containing a set of different candidates. Each candidate is represented by a number of integers which represents a randomly selected exchange reactions in the model. Following, mutation/crossover studies are performed where a number of exchange reactions are added, removed or replaced by other more suitable ones. Additionally, the crossover studies are based on merging two parent candidates to generate two other solutions by combining specific characteristics from the parent candidates. Upon generating these solutions, the model predicts the value of the objective function for each candidate using pFBA and the method assigns a fitness score that represents the value for which each candidate is scored. The latter generates a fitness value that can vary between 0 and 1, where the higher fitness

values correspond to candidates with a smaller size. The maximum candidate size is the maximum number of uptake reactions allowed by user. By default, this value is the number of exchange reactions present in the given model. Therefore, the fitness function is provided by:

$$\text{fitness} = \frac{\text{size(candidate)}}{\text{maximum of candidate size}}$$

This prediction workflow is continuously repeated evaluating different candidates and the results are obtained after several iterations. Additional details about the prediction protocol (pipeline) using optiModels and the different packages/tools used for the simulations are described in the annexes of this chapter. Following several optimization cycles, optiModels generated a combination (a minimal number) of the most important reactions that hold a promise in sustaining (high) growth of the cells. The resulted rates of key metabolites (substrates) were applied in the following medium optimization step.

The exchange rates of metabolites used for the optimization were described in Hefzi et al, 2016, and are referred along the thesis as the "original model constraints". Besides, the FBA objective is set towards growth optimization. A minimum value of 10% of the maximum value of biomass growth was appointed as the first simulation constraint. Furthermore, glucose was set as the unique carbon source for the cells. The predictions were performed using pFBA, minimizing the number of open exchange reactions. The optimal results holding the highest scores ranging from 0.7→1, were further investigated.

3.2.2. Medium optimization

3.2.2.1. Medium optimization pipeline

To consolidate the optiModels results, further screening based on literature studies was performed, excluding the reactions (or metabolites) that, although originated as a result from the evolutionary algorithm, have unclear biological significance. For mapping the metabolic landscape of the cells when using the optimized medium formulation, pFBA was performed to predict the growth rate values in each tested condition using Optflux.

In figure 3.1, we can observe a scheme representing the medium optimization pipeline, where the prediction results obtained with optiModels, combined with data based on literature, were used to predict the best candidates that can play a role in boosting cell growth and decrease the process related

by-products. In these predictions, the constraints of key metabolites were based on data in Hefzi et al, 2016 [35] combined with potential candidates predicted by optiModels. In addition, two different biomass equations were employed, and various environmental conditions were tested. The latter are expressed in mmol gDW^{-1} hr^{-1} and refer to the exchange rates values in steady state.

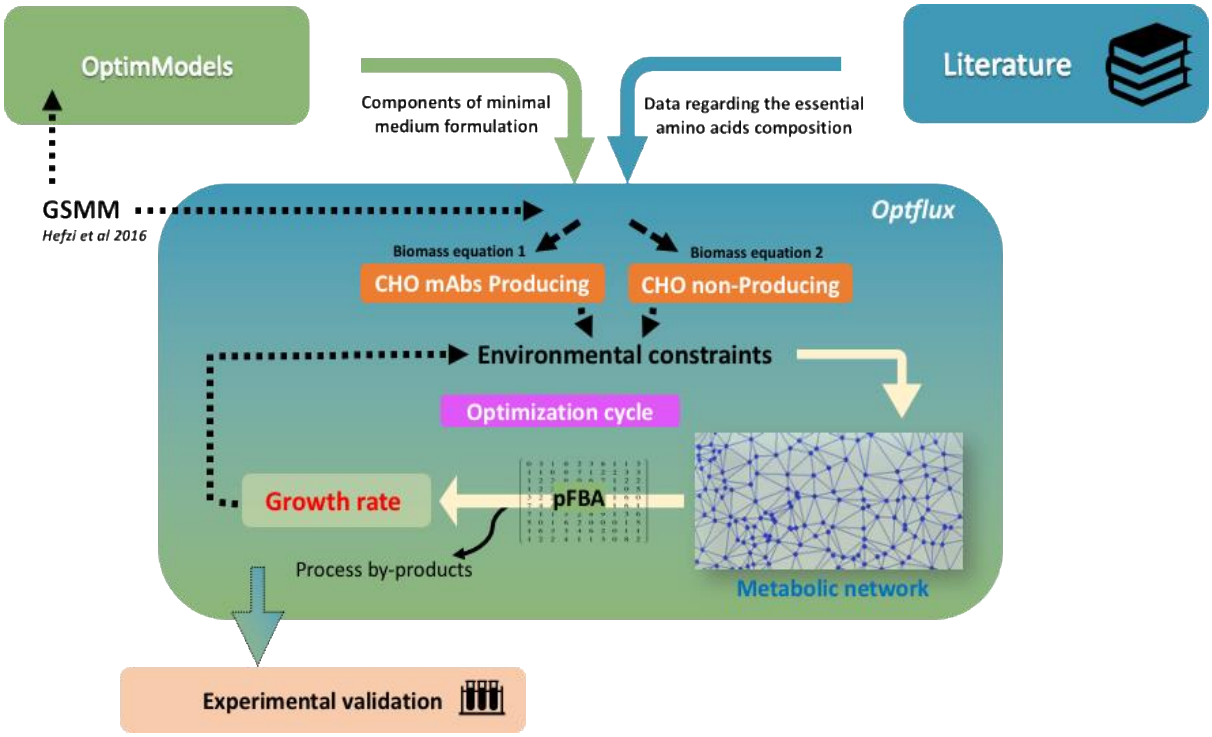

Figure 3.1 Medium Optimization: Prediction workflow. GSMM (Genome scale metabolic model), pFBA (Parsimonious flux balance analysis).

Multiple optimizations were performed and the values of growth rate and by-products (ammonium) were compared to the results with original constraints of the model which was constructed based on different simulations and omics data described in previous published reports [35,37,38]. This optimization pipeline can be very helpful to screen the effect of balancing the levels of different compounds in the growth medium, through pFBA.

Based on the literature, the amino acids uptake levels were optimized to maximize cell growth and particularly to overcome the secretion of process by-products such as ammonium. To better compare/understand the results, the values of both growth rate and growth yield were calculated in order to assess/compare the prediction results. Growth yield values were calculated according to the following equation where μ represents the predicted specific growth rate and $qGlc$ is the predicted specific consumption rate of glucose.

$$Growth\ yield = \frac{\mu}{qGlc}$$

3.2.2.2. Maximizing growth and recombinant protein production using FVA

In order to predict the maximum antibody production capacity using the original model constraints (Hefzi et al., 2016) and the in-house optimized constraints, flux variability analysis (FVA) was used. The latter tool was used to calculate the maximum possible value of a selected flux, in our case, the specific reaction for the production of IgG described in the model as (DM_igg[g]), for a range of fixed values of the biomass reaction.

3.3. Optimization results and discussion

3.3.1. Optimization of minimal medium using optiModels

The optimal set of reactions that are capable to sustain CHO growth were determined. Several combinations of reactions were determined with a fitness score restrained between 0.7 to 1, as described in materials and methods.

Within the solutions obtained using optiModels, we determined the 30 most frequent reactions that resulted from the predictions, relying on both biomass reactions, for producing and non-producing CHO cells. The comparison is highlighted in figure 3.2. In this representation, we observe the percentage of frequency versus the corresponding exchange reaction, where “R_EX” means Exchange reaction and at the end of the naming of the exchange reaction, “_e” means extracellular.

Several exchange reactions of amino acids such as tryptophane, valine and isoleucine were part of the prediction solutions. Exploring these results deeper, we noticed that the solutions with the optimal fitness scores also included many reactions involving the catabolism of several complex structures of molecules, mainly vitamins, oligopeptides and polypeptides. Among these solutions, kinetensin 1-8, corresponding to the exchange reaction (R_CE5789_e) appeared in more than 70 % of the optimal results obtained by optiModels.

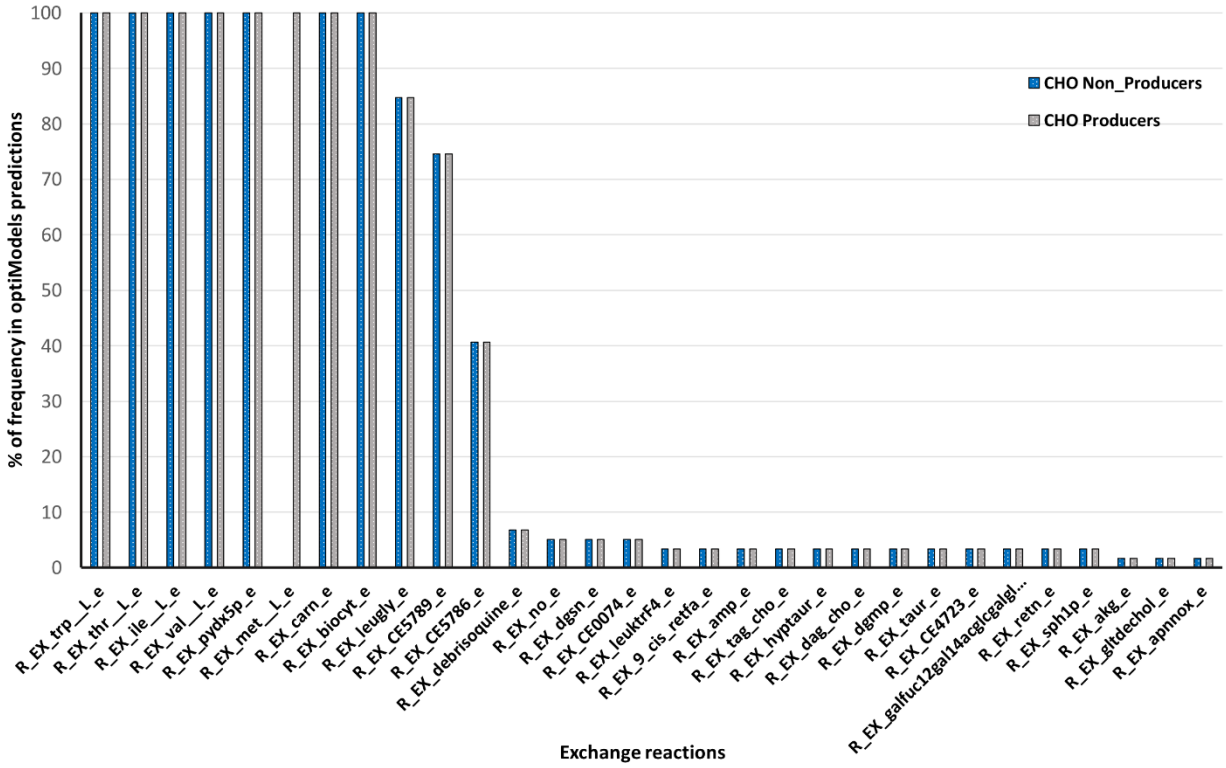

Figure 3.2 Frequency of reactions obtained by optiModels algorithm when minimizing the number of exchange reactions ensuring growth. trp_L (Tryptophan), thr_L (Threonine), Ile_L (Isoleucine), val-L (Valine), pydx5p (Pyridoxal-5-Phosphate), met_L (Methionine), cern (Carnitine), biocyt (Biocytin), leugly(Leucylglycine), CE5789 (Kinetensin 1-8), CE5786 (Kinetensin), debrisoquine (Debrisoquine), no (Nitric oxide), dgsn (Deoxyguanosine), CE0074 (Alloxan), leuktrF4 (Leukotriene F4), 9_cis_retfa (Fatty acid 9-cis-retinol), amp (Adenosine monophosphate), tag_cho (Triacylglycerol (cho)), dgmp (Deoxyguanosine monophosphate), taur (Taurine), CE4723 (Neocasomorphin (1-5)), galfuc12gal14acglcgalgl ((Gal)3 (Glc)1 (GlcNAc)1 (LFuc)1 (Cer)1), retn (Retinoate), sph1p (Sphinganine 1-phosphate), akg (α-ketoglutarate), gltdechol (beta glucan-taurodeoxycholic acid complex), apnnox (Alpha-Pinene-oxide).

The latter oligopeptide is chemically composed by several amino acids, in this case L-Isoleucinele-L-Alanine-L-Arginine-L-Arginine-L-Histidine-L-Proline-L-Tyrrosine-L-Phenylalanine-L-Leucine. This high molecular weight molecule ($C_{50}H_{76}N_{16}O_{10}$) [39] is not a potential candidate for cell culture medium component, not only because of its complex chemical structure, but also because its high price in the market and the lack of commercial availability.

In fact, these results are expected, since the model does not account for the biological feasibility of these solutions, but calculates, mathematically, the easiest metabolic path through pFBA towards growth and determines the best candidates with minimal medium components. Subsequently, further filtering of the data has to be performed based on literature studies to choose the best set of candidates proposed by the model that can be consumed by CHO. Nevertheless, it is important to emphasize that

the prediction results are merely an indication of which pathway should be targeted in terms of experimental implementation.

Based on the results described in figure 3.2, for example, pyridoxal-5'-phosphate (P5P) appears to be a potential supplement for cell culture medium. This vitamin, B_6, is essential for many mammalian metabolic reactions, acting as a coenzyme for several transamination reactions, mainly involved in the decarboxylation of amino acids [40].

Within the optimal solutions, several reactions are appealing from a biological feasibility point of view, namely amino acids and vitamins. In this study, our target is to narrow the solution space and focus on amino acids, together with other nutrients that are directly involved in the central metabolism. Future studies should be focused on exploring the impact of the other candidates obtained by optiModels, for instance vitamins. Studying the impact of changing the levels of vitamins in the media and their influence on metabolism can thus be very interesting to explore in future studies.

3.3.2. *In silico* CHO cell culture media optimization

As described in the previous section, the genome scale metabolic model of CHO constructed by Hefzi et al, 2016, was combined with an in-house developed algorithm in order to determine the minimal and most essential components for CHO cells growth. Two different biomass reactions were used in the *in silico* predictions, (R_biomass_cho) and (R_biomass_cho_producing), respectively, for producing and non-producing CHO cells as previously described. Several media components were predicted for both cell types. In the following step we used generated data in order to build the optimal amino acids formulation to sustain CHO growth.

3.3.2.1. Medium optimization for non-producing CHO cell lines

In this step, various *in silico* predictions were performed in order to improve the specific growth rate and decrease cell culture by-products using as basis the results from the previous section. The prediction results were based on varying the environmental conditions, i. e., changing the boundaries of uptake fluxes at which certain metabolites are consumed. This strategy can be used to define the components of cell culture media of a specific cell line, in our case, CHO cells. Multiple simulations were performed and the results were compared to the original constraints of the model which was constructed based on different simulations and omics data described in previous published reports [35,38,41]. The in-house simulations were performed in Optflux. The simulations were performed based on the biomass equation

(R_biomass_cho) specific for the prediction of growth rate of non-producer CHO cell lines. The input data were based on literature studies and also on different data generated using the Python-based algorithm (optiModels) previously described.

Using this approach, the optimal combinations of amino acids used for the growth of non-producer CHO cells were determined. The uptake reaction bounds (or constraints) referred in the model developed by Hefzi et al, 2016 and the in-house optimized constraints are referred in Figure 3.3 (Supplementary data in annexes). The values in Table 3.2 in Annexes are expressed in (mmol gDW^{-1} hr^{-1}) and refer to the rate at which the cell consumes certain metabolites for growth (in steady state). The prediction results account for the consumption (Figure 3.2) and production (Figure 3.4) fluxes of different metabolites, as well as the growth rate value (Figure 3.3).

Various differences were observed between the constraints developed in-house and to the original ones. In our work, we increased the uptake for different metabolites such as glucose and other amino acids, since they play a role in increasing cellular growth rate. On the other hand, we limited the consumption of a number of metabolites since their degradation in cell culture may promote the secretion of toxic by-products that can inhibit cell growth. As an example, in order to decrease ammonium accumulation in cell culture medium, glutamine was removed from the optimized formulation. This amino acid is known to be a major source of ammonium which is considered as a toxic metabolite (at high concentrations: approximately over 4mM) for the cells (as described in chapter 2). Ammonium diffusion across the cell membrane play a major part in disturbing intracellular pH in addition to the electrochemical gradients [42]. Its build-up in the cell culture medium can significantly inhibit cell growth and final cell densities [43,44]. Removal of glutamine from the cell culture medium was previously studied. According to the literature, cells cultured with a glutamine substitute in the feed medium generated a decrease in cell growth and also a decrease in ammonium levels [45,46].

On the one hand, additional amino acids were removed from the in-house medium formulation such as serine and asparagine. Serine is considered also as a potential source of ammonium secretion, since its degradation using serine dehydratase, also called serine ammonia lyase (SDH) can generate pyruvate and also ammonia [47]. Another study proved that serine presence in the cell culture medium did not improve the growth rates of the cells [42].The in-house *in silico* predictions using Optflux proved also that when forcing the over-consumption of serine, ammonium secretion rates increased significantly, and no improvements were observed regarding cell growth. Subsequently, we can presume that the *in silico* results are in accordance with the published data in Chen et al 2005 [42].

On the other hand, asparagine supplementation and consumption was proven to be linked to ammonia and alanine accumulation in the culture medium during growth [37]. Alanine accumulation during culture can also inhibit cell growth through repressing the TCA cycle, one of the most important pathways of the cellular machinery [48]. Our strategy was based on finding alternatives to glutamine, serine and asparagine that can improve cell growth and mitigate the effect of ammonium. The *in silico* predictions based on optiModels definitely played a role in facilitating this task. As part of the optimization strategy, the consumption rates of different groups of amino acids were set out to the minimum allowed levels. Tryptophan and methionine consumption rates were also set to the minimum since, as described in previously published reports by Pfzier, their presence in the cell culture medium in higher levels can generate a variety of toxic metabolites, considered as putative growth inhibitors for CHO cells. Examples can be indole 3-lactate and 2-Hydroxybutyrate [49]. On the other hand, disparate amino acids play a major part in improving CHO growth rate, for instance, proline and threonine [42]. Proline supplementation to the cells cultured in proline-free medium was previously studied, and it demonstrated a positive effect on cell growth [50].

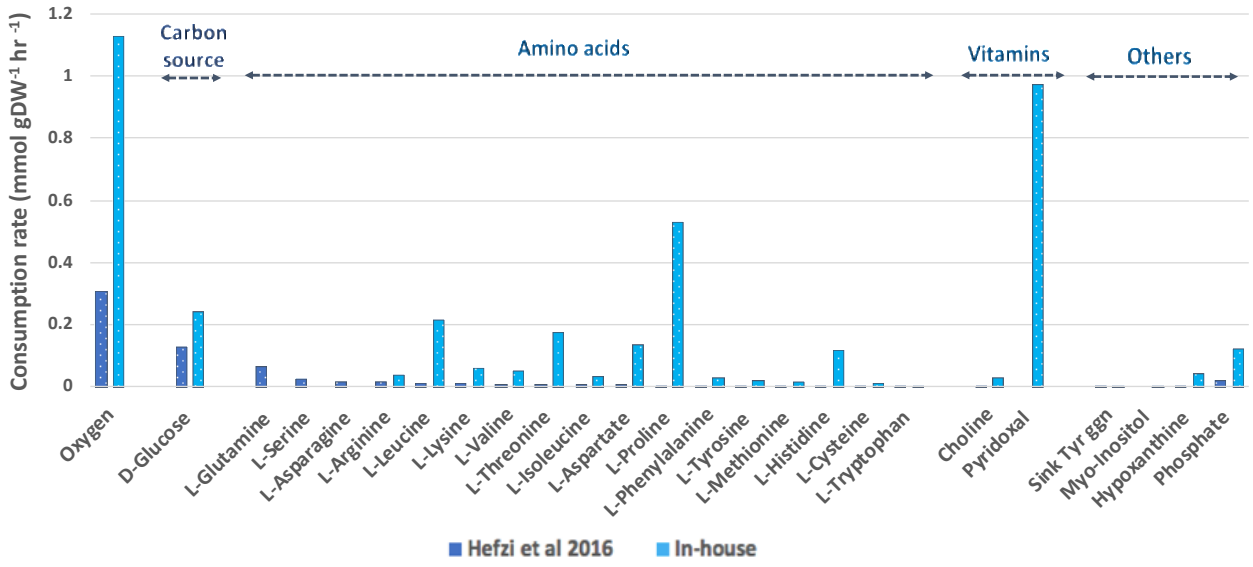

Figure 3.3 Predicted optimal metabolites uptake rates. Comparison between the prediction results of Hefzi et al., 2016 and the results of the in-house optimizations for non-producer CHO cell line. Sink Tyr ggn - Sink reaction for Tyr-194 Of Apo-Glycogenin Protein (Primer for Glycogen Synthesis).

As commonly known, the genetic makeup of the cell lines, their expression profile as well as the environment in which cells are present, can obviously influence the consumption rates and the metabolic fluxes of amino acids. The rationale behind this optimization strategy is to develop a formulation which includes not only the amino acids that are easily consumed by the cells, but also

other metabolites that can retrofit the metabolism of the cells towards more efficiency. Subsequently, higher growth and extended viability of the cells is expected.

As shown in figure 3.3, higher oxygen and glucose consumption were noticed as part of the predictions results when using our in-house optimized constraints. These results align with the fact that more oxygen and carbon source are needed as a means to achieve higher cell densities. We can also observe that higher amounts of valine, leucine, aspartate, proline and histidine have to be supplemented in the optimized cell culture medium to accommodate higher glucose consumption and the decrease in other amino acids.

Concerning vitamins uptake values, a slightly higher uptake rate of choline was observed in the optimized medium formulation. Also, based on the results obtained, pyridoxal, a form of vitamin B_6, positively impacts cell growth and plays a role in the decrease in ammonium accumulation in the cell culture medium. Vitamin B_6 has a central role in the metabolism of amino acids. As an example, the cofactor Pyridoxal-5'-Phosphate plays an important role in the catalysis of many important steps in the metabolism of amino acids, such as transamination, racemization, decarboxylation, and α,β-elimination reactions [51]. According to the stoichiometric matrix described in the GSMM, pyridoxal plays a role in transamination through pyridoxal kinase and in the use of ammonium to produce pyridoxine. Pyridoxal potential in decreasing ammonium levels in the cell culture medium is promising. Further experimental validations have to be performed in order to ratify this hypothesis.

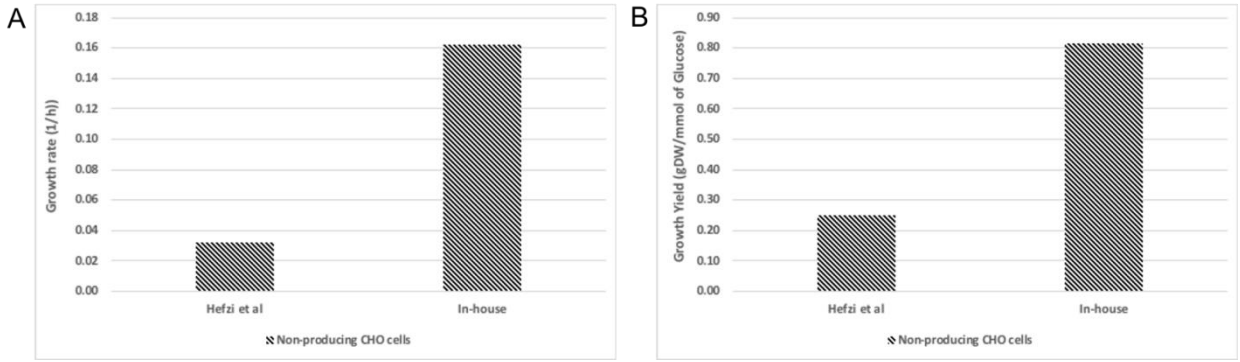

Figure 3.4 Specific growth rate and growth yield values based on the in-house-based constraints for CHO non-producing cell line.

Ensuing, myo-inositol was added to the optimized model constraints. In the predictions, myo-inositol was consumed by the cells and its uptake may hold a promise regarding the targeted improvements.

Finally, higher uptake rates of phosphate and hypoxanthine were observed comparing to the results based on Hefzi et al., 2016 predictions.

A higher specific growth rate was observed using the optimized uptake rates, comparing to the values obtained using the constraints of Hefzi et al., 2016 (figure 3.4). We were able to increase the growth rate up to 5 times comparing to the prediction results based on the constraints of Hefzi et al., 2016. However, since glucose uptake was also increased, the comparison of specific growth rates can be misleading. Therefore, growth yields on glucose were also compared, and a significant increase was also observed, around 3.3 times. Thus, in principle, higher uptake rates of certain amino acids and vitamins can hold a promise for improving the growth rate/yield. However, higher nutrients uptake has to account for transporters capacity within the cell.

Using the in-house optimized uptake constraints of various metabolites, interesting results were observed regarding the secretion rate of toxic metabolites such as ammonium (Figure 3.5). Relying on the in-house optimized constraints, ammonium secretion was eliminated comparing to the predicted results of Hefzi et al., 2016.

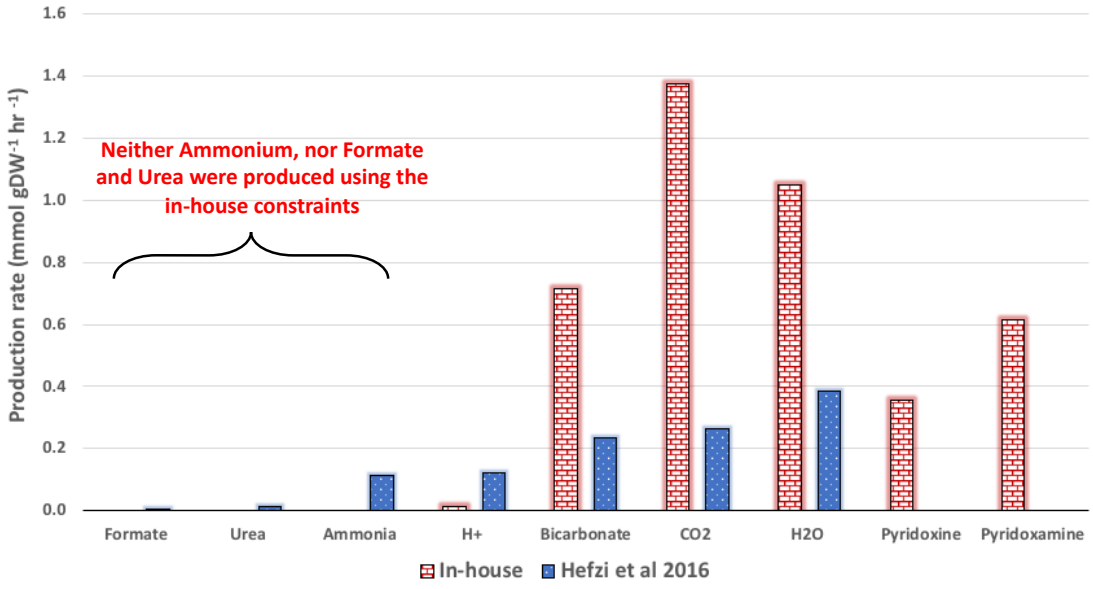

Figure 3.5 Metabolites secretion values for optimum uptake conditions. Comparison between the in-house prediction results and the results obtained using Hefzi et al 2016 constraints. H+ (hydrogen ion), CO2 (Carbon dioxide), H20 (Water).

Looking at the *in silico* results in figure 3.5, ammonium, formate and urea were not secreted by the cells when using the in-house optimized constraints, when maximizing for growth. Serine and asparagine were present in the formulation described in Hefzi et al., 2016, which can explain the

accumulation of these toxic metabolites as a result of amino acids breakdown. Formate secretion is directly linked to serine presence in the constraints of Hefzi et al., 2016 since formate is considered as a product of serine metabolism [48]. Finally, pyridoxamine and pyridoxine were secreted in our results as part of vitamin B_6 metabolism.

As a conclusion, we can affirm that the improvements made using Optflux based on the minimal components developed in-house were successful in improving the non-producing cell's growth rate. The biomass yield was more than tripled comparing to the results described in previously published data. Another major improvement is that the toxic by-products such as ammonium were decreased to their lowest levels.

In the next section, we will discuss the *in silico* efforts made to improve the growth parameters for CHO producer cell lines, as well as the improvements made to enhance recombinant proteins production.

3.3.2.2. Optimization results of CHO producing cell lines

In order to predict the best medium components that can be used to improve the growth rate of CHO producing cell lines, the uptake fluxes were also optimized based on literature and also the minimal components predictions obtained using optiModels, previously described. The predictions were performed based on the biomass equation (R_biomass_cho_producing) specific for predicting growth rate of producer CHO cell lines. The prediction results account for the consumption and production rates of different metabolites, as well as the growth rate and yield values, specific for each condition tested. In this section, we were able to improve the growth rate by 5 times comparing to the growth value based on the use of Hefzi et al., constraints. Additionally, the growth yield value increased by 2 times. These results are described in figure 3.6.

In this part of the work, not only improving the growth rate/yield and reducing cell culture by-products is targeted, but also improving the production of mAbs is a major ambition. The obtained results were used to determine the effect of flux constraints on the production of recombinant proteins in producer cell lines. Two different sets of constraints were obtained depending on the goal of the use of CHO producing cell lines. The first set of results is based on improving the growth rate and decreasing by-products levels. The second set of constraints was based on improving the mAbs production with maintaining the cell culture by-products at their lowest levels. The reason behind generating two different sets of environmental constraints in this part of the study, is that the cells use their resources

(Carbon source, amino acids, *etc.*) jointly for maximizing growth and maximizing the recombinant proteins production at the same time, as part of the mammalian growth-uncoupled phenomena, previously described in chapter 2. In this prediction studies we were able to improve the production rate of recombinant proteins comparing to different published data, described in Carinhas et al., 2013 and Selvarasu et al., 2012 [38,41]. In table 3.3 (Supplementary data), we can observe the lowest bounds (uptake) of several metabolites referred in the reference model (original model constraints) and also the in-house optimized constraints aiming at increasing growth rate/yield and mAbs production rate.

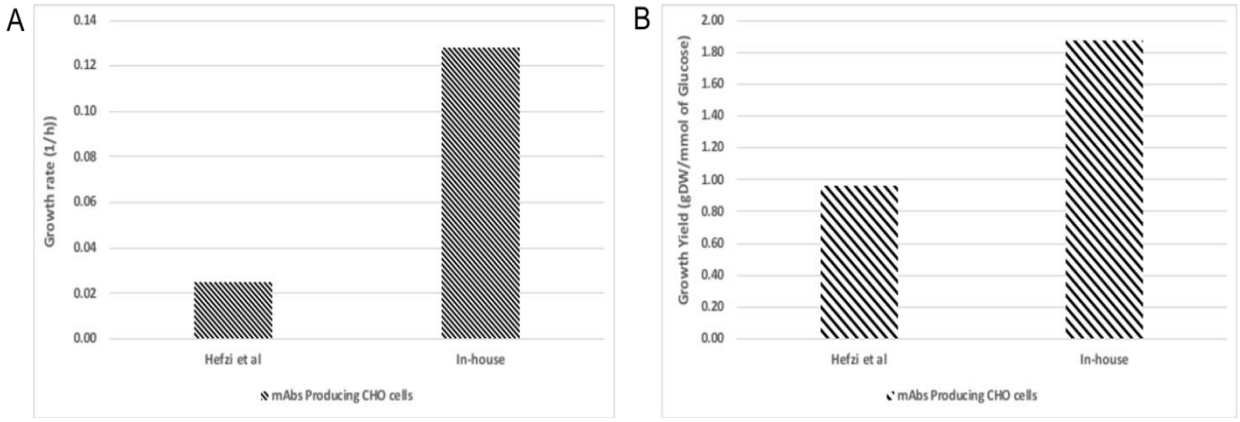

Figure 3.6 Specific growth rate and growth yield values based on the in-house-based constraints for CHO producing cell line.

Predictions in Optflux targeting the enhancement of cell growth rate and the decrease of cell culture toxic metabolites were performed using pFBA. For predicting maximum protein production, different solutions were obtained by maximizing the flux through the DM_igg[g] reaction, specific for the production of Immunoglobulin G (IgG) in the model.

On the one hand, the same constraints used for predicting the optimal growth rate for CHO non-producing cell lines, described in the previous section, were used in this study. Some minor changes were performed in order to reduce the predicted secretion rate of ammonium, since the metabolic behavior of the producing cells was different regarding the use/secretion of some amino acids, pyridoxal-5-phosphate (P5P) and other metabolites. For that, hypoxanthine and P5P were removed from the optimized uptake constraints of CHO producing cell lines. Hypoxanthine was linked to ammonium secretion in this part of predictions. Also, in this case, serine uptake showed a positive effect on

increasing cell growth rate for CHO producing cell lines. In this part of the work, serine removal from the optimized constraints generated high secretion of ammonium and acetate.

On the other hand, for maximizing IgG production, we exerted the same constrains used for predicting the CHO producing cells growth rate with minor modifications in order to balance the aptitude of the cell in sharing its nutrients for both growth and production of recombinant proteins. The optimum constraints obtained in this study are also shown in table 3.3 (Supplementary data).

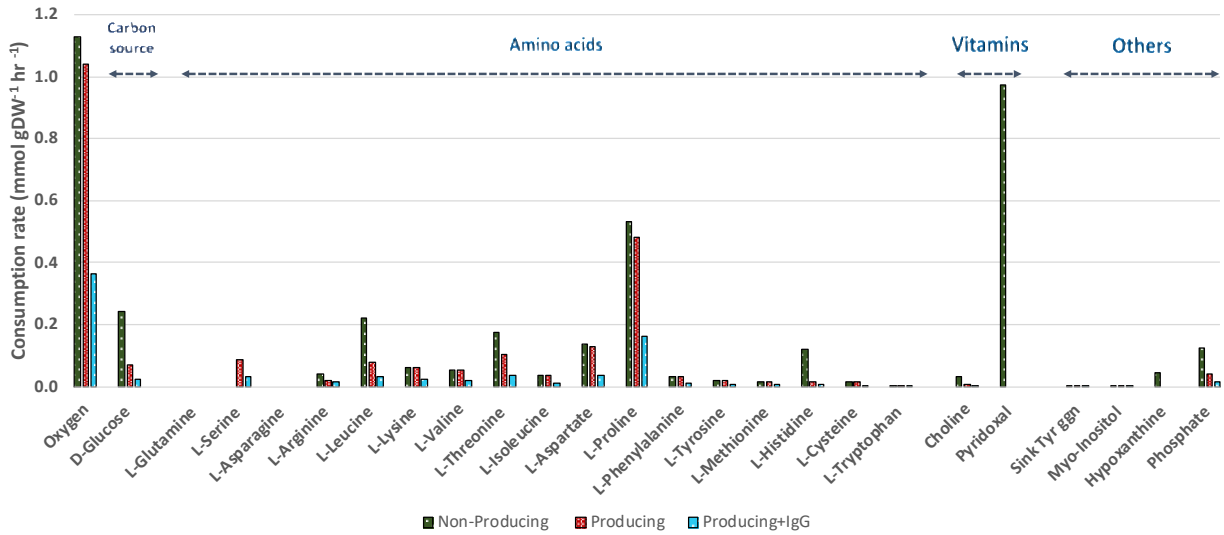

Figure 3.7 Predicted metabolites optimal uptake rates. Comparison between the prediction results of non-producing cell line, producing cell line and the producing cell line targeting the IgG production. Sink Tyr ggn (Sink reaction for Tyr-194 of Apo-Glycogenin protein (Primer for glycogen synthesis).

The predictions regarding the consumption rates of the metabolites applying the previously described constraints are referred in figure 3.7, where we can observe a comparison between the consumed metabolites of non-producing cell line, producing cell line and producing cell line targeting the production of recombinant proteins.

Targeting growth maximization in producer cell lines, the latter consumed high levels of serine and much lower levels of histidine than the non-producer cell lines. In the predictions using CHO producing cell lines targeting the production of mAbs, we can observe that the cells used less resources, given that the production of recombinant proteins is less demanding than the production of biomass. . Moreover, higher ammonium and formate production rates were observed in the predictions using the CHO producing cell lines comparing to the non-producer ones. A comparison between the production

rates of metabolites in the conditions tested (non-producing, producing and producing+IgG) is highlighted in figure 3.89.

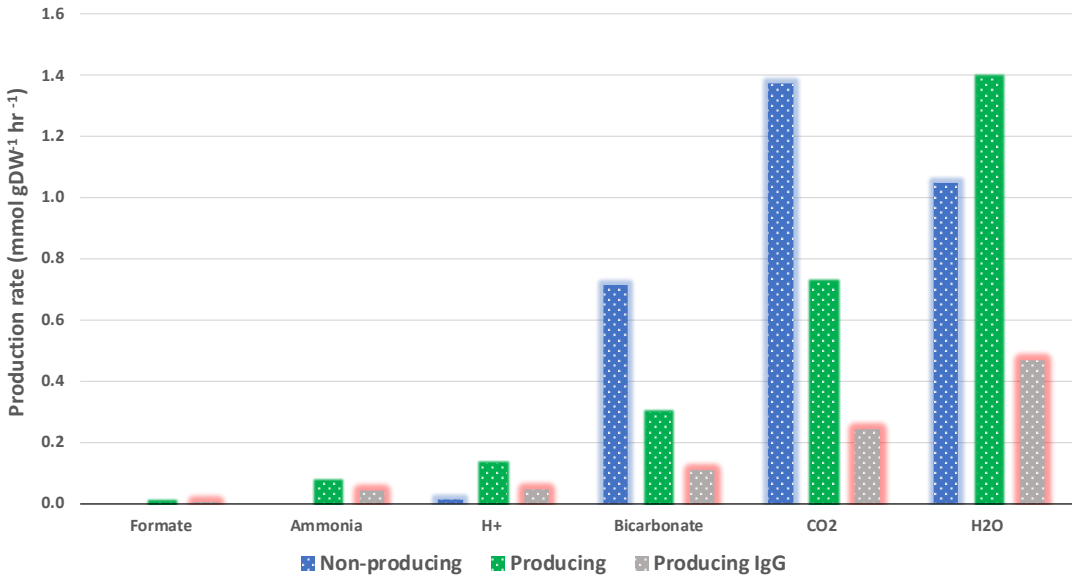

Figure 3.8 Metabolites production rates for optimum uptake rates. Comparison between the in-house prediction results of non-producing cells, producing cells and producing targeting the generation of mAbs**.** H+ (hydrogen ion), CO2 (Carbon dioxide), H20 (Water).

As described, recombinant protein production competes with biomass. Therefore, the results above assume the production of one or the other.

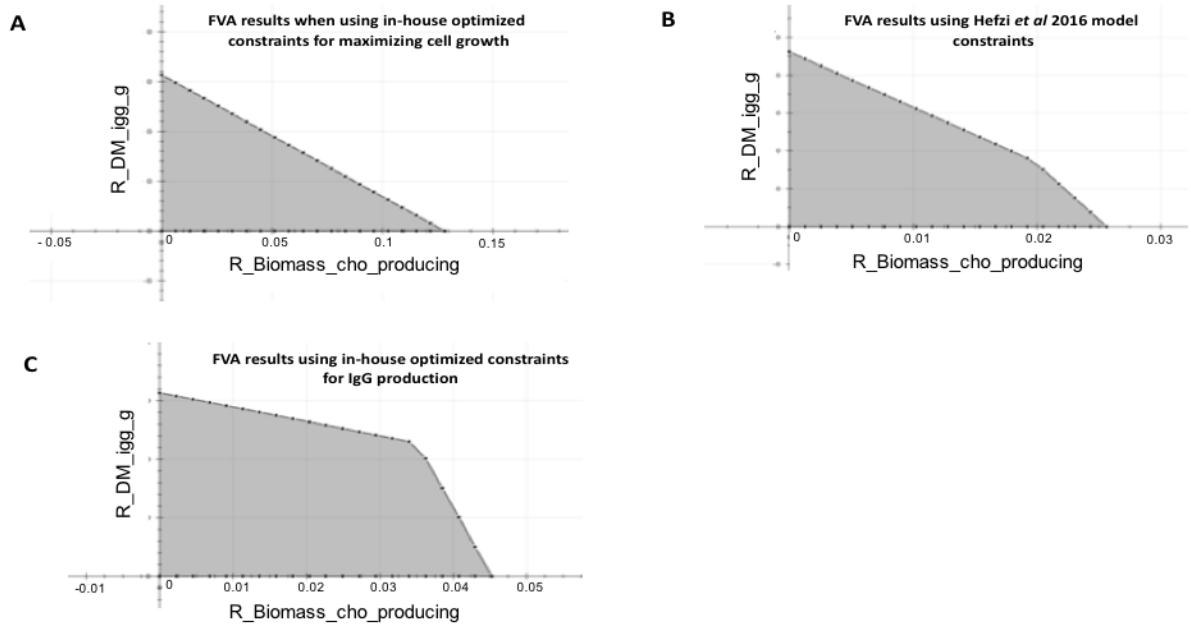

Figure 3.9 FVA prediction results. (A): FVA results using the in-house optimized constraints for maximizing cell growth for producer cell lines. (B): Prediction results using Hefzi et al., 2016 model constraints. (C): Prediction results using the in-house optimized constraints targeting IgG production.

To illustrate this competition, a Flux Variability Analysis (FVA) was performed (Figure 3.9). FVA results show that the best predicted production rates of mAbs are the ones based on the constraints optimized targeting the optimization of protein production instead of the constraints targeting the optimization of growth rate.

The optimal conditions can be studied and validated using cell culture and metabolomics studies, since they are just an estimation about the possible exchange values, but not taking into consideration any information regarding affinity of the cells towards these compounds or toxicity, neither about transporters capacities regarding the uptake of these candidates.

3.3.3. Investigating α-ketoglutarate (AKG) effect on CHO metabolism *in silico*

From the optiModels results, besides amino acids and vitamins, one compound that was part of the metabolites making part of the minimal components was further investigated: α-ketoglutarate (AKG). For this purpose, various predictions in Optflux using pFBA were performed. The growth rate, exchange rates of key metabolites, as well as values for the sum of fluxes that have been used in the network were determined, together with the values of growth yields in reference to glucose. In these

experiments, three different uptake rates of AKG were tested, based on 3 different concentrations: 4mM, 8mM and 12 mM. The uptake values of AKG corresponding to the described concentrations were determined experimentally in the laboratory (Data shown in chapter 4). Therefore, these predictions were based on feeding experimental AKG uptake data to the model.

In these experiments, we noticed that, when the cells consume AKG at different rates, the values of the sum of the fluxes decrease slightly and ammonium secretion rate also decreases, for both producer and non-producer cell lines. Relying on the use of the constraints described in Hefzi et al., the predicted growth rate was equal to 0.0323 h^{-1} in the case of non-producer cells. When forcing the uptake of AKG during the simulations, we can observe that the growth rate values decreased to 0.0243 h^{-1} (Figure 3.10/A). The same effect was observed in the case of producing cells (Figure 3.10/C). Regarding the calculated growth yield, interesting results were obtained. We can observe in figures 3.10/B and 3.10/D, that in the 4mM condition, the growth yield decreases comparing to the growth yield calculated using the original model constraints.

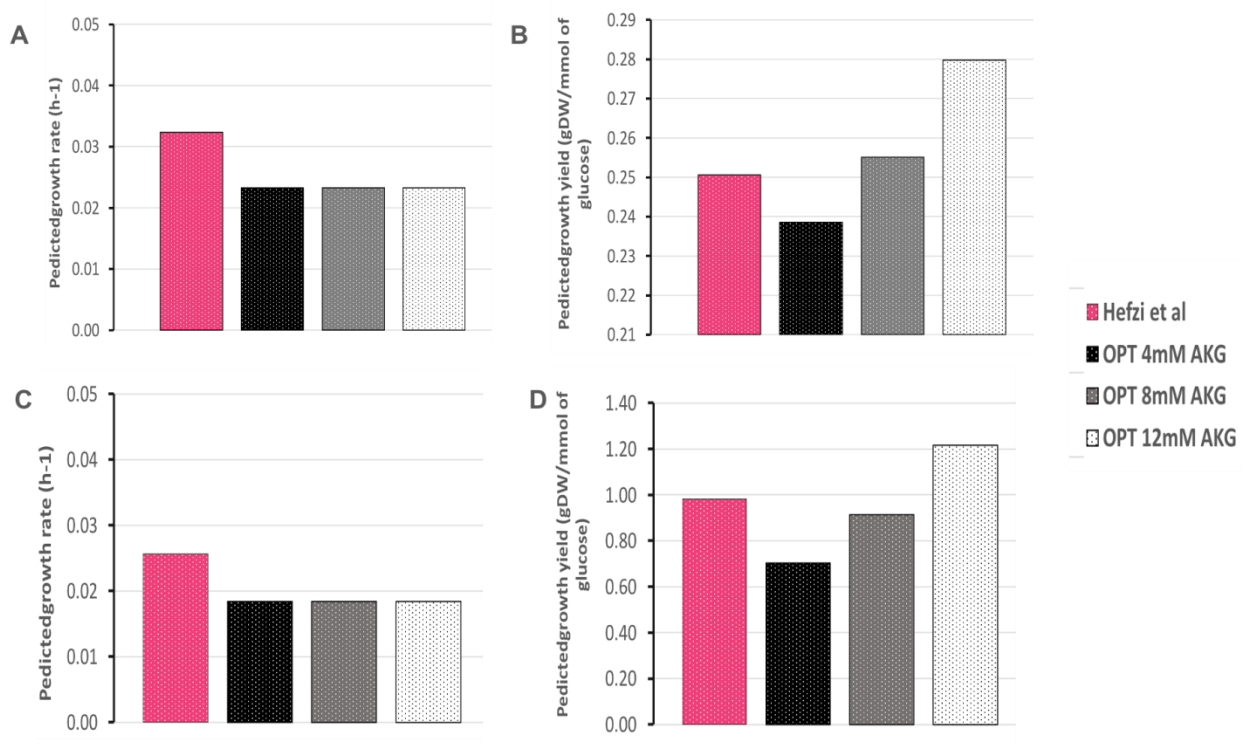

Figure 3.10 Comparison of predicted growth rate and growth yield values between non-producer and producer CHO cells lines when varying AKG uptake rate. 3.12/A describe the predicted growth rate of naïve CHO cells. 3.12/B describe the predicted growth yield of naïve CHO cells. 3.12/C describe the predicted growth rate of producer CHO cells. 3.12/D describe the predicted growth yield of producer CHO cells.

However, in the conditions of 8mM AKG and 12mM AKG, the growth yield increases with the increase of AKG uptake.

In order to further understand these results, we looked deeper at the exchange rates of metabolites and we tried to understand the response of the metabolism to the uptake of AKG. In figure 3.11, we can observe the values of the different exchange rates of key metabolites when varying the uptake levels of AKG, for both naïve (Figure 3.11/A) and producer cell lines (Figure 3.11/B). We can observe that, in the majority of the cases, the flow of different metabolites was similar among different cell types (Producers or not). Few differences were observed especially for the case of glucose consumption, which decreased when increasing the AKG uptake. These results might explain the lower growth rate observed where AKG was consumed by the cells. It should also be emphasized that AKG is also a relevant carbon source, which in part explains changes in growth rates and yields.

The exchange rates of key amino acids were also studied and the results are also shown in Figure 3.11. When maximizing for growth, we noticed, among producer and non-producer cells, many differences in the uptake rate values of amino acids, as a result of varying the consumption rates of AKG in the model. The uptake of asparagine, in the case of naïve cells and serine/leucine in case of producer cell lines, were different when varying the uptake rate of AKG.

On the other hand, lower levels of byproducts were observed in both cell types. In the case of naïve cells (Figure 3.11/D) we observed a decrease in ammonium secretion when increasing AKG concentration. Besides, the secretion rates of formate and urea decreased also. Regarding bicarbonate, its production rates increased significantly when increasing AKG uptake. In the case of producer cells, ammonium levels were decreased when increasing AKG uptake until observing a 0-ammonium secretion in the case of 12mM AKG condition (Figure 3.11/E). Formate and urea secretion rates were also decreased comparing to the predictions based on Hefzi et al. constraints. Finally, a different metabolic profile of bicarbonate was observed, where its secretion levels decreased when increasing AKG uptake, on the contrary of the metabolic behavior of the naïve cells.

These prediction results are very promising towards the decrease of process byproducts such as ammonium and for future experimental validation, where we can test, experimentally, different concentrations of AKG that can be supplemented to CHO cultures. In principle, higher concentrations of AKG in the medium tend to force the production of glutamate in the cell, which is driven by the consumption of ammonium available in the medium. This reaction is catalyzed by glutamate dehydrogenase (GDH). The formed glutamate can be further aminated to produce glutamine, under the

control of glutamine synthase (GS), consuming free ammonia molecules [52]. Observing a decrease in the *in silico* ammonia secretion rate when AKG is consumed, correlates with idea of AKG being converted to glutamine and glutamate.

According to the prediction results, we can conclude that adding AKG to the medium can play a role in influencing the amino acids metabolism (Asparagine-aspartate metabolism) and impact also the ammonium secretion levels.

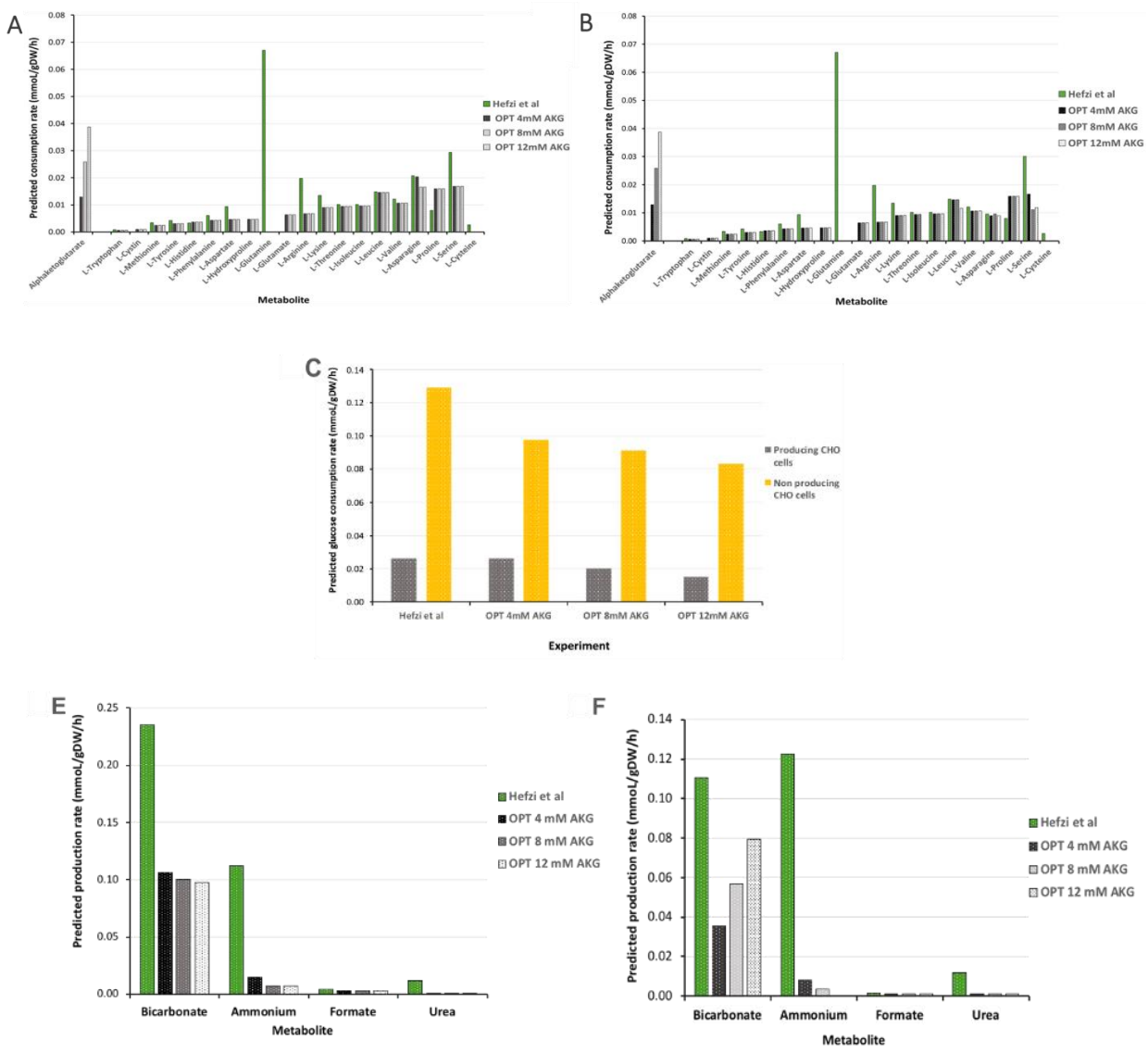

Figure 3.11 Comparison of predicted exchange rates of metabolites between non-producer and producer CHO cells lines when varying AKG uptake rate. 3.11/A and 3.11/B describe the different predicted uptake rates of key metabolites for CHO naïve and producer cells. 3.11/C describes the difference of glucose uptake between non

producing and producing CHO cells. 3.11/D and 3.11/E describe the different predicted secretion rates of key metabolites for CHO naïve and producer cells.

Looking deeper into the literature, it was previously discussed that alpha-ketoglutarate supplementation can play a role in both decreasing ammonia levels in the culture and increasing productivity of the cells towards the production of recombinant proteins [53,54].

Additionally, cells supplemented with AKG exhibited lower growth rate comparing to glutamine-fed cells. This observation was highlighted in the work published by Tae Kwang Ha, Gyun Min Lee, 2014. In this study, the growth rate was recovered after several passages [54]. This strategy is further investigated along the thesis and described in the following chapters. According to the prediction results, it is relevant to understand experimentally the optimal levels of AKG to be added in the media.

Being a very important intermediate in the TCA cycle, understanding AKG metabolism in both producer and non-producer cell lines, how does it impact the different metabolic pathways of the cell and what metabolic mechanisms are behind the boost in productivity will be targeted in the next chapter and consolidated with experimental data.

3.4. Conclusions and following work

Evolutionary algorithms hold a potential in improving various industrial organisms through its combination with genome scale metabolic models. On this regard, several efforts have been performed to explore various optimization strategies using several microbial organisms. Nonetheless, few examples employed mammalian production platforms (e.g., CHO) due to their genetic and metabolic complexity. In this study, we were successful in using the GSMM of CHO combined with optiModels, a novel evolutionary algorithm, that aids in exploring CHO metabolic network and understanding how to develop an optimal medium formulation, that can sustain (high) growth of CHO. Then, higher production titers and/or productivity can be achieved. Higher growth yield (3.3 times) was observed when using the in-house optimized constraints comparing to predictions using the original model constraints (Hefzi et al., 2016). Furthermore, based on FVA predictions, it was demonstrated that when using the optimized media formulation (based on the optimized in-house constraints), higher production rate of IgG was observed comparing to the conditions used for non/producing cells.

In the second part of the study, we demonstrated that the supplementation of several candidates, determined using optiModels, might hold a potential in improving both growth and productivity of the

cells. Theoretically, AKG, as a very important intermediate in the TCA cycle, holds a huge potential in improving growth and productivity. Different uptake rates of AKG were tested *in silico* and the metabolic flux distribution was mapped based on the use of pFBA for both CHO producing and naive cells. Very interesting results were obtained regarding the supplementation of AKG to the medium, reflected by an increase in growth yield by 1.1 and 1.2 times, respectively, for naïve and producing CHO cells. In addition, a significant decrease in by-products secretion (Ammonia) was observed comparing to the results obtained using Hefzi et al. constraints.

As conclusion, using the minimal media formulation described in this chapter as well as supplementing AKG might hold a tremendous potential in improving CHO bioprocesses. However, it should be pointed out that these results are merely an indication of a pathway forward in terms of experimental implementation. In fact, the results obtained using the GSMM do not take into consideration any kinetic limitations of the transporters and/or enzymes involved. This implies that the optimal uptake rates *in silico* might be not achievable in real conditions due to rate limitations. However, even if this is not fully possible, one can take the obtained results as leads for further metabolic or enzyme engineering approaches. The next steps will be focused on validating some of the *in silico* results experimentally.

3.5. Annexes

A) OptiModels

This tool was developed by Sara Correia and Sophia Santos at Minho University.

The framework uses three other open-source Python frameworks, namely:

- **FRAMED** (Framework for Metabolic Engineering and Design): A Python package for analysis and simulation of metabolic models that is used to load metabolic models from SBML files (https://github.com/cdanielmachado/framed).
- **Odespy**: offers a unified interface to a large collection of software for solving systems of ordinary differential equations (ODEs) (https://github.com/hplgit/odespy).
- **Inspyred**: an open-source framework for creating biologically-inspired computational intelligence algorithms in Python, including evolutionary computation (https://github.com/aarongarrett/inspyred).

This framework was implemented to be able to use parallel computing during the optimization tasks taking advantage of high-performance computing resources. Moreover, the user can specify the maximum time allowed for each simulation. The main entities involved in the simulation process are shown in figure 3.12. The framework files are divided into 4 main packages: Model, simulation, optimization and utils, and the complete description of each one of them is described below:

- **Model:** This package contains functions to load and manipulate the models. Loading the used model in SBML format, is based on the methods and classes present in the FRAMED framework.
- **Simulation:** This package contains the classes and functions used to simulate different types of models.
- **Optimization:** This package contains all required entities to perform the targeted optimization based on evolutionary computation. The inspyred framework is used for creating biologically inspired computational intelligence algorithms in Python, including evolutionary computation and simulated annealing.
- **Utils:** This tool holds a set of generic and auxiliary functions, constants and configurations used by the methods developed in the framework.

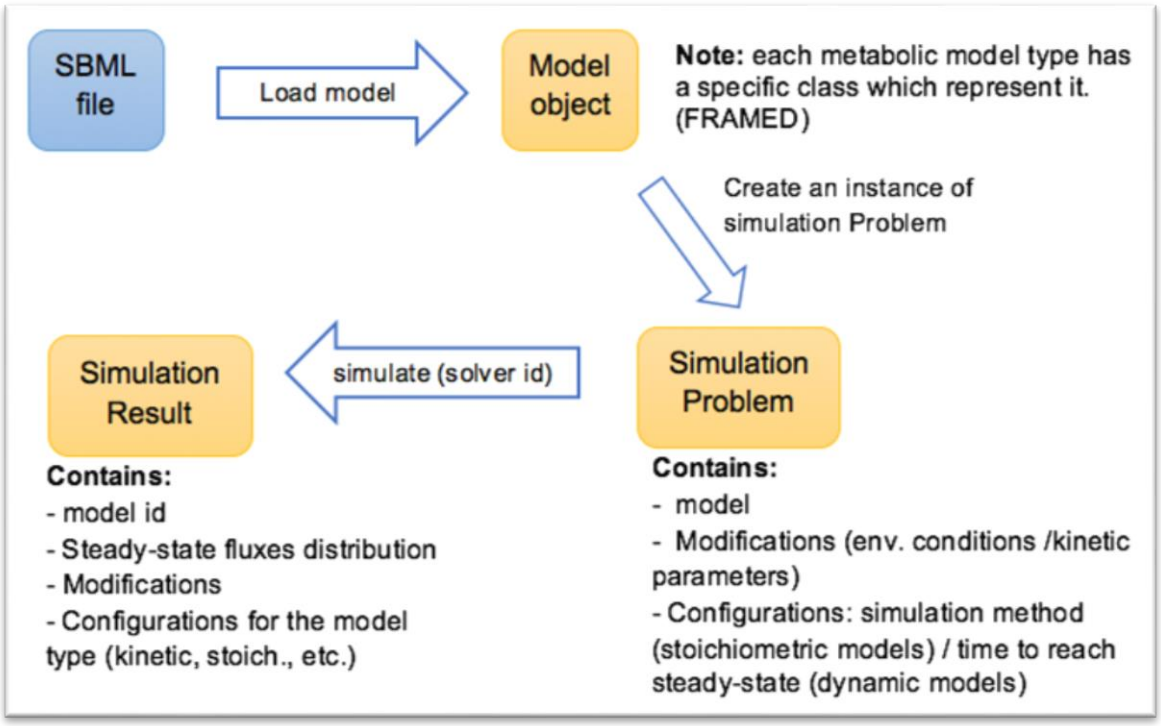

Figure 3.12 OptiModels simulation workflow.

3.5.1. Model handling and simulation

The data obtained from the SBML file is stored as an instance class according to its own model type. The *CBModel* and *ODEModel* classes are extensions of the generic class *Model*. All of these classes are present in the FRAMED framework.

To simulate the problem, the different simulation problem classes have all the information required to perform a phenotype simulation. Different parameters are required for each simulation problem class, depending on the model type (stoichiometric, kinetic, etc.). All these classes extend the abstract class *SimulationProblem*, and implement the abstract methods *get_model* and *simulate.* Thus, the implementation of new types of simulation problems must extend the abstract class *SimulationProblem* and implement the abstract methods. This allows the usage of any simulation problem by the optimization layer since all the classes have the required methods implemented. Following, for the simulation results, the instances of these classes stores the results of phenotype predictions. Depending on the model type, different data should be saved. All classes must extend the abstract class *SimulationResults* and implement the method *get_fluxes_distribution*, which return the steady-state flux distribution of the phenotype simulation. The flux distribution values will be used by the objective function to calculate the fitness of each candidate in the optimization process.

Meta-heuristics algorithms, including Evolutionary Algorithms and Simulated Annealing, are used by this framework to identify genetic modifications (strain design) and infer minimal medium composition that can improve production yields for relevant industrial compounds. Although these algorithms do not guarantee the convergence to global optima, they have the necessary flexibility, use lower computational power than exact solvers and also provide a family of optimal or sub-optimal solutions that can be further studied in order to determine the optimal one. Figure 3.13 depicts the workflow of the optimization process using Evolutionary Algorithms and the important points of the implementation are explained in the following.

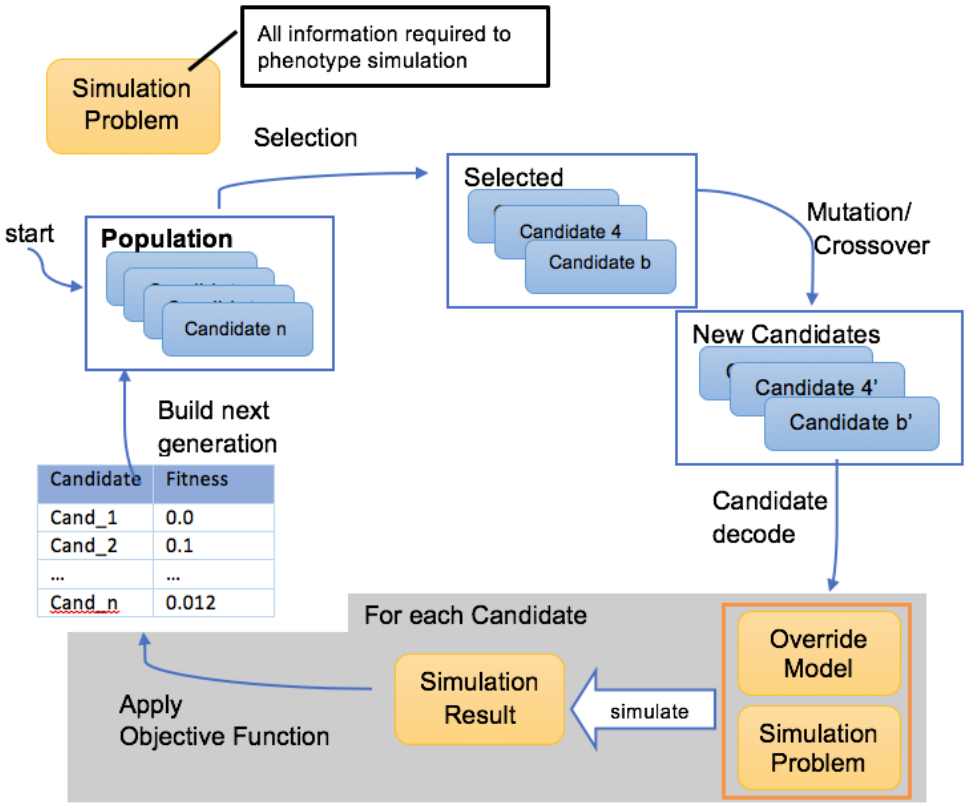

Figure 3.13 OptiModels optimization pipeline.

- **Candidate representation**

In this framework there are two types of candidate representations:

- **Set representation**: this kind of representation can be used to simulate gene/reaction knockouts and infer minimal medium composition. In this case, each candidate element represents the index of the gene/reaction that will be knocked-out in the phenotype simulation.

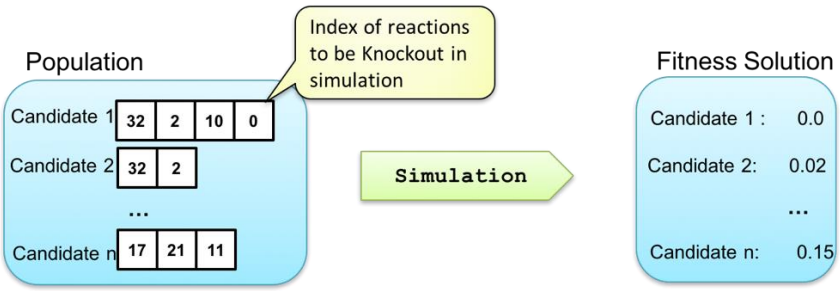

Figure 3.14 Candidates representation – Set representation.

- **Set of tuples:** this representation is used to perform the simulation of under/over expression. Each element of the candidate solution is a tuple of 2 integers. The first identifies the reaction to manipulate and the second the level of expression.

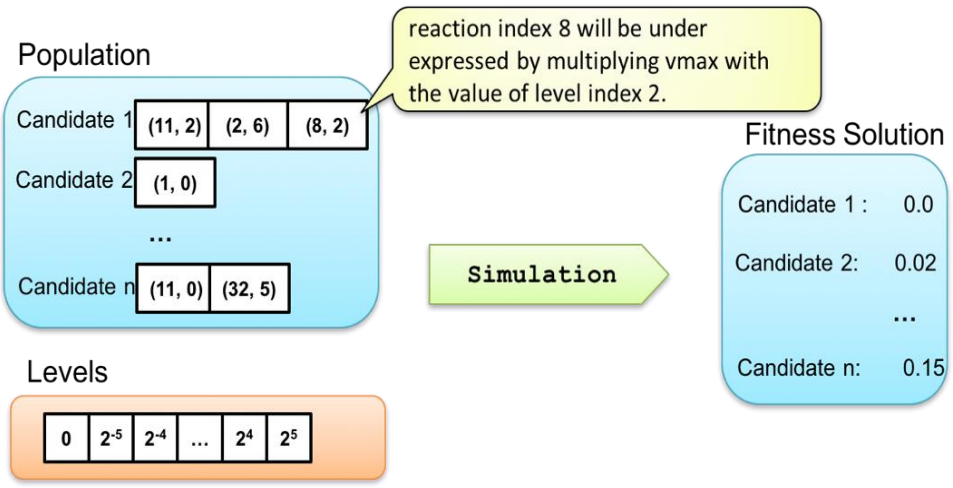

Figure 3.15 Candidate representation - Set of tuples.

These representations are the result of the functions *generator_intSetRep* and *generator_intTupleRep* used in the optimization workflow to generate new candidates for the population.

- **Operators**

For reproduction purposes within the Evolutionary Algorithm, the following operators have been implemented:

- **Mutation operations**
  - *Grow*: insert a new element (integer/tuple) in the candidate solution.
  - *Shrink*: remove an element from the candidate solution.
  - *Replace*: replace one element for a new one randomly generated.
- **Crossover**: using two candidates (parents) build 2 children:
  - Elements present in both parents will be present in both children.
  - Elements present in only one parent have equal probability to be present in child 1 or child 2.
  - *note*: children can be equal to the parents

- **Objective Functions**

The objective function has the role of evaluating each candidate of the population calculating the corresponding fitness value. The most common objective function is the flux value of a target compound, implemented in the framework as *targetFlux* class. All objective functions on the framework must extend the abstract class *objectiveFunction* and implement the methods:

- *get_name:* returns a string with the method name.

- *method_str:* returns a string with the method formulation.
- *get_fitness:* returns the fitness value considering the simulation result given as argument.

- **Decoders**

The decoders are responsible to convert a candidate representation into an OverrideModel which contains the modification that will be used over the simulation problem in the phenotype simulation. As example, the candidate represented by a set of integers (1,3,5) given as argument to the method *get_override_simul_problem* of the *decoderReactionsKnockouts* class, will retrieve a list of modifications that must be imposed to the simulation problem, in order to knockout the reactions represented by 1, 3 and 5 indexes.

- **Applications**
  - Strain design

Strain design through reaction knockouts or under/over expressed enzymes is implemented in our framework for single and multi-organism models. The approach used is the same for the two cases. The solution candidates can be represented as a set representation or as set of tuples, as described above. The operators (mutation and crossover) used in EA are the ones described previously.

Two evaluation functions were implemented in the framework under the scope of strain design:

- **TargetFlux**: The fitness is given by the flux value of the target reaction.
- **BPCY**: "Biomass-Product Coupled Yield" objective function (Patil *et al.*, 2005). The fitness is given by the equation:

$$fitness = \frac{biomass\ flux\ \times product\ flux}{uptake\ flux}$$

  - **Minimal Medium optimization**

The goal of minimal medium optimization is to find the best medium composition for a given objective function, such as growth or the production of a target compound. EA are used by our framework to identify the smallest set of uptake compounds that can improve a given objective function for single and multi-organism models.

The solution candidates can be represented as a set, where each element represents an uptake exchange reaction. The operators (mutation and crossover) used in EA are the ones described previously.

For medium optimization purposes, two evaluation functions are available in the framework:

- **BP_MinModifications**: this evaluation function is based on the "Biomass-Product Coupled Yield" objective function (Patil *et al.*, 2005) but considering the candidate size. The fitness is given by the equation:
-

$$fitness = \frac{biomass\ flux\ \times product\ flux}{uptake\ flux}$$

- **MinNumberReac**: this function returns a fitness value between 0 and 1. Higher fitness values correspond to candidates with a smaller size.

$$fitness = \frac{size(candidate)}{maximum\ of\ candidate\ size}$$

The maximum of candidate size is the maximum number of uptake reactions allowed by user. By default, this value is the number of exchange reactions present in the given model.

B) Additional Material

Table 3.2 Original model constraints vs in-house optimized constraints for non-producer cell lines.

Reaction ID	Metabolite	Hefzi et al 2016	In-House
R_EX_glc__D_e	D-Glucose exchange	-0.19835	-0.3
R_EX_his__L_e	L-Histidine exchange	-0.00330	-0.2
R_EX_trp__L_e	L-Tryptophan exchange	-0.00408	-0.00408
R_EX_cys__L_e	L-Cysteine exchange	-0.00522	-0.05
R_EX_met__L_e	L-Methionine exchange	-0.00604	-0.02
R_EX_phe__L_e	L-Phenylalanine exchange	-0.00604	-0.1
R_EX_pro__L_e	L-Proline exchange	-0.00797	-0.7
R_EX_asp__L_e	L-Aspartate exchange	-0.00934	-0.2
R_EX_tyr__L_e	L-Tyrosine exchange	-0.00934	-0.05
R_EX_ile__L_e	L-Isoleucine exchange	-0.01016	-0.05
R_EX_thr__L_e	L-Threonine exchange	-0.01016	-0.2
R_EX_val__L_e	L-Valine exchange	-0.01209	-0.1
R_EX_lys__L_e	L-Lysine exchange	-0.01346	-0.08
R_EX_leu__L_e	L-Leucine exchange	-0.01484	-0.3
R_EX_arg__L_e	L-Arginine exchange	-0.01978	-0.1
R_EX_asn__L_e	L-Asparagine exchange	-0.04038	0
R_EX_ser__L_e	L-Serine exchange	-0.04780	0
R_EX_gln__L_e	L-Glutamine exchange	-0.06703	0
R_EX_chol_e	Choline exchange	-0.02029	-0.05
R_EX_pydxn_e	Pyridoxine exchange	-0.00017	-0.00017
R_EX_fol_e	EX fol e	-0.00046	-0.00046
R_EX_pydx_e	Pyridoxal exchange	0	-1
R_EX_hxan_e	Hypoxanthine exchange	-0.00619	-0.1
R_EX_inost_e	Myo-Inositol exchange	0	-0.001
R_EX_o2_e	O2 exchange	-1.12747	-1.12747
R_EX_so4_e	Sulfate exchange	-1000	-1000
R_EX_h2o_e	H2O exchange	-1000	-1000
R_EX_pi_e	Phosphate exchange	-1000	-0.5
R_SK_pre_prot_r	Sink pre prot LPAREN er RPAREN	-1000	-1000
R_EX_h_e	H+ exchange	-1000	-1000
R_SK_Ser_Thr_g	Sink Ser/Thr[g]	-0.1	-0.1
R_SK_Tyr_ggn_c	Sink Tyr ggn	-0.1	-0.1
R_SK_Asn_X_Ser_Thr_r	Sink Asn X Ser/Thr[r]	-0.1	-0.1
R_EX_fe2_e	Fe2+ exchange	-1	-1
R_EX_hco3_e	EX hco3 LPAREN e RPAREN	-1	-1

Table 3.3 Comparison between the constraints used for predictions using producer cell lines.

Reaction ID	Metabolite	Hefzi et al 2016	Growth rate optimization	IgG maximization
R_EX_glc__D_e	D-Glucose exchange	-0.19835	-0.3	-0.3
R_EX_his__L_e	L-Histidine exchange	-0.00330	-0.2	-0.2
R_EX_trp__L_e	L-Tryptophan exchange	-0.00408	-0.00408	-0.00408
R_EX_cys__L_e	L-Cysteine exchange	-0.00522	-0.05	-0.05
R_EX_met__L_e	L-Methionine exchange	-0.00604	-0.02	-0.00604
R_EX_phe__L_e	L-Phenylalanine exchange	-0.00604	-0.1	-0.1
R_EX_pro__L_e	L-Proline exchange	-0.00797	-0.7	-0.7
R_EX_asp__L_e	L-Aspartate exchange	-0.00934	-0.2	-0.2
R_EX_tyr__L_e	L-Tyrosine exchange	-0.00934	-0.05	-0.05
R_EX_ile__L_e	L-Isoleucine exchange	-0.01016	-0.05	-0.05
R_EX_thr__L_e	L-Threonine exchange	-0.01016	-0.2	-0.2
R_EX_val__L_e	L-Valine exchange	-0.01209	-0.1	-0.1
R_EX_lys__L_e	L-Lysine exchange	-0.01346	-0.08	-0.08
R_EX_leu__L_e	L-Leucine exchange	-0.01484	-0.3	-0.3
R_EX_arg__L_e	L-Arginine exchange	-0.01978	-0.01978	-0.01978
R_EX_asn__L_e	L-Asparagine exchange	-0.04038	0	0
R_EX_ser__L_e	L-Serine exchange	-0.04780	-0.1	-0.04780
R_EX_gln__L_e	L-Glutamine exchange	-0.06703	0	0
R_EX_chol_e	Choline exchange	-0.02029	-0.05	-0.05
R_EX_pydxn_e	Pyridoxine exchange	-0.00017	-0.00017	-0.00017
R_EX_fol_e	EX fol e	-0.00046	-0.00046	-0.00046
R_EX_pydx_e	Pyridoxal exchange	0	0	0
R_EX_hxan_e	Hypoxanthine exchange	-0.00619	0.00000	0
R_EX_inost_e	Myo-Inositol exchange	0	-0.001	-0.001
R_EX_o2_e	O2 exchange	-1.12747	-1.12747	-1.12747
R_EX_so4_e	Sulfate exchange	-1000	-1000	-1000
R_EX_h2o_e	H2O exchange	-1000	-1000	-1000
R_EX_pi_e	Phosphate exchange	-1000	-1000	-1000
R_SK_pre_prot_r	Sink pre prot LPAREN er RPAREN	-1000	-1000	-1000
R_EX_h_e	H+ exchange	-1000	-1000	-1000
R_SK_Ser_Thr_g	Sink Ser/Thr[g]	-0.1	-0.1	-0.1
R_SK_Tyr_ggn_c	Sink Tyr ggn	-0.1	-0.1	-0.1
R_SK_Asn_X_Ser_Thr_r	Sink Asn X Ser/Thr[r]	-0.1	-0.1	-0.1
R_EX_fe2_e	Fe2+ exchange	-1	-1	-1
R_EX_hco3_e	EX hco3 LPAREN e RPAREN	-1	-1	-1

3.6. References

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CHAPTER 4

Experimental validation of in silico results

The information presented in this Chapter is being prepared for submission to a peer reviewed journal:

Hamdi A., Borth, N., Zanghellini, J., Rocha I.; *In silico*-based approach for medium optimization of CHO cells.

4.1. Introduction

Mammalian metabolic network is very complex, and its operative mode is far away from being optimal [1]. Metabolic requirements vary between different industrial bio-production platforms (e.g., mammalian cells, yeast or bacteria) which reflects a complex divergent metabolome between species [2]. Within the mammalian metabolic network, biochemical pathways are tightly interconnected with strong crosstalk driven by cellular signaling [3] to regulate cellular activity [4], for instance, between glutaminolysis and glycolysis [5]. In the case of CHO, as previously described in chapter 2, glucose and glutamine represent two of the most important nutrients and energy sources of the cell [6]. In culture, these metabolites are drained rapidly and are consumed at higher rates compared to other media components [7,8]. Along with other nutrients, these two metabolites are the main responsible for delivering key atoms (e.g., carbon and nitrogen), allowing the cells to support their basic biological functions. Nevertheless, the fate of these two metabolites can be diverted towards the generation of high levels of toxic by-products [1,9,10], that can not only alter the growth rate of the cells but also decrease the final cell number and alter the productivity of recombinant proteins together with their post-translational patterns [11,12]. Moreover, amino acids are essential to the cell, being the core base of protein synthesis and cellular function, constituting up to 70 % of the cells dry mass [13,14]. The deamination of several amino acids was proven also to play a role in by-products accumulation during the culture period, such as ammonium (Chapter 2).

Glutamine is a crucial amino acid and a substantial metabolite in nitrogen metabolism, producing cellular glutamate by glutamine synthase. This amino acid is involved in several anaplerotic reactions

(e.g., producing oxaloacetate and α-ketoglutarate) and also in energy formation [15]. However, its degradation yields high levels of free ammonium in the culture, which can heavily impact bioprocesses. Beyond that, glutamine instability in liquid media is very well known and it poses several issues for media manufacturers. Supplementing glutamine to the chemically defined media formulations can radically decrease the shelf life of the produced media [16]. Therefore, finding an alternative to glutamine in the cell culture media can definitely mitigate several bioprocessing problems mainly regarding the media validity and most importantly, ammonia accumulation. Moreover, not only glutamine degradation is a source of high ammonium concentrations, but also other amino acids, for instance, asparagine and serine are associated with this phenomenon [8].

It is very important to highlight that different amino acids in the culture medium are consumed at different rates [17]. These values depend on the CHO platform in use, nutritional needs, culture condition, medium composition, the produced recombinant protein [13] and most importantly the transporters capacity within the cell, towards the exchange of metabolites between the extracellular and the intracellular environment [18]. In order to overcome the overproduction of ammonium and lactate and optimize the culture parameters, several approaches are being employed but few were able to fulfill the desired results [19,20].

One of the most important approaches for optimizing bioprocesses is focused on media optimization. Previously, methodologies were based on using conventional practices, for instance mixing different media formulations or titration of the existing components, which, experimentally represents a hurdle [21]. In fact, blending different media formulations is commonly used. This procedure relies mostly on the use of design of experiment (DoE) strategies that can help depicting the optimal media formulation among the tested ones. This procedure is efficient; however, the chemical composition of the resulting optimal media formulation selected remains unknown. Modern media optimization strategies are focusing mainly on statistical design and on the use of high-throughput screening of different potential media candidates, typically based upon scaling down cell culture models [22], where erlenmeyers and also spin tubes [23] are commonly used as small scale cell culture methods. Alongside, the emergence of novel high throughput automated robots, for instance ambr® microbioreactors and its combination with powerful DoE approaches is a robust tool for rapid and efficient process and media screening and optimization [24]. These approaches hold several advantages, for instance, accelerating the optimization timeline.

On the other hand, cell line development strategies have been employed to construct highly characterized and efficient stable cell lines for recombinant proteins production [25]. For large scale manufacturing of recombinant biotherapeutics using CHO platforms, several optimized CHO strains have evolved. These platforms are known for increased production titers comparing to the standard CHO cells commonly in use. Among these newly established cell lines, dihydrofolate reductase DHFR (-) and glutamine synthetase GS (-) cell lines are the most known [26]. Using the GS-knockout CHO cell line is very beneficial when it comes to overcoming the accumulation of ammonia in culture. These cells can grow independently of glutamine presence in the medium [26,27], considering that glutamine synthase catalyzes the consumption of glutamate to biosynthesize glutamine in the cell [28,29].

A strategy built on re-channeling ammonium through several pathways within the cell can also be possible, even knowing that the CHO-GS metabolism is poorly known [17]. Indeed, metabolic engineering strategies based on directing the precursors of these byproducts inside the cell through other pathways combined with optimizing flux distributions is a very promising approach, taking advantage of the versatility of the cell's metabolic network [30]. For that, balancing the levels of metabolites in the culture and controlling their exchange rates is an interesting approach, particularly emphasizing on medium optimization and customizing it to the cells exact nutritional need. In this case, the cells will evolve a more effective metabolism, by reusing the accumulated metabolites to fuel the cells with more energy and/or important metabolites that can improve both growth and quality attributes of products.

α-ketoglutarate (AKG) or also called 2-oxoglutarate is a very important TCA cycle intermediate and a crucial compound for cell's metabolism [31]. It is a precursor in amino acids biosynthesis, also involved in signaling processes in the cell, ATP production, generation of reducing equivalents (NAD+/NADH), also playing a role in regulating various epigenetic mechanisms in the mammalian cell [32]. During cell culture, α-ketoglutarate is a potential additive for cell culture medium, since it can mutually replenish the TCA cycle and trigger its different metabolic intermediates to produce energy and replace glutamine, producing cellular glutamate by glutamate dehydrogenase (GDH). In theory, accumulated glutamate can play a role in the *de novo* biosynthesis of cellular glutamine by glutamine synthase, using the free ammonium in the culture [28,29].

As well, α-ketoglutarate acts as an antioxidant instead of glutamine in many cellular processes and besides, it is more affordable and chemically more stable (in culture and storage) comparing to glutamine [33]. Previous reports studied the effect of supplementing AKG and different TCA cycle

intermediates, as a replacement of glutamine on CHO cells. In one study, they used CHO-DG44 DHFR (-) cell line where AKG was supplemented at a concentration of 4 mM. In this study, the authors stated the advantages of supplementing AKG to culture. Among these, an increase in productivity as well as a significant decrease in ammonia accumulation in the media were observed [34]. Following this idea, it is not yet clear how the substitution of glutamine by α-ketoglutarate modifies the metabolic network of CHO cells. It is a fact that supplementing α-ketoglutarate to the culture is not enough to channel most of the by-products in the cell, but concurrently balancing the amino acids levels inside of the culture medium, in the presence of α-ketoglutarate, might restructure the cells metabolism and overcome the accumulation of toxic metabolites that can hinder both growth and productivity. This hypothesis drove us to think about engineering strategies based on balancing the levels of nutrients in the medium in order to sustain and rewire the metabolism towards optimality. For that, balancing the amino acids levels together with testing different AKG concentration on CHO cells is very interesting, especially tackling GSneg CHO cell lines, since they hold a huge potential in modern bioproduction processes.

It is a hurdle to rely just on experimental setup for optimization. As a solution, metabolic modeling approaches comes to support faster and accurate predictions towards optimizing the culture parameters by studying biochemical pathways *in silico* and applying the prediction results to be tested experimentally.

Based on the results presented in chapter 3, predictions based on the use of GSMM of CHO and the evolutionary algorithm (optiModels) drove us to think about validating those hypotheses experimentally.

4.2. Materials and Methods

4.2.1. Experimental setup

4.2.1.1. Cell culture

Three different CHO cell lines (two producer and one non-producer) were used in this work. First, CHO-K1 (ECACC 85051005) was employed in this study representing a naïve cell line, non-modified to produce recombinant proteins. Second, CHO-EpoFc represents a CHO DHFR (-) strain, a producer cell line engineered to produce EpoFc fragments (one molecule of erythropoietin joined to each hinge region of huIgG1Fc). CHO-EpoFc is known as a low producer that was established following the protocol previously described in Lattenmayer et al 2007 [35]. These cells were adapted internally to growth in serum-free and glutamine-free medium in the laboratory, prior to the use in this study. Finally, CHO-HyC

cells correspond to an antibody expressing CHO cell line provided by Cytiva, Uppsala, Sweden. The latter is GSneg cell line and known as a high-producer industrial clone, producing Trastuzumab, a monoclonal antibody under the commercial name of Herceptin.

All the CHO clones were cultivated in suspension mode in chemically defined serum-free conditions using CD CHO medium (Gibco, Invitrogen, Carlsbad, CA, USA). After thawing, the cells were routinely cultivated in 50 mL TPP® TubeSpin bioreactors (Techno Plastic Products AG, Trasadingen, Switzerland) at a maximal working volume of 25 mL. The cells were incubated in 37°C in 80 % humidified air with 7 % CO_2, shaking at a speed of 220 rpm (rotation per minute). The cells were passaged every 3-4 days and the viable cell concentrations, viabilities and the values of the average cell diameters were determined using Vi-CELLXR (Beckman Coulter, USA).

In standard conditions, CHO-K1 cells were grown in glutamine-free CD CHO medium (Gibco TM, MA, USA) and supplemented with 0.2% anti-clumping agent (ACA) (Thermo Fisher Scientific). CHO-EpoFc cells were grown in glutamine-free CD CHO medium and supplemented with 0.096 μM methotrexate (MTX) (Thermo Fisher Scientific).

CHO-HyC cells were originally cultivated in CD CHO medium supplemented with 8 mM glutamine, 75 μM of L-Methionine sulfoximine (MSX) (Thermo Fisher Scientific) and 0.2 % ACA (Thermo Fisher Scientific). An adaptation process to glutamine-free conditions was performed and consists of a sequential adaptation of the cells to different ratios of the CD CHO media containing 8mM glutamine and CD CHO glutamine-free medium. The sequential adaptation process is described in table 4.1.

Table 4.1 Sequential adaptation of CHO-HyC cells to glutamine-free conditions.

Adaptation step	Ratio of CD CHO medium containing 8mM glutamine to CD CHO glutamine-free media	Criteria to fulfill
A	75:25	Viability ≥90% and normal doubling time for 2 passages
B	50:50	Viability ≥90% and normal doubling time for 2 passages
D	0:100	Viability above 90% of cells grown in glutamine-free medium and doubling time for 2 passages

As a result of this adaptation strategy, the cells were grown in glutamine-free media. A working cell bank was stored for further experiments.

In all the experiments the cells were grown in glutamine-free conditions using CD CHO cell culture media. These cells were supplemented with different concentrations of AKG (4mM, 8mM and 12mM). For the experimental setup, batch cultures were performed in 125 mL non-baffled Erlenmeyer shake flasks at a working volume of 50 mL, incubated in 37°C in 80 % humidified air with 7 % CO_2, shaking at a speed of 140 rpm. All the experiments were performed in triplicates, inoculated at the beginning of the experiment, at the same time and cell concentration. Samples were taken every 24h. The culture continued until reaching a cell viability lower than 60 %.

4.2.1.2. Extracellular Metabolites

For the analysis of the extracellular metabolites, samples were collected every 24h along the culture period. The cells were removed by centrifugation 10 min at 200 rcf (relative centrifugal force) and the supernatant was stored at -20°C. Metabolomics analyses were performed shortly after sampling, analyzing glucose, lactate and ammonia using Bioprofile 100 Plus (Nova Biomedical, MA, USA). Amino acids levels were quantified using HPLC with fluorescence detection (Dionex 3000 HPLC, Thermo Fisher Scientific, Waltham, Massachusetts, US). The HPLC was equipped with an AdvanceBio AAA column (4.6 x 100 mm, 2.7 µm, Agilent Technologies, Santa Clara, CA, USA) and a pre-column UPLC guard column, AdvanceBio AAA (4.6 x 5 mm, 2.7 µm, Agilent Technologies, Santa Clara, CA, USA). The column temperature was set to 37°C. The mobile phase consisted of A: 40 mM Na_2 HPO_4 in 0.02 % NaN_3 and B: ACN/MeOH/H_2O (45:45:10) (v/v). O-phthalaldehyde (OPA)-derivatized amino acids were detected at 340ex and 450em nm and 9-fluorenylmethyloxycarbonyl (FMOC)-derivatized amino acids at 266ex and 305em nm [13]. Data were processed by Chromeleon software (Thermo Fisher Scientific, Waltham, MA, US). Cysteine could not be quantified due to sensitivity issues in the method, so the results are only qualitative.

4.2.1.3. α-ketoglutarate quantification

α-ketoglutarate levels were quantified using a colorimetric analytical method, following the manufacturer protocol. The quantification kit was purchased from Sigma Aldrich (MAK054).

The supernatant samples were deproteinized using 10 kDa MWCO Amicon Ultra-0.5 centrifugal filter units (Merck Millipore, MA, USA) and diluted using the assay buffer of the kit. Several dilutions were performed in order to fit our samples concentrations into the standard curve, not forgetting the negative control samples without adding AKG converting enzyme into the reaction mix. The AKG standard curve was established with concentrations ranging from 0 to 10 nmole/well. Every reaction contained 50 µL of reaction/well. The plates were incubated for 30 min at 37°C with a shaking speed of 330 rpm to homogenize the reaction mix during the incubation time. Ensuing, the absorbance was measured at 570 nm.

4.2.1.4. Product quantification

Product concentration was determined using Octet® QKe (Port Washington, NY), equipped with Dip and ReadTM Protein A Biosensors (Pall corporation, Port Washington, NY) according to the manufacturer's recommendations. The supernatant was diluted with CD CHO medium prior to measurements in order to fit the samples concentrations within the standard curve ranging between 0-100 µg/mL of Trastuzumab (BioVision, Milpitas, CA). A negative control consisting of cell culture medium was included. This method is based on biomolecular interactions, measuring the binding intensity of our product of interest to an immobilized ligand.

4.2.1.5. α-ketoglutarate toxicity assay

To evaluate the toxicity of supplementing different concentrations of α-ketoglutarate on CHO cells, Cell Titer 96 AQueousOne Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used. The latter is a colorimetric assay, used to determine the number of viable cells for cytotoxicity assays. It is based on the use of tetrazolium compound called [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) or simply MTS, which is converted by the cells to generate formazan. This process is mediated by NADPH or NADH produced by dehydrogenase enzymes in viable cells. The levels of formazan determined by absorbance at 490 nm are directly proportional to the number of viable cells in culture.

In our case, 100 µL of CHO-HyC cells, at a concentration of $2x10^5$ cells/mL, were inoculated in coated 96 well plate for cell culture. The cells were supplemented with 10 µL of AKG at different concentrations, ranging between 20mM and 100mM. The cells were incubated at 37°C for 48 h. Ensuing, Cell Titer 96 reagent was added to the samples, and the plate was incubated 1h at 37°C with

a shaking speed of 130 rpm. Following, the absorbance was measured at 490 nm in order to evaluate the level of formed formazan and assess the number of viable cells after incubation with AKG.

4.2.2. Culture characterization

Growth data were determined based on Vi-CELL XR data and specific growth rates values were calculated as a function of time according to the following equation, knowing that X is the viable cell concentration at a specific time point (t), X_0 is the initial viable cell concentration and μ represents the cell growth rate.

$$X = X_0 e^{\mu t}$$

The viable cell concentration described as viable cell density (VCD) given in viable cells/mL was measured using Vi-CELL XR. The viable cell volume VCV was calculated as follows. First, the values of the volume per cell described as (μm^3/cell) were calculated according to equation 1 (Eq 1).

$$\boldsymbol{Eq\ (1)}: \qquad Volume\ per\ cell = \frac{4}{3}\,\pi\,\left(\frac{Viable\ cell\ diameter}{2}\right)^3$$

The values determined in Eq 1 were used to calculate the VCV values as follow in Eq (2), using the diameters obtained also from Vi-CELL XR data. The VCV values are given in (mm^3/mL).

$$\boldsymbol{Eq\ (2)}: \qquad VCV = Volume\ per\ cell.10^{-9}.VCD$$

Pearson's correlation coefficients were determined for linear correlations between Ln-transformed VCD and the culture time, starting from the first time point analyzed (TP00) and including at least 5 time points. For each sample the highest correlation coefficient (r_{MAX}) and the time point (TPXY) of its occurrence were determined. The growth rates were calculated as slopes in simple linear regressions of the ln-transformed VCD (or VCV) versus the interval (TP00–TPXY).

Following, cumulative viable cell days (CCD_{CD}), (CCD_{CV}) were calculated based on different values of VCD and VCV respectively and described as (cells*days). This method was previously described at Klanert et al., 2019 [36] and adopted in this study. The different CCD_{CD} and CCD_{CV} values were determined based on the following equations where t represents the hours post-inoculation, and n the number of time points analyzed per batch.

Eq (3): $$\mathrm{CCD}_{CD} = \sum_{i=1}^{n-1} \frac{(VCD_{i+1} - VCD_i).(t_{i+1} - t_i)}{(\ln(VCD_{i+1}) - \ln(VCD_i)).24}$$

Eq (4): $$\mathrm{CCD}_{CV} = \sum_{i=1}^{n-1} \frac{(VCV_{i+1} - VCV_i).(t_{i+1} - t_i).10^{-3}}{(\ln(VCV_{i+1}.10^{-3}) - \ln(VCV_i.10^{-3})).24}$$

Pearson's correlation coefficients were determined for linear correlations between CCD_{CD} and CCD_{CV} and the product titers starting from the second measurement (TP02) (Taking into consideration that TP00 is the first measurement of time of inoculation) and including at least 6 time points. For each sample the highest correlation coefficient (r_{MAX}) and the time point of its occurrence (TPXY) were determined. The specific productivities of product (qP) were calculated as slopes in simple linear regressions of the CCD_{CD} (or CCD_{CV}) versus the titers for the interval (TP02 – TPXY), representing the exponential phase of the cultures.

4.2.3. Mathematical fitting of exchange rates of metabolites

The calculated specific growth rates and the initial cell concentrations of different experiments were used to calculate the exchange rates of different metabolites, for instance, glucose, lactate, ammonia and other amino acids, relying on the following equation (Eq (5)) described in Széliová et al., 2020 [13]:

Eq (5): $$[i] = [i]_0 + \frac{q_M B_0}{\mu}(e^{\mu t}\text{-}1)$$

where $[i]_0$ and $[i]$ are the concentration of metabolite *i* at the beginning and during the exponential phase, respectively, q_M is the specific uptake or secretion rate of metabolites, and B_0 is the initial amount of biomass, calculated from the initial cell concentration X_0 and the data of dry mass per cell. The latter was obtained internally in the lab and partially adapted from Széliová et al., 2020 [13]. This equation was used to estimate the exchange rates of metabolites in (mmol/gDW/h), using non-linear regression function.

4.3. Results and discussion

As previously mentioned in the materials and methods section, three different cell lines were cultivated in CD CHO medium without glutamine supplemented with ACA. Different concentrations of α-ketoglutarate (4mM, 8mM and 12mM) were added in order to study the effect of α-ketoglutarate (AKG)

on growth and also its impact on the production of EpoFC recombinant proteins and Trastuzumab. Negative control batches also were performed, where the cells were not supplemented with α-ketoglutarate but just grown with CD CHO medium without glutamine. Cell density, viability and metabolic profiles of the cells were monitored over time and compared between the different conditions.

4.3.1. Growth characteristics

Throughout the results and looking at figure 4.1, we can observe the growth profiles and the viability trends of CHO cell lines tested in this study. A clear difference in growth profiles was observed among the tested conditions, employing both producer and non-producer CHO cell lines. These results were mainly focused on comparing the batch periods, the viability profiles, as well as the maximal cell densities reached in each tested condition when varying the supplemented AKG concentrations.

Higher specific growth rate values and maximal cell densities were observed in cultures non-treated with AKG comparing to the treated cells, noticing also a decrease in CHO growth rate when increasing the AKG concentration in the medium. In contrast to these observations, the viability/life span of the cells was extended in all clones treated with AKG comparing to the non-treated cells (Figure 4.1 B/C/D).

It is clear that AKG supplementation is associated with increasing the viability of the cells and prolonging the stationary phase of the culture, which might be interesting from a bioprocessing standpoint. In fact, maintaining a high viability of the cells over time is a standard approach to increase the volumetric productivity of the cells, previously highlighted in literature [37]. Furthermore, several studies focused on controlling cells proliferation to improve mammalian cells productivity were previously discussed [38]. Within these approaches, medium optimization and metabolic rewiring of the cells holds a promise in controlling cell's proliferation and consequently improving the product's final titer [39,40]. In fact, the increase of production of recombinant proteins in stationary phase was previously observed [41]. To achieve this behavior, several approaches were already used to control the proliferation of the cells as mean of increasing its productivity in bioprocesses [42]. Strategies based on culturing the cells at lower temperatures (e.g., 32° C), chemical treatments (e.g., sodium butyrate) or also cell cycle arrest strategies, were previously described in the literature [43–46].

Ensuing, in figure 4.2, we can observe a summary describing the different values of the specific growth rate of the 3 different CHO cell lines tested in this study. According to these results, no significant differences in growth rate values were observed in non-treated cells and when treated with 4mM AKG.

When adding higher concentrations (8mM and 12mM of AKG) a more important difference in growth rate was observed among these cell types. These experimental results are comparable to the data previously published in Kwang Ha et al., 2014, where they tested different TCA cycle intermediates that can substitute glutamine in the cell culture medium. They also observed a clear decrease in growth rate of CHO cells with AKG supplementation. The latter was recovered after various passages [34].

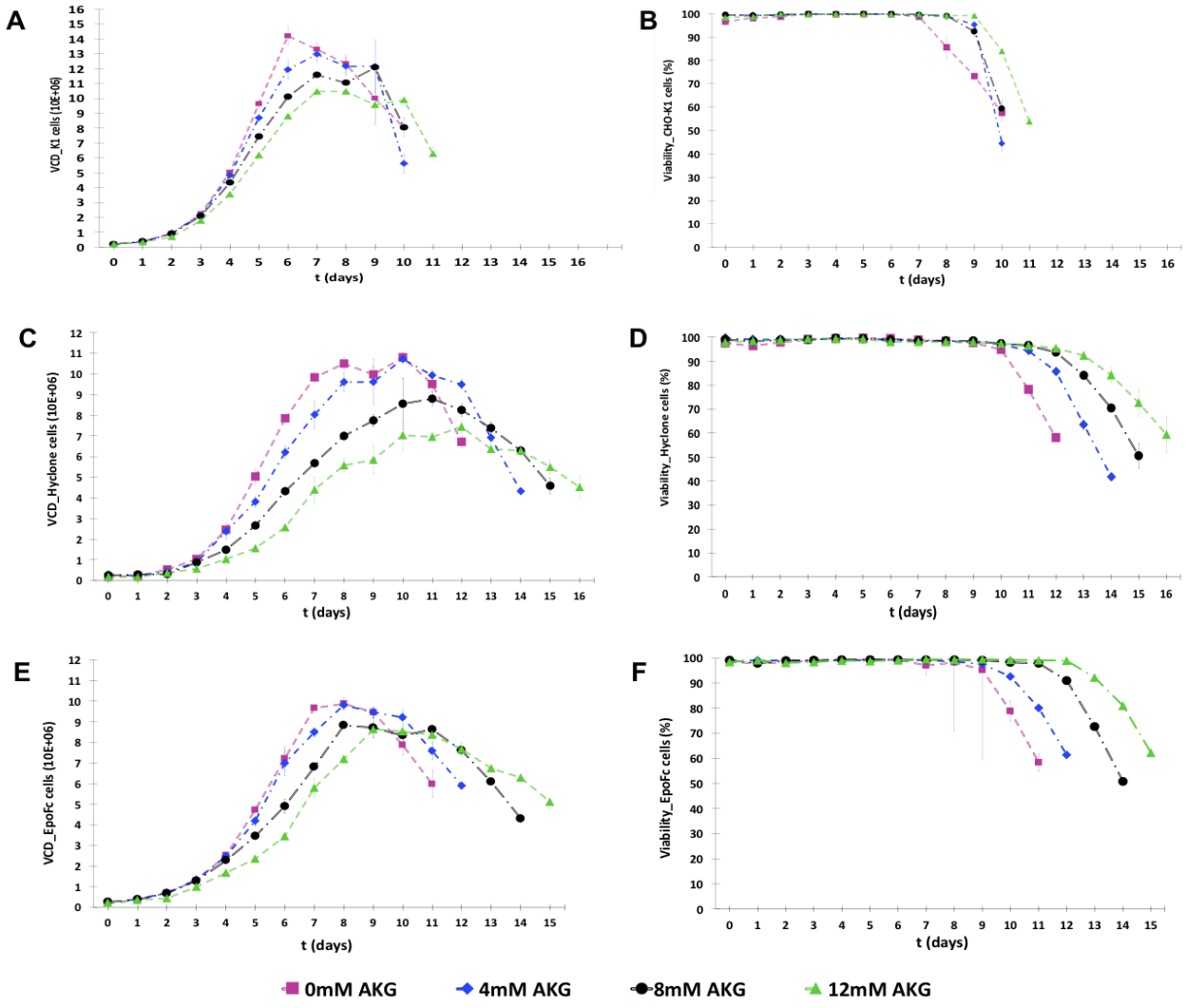

Figure 4.1 Growth profiles of three different CHO cell lines used in the study. (A), (C) and (E) represent respectively the growth profiles of CHO-K1, CHO-HyC and CHO-EpoFc cell lines non supplemented with AKG (Purple squares), supplemented with 4mM AKG (Blue inclined squares), supplemented with 8mM AKG (Black circles) and supplemented with 12mM AKG (Green triangles). (B), (D) and (F) represent respectively the viability of the cells along the cultures of CHO-K1, CHO-HyC and CHO-EpoFc cell lines non supplemented with AKG (Purple squares), supplemented with 4mM AKG (Blue inclined squares), supplemented with 8mM AKG (Black circles) and supplemented with 12mM AKG (Green triangles). The error bars represent the standard deviation between replicates.

This is explained by the fact that the cells tend to adapt its metabolism to the new media formulation and adjust it according to the available nutrients, which will generate a distinct metabolic flux distribution within the cell. A higher amount of a specific metabolite can change the utilization of metabolic pathways and influence growth parameters mainly the specific growth rate, doubling time and the maximal cell density.

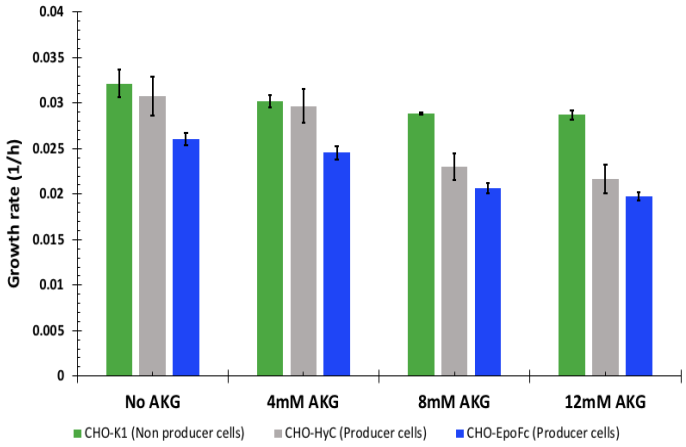

Figure 4.2 Comparison of growth rate values between different CHO cell lines (Producer and non-producer cells). The green bars represent the growth rate values of CHO-K1 non-producer cells. The grey and blue bars represent respectively the growth rates of two producer cells CHO-HyC and CHO-EpoFc. The error bars represent 95% confidence interval.

4.3.2. Product titer and CHO productivity for producer cells

When supplementing AKG, the production titers of Trastuzumab and EpoFc recombinant proteins obtained by different producer strains of CHO cells were monitored and described in figure 4.3. According to these results, we noticed an increase in the final batch titer when increasing the concentration of AKG in the medium. Regarding CHO-EpoFc and CHO-HyC cell lines, the final titer of the recombinant product in cells non-treated with AKG was respectively 108.25±1.06 µg/mL and 686.9±20.57 µg/mL. When increasing AKG concentration in the medium up to 12mM, the final titer increased by 1.5-fold, reaching a concentration of 170.7±9.52 µg/mL and 1023±47 µg/mL respectively for CHO-EpoFc and CHO-HyC cell strains.

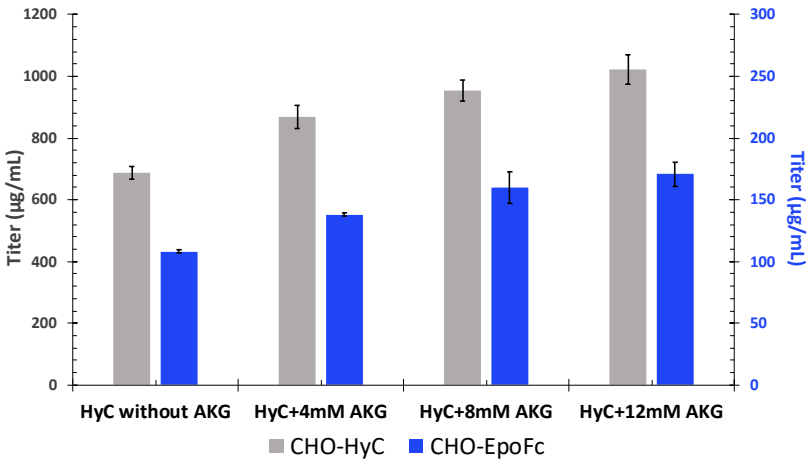

Figure 4.3 Comparison of final batch product titers between 2 different CHO cell lines (CHO-EpoFc and CHO-HyC) when varying AKG supplemented concentration. The barplots describing product titers of CHO-HyC cells were highlighted in grey. The barplots describing product titers of CHO-EpoFc cells were highlighted in blue. Error bars represent 95% confidence interval.

Furthermore, during the exponential phase of the culture, the specific productivity (qP) values of both producer cell lines were calculated and different qP values were determined. The first is related to the viable cell density values (VCD) and the second is related to the corresponding cell volume (VCV), described respectively in (pg/cell/day) and (mg/mL/day). The mathematical description is described in materials and methods section. In figure 4.4, we can observe the different qP values for CHO-EpoFc (4.4/A) and CHO-HyC (4.4/B). In the case of CHO-EpoFc cells (Left plot), no significant changes were observed regarding the productivity of the cells. However, for CHO-HyC (Right plot), the specific productivity, in the exponential phase of the culture, increased when supplementing AKG. For the low producer cell line (CHO-EpoFc), the specific productivity was estimated as 2.66 pg/cell/day and 1.7 mg/ml/day in the case of cells non-treated with AKG, while the qP values remained relatively unchanged, corresponding to 2.53 pg/cell/day and 1.55 mg/mL/cell for the case of CHO-EpoFc cells treated with 12mM AKG. Thereafter, focusing on the high-producer cell line, the specific productivity was estimated as 20.0 pg/cell/day and 12.7 mg/mL/day in the case of CHO-HyC cells non-treated with α-ketoglutarate increasing around 1.9-fold, corresponding to 38.1 pg/cell/day and 1.87-fold with a value of 21 mg/mL/cell for the case of CHO-HyC cells treated with 12mM AKG.

Taking into consideration these differences in productivities during the exponential phase and the increase in the final titer values when increasing α-ketoglutarate concentration for both lines, we can assume that for the case of CHO-EpoFc cells treated with α-ketoglutarate, the production rate of the recombinant EpoFc proteins increased significantly in the stationary phase.

Conversely, the production rate of Trastuzumab clearly was improved in both exponential and the stationary phase, for CHO-HyC cells. The latter is a logical observation knowing that, in the exponential phase of the culture, there might be a clear competition between growth and production of recombinant proteins from nutrients standpoint [8], especially for the case of CHO-HyC.

As previously described, CHO-HyC is a GSneg cell line. The latter seems to benefit more from the supplementation of AKG to the cell culture media, which can be explained by the fact that this high producer strain tends to biosynthesize glutamine due to its lack in the medium. In this construct, the glutamine is mainly produced by the available glutamate in the culture, which can be generated using AKG. Of course, this reaction is possible due to the GS gene inserted in the cells together with the transgene of interest.

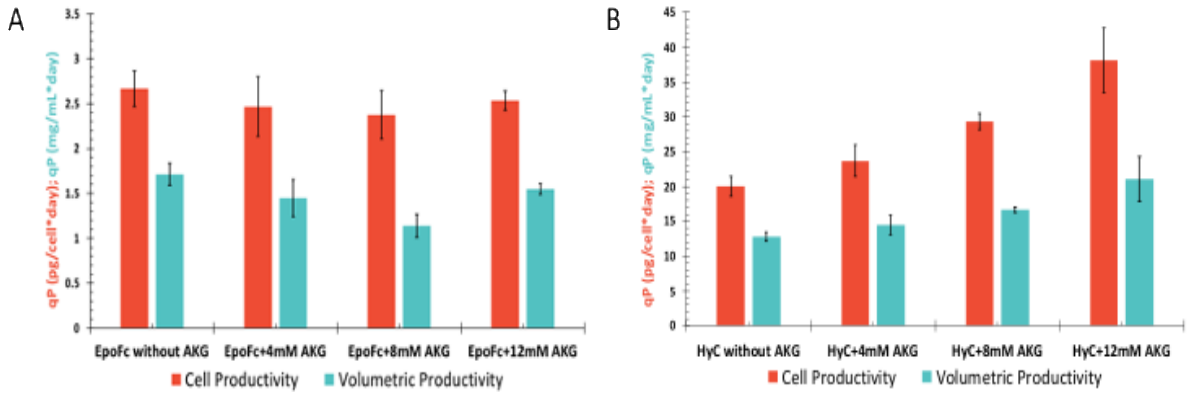

Figure 4.4 qP values for CHO-EpoFc (A) and CHO-HyC (B) and comparison between different AKG conditions. The qP of batch cultures were calculated by VCD (Red bars, pg/(cell*day)) or VCV (Green bars, mg/(cm³*day)). The error bars represent 95% confidence interval.

In summary, we clearly observed that CHO production increased when supplementing AKG. Interesting results were observed mainly in CHO treated with 8mM and 12mM α-ketoglutarate, where the final titers were improved significantly. Even though the maximal cell densities were lower in treated cells, productivities were higher than previously published data [36]. Previous study showed also that α-ketoglutarate is efficient in improving the productivities of CHO cells [34]. The results obtained in this work clearly consolidate the previously published data by exploring the effect of different concentration of AKG on two different producer CHO cell lines, DHFR and GS constructs.

4.3.3. Metabolic Impact of AKG supplementation

As previously described, supplementing α-ketoglutarate had a clear influence on the growth characteristics of CHO. In order to understand the metabolic behavior of the cells in the presence of different concentrations of α-ketoglutarate in the cell culture media, the levels of several extracellular metabolites (glucose, lactate, ammonium and amino acids) were monitored over the culture period focusing on both non-producer (CHO-K1) and high-producer CHO (CHO-HyC) cell lines, to understand and compare the metabolic behavior of these two platforms, in the presence of AKG. In this part of the study, we decided to compare the data of both non-producer and producer CHO cells. CHO-EpoFc were excluded from this study since our focus is mainly centered around understanding what metabolic pathways are involved in obtaining high titers of recombinant proteins emphasizing more on the industrially preferred GSneg CHO cell lines (CHO-HyC). In this section, the metabolic profiles of CHO-K1 and CHO-HyC cells treated or not with AKG are shown respectively in figure 4.5 and 4.6. In these figures, a number of time points especially concerning AKG and aspartate were not analyzed due to limiting access to the analytical equipment. Following, in figure 4.7 and 4.8, we can observe the different exchange rates of metabolites for both CHO-HyC and CHO-K1 cells cultured with different AKG concentrations.

4.3.3.1. Effect of α-ketoglutarate on glucose and lactate metabolism

During the exponential phase of the culture, we previously highlighted that the growth rate of CHO decreases when increasing the levels of α-ketoglutarate in the media. Looking at the consumption of glucose during culture, we can observe that, for non-treated cells, glucose depletes earlier comparing to the conditions where AKG is supplemented (Figure 4.5A/4.6A). However, looking at the specific consumption of glucose per cell, we can affirm that, in the presence of AKG, cells consume glucose at higher rates for both CHO-K1 and CHO-HyC cells. Data regarding the consumption and production rates of different metabolites are provided in annexes (Table 4.2/4.3). In the case of CHO-HyC cells grown with 12 mM AKG, glucose consumption increased by 1.4-folds comparing to the non-treated cells and 1.3-folds comparing to the cells supplemented with 4mM AKG. For CHO-K1 cells, the latter was slightly increased by 1.1-folds comparing the non-treated cells and the cells supplemented with 4mM AKG.

Focusing on the same conditions, AKG consumption rates increased when increasing its concentration in the media. The consumption rate of AKG in CHO-HyC cells treated with 12mM AKG increased 5 times comparing to the cells treated with 4mM AKG. The same behavior is observed for CHO-K1 cells,

where cells treated with 12mM AKG increased 2.1 times comparing to the cells treated with 4mM AKG. Throughout these results we can observe that, in the case of CHO-HyC cells, the consumption of glucose and AKG, in the exponential phase, is higher comparing to the CHO-K1 cells. This suggests that these resources are diverted to produce higher titers of Trastuzumab, correlating with the results illustrated in the previous section 4.4.2.

The low (non-significant) increase in glucose consumption rate in case of CHO-K1 cells is reasonable since the uptake rate of AKG in the exponential phase was very low. A hypothesis might be linked to the fact that the available nutrients in the media are able to sustain the growth of CHO-K1 cells. Thus, AKG play a role as an alternative metabolite in case of CHO-K1 cells, where it is consumed when the nutrient concentrations during culture are very low or depleted. In the case of CHO-K1 cultures, results suggest that AKG starts being consumed just at the stationary phase of the culture, because glutamate levels increased starting from day 9 post-inoculation (Figure 4.5/A and 4.5/C). Another hypothesis can be based on the idea that the cells need more time to adapt the metabolism to the consumption of AKG.

Furthermore, during the exponential phase, the levels of lactate are lower in cultures supplemented with AKG (Figure 4.5/B and 4.6/B), an effect that is more pronounced for the K1 cells. The different production rates of lactate for all the tested conditions are described in Table 4.2 and 4.3 in annexes, respectively representing CHO-K1 and CHO-HyC cells. CHO-HyC cells produce lactate at a higher rate in cultures supplemented with α-ketoglutarate.

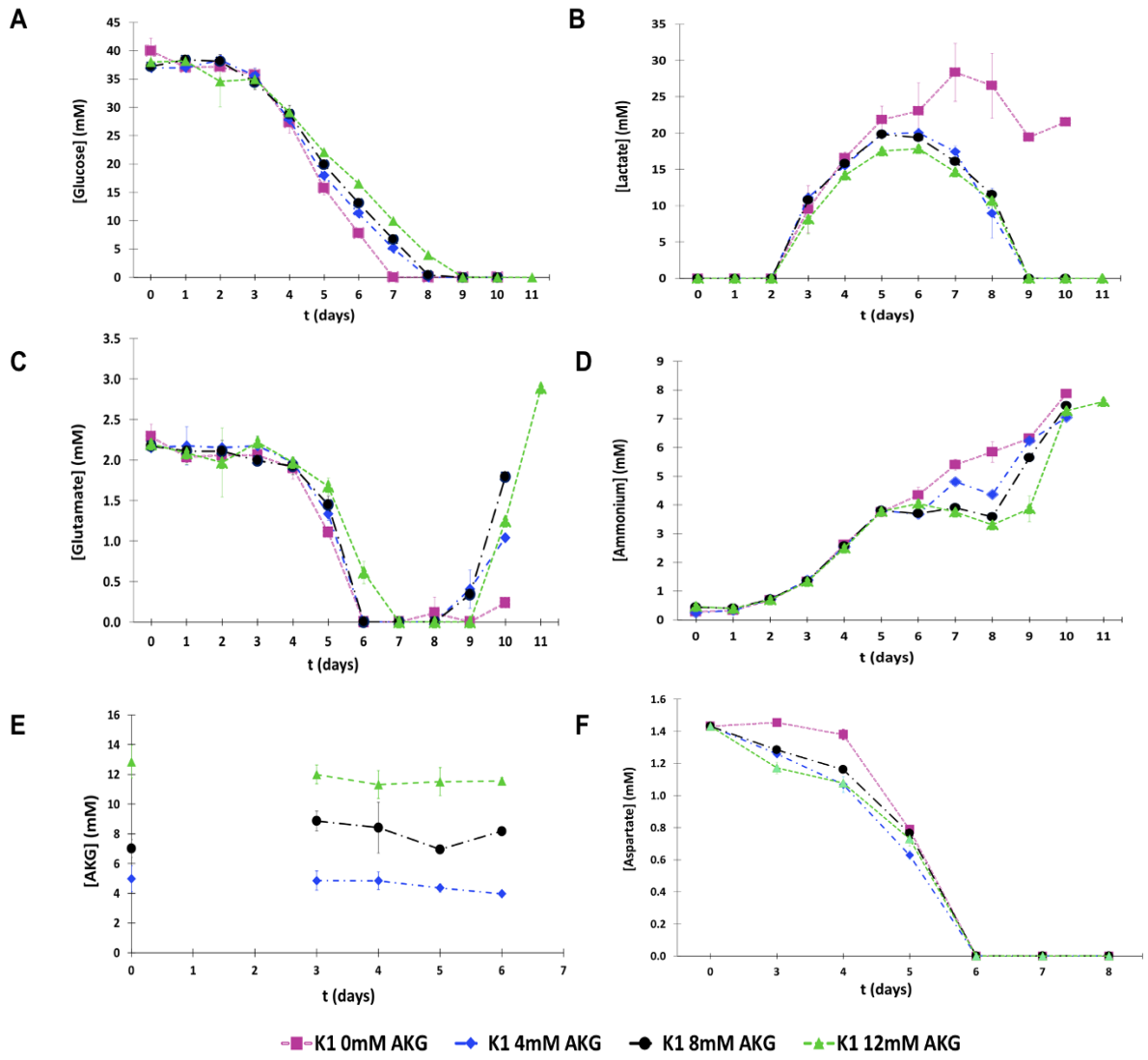

Figure 4.5 Metabolic profiles of CHO-K1 cultures treated or non-treated with AKG. (A) represents the glucose profile over time, (B) represents lactate profile over time, (C) represents glutamate profile over time, (D) represents ammonium profile over time, (E) represents AKG profile over time and (F) represents aspartate profile over time.

For instance, we observe that for CHO-HyC cells supplemented with 12mM AKG, lactate production rate increases by 2-folds comparing to the non-treated cells, whereas, for CHO-K1 cell lines, a small increase by ~1.2-folds in lactate production was observed. Despite the fact that lactate secretion rates increased when supplementing AKG, the concentrations in the culture remains lower at the AKG treated cultures.

Following, at the late exponential phase, a metabolic switch ceases lactate production and alternates it to consumption at the stationary phase of the culture until lactate is fully drained (Figure 4.5/B and 4.6/B). As an exception, CHO-K1 cells consume lactate at the late exponential phase, just in the AKG treated cultures. For the non-treated cells, lactate accumulates during the exponential phase at higher

levels comparing to the treated cells together with a slight consumption of lactate when glucose is drained.

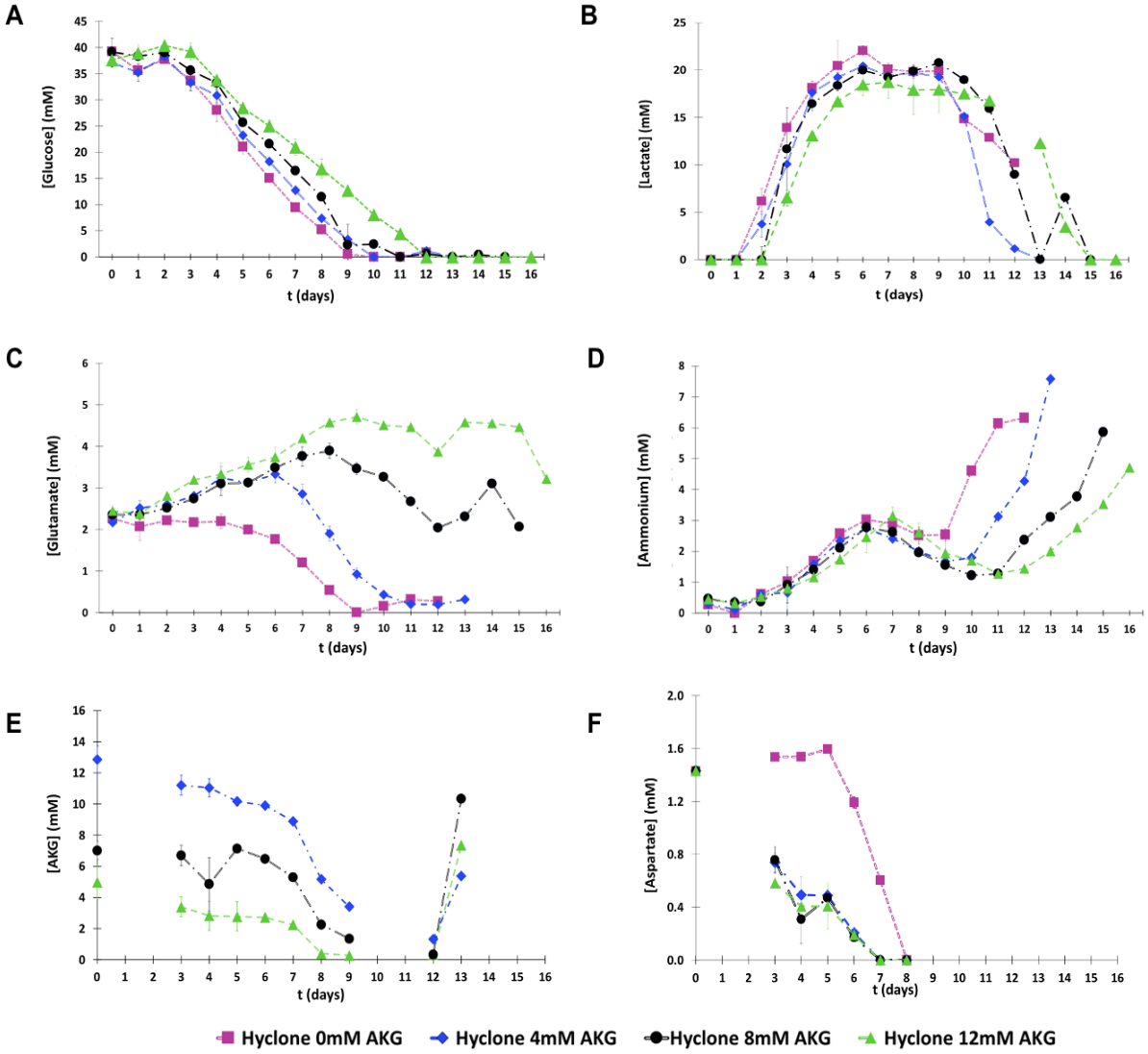

Figure 4.6 Metabolic profiles of CHO-HyC cultures treated or non-treated with AKG. (A) represents the glucose profile over time, (B) represents lactate profile over time, (C) represents glutamate profile over time, (D) represents ammonium profile over time, (E) represents AKG profile over time and (F) represents aspartate profile over time.

In fact, the high level of AKG in the medium replenishes the TCA cycle of CHO and in parallel seems to trigger the glycolytic pathway. Concerning the metabolic switch that forces lactate consumption, in some cases, this phenomenon can be triggered by glucose depletion in the medium, change in pH of the culture or also the difference in lactate levels in intra and extra cellular environment [47]. In fact, in this process, lactate is oxidized to pyruvate, and can take part of 4 metabolic pathways, being converted

to acetyl-CoA, oxaloacetate, malate or alanine, generating NADH that can play a role in the oxidative phosphorylation mechanism leading to ATP production [47].

This mechanism of lactate switch in mammalian cultures is, in fact, an efficient metabolic process and was previously discussed in literature [48], knowing that high levels of lactate are toxic to the cells, consuming it can alleviate this toxicity and can be a source of energy to the cells. This phenomenon was previously discussed in various studies, but an exact explanation about the mechanisms that drives this switch are still unknown.

According to these results, we can conclude that, especially regarding the high producer cell line (CHO-HyC), glucose consumption rate increased during the exponential phase of the culture when increasing the levels of AKG (Table 4.2). Jointly, during the late exponential phase, even though lactate production rate was higher (Table 4.2), lactate switched to be consumed which decreases its amount in culture, overcoming the problem of lactate accumulation and its toxicity.

4.3.3.2. Amino acids and ammonium

When supplementing α-ketoglutarate, a major shift in the exchange rates of several amino acids such as glutamine, glutamate, asparagine, aspartate and ammonium was observed comparing to the standard condition (where CHO cells were grown in CD CHO medium without glutamine and without α-ketoglutarate) (Figure 4.7 and 4.8). The different exchange rates of ammonia and amino acids in all the tested conditions are observed in table 4.2/4.3 in annexes.

At first, in the presence of high levels of α-ketoglutarate in the medium, glutamate is produced, and its rate increases when increasing α-ketoglutarate concentration in the medium. Yet, its secretion period was also extended in cultures supplemented with 12mM α-ketoglutarate comparing to the ones with 4mM α-ketoglutarate (Figure 4.5/C and 4.6/C). The amount of glutamate produced in the treated conditions was equivalent to the levels of α-ketoglutarate available in the medium. When reaching a level of (~2mM) of α-ketoglutarate, glutamate production from AKG is halted. For the case of CHO-HyC cells and looking to the flow of these metabolites over time, we observe that aspartate was the main driver of the conversion of α-ketoglutarate to glutamate as its concentration decreases gradually over time in the treated cells comparing to the non-treated ones (Figure 4.10). This was not observed for CHO-K1 cells (Figure 4.9). Several pathways are driving in the conversion α-ketoglutarate to other metabolites such as glutamate. This involves several reactions using specific amino acids such as

aspartate, tyrosine, phenylalanine, *etc.* [49,50]. In our case, we suggest that glutamate production from AKG is mediated by two main reactions, the first is the direct conversion of AKG to glutamate, using ammonium and the second is relying on the conversion of AKG using aspartate, secreting oxaloacetate. Since the extracellular ammonium levels are very low at the beginning of culture, direct conversion of AKG to glutamate is metabolically rather difficult. We suggest that AKG is using the available aspartate to produce glutamate. Investigating this hypothesis, we noticed that aspartate is consumed at higher rates in AKG treated cells.

In addition, in figure 4.7 and 4.8 we can observe a comparison between the different tested conditions. When cells are not treated with AKG, we observe a different metabolic profile of aspartate where it is being produced, certainly, as a consequence of asparagine degradation [9], then consumed at the early/mid exponential phase. During growth of producer cells, when aspartate is consumed at high rate, hypothetically, asparagine comes to support the production of aspartate. Aspartate consumption rates increased by 10 folds when supplementing 12mM AKG to the cultures. In addition, asparagine consumption rates increased by 3 folds when increasing AKG concentrations in the medium. In the case of non-producer cells, the latter increased by 1.5 folds. As described in the literature, aspartate uses α-ketoglutarate to produce oxaloacetate and glutamate through transamination [9]. According to the experimental data, we observed that α-ketoglutarate metabolism in CHO is directly linked to the aspartate-asparagine metabolism.

One of the possible theories can be that glutamate is also produced by direct conversion of α-ketoglutarate to glutamate using the free ammonium in the culture and the cofactor NADH. This hypothesis is based on the results that show that, although aspartate is depleted, the production of glutamate continues, relying on other metabolites available in the medium. To investigate this hypothesis, looking at the experimental data, we can observe that, when aspartate is exhausted, CHO starts to consume ammonium from the medium. We can observe that ammonium starts to be consumed at ~day 6/7 of the experiment, exactly when aspartate depletes from the medium (Figure 4.6/D, 4.7/D, 4.9 and 4.10). In the case of non-treated cells, the ammonium levels are rather stationary, which is probably related to the low levels of amino acids in the medium (e.g., glutamine, glutamate, asparagine, aspartate and serine).

In cultures treated with α-ketoglutarate, we observe a production of α-ketoglutarate in the stationary phase, after it was depleted in the mid exponential phase. This production of α-ketoglutarate in the stationary phase, comes with a high increase of ammonium levels in the medium. The latter

phenomenon is an indication that the produced glutamate during the exponential phase, degrades to produce α-ketoglutarate, which will fuel the TCA cycle producing energy and boosting the production of recombinant proteins of interest.

Finally, we can observe that for the cells treated with α-ketoglutarate, glutamine production rate increases also with the increase of α-ketoglutarate concentration in the medium. Looking to figures 4.7 and 4.8, we can observe that the biosynthesis rate of glutamine is clearly higher in treated cells comparing to the non-treated cells, with a production rate 12 folds higher than the non-treated cells. Looking deeper to the metabolic flow of glutamine during culture, we can observe that glutamine is produced at high rate in the early exponential phase. In the mid/late exponential phase, the produced glutamine is consumed rapidly. The production/consumption rate of glutamine is higher in the case of CHO supplemented with 12mM α-ketoglutarate comparing to the non-treated cells.

These results are in accordance with the hypothesis targeting the use of α-ketoglutarate supplementation to overcome the lack of glutamine in the medium. This way, the cells will rely in its metabolic capabilities to biosynthesize the required metabolites. In addition, glutamine production plays a role in detoxifying the cells from the free ammonium in the culture during growth instead of secreting it when glutamine is initially present in the medium. We can affirm that this strategy is more efficient from bioprocess standpoint overcoming the drawbacks of the initial supplementation of glutamine to the medium but not ignoring its essentiality for the cells, as a nitrogen supply, fueling biosynthesis, energy generation [51] and for the produced recombinant proteins.

Another interesting result is the high production of glycine when increasing the AKG concentrations in the medium. Usually, glycine is a product of serine catabolism in culture. Its accumulation in culture indicates a positive effect [8]. Furthermore, alanine secretion rates increase when increasing the level of AKG in cultures. Even if its rate increases, alanine levels in CHO-HyC cultures were lower for the treated cells comparing to the non-treated (Figure 12). A different alanine profile was observed in CHO-K1 cultures. On the other hand, serine consumption rates were very high in the case of CHO-HyC cultures while in the case of CHO-K1 cells, rather a small change in its uptake rate was observed (Figure 4.7 and 4.8).

Following, the uptake rates of the essential amino acids, for instance leucine, isoleucine, lysine and valine increased when increasing AKG concentrations in CHO-HyC cells. Conversely, these values were not influenced for CHO-K1 cells. This might be related to the fact, that these amino acids play an

important role in the production of recombinant proteins in the case of CHO-HyC cells. Higher productivity and titers need to be supported by higher levels of amino acids.

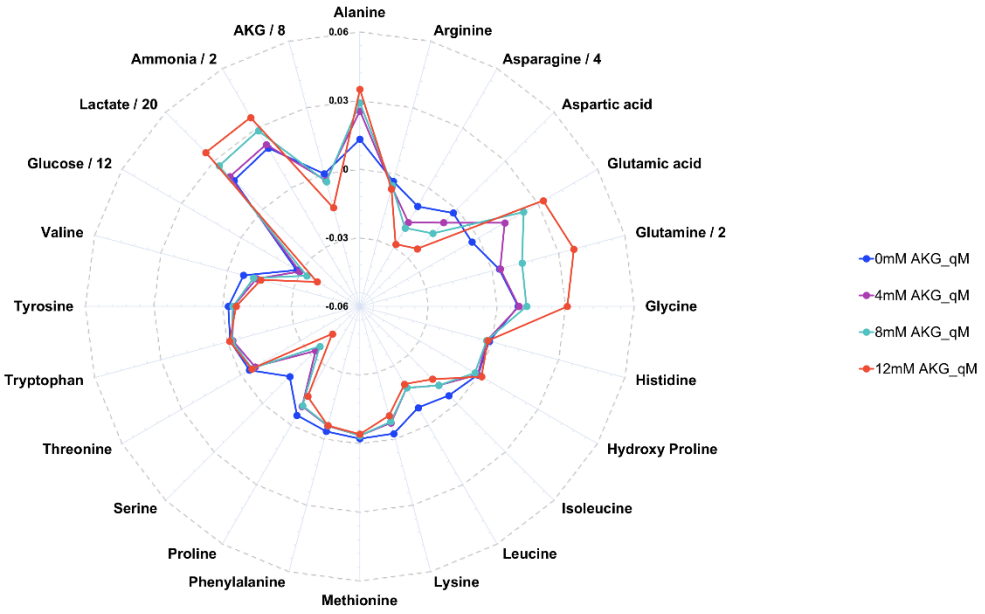

Figure 4.7 Exchange rates of key metabolites during culture of CHO-HyC cells treated or not with α-ketoglutarate. The negative and positive value indicate, respectively, the uptake and secretion rates of the corresponding metabolite. The values of the exchange rates of metabolites are expressed in mmol/gDW/h. Note that the rates of glucose, lactate and other metabolites were scaled down to fit the plot (indicated by the numbers after "/").

Regarding ammonium, its levels in culture were lower when increasing α-ketoglutarate concentration in the medium, for both CHO-HyC and CHO-K1 cells (Figure 4.5.D and 4.6.D). Yet, we noticed that the secretion rates of ammonium are higher when increasing AKG concentrations in the medium. For both cell lines, ammonium levels were slightly lower in culture during the early/mid exponential. Following, in the late exponential phase of the culture, ammonium is consumed and afterwards produced in the stationary phase of the culture, probably as a consequence of oxidative deamination of glutamate via GDH, generating α-ketoglutarate, that fuels the TCA cycle and produces ATP. This process also generates NADH or NADPH, important cofactors for oxidative phosphorylation [52].

These results are very interesting from bioprocess standpoint, since adding AKG to the culture acts on 2 different metabolic related bottlenecks at the same time: decreasing lactate concentrations in the culture, triggering its consumption by fueling the TCA pathway generating more ATP, and also detoxifying the cells from the free ammonium accumulated in the culture through two different reactions.

For CHO-K1 cells, we observed a different metabolic behavior of the cells towards the use of the available α-ketoglutarate in the medium

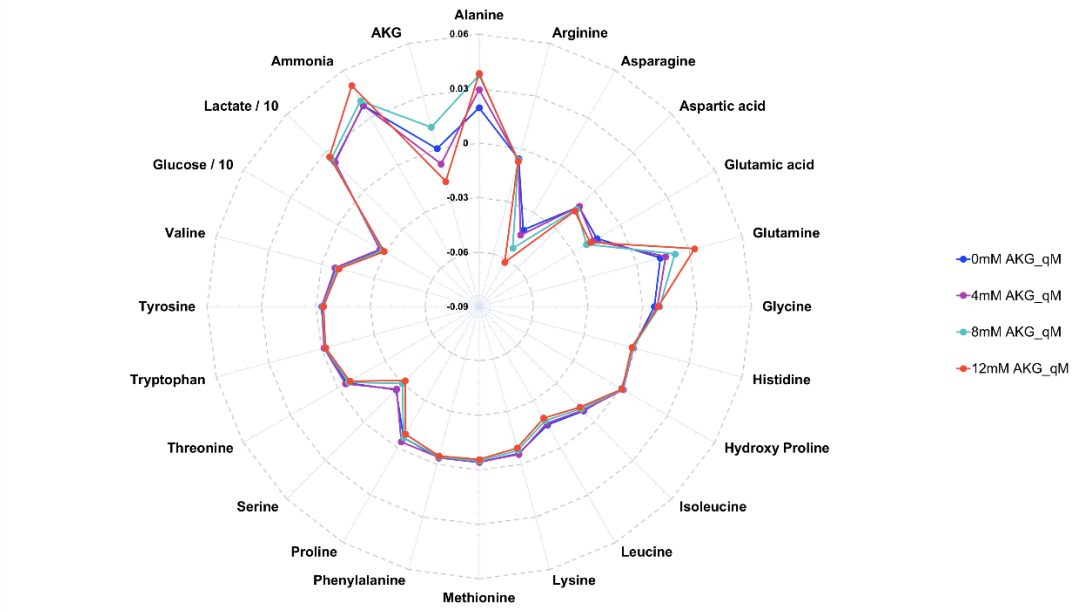

Figure 4.8 Exchange rates of key metabolites during culture of CHO-K1 cells treated or not with α-ketoglutarate. The negative and positive value indicate, respectively, the uptake and secretion rates of the corresponding metabolite. The values of the exchange rates of metabolites are expressed in mmol/gDW/h. Note that the rates of glucose and lactate were scaled down to fit the plot (indicated by the numbers after "/").

At the stationary phase, we notice that α-ketoglutarate starts being produced, as a result of degradation of glutamate that was produced in the exponential phase.

4.4. Conclusions

Based upon the *in silico* results described in chapter 3, we recurred in this chapter to test experimentally the effect of supplementing different concentrations of AKG to the culture medium, evaluating its effect on growth, productivity and accumulation of by products during culture. This experimental validation was performed using different CHO strains (producer and nonproducer cells).

In this context, we can conclude that AKG is a valuable additive to the culture media and can play a role in substituting glutamine in the formulation. Glutamine is generated due to AKG conversion to glutamate and the latter to glutamine.

In addition, we were able to prove that the conversion of AKG to glutamate was mainly based on the use of available aspartate in the medium. Besides, we deduce that when increasing the AKG concentrations

in the medium, the final product titer of increased significantly. The latter is 1.9 folds higher comparing to the titer obtained in standard conditions. Obtaining higher titers may be correlated with the fact of exhibiting a more effective metabolism, fueling the TCA cycle and allowing lower accumulation of ammonia and lactate during culture.

In addition, we were able to underline the different metabolic mechanisms involved in converting the supplemented AKG to CHO cultures. This knowledge is a valuable asset for developing optimization strategies, especially targeting cell culture media optimization. One approach can be based on increasing the aspartate levels in the media due to its potential in driving the conversion of AKG to glutamate. Another optimization strategy can be based on supplementing traces of ammonium in the culture in order to boost other metabolic reactions based on converting directly AKG to glutamate.

4.5. Annexes:

Table 4.2 Exchange rates of metabolites for CHO-HyC cells treated or not with AKG (mm/gDW(h).

	CHO-HyC_0mM AKG		**CHO-HyC_4mM AKG**		**CHO-HyC_8mM AKG**		**CHO-HyC_12mM AKG**	
	qM	**Standard error**	**qM**	**Standard error**	**qM**	**Standard error**	**qM**	**Standard error**
Alanine	0.01304	0.00133	0.02543	0.00050	0.02892	0.00148	0.03488	0.00430
Arginine	-0.00336	0.00015	-0.00586	0.00088	-0.00534	0.00088	-0.00682	0.00096
Asparagine	-0.03790	0.00467	-0.07042	0.00446	-0.08159	0.00264	-0.11450	0.00637
Aspartic acid	-0.00227	0.00080	-0.00816	0.00055	-0.01488	0.00345	-0.02440	0.00679
Glutamic acid	-0.00350	0.00019	0.01314	0.00469	0.02264	0.00486	0.03249	0.00446
Glutamine	0.00612	0.00464	0.00705	0.00056	0.02686	0.01273	0.07362	0.02515
Glycine	0.00914	0.00541	0.00954	0.00209	0.01289	0.00239	0.03061	0.00510
Histidine	-0.00148	0.00027	-0.00248	0.00048	-0.00262	0.00095	-0.00209	0.00084
Hydroxy Proline	-0.00036	0.00067	-0.00052	0.00118	-0.00191	0.00285	0.00139	0.00087
Isoleucine	-0.00498	0.00025	-0.01126	0.00163	-0.01130	0.00220	-0.01507	0.00280
Leucine	-0.00895	0.00011	-0.01901	0.00243	-0.01894	0.00269	-0.02084	0.00221
Lysine	-0.00247	0.00187	-0.00730	0.00251	-0.00784	0.00262	-0.01062	0.00669
Methionine	-0.00227	0.00026	-0.00356	0.00028	-0.00351	0.00039	-0.00418	0.00070
Phenylalanine	-0.00347	0.00041	-0.00594	0.00050	-0.00607	0.00088	-0.00618	0.00070
Proline	-0.00507	0.00049	-0.00961	0.00193	-0.01000	0.00276	-0.01460	0.00301
Serine	-0.01674	0.00297	-0.03266	0.00353	-0.03533	0.00237	-0.04302	0.00826

Threonine	-0.00436	0.00034	-0.00721	0.00117	-0.00568	0.00238	-0.00545	0.00306
Tryptophan	-0.00139	0.00025	-0.00251	0.00060	-0.00208	0.00091	-0.00114	0.00652
Tyrosine	-0.00258	0.00027	-0.00466	0.00056	-0.00451	0.00079	-0.00596	0.00091
Valine	-0.00741	0.00044	-0.01329	0.00182	-0.01193	0.00265	-0.01525	0.00229
Glucose	-0.3380	0.0202	-0.3557	0.0192	-0.3992	0.0154	-0.4620	0.02854
Lactate	0.3545	0.0469	0.4057	0.0424	0.5378	0.0504	0.7030	0.0455
Ammonia	0.0398	0.0029	0.0434	0.0026	0.0573	0.0018	0.0707	0.00136
AKG	0	0	-0.0238	0.0015	-0.0292	0.0075	-0.1228	0.01583

Table 4.3 Exchange rates of metabolites for CHO-K1 cells treated or not with AKG (mmol/gDW/h).

	CHO-K1_0mM AKG		**CHO-K1_4mM AKG**		**CHO-K1_8mM AKG**		**CHO-K1_12mM AKG**	
	qM	**Standard error**	**qM**	**Standard error**	**qM**	**Standard error**	**qM**	**Standard error**
Alanine	0.01938	0.00179	0.02950	0.00164	0.03737	0.00040	0.03831	0.00135
Arginine	-0.00587	0.00072	-0.00645	0.00148	-0.00660	0.00050	-0.00725	0.00054
Asparagine	-0.04132	0.00645	-0.04456	0.01076	-0.05297	0.00656	-0.06185	0.00663
Aspartic acid	-0.01229	0.00020	-0.01201	0.00036	-0.01420	0.00030	-0.01558	0.00046
Glutamic acid	-0.01520	0.00024	-0.01737	0.00141	-0.02175	0.00220	-0.01936	0.00201
Glutamine	0.01326	0.00151	0.01624	0.00161	0.02161	0.00255	0.03280	0.00433
Glycine	0.00644	0.00166	0.00810	0.00354	0.00933	0.00208	0.00870	0.00244
Histidine	-0.00229	0.00024	-0.00219	0.00054	-0.00248	0.00037	-0.00304	0.00021
Hydroxy Proline	0.00059	0.00030	0.00161	0.00051	0.00123	0.00092	0.00083	0.00052
Isoleucine	-0.00879	0.00084	-0.00930	0.00108	-0.01054	0.00026	-0.01169	0.00039
Leucine	-0.01477	0.00138	-0.01569	0.00174	-0.01738	0.00072	-0.01911	0.00049
Lysine	-0.00634	0.00176	-0.00577	0.00300	-0.00801	0.00145	-0.00934	0.00185
Methionine	-0.00428	0.00029	-0.00438	0.00039	-0.00500	0.00019	-0.00578	0.00022
Phenylalanine	-0.00381	0.00042	-0.00405	0.00094	-0.00434	0.00026	-0.00484	0.00034
Proline	-0.00662	0.00087	-0.00402	0.00244	-0.00670	0.00112	-0.00881	0.00077
Serine	-0.02522	0.00273	-0.02571	0.00380	-0.03006	0.00242	-0.03228	0.00219
Threonine	-0.00591	0.00045	-0.00501	0.00090	-0.00679	0.00082	-0.00781	0.00064
Tryptophan	-0.00192	0.00024	-0.00158	0.00039	-0.00217	0.00020	-0.00244	0.00019
Tyrosine	-0.00317	0.00028	-0.00339	0.00035	-0.00385	0.00025	-0.00423	0.00030

Valine	-0.00810	0.00093	-0.00771	0.00142	-0.00931	0.00066	-0.00999	0.00077
Glucose	-0.27302	0.01453	-0.26835	0.01476	-0.28437	0.01331	-0.29501	0.01855
Lactate	0.22692	0.02506	0.22269	0.02658	0.25120	0.02918	0.26569	0.02838
Ammonia	0.03775	0.00305	0.03802	0.00385	0.04071	0.00366	0.05037	0.00373
AKG	0	0	-0.00874	0.00070	0.01220	0.00592	-0.01874	0.00440

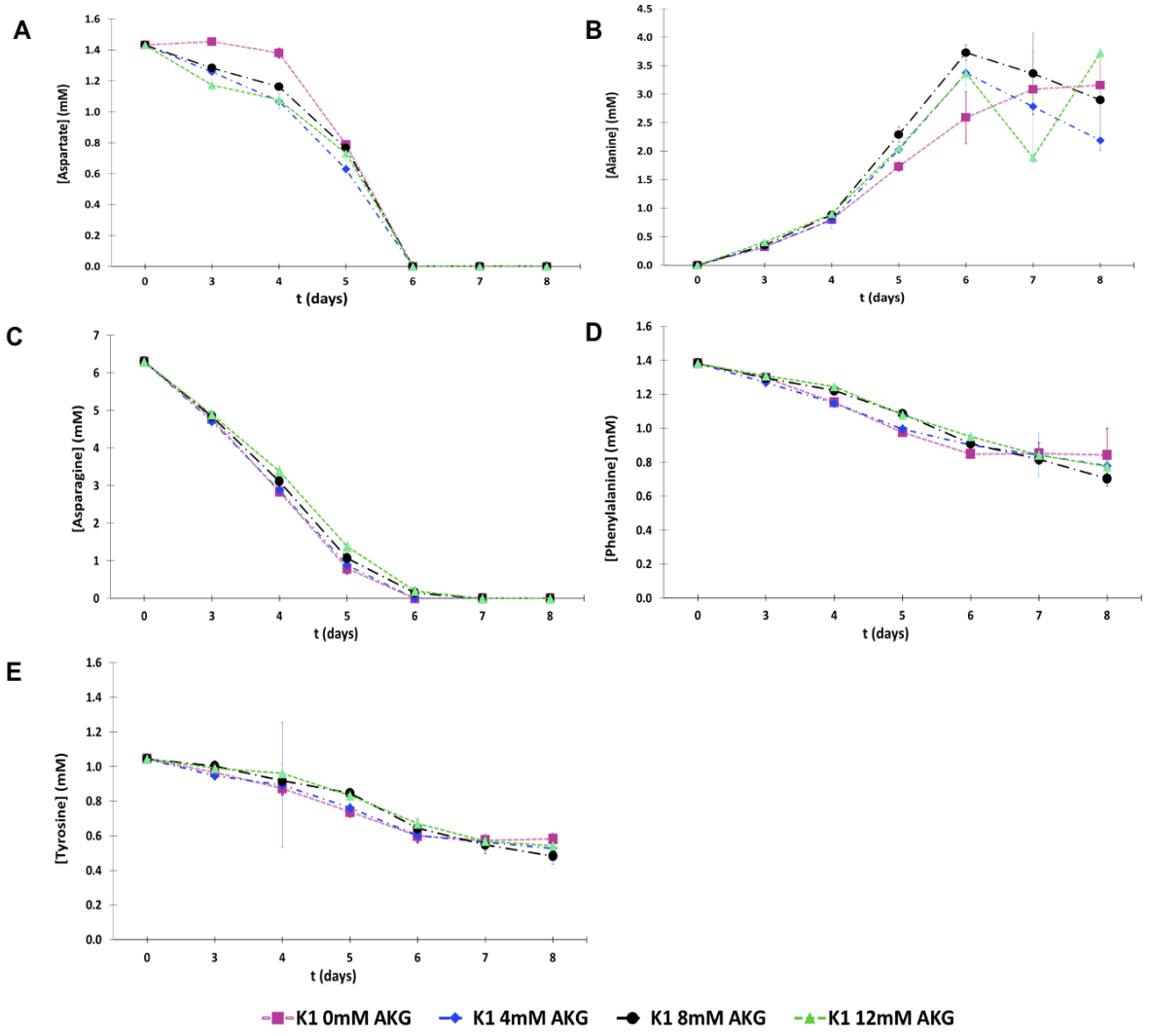

Figure 4.9 Metabolic flow of metabolites directly involved in the conversion of AKG to glutamate for CHO-K1 cells. (A) represents the aspartate profile over time, (B) represents alanine profile over time, (C) represents asparagine profile over time, (D) represents phenylalanine profile over time, (E) represents tyrosine profile over time.

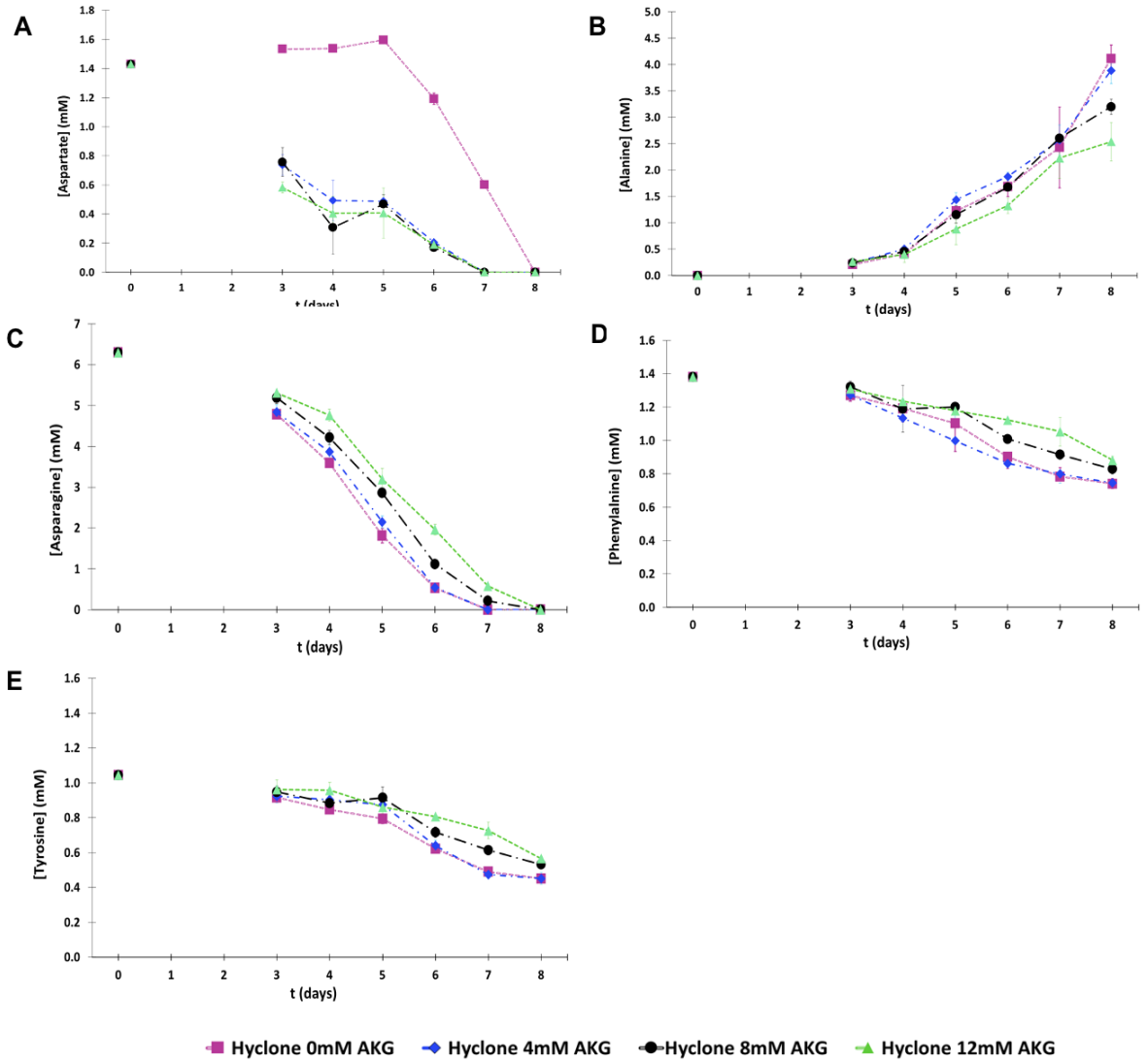

Figure 4.10 Metabolic flow of metabolites directly involved in the conversion of AKG to glutamate for CHO-Hyc cells. (A) represents the aspartate profile over time, (B) represents alanine profile over time, (C) represents asparagine profile over time, (D) represents phenylalanine profile over time, (E) represents tyrosine profile over time.

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CHAPTER 5

Cell culture media optimization for CHO cells

The information presented in this Chapter is being prepared for submission to a peer reviewed journal:

Hamdi A., Borth, N., Zanghellini, J., Rocha I.; *In silico*-based approach for medium optimization of CHO cells.

5.1. Introduction

Based on the recent advances in metabolic engineering and bioinformatics, several efforts are targeted at improving mammalian bioprocesses [1,2] and translating the predictive pipeline for bioprocess optimization from academic research to industry. Various studies highlighted the benefits of mathematical modeling for bioprocess optimization [3]. To achieve these goals, optimization strategies are being used employing CHO specific genome scale models, to predict cell growth, metabolic features of the cells and optimize the productivity/quality of the produced recombinant proteins (e.g., monoclonal antibodies) [4]. These predictions are based on combining genome scale metabolic models, experimental omics data and constraint-based modeling approaches to consolidate fluxomics studies. Omics data are very important asset to calculate, *in silico*, cellular flux distributions in an accurate manner [5] and consequently infer about the cell metabolism when varying culture conditions. Assuming steady state or a dynamic mode, these tools can be very useful for metabolic engineering and media design/optimization strategies.

Along the years, several approaches for media optimization of mammalian cells, especially for CHO, have been explored, aiming at improving cell culture conditions and production titers. As described in chapter 2 and 4, one of the most important highlights was the development of serum-free, animal-free, chemically defined media. These media were further optimized using different strategies such as media blending and high-throughput screening of the best candidates, relying on deterministic modeling approaches, for instance design of experiments (DoE) [6].

Moreover, to further improve the production yield, strategies such as fed-batch and perfusion technologies were used to control the levels of toxic metabolites and improve the production titers of recombinant proteins of interest by balancing nutrients levels in culture and improving feeding strategies [7]. Several efforts were performed for media and feed optimization, improving CHO cells productivity and also decreasing the hurdle of downstream processing [8–14].

Knowing that different strains within CHO have different growth requirements, feeding strategies have to be customized to the cell strain in use [15,16]. It is also relevant to address other topics, for instance, cells heterogeneity and epigenetic regulations.

Further, one of the most important aspects of media optimization is based on spent media analysis. This strategy is very useful to understand the flow of metabolites in the culture, which will facilitate designing the best media formulation for the cell line in use, and also determining optimal feeding tactics [17]. Accordingly, regular monitoring of glucose, amino acids, vitamins, *etc.* is very important in order to understand how the cells consume these nutrients and what are the limiting components that might influence the bioprocess performance.

Following this idea, balancing amino acids levels in the medium has a great potential in improving not only growth parameters of CHO, but also the final product titers. Previous studies showed that by optimizing the amino acids levels in the medium, CHO cells were able to increase titers of fusion proteins by almost 25% [18]. Other efforts based on employing statistical experimental design methodologies helped also in improving the titers of recombinant proteins in CHO by 70%. These studies allowed understanding the key amino acids essential for CHO growth and the important elements interfering with the production of recombinant proteins [19,20].

Amino acids are very important metabolites for the mammalian cell [21]. Besides providing essential building blocks molecules for cell metabolism and protein production, amino acids can also act as signaling molecules influencing cellular apoptosis [22] and also regulate the levels of osmolality in the medium and of other metabolites (e.g., ammonium). Therefore, it is mandatory to optimize the amino acids levels in the medium to fulfill the cellular needs. Within mammalian cells, it is important to optimize the concentrations of both essential amino acids (EAA) and non-essential amino acids (NEAA) in the medium, since they control, in a sophisticated manner, growth and recombinant proteins production. In fact, the medium has to contain high levels of EAA since they are consumed usually at very high rates [23]. Low concentrations of some EAA amino acids in culture, for instance, branched

amino acids (e.g., Leucine, isoleucine and Valine) or also phenylalanine, can heavily influence transporters activity in the cell, for instance, the L-transport system. Studies showed that Na^+ transport system increased activity by 3-4 folds when cells starved these amino acids [24]. In media design efforts, different amino acids often have to be added at higher concentrations than the values determined *in silico* or by fluxomics/metabolomics studies [25] and levels of other EAA (e.g., tryptophan or lysine) have to be optimized since their excess or deficiency can radically influence the process, for example by secreting toxic amino acids derivatives [26,27].

On the other hand, NEAA are also very important nutrients for various metabolic pathways within the cell. Even though NEAA can be synthesized by mammalian cells, they are substantial in the cell culture medium. As previously described (Chapter 4), we noticed that when supplementing AKG into the medium, a very significant shift was observed from the levels of NEAAs standpoint. Glutamine and glutamate were highly produced and aspartic acid was depleted at a very early phase of the culture. An important observation was also regarding the exchange rates of both alanine and glycine, reflecting that high levels of AKG in the medium can rewire the metabolism and alter its behavior. Asparagine and aspartic acid are particularly important in various mechanisms in the mammalian metabolic system. These NEAAs are typically consumed at very high rates, mainly at the exponential phase of the culture, playing an important role in energy and glutamine/glutamate metabolism [12,28]. Lack of asparagine in the cell culture medium can radically impact protein synthesis and also the process by altering the quality of monoclonal antibodies [29]. However, its lack in the medium can be compensated by higher uptake of other amino acids such as aspartate, glutamine or glutamate. Although asparagine is known to be highly aminogenic, ammonium concentrations can be further controlled by balancing the levels of asparagine, glutamine and/or glutamate in the medium [30].

When designing an optimized formulation of the cell culture media, it is important to focus on studying also the recombinant protein being produced. In our case, CHO-HyC cells are producing Trastuzumab, a humanized monoclonal antibody under the commercial name of Herceptin. The latter is an IgG1 kappa molecule, a very important biopharmaceutical for the treatment of HER2-positive metastatic breast cancer [31], developed by Genentech/Roche and targeting specifically the human epidermal growth factor receptor 2 (HER-2/neu) [32,33]. The amino acids sequence of both the heavy chain and the light chain of this molecule is described in the invention patent of Trastuzumab [34].

Supplementing α-ketoglutarate to CHO cultures showed a great potential in improving productivity and decreasing by-products accumulation. Subsequently, according to the metabolomics results obtained in

chapter 4, we will try to optimize cell culture media and apply it to CHO-HyC. However, there is a wide room for media optimization, since we noticed that in the presence of AKG, the amino acids levels in the standard medium are unbalanced. Aspartate was highly consumed in the presence of AKG in culture, together with a high production of glutamate. Following these observations, we decided to design a medium containing the same levels of essential amino acids as the standard used media CD CHO, together with altering the levels of NEAAs by removing glutamate from the formulation and replacing it by 8 mM AKG. Additionally, higher levels of aspartate were supplemented to boost the production of glutamate by AKG. Additionally, a decrease in asparagine levels was tested in the presence of high values of aspartate in the media. Finally, the effect of supplementing different concentration of ammonium was performed. This strategy is based on triggering the reaction of direct production of glutamate from AKG at the beginning of the culture, relying on ammonia. Since ammonia levels in the beginning of the batch are very low, this strategy was employed in order to force the production of glutamate to fulfill the metabolic need of CHO-HyC cells. Taking into consideration that the latter is a GSneg cell line where the GS gene was inserted with the transgene of interest, glutamine production is expected during culture to overcome its lack in the medium.

5.2. Materials and methods

5.2.1. Experimental setup

5.2.1.1. Cell culture

The CHO-HyC cell line was used in this work. These cells correspond to an antibody expressing CHO cell line provided by Cytiva, Uppsala, Sweden. The latter is GSneg cell line and known as a high-producer industrial clone, producing Trastuzumab, a monoclonal antibody under the commercial name of Herceptin.

CHO cells were cultivated in suspension mode in chemically defined serum-free conditions using CD CHO medium (Gibco, Invitrogen, Carlsbad, CA, USA). After thawing, the cells were routinely cultivated in 50 mL TPP® TubeSpin bioreactors (Techno Plastic Products AG, Trasadingen, Switzerland) at a maximal working volume of 25 mL. The cells were incubated in 37°C in 80 % humidified air with 7 % CO_2, shaking at a speed of 220 rpm (rotation per minute). The cells were passaged every 3-4 days and the viable cell concentrations, viabilities and the values of the average cell diameters were determined using Vi-CELLXR (Beckman Coulter, USA).

To perform the experiments described in this chapter, we recurred to the use of CD CHO media without amino acids, a pre-customized medium purchased from Thermo-Fisher Scientific (Reference: ME19349L1). The formulation contained the same components with concentrations similar to CD CHO media, used for the negative control experiments.

In order to design media formulation, non-essential amino acids levels were optimized. Amino acids stock solutions were prepared in the lab (see next section) and supplemented to the culture medium according to the desired levels in each experiment. Regarding essential amino acids, the levels were unchanged. Its concentration in the optimized media is equivalent to the values described in the CD CHO medium.

In these experiments, different levels of glutamate, aspartic acid and asparagine, were tested in this study. After thawing, the cells were passaged 3 times in the corresponding culture conditions. For the experimental setup, batch cultures were performed in triplicates using 50 mL TubeSpin bioreactors at a working volume of 28 mL, incubated in 37°C in 80 % humidified air with 7 % CO_2, shaking at a speed of 220 rpm. All the experiments were performed in triplicates, inoculated at the beginning of the experiment, at the same time and seeding density. Cell concentration and viability were monitored every 24h. In addition, samples for metabolomics evaluation were withdrawn every 24h. The culture continued until reaching a cell viability lower than 60 %.

5.2.1.2. Preparation of amino acids solutions

All the amino acids used in this study are from non-animal origin and suitable for cell culture experiments. The different amino acids used in these experiments were dissolved in CD CHO medium without amino acids, forming the stock solutions prior to the experiments. The reason behind dissolving these amino acids in the medium is to avoid the dilution of the other nutrients of the medium.

The pH of the stock solutions was adjusted to 7.2 (besides some amino acids which are stable in acidic or basic pH). In table 1, we find the list of the used amino acids, its solubility values, the stock concentration prepared and the manufacturer references jointly with the CAS-number.

Table 5.1 Amino acids used in this study.

Amino acid	Solubility*	Stock concentration	Reference**	CAS-Number
L-Arginine	H_2O: 100 mg/mL	70 mM	A8094	74-79-3
L-Asparagine	1 M HCl: 100 mg/mL	100 mM	A4159	70-47-3
L-Aspartate	1 M HCl: 100 mg/mL	70 mM	A7219	56-84-8
L-Cystin	1 M HCl: 100 mg/mL	30 mM	C7602	56-89-3
L-Glutamate	1 M HCl: 100 mg/mL	100 mM	G8415	56-86-0
L-Histidine	H_2O: 50 mg/mL	70 mM	H6034	71-00-1
L-Hydroxyproline	H_20	70 mM	H5534	51-35-4
L-Isoleucine	1 M HCl: 50 mg/mL	100 mM	I7403	73-32-5
L-Leucine	1 M HCl: 50 mg/mL	100 mM	L8912	61-90-5
L-Lysine	H_2O: 100 mg/mL	70 mM	L8662	657-27-2
L-Methionine	H_2O: 25 mg/mL	70 mM	M5308	63-68-3
L-Phenylalanine	1M HCl; 50 mg/mL	70 mM (Protected from light)	P5482	63-91-2
L-Proline	H_2O: 50 mg/mL	100 mM	P5607	147-85-3
L-Serine	H_2O: 50 mg/mL	100 mM	S4311	56-45-1
L-Threonine	H_2O: 1 g/10 mL, clear, colorless	70 mM	T8441	72-19-5
L-Tryptophan	1 M HCl: 10 mg/mL	70 mM (Protected from light)	T8941	73-22-3
L-Tyrosine	1 M HCl: 25 mg/mL	70 mM	T8566	60-18-4
L-Valine	H_2O: 25 mg/mL	100 mM	V0513	72-18-4

*According to manufacturer recommendations. **Sigma-Aldrich order reference.

5.2.1.3. Media preparation

In this study, two different media formulations were prepared in order to evaluate the effect of adjusting amino acids levels in the media, on high-producer CHO clones (CHO-HyC). The different media formulations are summarized in the following table:

Table 5.2 The levels of essential, non-essential amino acids and metabolites in the prepared cell culture media.

Essential amino acids	Concentration (g/L)	Non-essential amino acid	Concentration in the medium (g/L)		
			CD CHO (Standard medium)	Medium A	Medium B
L-Arginine	0.4	L-Aspartate	0.18	0.54	0.72
L-Cystin	0.1	L-Asparagine	0.9	0.9	0.45
L-Histidine	0.2	L-Glutamate	0.27	0	0
L-Isoleucine	0.4	L-Proline	0.54	0.54	0.54
L-Leucine	0.5	L-Hydroxyproline	0.18	0.18	0.18
L-Lysine	0.5	L-Glutamine	0	0	0
L-Methionine	0.1	L-Serine	0.54	0.54	0.54
L-Phenylalanine	0.2	**Metabolite**		**Condition A**	**Condition B**
L-Threonine	0.4	AKG		1.16	1.16
L-Tryptophan	0.2	Ammonia		*	*
L-Tyrosine	0.2	* In experiments using media A and B, different experiments were performed adding different levels of ammonium to the culture. The levels of ammonium tested were 0.01, 0.02 and 0.04 mM.			
L-Valine	0.4				

As previously described, the levels of essential amino acids were equivalent to the ones at CD CHO media. Instead, we adjusted the concentrations of the NEAA in the media, focusing mainly on aspartic acid, asparagine and glutamic acid levels. In medium A, aspartate levels were tripled, glutamate was removed, and the levels of asparagine kept constant. In medium B, aspartate levels quadrupled while

glutamate was removed and asparagine levels were reduced by half. Proline, hydroxyproline and serine levels were unchanged, and the concentrations were equivalent to CD CHO formulation. In medium B, asparagine was reduced by half in order to understand if the increased levels of aspartate will recover the lack of asparagine or not and how the growth and productivity will be affected in this case. Furthermore, AKG was added to all the media formulations at a concentration of 8 mM, replacing both glutamine and glutamate. In addition, ammonia was also added at different concentrations (0.01 mM, 0.02 mM and 0.04 mM), to evaluate its effect on growth and productivity on CHO-HyC cells. Negative control experiments without ammonia supplementation were also performed.

The growth parameters in these conditions were compared to the standard culture conditions. The latter represent CHO-HyC cells grown in CD CHO media without glutamine.

5.2.1.4. Osmolality and pH

For the prepared media solutions, pH was adjusted to 7.2, the same pH value of CD CHO cell culture media. Calibration was performed prior to the use of the pH meter. In addition, osmolarity was measured by freezing point depression using an Osmomat 030 (Gonotec). Osmolarity was adjusted by supplementing NaCl to the cultures. The values of osmolarity of each medium used for the experiments are described in the following table.

Table 5.3 Osmolarity and pH values of the media used in the experiments.

	CD CHO	Medium A				Medium B			
		mM NH_4				mM NH_4			
		0	0.01	0.02	0.04	0	0.01	0.02	0.04
Osmolality (mOsm/kg)	0.326	0.322	0.322	0.323	0.318	0.324	0.321	0.320	0.319
pH	7.2	7.2							

5.2.1.5. Extracellular Metabolites

For the analysis of the extracellular metabolites, the supernatant was collected every 24 h along the culture period. The cells were centrifuged 10 min at 200 rcf (relative centrifugal force) and the supernatant was collected and stored at -20°C. Analysis of key metabolites (glucose, lactate, glutamine, glutamate and ammonium) were performed shortly after sampling, using Bioprofile 100 Plus (Nova Biomedical, MA, USA).

5.2.1.6. Product quantification

Product concentration was determined using Octet® QKe (Port Washington, NY), equipped with Dip and ReadTM Protein A Biosensors (Port Washington, NY) according to the manufacturer's recommendations. The supernatant was diluted with CD CHO medium prior to measurements in order to fit the samples concentrations within the standard curve ranging between 0-100 µg/mL of Trastuzumab (BioVision, Milpitas, CA). A negative control consisting of cell culture medium was included. This method is based on biomolecular interactions, measuring the binding intensity of our product of interest to an immobilized ligand.

5.2.2. Culture characterization

Growth data were determined based on Vi-CELL XR data and specific growth rates values were calculated as a function of time according to the following equation, knowing that X is the viable cell concentration at a specific time point (t), X_0 is the initial viable cell concentration and μ represents the cell growth rate.

$$X = X_0 e^{\mu t}$$

The viable cell concentration described as viable cell density (VCD) given as viable cells/mL was measured using Vi-CELL XR. The viable cell volume VCV was calculated as follows. First, the values of the volume per cell using the diameters obtained also from Vi-CELL XR data described as (µm³/cell) were calculated according to equation 1 (Eq 1).

$$\boldsymbol{Eq\ (1)}: \qquad Volume\ per\ cell = \frac{4}{3}\,\pi\,\left(\frac{Viable\ cell\ diameter}{2}\right)^{3}$$

The values determined in Eq 1 were used to calculate the VCV values as follow in Eq (2). The VCV values are described in (mm³/mL).

$$\boldsymbol{Eq\ (2)}: \qquad VCV = Volume\ per\ cell.10^{-9}.VCD$$

Pearson's correlation coefficients were determined for linear correlations between Ln-transformed VCD and the culture time, starting from the first time point analyzed (TP00) and including at least 5 time

points. For each sample the highest correlation coefficient (r_{MAX}) and the time point (TPXY) of its occurrence were determined. The growth rates were calculated as slopes in simple linear regressions of the ln-transformed VCD (or VCV) versus the interval (TP00–TPXY).

Following, cumulative viable cell days (CCD_{CD}), (CCD_{CV}) were calculated based on different values of VCD and VCV respectively and described as (cells*days). This method was previously described at Klanert et al., 2019 [35] and adopted in this study. The different CCD_{CD} and CCD_{CV} values were determined based on the following equations where *t* represents the hours post-inoculation, and *n* the number of time points analyzed per batch.

$$\textbf{Eq (3)}: \qquad \mathrm{CCD}_{CD} = \sum_{i=1}^{n-1} \frac{(VCD_{i+1} - VCD_i).(t_{i+1} - t_i)}{(\ln(VCD_{i+1}) - \ln(VCD_i)).24}$$

$$\textbf{Eq (4)}: \qquad \mathrm{CCD}_{CV} = \sum_{i=1}^{n-1} \frac{(VCV_{i+1} - VCV_i).(t_{i+1} - t_i).10^{-3}}{(\ln(VCV_{i+1}.10^{-3}) - \ln(VCV_i.10^{-3})).24}$$

Pearson's correlation coefficients were determined for linear correlations between CCD_{CD} and CCD_{CV} and the Trastuzumab titers starting from the second measurement (TP02) (Taking into consideration that TP00 is the first measurement of time of inoculation) and including at least 6 time points. For each sample the highest correlation coefficient (r_{MAX}) and the time point of its occurrence (TPXY) were determined. The specific productivities of Trastuzumab (qP) were calculated as slopes in simple linear regressions of the CCD_{CD} (or CCD_{CV}) versus the titers for the interval (TP02 – TPXY), representing the exponential phase of the cultures.

5.2.3. Mathematical fitting of growth and exchange rates of metabolites:

The calculated specific growth rates and the initial cell concentrations of different experiments were used to calculate the exchange rates of different metabolites, for instance, glucose, lactate, ammonia and other amino acids, relying on the following equation (Eq (5)) described in Széliová et al., 2020 [36]:

$$\textbf{Eq (5)}: \qquad [i] = [i]_0 + \frac{q_M B_0}{\mu}(e^{\mu t}\text{-}1)$$

where $[i]_0$ and $[i]$ are the concentration of metabolite *i* at the beginning and during the exponential phase, respectively, q_M is the specific uptake or secretion rate of metabolites, and B_0 is the initial

amount of biomass, calculated from the initial cell concentration X_0 and the data of dry mass per cell. The latter was obtained internally in the lab and partially adapted from Széliová et al 2020 [36]. This equation was used to estimate the exchange rates of metabolites in (mmol/gDW/h), using non-linear regression function, based on computational analysis following an R script described in annexes.

5.3. Results and discussion

As previously described in materials and methods, two media formulations were tested using CHO-HyC GSneg cell line. Growth characteristics, as well as productivity of the cells were evaluated. In addition, the metabolic profiles of these cells during culture were studied focusing mainly on key metabolites, glutamine, glutamate, glucose, lactate and ammonia. Metabolomics studies based on profiling amino acids levels during culture was not assessed in this part of study due to limitation to access the analytical tools at the university.

5.3.1. Growth characteristics

Throughout the results and looking at figure 5.1, we can observe the different growth profiles, as well as the viability trends of the different tested conditions using both medium A and B. Looking at figure 5.1/A we can observe that no significant differences were observed regarding cell growth when supplementing different concentrations of ammonia to the cells cultured in medium A. Similar results were observed in Figure 5.1/C, where ammonia supplementation did not have an effect on the growth of CHO-HyC cells cultured in the second medium, B. On the other hand, we can notice that the maximal cell density of the cells in both media A and B was reduced by half comparing to the results of the cells grown in CD CHO medium without AKG and without glutamine (Results described in chapter 4). As we can see in figure 5.1 A/C, the maximal cell density of CHO-HyC cells reached $5x10^6$ cells/mL, while for the cells grown in CD CHO media without AKG and without glutamine, cells reached almost $11x10^6$ cells/mL. The decrease in maximal cell density is not related to the presence of ammonia in the culture, since the negative control culture, grown in both media A and B without ammonia (Highlighted in purple in figure 5.1A/C) showed similar growth trends.

In literature, few studies were performed in order to assess the ammonia influence on growth of CHO cells. Results published in Mio-Sam Lao and Derek Toth, stated that higher levels of ammonia in the culture did not influence cell growth neither productivity of glycoproteins in CHO cells [37]. On the other hand, a study performed by Yang and Butler, 2000 suggested that supplementing concentrations of

ammonia chloride above 5 mM can impact growth [38]. Knowing that in these experiments, the ammonia levels supplemented to the media were of a maximum concentration of 0.04 mM, it is excluded that ammonia is responsible for the lower cell densities using Media A and B.

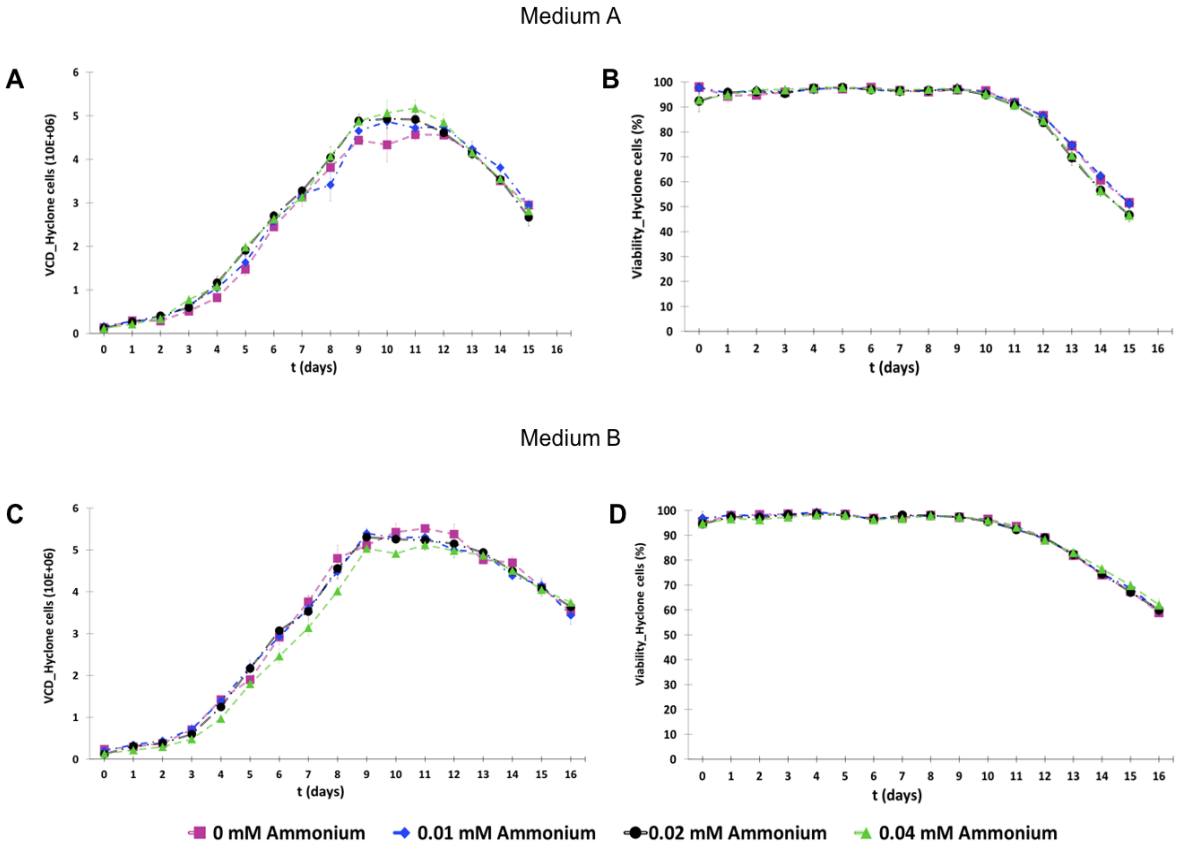

Figure 5.1 Growth parameters of CHO-HyC cells grown in tested medium A and B.

(A) represents the growth profiles of CHO-HyC cells grown in medium A supplemented or not with ammonium describing the VCD of the cells over time (days). (B) represents the viability in (%) profiles of CHO-HyC cells grown in medium A supplemented or not with ammonium. (C) represents the growth profiles of CHO-HyC cells grown in medium B supplemented or not with ammonium describing the VCD of the cells over time (days). (D) represents the viability in (%) profiles of CHO-HyC cells grown in medium B supplemented or not with ammonium.

As a matter of fact, the observed decrease in maximal cell density is maybe influenced by the levels of amino acids in the cell culture media. These media formulations might have triggered metabolic pathways that are responsible for upregulating the phenomena of growth-uncoupled production. The only way to validate this hypothesis is to look at the production capacity of these cells, the final titer of the batches, as well as the specific productivity of the cells.

Besides, the viability trends are similar among the different tested conditions, regarding the two media formulations A and B. No influence was observed regarding ammonia supplementation nor the

optimized amino acids formulation. Although the trend was similar, the cells grown in medium B lasted 1 day longer in the culture (Figure 5.1/B and 5.1/D).

In addition, looking at the growth rates values in figure 5.2, we can observe that the growth rates in different conditions using medium A and B (Bars in green and red) are lower than the growth rate of cells grown in standard conditions using CD CHO medium (Blue bar). Additionally, we can also observe that, among the different conditions within medium A and B, ammonium concentration did not influence the growth rate values in the experiments.

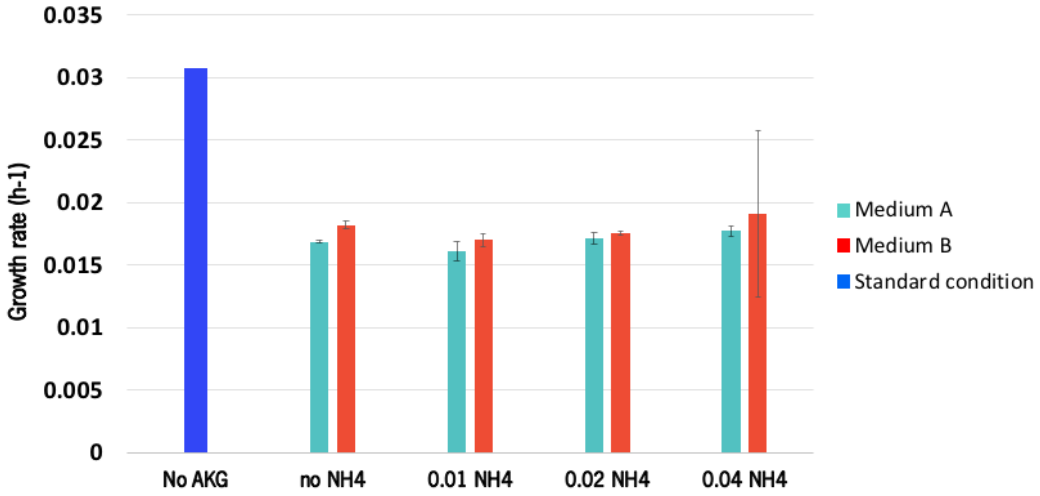

Figure 5.2 Growth rates values of different conditions tested among medium. A (Green bars) and B (Red bars), compared to growth rate values obtained using CHO-HyC cells grown in CD CHO media without AKG, referred as standard condition (Blue bar). The error bars represent 95% confidence interval.

5.3.2. Trastuzumab titer and CHO productivity for producer cells:

In order to evaluate the production efficiency of CHO-HyC cells towards the production of Trastuzumab while using both media formulations with different ammonia concentrations in the media, the final batch titers for the different tested conditions are shown in figure 5.3. According to these results, we do not observe any significant difference in final titer among the tested conditions. Following, we can affirm that increasing the level of ammonium up to 0.04 mM in the medium did not influence the final product concentration in the batches. Furthermore, removing glutamate from the medium in the presence of AKG did not influence the final titers, knowing that the values obtained in this part of the study are similar to the values described in the previous chapter 4. However, it is interesting to observe that, even when the cell density was reduced by half when optimizing the media formulation, the final titers obtained in the batches were similar.

Furthermore, looking at the specific productivities values described in figure 5.4, we can observe that the latter were also identical to the previously shown results in chapter 4. qP values based on VCD and VCV were comparable among the tested conditions. We can observe that ammonium supplementation to medium A, slightly influenced the productivity per cell since we observed a slightly higher qP value for the condition where ammonia was not supplemented to the culture. In medium B, we can observe that ammonium slightly improved the qP values as we can observe on figure 5.4/B. A hypothesis is maybe related to the different levels of aspartate and asparagine in the two different media formulations. About volumetric qP values, no significant changes were observed.

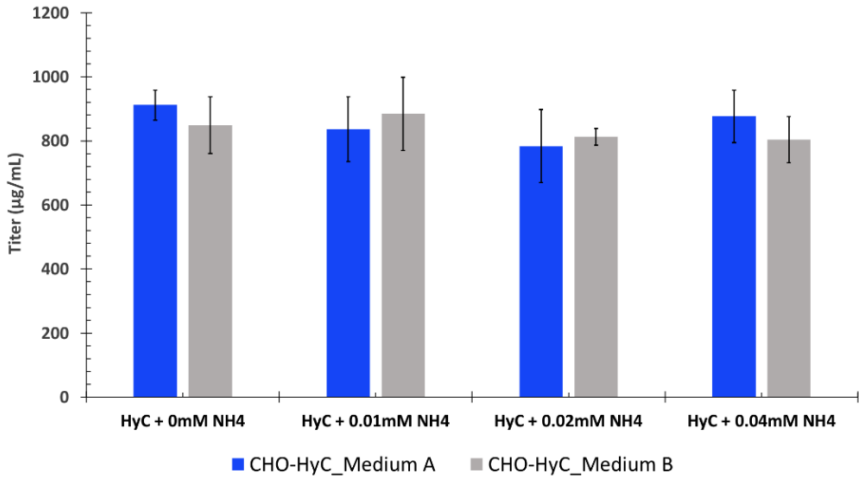

Figure 5.3 Comparison of the final product titers for CHO-HyC cells grown in modified media A and B supplemented with different concentrations of ammonium. The barplots describing Trastuzumab titers of CHO-HyC cells cultivated in medium A are highlighted in blue. The barplots describing Trastuzumab titers of CHO-HyC cultivated in medium B are highlighted in grey. Error bars represent 95% confidence interval.

Interesting conclusions are based on the idea that even with the lack of glutamine and glutamate in the medium, the final titers, as well as the specific cells productivity were not altered.

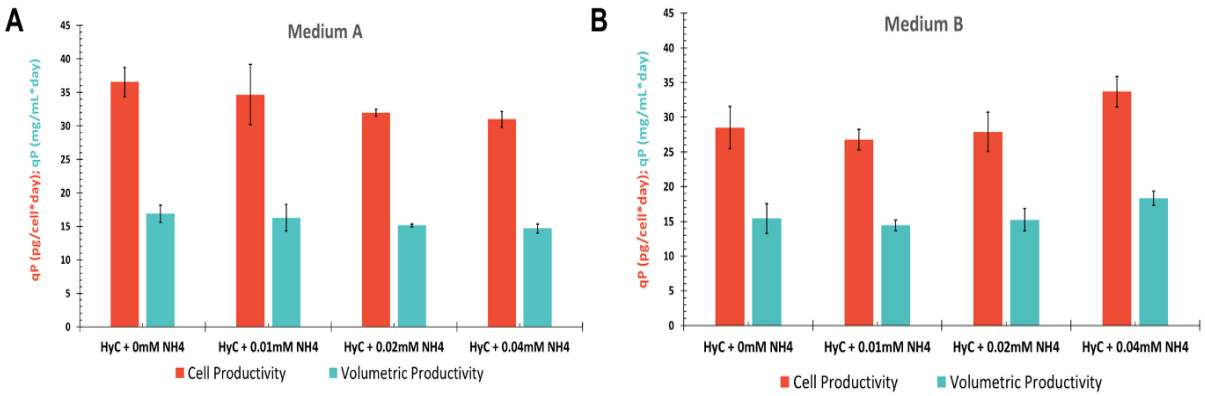

Figure 5.4 Comparison between the different qP values for CHO-HyC cells cultured in both medium A and B when supplementing different ammonium concentrations into the medium. The qP of batch cultures were calculated by VCD (Red bars, pg/(cell*day)) or VCV (Green bars, mg/(cm3*day)). The error bars represent 95% confidence interval.

In the presence of 8 mM of AKG in the medium together with an increased aspartate concentration in medium A and B, CHO-HyC cells overcame the lack of glutamine and glutamate. A hypothesis is based on the idea that AKG, available in the 2 different media formulations, was converted to cellular glutamate and then to glutamine. This hypothesis can be validated by looking at the metabolites flow during culture, described in the next section of this study. On the other hand, ammonium did not influence the titers in these experiments, certainly because its levels are below the toxic limit for the cells.

5.3.3. Metabolic overview

As described in chapter 4, AKG supplementation to the culture medium played an important role in boosting the productivity of CHO cells by improving the final product titers in the tested batches. AKG conversion to glutamate was highlighted. The latter was the main source of the produced glutamine in the culture. In this part of the study, we modified the formulation of CD CHO medium from the NEAA point of view. Glutamate was removed from the medium and serine, proline, hydroxyproline levels were kept constant. Higher aspartate levels were added in medium A and B while asparagine levels were constant in medium A but reduced by half in the case of Medium B.

In figure 5.5 and 5.6, we can observe the metabolic flow of different metabolites (Glucose, lactate, glutamine, glutamate and ammonia) for CHO-HyC cells cultivated in medium A and B with different concentration of ammonia. In figure 5.5, few data (time points) of metabolites are missing because of analytical problems where the sample could not be analyzed.

Following, in figure 5.7 and 5.8, we can observe a comparison between the different exchange rates of key metabolites in the standard condition and medium A (Figure 5.7) and medium B (5.8). In the standard condition, CHO-HyC cells were grown in CD CHO medium without glutamine and without AKG and the data were described in chapter 4. Finally, we can observe in table 5.9 and 5.10, the different values of the exchange rates of different metabolites that were analyzed in this study. It is important to highlight that fitting errors might be impact to the calculation of the exchange rates of metabolites. In table 5.9 and 5.10, in annexes, where we can observe the standard error values related to the metabolites exchange rates estimation for each condition tested experimentally.

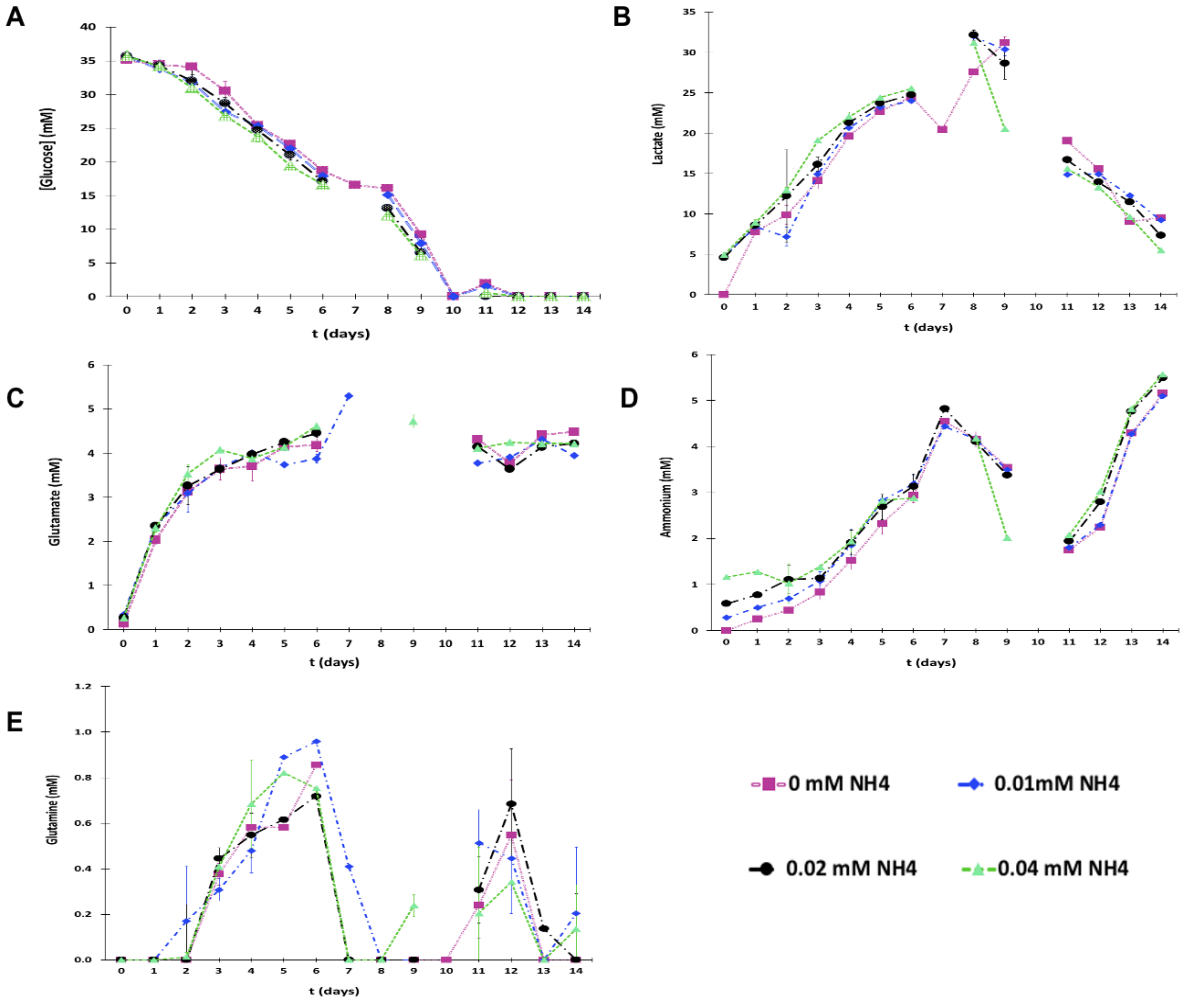

Figure 5.5 Metabolic profiles of CHO-HyC cultures in medium A. (A) represents the glucose profile over time, (B) represents lactate profile over time, (C) represents glutamate profile over time, (D) represents ammonium profile over time and (E) represents glutamine profile over time. The different conditions using different concentrations of NH4 are highlighted as follow: Purple (No ammonia was supplemented), blue (0.01 mM ammonia was supplemented), black (0.02 ammonia was supplemented), green (0.04 mM ammonia was supplemented).

Looking at the metabolic flow of glucose along the batch period, we can observe that the levels were similar, when using both medium A and B with different NH4 concentrations (Figure 5.5/A and 5.6/A). On the other hand, glucose consumption rate increased when increasing the NH4 concentration by 0.04 mM in medium A. Similarly, glucose consumption also increased in the media supplemented by 0.04 mM of ammonia comparing to the cells cultured in medium B non-supplemented with ammonia. Furthermore, comparing the two different conditions supplemented with the highest ammonia concentration, we can also observe an increase in glucose uptake by 1.3 folds when the cells are cultured in medium A.

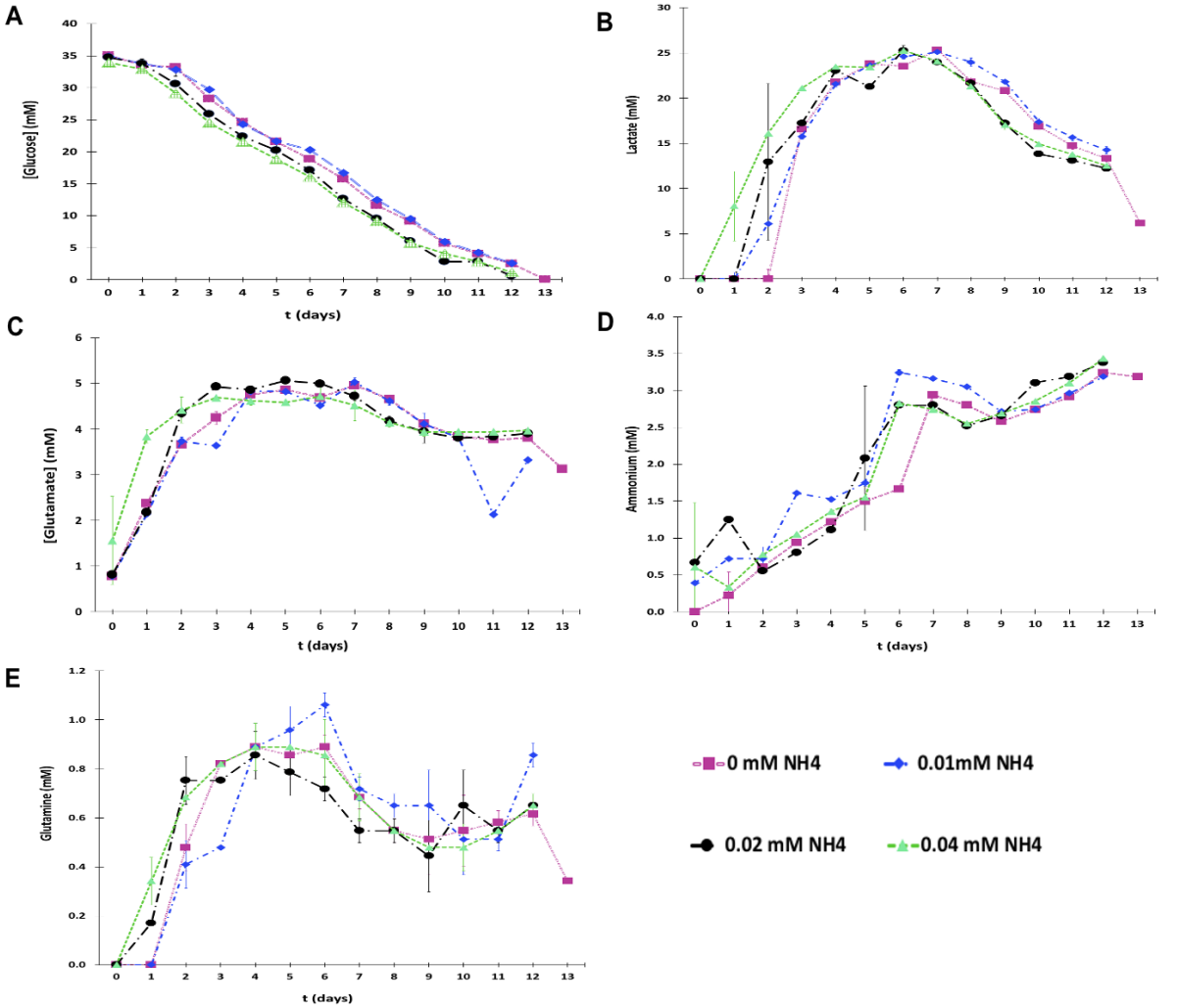

Figure 5.6 Metabolic profiles of CHO-HyC cultures in medium B. (A) represents the glucose profile over time, (B) represents lactate profile over time, (C) represents glutamate profile over time, (D) represents ammonium profile over time and (E) represents glutamine profile over time. The different conditions (using different concentrations of NH4 are highlighted as follow: Purple (No ammonia was supplemented), blue (0.01 mM ammonia was supplemented), black (0.02 ammonia was supplemented, green (0.04 mM ammonia was supplemented).

Regarding lactate concentrations over time in culture, it is observed that the levels were also comparable among the tested conditions. Following, the metabolic switch from lactate production to consumption was observed (Figure 5.5/B and 5.6/B), where lactate starts being consumed 7-8 days post-inoculation, when glucose levels are very low. On the other hand, lactate production rate increased with increasing ammonia concentration in the medium. In medium A, the production rate increased by significantly between the cells supplemented with 0.04 mM NH4 and the ones non-treated with ammonia. For medium B, the secretion rate increased also. Although the secretion rate of lactate increased more in medium B, the rate of production of lactate in medium A was 1.3 higher than the values obtained for medium B.

Furthermore, interesting results were observed concerning glutamate production profile. The latter started being produced 24h post-inoculation, certainly, as consequence of AKG catabolism as described in chapter 4. High values of glutamate were produced, reaching up to 5 mM using both media formulations. No difference was observed between the cells treated and non-treated with ammonia. According to the results observed in figure 5.5/C and 5.6/C, the ammonia supplemented to the culture did not boost the production of glutamate comparing to the non-treated cells, since the levels in the culture were quite comparable.

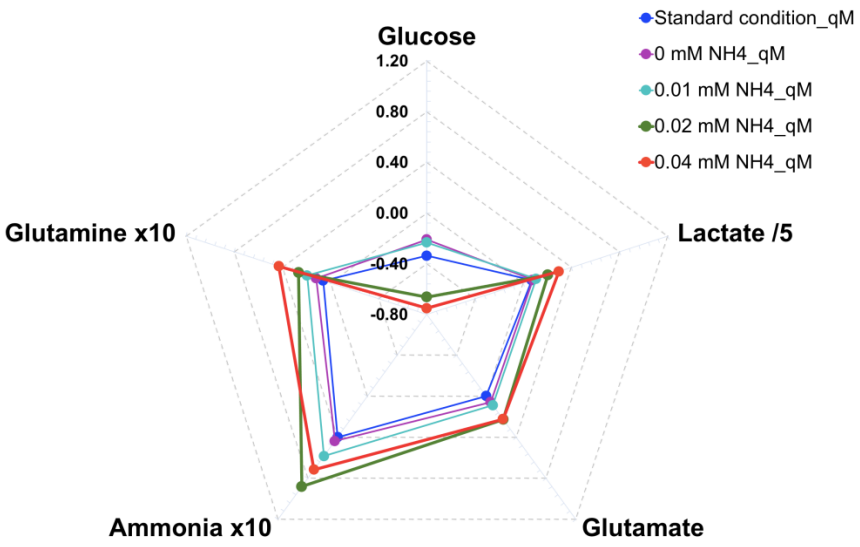

Figure 5.7 Comparison of the exchange rates of key metabolites during culture of CHO-HyC cells in standard condition and medium A with different ammonia concentrations. The standard condition is highlighted in dark blue (Standard condition_qM). The negative and positive value indicate, respectively, the uptake and secretion rates of the corresponding metabolite. The values of the exchange rates of metabolites are expressed in mmol/gDW/h. Note that the rates lactate was scaled down and glutamine together with ammonia were scaled-up to fit the plot (indicated by the numbers after "/" for scaling down and "x" for scaling up).

On the other hand, looking at the rate of production of glutamate, calculated at the early exponential phase of the culture, we can observe that, when cells are grown in both media conditions, we can observe that the production rate of glutamate increased by 4 folds when the cells grown in medium A supplemented with 0.04 mM of ammonia. This production rate was compared to the glutamate production capacity of the cells supplemented with 0.01 mM of ammonia. When the cells are grown in medium B, production rate of glutamate increased by 3.7 folds comparing the same conditions previously mentioned. According to these results, the presence of ammonia reinforced the production of glutamate in the culture, since a direct conversion of AKG to glutamate is possible, when NH4 is consumed. In fact, the supplementation of ammonia was performed to fulfill this objective, so we can conclude that the hypothesis based on the idea of boosting AKG conversion to glutamate via supplementation of ammonia is confirmed.

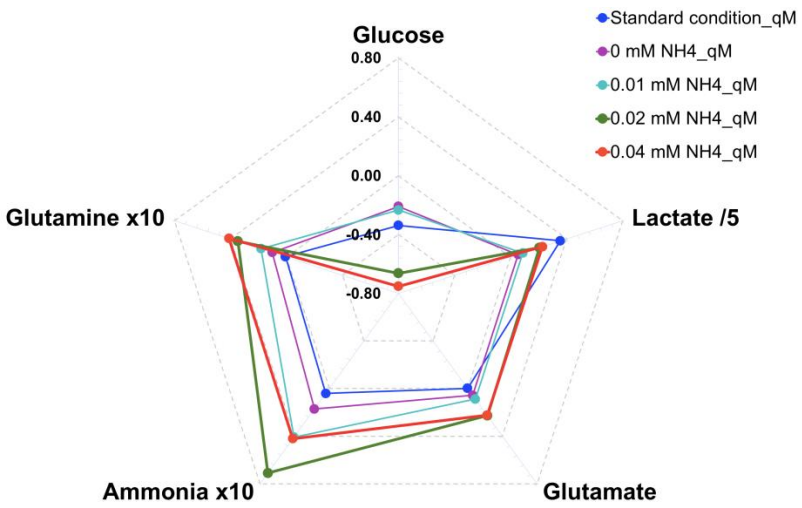

Figure 5.8 Comparison of the exchange rates of key metabolites during culture of CHO-HyC cells in standard condition and medium B with different ammonia concentrations. The standard condition is highlighted in dark blue (Standard condition_qM). The negative and positive value indicate, respectively, the uptake and secretion rates of the corresponding metabolite. The values of the exchange rates of metabolites are expressed in mmol/gDW/h. Note that the rates lactate was scaled down and glutamine together with ammonia were scaled-up to fit the plot (indicated by the numbers after "/" for scaling down and "x" for scaling up).

When glutamate is highly produced in the culture, a high production of glutamine is expected. Looking at figure 5.5/E and 5.6/E, we can observe that glutamine levels increase during the cultures when using both medium A and B. Regarding its secretion rate, when looking at the results described in annexes, we can observe that the secretion rate of glutamine was improved by 3.6 folds when the cells

grown in medium A supplemented with 0.04 mM of ammonia comparing to the cells supplemented with 0.01 mM of ammonia. On the other hand, the rate of biosynthesis of glutamine in medium B was improved by 4 folds. We can understand from these results, that when higher concentrations of glutamate are available in the culture, higher levels of glutamine are produced. The reactions of glutamate and glutamine production are very important in the culture and generally in bioprocesses. The reactions of conversion of AKG to glutamate catalyzed by glutamate dehydrogenase while the conversion of glutamate to glutamine is driven by glutamine synthase, consume ammonia molecules which helps in overcoming its accumulation in the culture, and by than avoiding its toxic effects on the cells. Looking at the results described in figure 5.5/D and 5.6/D, we can observe that, even when, initially supplementing high levels of ammonia in the culture, the levels along the culture remained similar in all the conditions.

Previous study published by Mio-Sam Lao, Derek Toth [37], showed that when supplementing high amount of ammonium chloride to CHO cultures, the specific ammonia production decreased by 55%. Looking at the secretion rates of ammonia described in figure 5.7 and 5.8 respectively for the experiment using media A and B, we can observe that when supplementing 0.04 mM of ammonia, slight increase in secretion rate of ammonia observed. The latter increased by 1.6 folds in medium A condition and 2.4 times in medium B condition. Higher ammonia supplementation to the culture did not generate higher uptake of the latter in the tested media conditions. An explanation is based on the fact that, our study was based on a media formulation, totally different from the one that was used in [37].

The levels of amino acids and other nutrients in the media impact the metabolism of the cells towards the consumption and production of different metabolites. As an examples, adjusting feed rates in fed batch cultures, can play a role in changing the consumption rates of several nutrients during the culture [39].

In fact, developing an animal-free chemically defined cell culture medium that can support the growth and boost the production of recombinant proteins without recurring to the supplementation of glutamine holds a tremendous promise in improving bioprocesses. This strategy can decrease bioprocess related by-products such as ammonia, increase the shelf life of the produced media and also allow the production of cheaper media formulations allowing the reduction of upstream processing cost of goods and decrease in the price of the drugs in the market.

5.4. Conclusions:

Optimizing cell culture media is the core base of cell culture engineering, due to its potential for improving bioprocesses. However, knowing that the formulation contains several components to balance, finding the optimal recipe of cell culture media is still a hurdle. Focusing on balancing amino acids levels, we can affirm that removing glutamine and glutamate from the cell culture medium formulation and substituting those by AKG showed potential in improving the final titer as well as the specific productivities of the cells, comparing to the cells grown in CD CHO media without glutamine (standard condition) and maintaining the levels of unwanted metabolites below the toxicity threshold. Further, we concluded that when increasing the concentration of ammonia in the medium, glucose consumption rate slightly increased. The latter were unchanged among the two tested media formulations (A and B).

In fact, based on previous assumptions and the results described in chapter 4, we infer that adding higher levels of aspartate comparing to the levels in original media (Tripled in medium A and quadrupled in medium B) drove higher production of glutamate from AKG using CHO-HyC cells comparing to the results observed in Chapter 4. It is not known, in these tested conditions if aspartate was in fact the main driver of AKG conversion to glutamate, due to the lack of analytical data, or other pathways were triggered in the case using this media formulation. Further studies can be performed based on analyzing the levels of amino acids during the culture. Although, in these experiments, glutamate secretion levels were increased by 3.6 and 4 folds, respectively for medium A and B, ammonia secretion levels increased by 1.6 folds in medium A condition and 2.4 times in medium B. This increase in ammonia secretion is not problematic from bioprocess standpoint, since its levels in the culture remained below the toxicity levels.

5.5. Annexes:

Table 5.4 Exchange rates values of metabolites for CHO-HyC cells cultured in standard condition and in Medium A with different NH4 concentration. The standard condition is highlighted as (0mM AKG_qM).

	CHO-HyC_0mM AKG		0mM NH4		0.01mM NH4		0.02mM NH4		0.04 mM NH4	
	0 mM AKG_qM	Std error	0 mM NH4_qM	Std error	0.01 mM NH4_qM	Std error	0.02 mM NH4_qM	Std error	0.04 mM NH4_qM	Std error
Glucose	-0.3380	0.0202	-0.2559	0.0261	-0.3556	0.0340	-0.6426	0.0581	-0.9552	0.0928
Lactate	0.3545	0.0469	0.4040	0.0666	0.5369	0.0839	1.0229	0.1562	1.4778	0.2753
Glutamate	-0.0035	0.0002	0.0728	0.0170	0.0928	0.0214	0.1893	0.0365	0.2706	0.0637
Ammonia	0.0398	0.0029	0.0435	0.0038	0.0583	0.0045	0.0880	0.0067	0.0716	0.0044
Glutamine	0.0061	0.0046	0.0119	0.0008	0.0194	0.0017	0.0265	0.0035	0.0429	0.0058

Table 5.5 Exchange rates values of metabolites for CHO-HyC cells cultured in standard condition and in Medium B with different NH4 concentration. The standard condition is highlighted as (0mM AKG_qM).

	CHO-HyC_0mM AKG		0mM NH4		0.01mM NH4		0.02mM NH4		0.04 mM NH4	
	0 mM AKG_qM	Std error	0 mM NH4_qM	Std error	0.01 mM NH4_qM	Std error	0.02 mM NH4_qM	Std error	0.04 mM NH4_qM	Std error
Glucose	-0.3380	0.0202	-0.2093	0.0177	-0.2341	0.0231	-0.6631	0.0619	-0.7522	0.0692
Lactate	0.3545	0.0469	0.2636	0.0510	0.4315	0.0633	1.0284	0.1519	1.1324	0.1846
Glutamate	-0.0035	0.0002	0.0561	0.0146	0.0870	0.0187	0.2259	0.0469	0.2224	0.0532
Ammonia	0.0398	0.0029	0.0170	0.0028	0.0407	0.0034	0.0709	0.0071	0.0420	0.0098
Glutamine	0.0061	0.0046	0.0101	0.0023	0.0180	0.0024	0.0346	0.0078	0.0409	0.0072

5.6. References:

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CHAPTER 6

General conclusions and future work

The main objective of this work was to improve cell culture media for the cultivation of CHO cells to achieve better growth parameters and production capacity of biopharmaceuticals. More specifically, this study aimed at contributing to solve the current bottlenecks related to mammalian bioprocesses by developing a pipeline for optimal media formulation for CHO strains. This strategy employed *in silico*-based approaches namely constraint-based genome-scale models and flux balance analysis (FBA). Subsequently, some of the results regarding the optimal cell culture media formulation were tested using both producer and nonproducer CHO cell lines. To achieve the proposed motivation, the targeted research aims were elaborated in chapter 1. The main conclusions achieved, and the future research aims are presented below.

Chapter 2 contains a very detailed literature review regarding the state-of-the-art methodologies for improving CHO bioprocesses. In this chapter, various concepts regarding the importance of CHO platforms in the biopharmaceutical industry were highlighted, as well as the basic features of CHO metabolism and its differences compared to microbial production platforms. Moreover, the different methodologies currently used to optimize growth parameters and production performance of CHO platforms were highlighted. Finally, modern engineering strategies based on systems biology approaches were described, focusing on the importance of constraint-based modeling and genome-scale metabolic models and its great value for developing robust *in silico*-based approaches for bioprocess optimization, mainly media design and improvement.

Knowing that the metabolic network of mammalian platforms is very complex, it is essential to deeply understand the different metabolic mechanisms of CHO, focusing on the different pathways and reactions, involved in cell growth and production of recombinant proteins. In chapter 3, we applied the universal genome-scale metabolic model of CHO in combination with an evolutionary algorithm (optiModels framework) to understand the metabolic demands of CHO and to design an improved version of cell culture media. As an outcome from these optimization strategies, we were able to:

- Predict the minimal media formulation that can sustain CHO growth based on the use of optiModels framework. After various optimization steps, this tool allowed designing an approach to further improve growth parameters of CHO.
- Optimize the environmental constraints previously published in Hefzi et al., 2016 based on the results using optiModels, flux balance analysis simulations and literature. This approach was focused on identifying the most important metabolites that could influence growth parameters of CHO, as well as impact the secretion of by-products such as ammonium. Based on the optimized constraints, we were able to improve *in silico* the growth yield of CHO, as well as reduce ammonium secretion levels to its lowest limit.
- Determine potential candidates (e.g., AKG) that hold a potential in further boosting CHO lifespan and increasing the duration of the batch process. AKG could replace glutamine in the culture medium due to its potential in producing cellular glutamate that can be used to biosynthesize glutamine in case of its lack in the cell culture medium.
- Design experimental validation based on exploring the effect of supplementing AKG to the culture medium and validate the *in silico* results.

In the last few years, the effect of glutamine supplementation into the cell culture media was discussed and several alternatives to glutamine have been explored due to several drawbacks resulting from its fast degradation. As a result of the prediction pipeline described in chapter 4, we experimentally tested the effect of supplementing different concentrations of AKG into the standard cell culture media (CD CHO). As an outcome of these experiments, we can conclude that:

- The presence of AKG in the culture reduced specific growth rate compared to the standard culture conditions (Cells cultured in CD CHO media deprived of glutamine).
- Supplementing AKG to different CHO cultures increased the lifespan of the cells during the batch cultures and improved the final product titers of the process. The final batch titer was 1.9-fold higher compared to the titers obtained in standard culture conditions.
- The presence of AKG in the cell culture medium influenced several metabolic pathways, for instance aspartate and glutamate metabolism. Aspartate consumption increased 10-fold when cultivating the producer cells in media containing 12 mM of AKG. However, its consumption increased only by 1.5 times in case of non-producer cells (CHO-K1) cultivated in the same conditions. According to the results, aspartate might play an important role in converting AKG to glutamate, mainly in the exponential phase of the cultures and therefore is an important

metabolite for increasing growth and qP. Subsequently, glutamate secretion rate increased proportionally to the level of AKG in the media. We can conclude that higher levels of AKG in the medium positively correlated with higher aspartate uptake rate and glutamate secretion rate. These results are interesting since glutamate production from AKG is an ammonium detoxifying reaction. This reaction is metabolically efficient since it plays a role in consuming the free ammonium in the culture to produce glutamate molecules.

- AKG supplementation clearly influenced the exchange rates of various metabolites for both CHO-K1 and CHO-HyC cells cultures. However higher impact on metabolism was observed in case of high producer cells (CHO-HyC), where glucose consumption and lactate production rates increased compared with the uptake/secretion rates of these metabolites when cells cultured in the standard media formulation. This phenomenon is probably associated with the highest demands for recombinant protein production.
- Despite the higher secretion rates of lactate and ammonia during the process, the concentrations of these metabolites during the batch were slightly lower than the concentrations in standard batches.

Finally, the strategy adopted in chapter 5 is inspired from the results obtained in chapter 3 and 4, where the formulation of cell culture media was optimized and tested experimentally. In these experiments, the levels of non-essential amino acids (Aspartate, asparagine and glutamate) were varied in the media formulations. In addition, different concentrations of ammonium were supplemented to the cultures. Consequently, the metabolic effects of these media optimization strategies were studied using CHO-HyC cells. In this chapter we conclude that:

- Growth and viability profiles of CHO-HyC cells cultured in modified media A and B (Table 5.2) were identical. The growth trends were also similar to the cells grown in CD CHO media supplemented with 8mM AKG (Results shown in chapter 4).
- Removing both glutamine and glutamate from the media formulation did not influence the final titer of the process. The final batch concentrations were equivalent to the titers of the reference cultures grown in standard media supplemented with AKG (Results described in chapter 4). Consequently, we can conclude that both amino acids are not so relevant when the media contains high concentrations of AKG and aspartate. Subsequently, we hypothesize that aspartate is converted to oxaloacetate, boosting the TCA cycle together with AKG.

- We observed no differences in the final product titers in the case of CHO-HyC cultured in medium A and B. However, the specific productivity of the cells was slightly higher when using medium B supplemented with 0.04 mM ammonia compared to the other tested conditions. The latter might be explained by the adjustment of aspartate/asparagine levels in the media.
- The increase of aspartate levels in the media formulations correlated with the increase in the levels of glutamate produced during cultures, using both medium A and B. This result is associated with the possible fact that the available AKG molecules in the media are directly converted to glutamate using glutamate dehydrogenase. Another hypothesis might be that the produced glutamate is based on the conversion of aspartate and AKG to glutamate under the control of aspartate amino transferases.
- Higher secretion rates of ammonia and lactate were observed, but the concentrations during culture were below the levels determined in standard conditions and the toxicity levels at culture.
- Overall, the conditions evaluated regarding aspartate, glutamate and ammonium did not provoke significant differences in the variables evaluated, comparing with the results obtained with AKG addition in the previous chapter, indicating that, at least in the range of concentrations testes, AKG was the most significant variable evaluated.

Finally, we can conclude that using GSMMs combined with constraint-based modeling approaches such as FBA hold a unique potential in exploring cells metabolic features and simulating metabolic states of the cells *in silico* under specific environmental constraints, for instance media formulation. These tools are very promising in designing engineering approaches aiming at improving bioprocesses, for instance media optimization. This approach is not only important strategy for improving the titers of the producer of recombinant proteins but also improving the product quality.

A follow up of this work should be focused on integrating the experimental data obtained in chapter 4 and 5 into the CHO GSMM (iCHO1766) and compare its prediction capability to the experimental values. This would open various possibilities also for curating the existing model in order to increase its prediction accuracy. In addition, understanding the metabolism of amino acids in the optimized media formulations (A and B) is needed in order to further optimize the media formulations to retrofit the metabolic/nutritional need of the clone of interest (Producer or non-producer clone), improving growth parameters and production of recombinant proteins.

Based on optiModels predictions, various candidate metabolites, besides AKG, were determined and can be supplemented to the media formulation and evaluate its effect on improving growth parameters, process titers and product quality attributes.

Future perspective regarding this project could be focused on improving the in-house evolutionary algorithm (optiModels) and improving its prediction accuracy towards determining additional supplementation candidates. Additionally, future work should be focused on further optimizing CHO strains at the cell line development stage due to their importance in biomanufacturing. Moreover, knowing that cellular resources are limited and need to be shared between growth and recombinant protein production, it is important to further study CHO metabolism and determine possible strategies to improve production strains through ameliorating its metabolic machinery. Besides metabolism, other bottlenecks should be addressed, for instance, improving the secretion capacities of the cells, better study/improve predictions towards designing custom post translational modifications patterns (e.g., glycosylation), folding, etc. These further improvements are essential for better CHO performance.

Following, focusing on cell line development through designing a preoptimized modular CHO strain (a mammalian chassis strain) can reduce genetic instability of the cells which is one of the main sources of a large variation in product titers and fluctuation in product quality. The most important obstacle here is our lack of knowledge on many regulatory mechanisms within the cells that enable it to respond to different conditions and challenges. Subsequently, in the attempt to reach a fully designed chassis cell line rather than an optimized or engineered one, designing model-based predictions and computational strategies for the identification of the most useful engineering strategies (e.g., stable transgene integration sites) will play a key role. A challenge that still needs to be addressed here is the availability of tools to combine and correlate the different omics data sets in a comprehensive and automated way. A proper connection should link different omics layers to obtain a complete picture of their interrelationship and its combined influence on the system. So far, several algorithms have been developed to integrate omics data, such as transcriptomics, proteomics or metabolomics into GSMMs, but no truly comprehensive solution is yet available. Nevertheless, with the rapid advances that have been achieved over the last years, both in our basic understanding of cellular mechanisms and regulatory circuits, and with the new tools that have emerged, we are today in a much better position to aim for the design of mammalian chassis cell lines with defined characteristics, even though several challenges still need to be resolved.