

QUANTIFYING THE IMPACT OF MEDIA SUPPLEMENTATION  
ON CELL GROWTH AND PRODUCT YIELD IN CHINESE  
HAMSTER OVARY (CHO) CELLS

by

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## Abstract

The optimization of cell growth and productivity is a major concern in the production of recombinant proteins in Chinese hamster ovary (CHO) cell cultures. Despite the frequency of media optimization in literature, there have been few attempts to comprehensively assess the overall effectiveness of media additives. This thesis aims to document media optimization (of CHO cell cultures) over the last 20 years and quantitatively assess the impact of media optimization on cell culture performance. A review of 78 studies identified 238 unique additive components, of which, trace elements stood out as having a positive impact on cell density while nucleosides show potential for increasing titer, with commercial supplements benefiting both. However, the impact of specific additives was found to be more variable than commonly perceived. With relatively few media studies considering multiple cell lines or multiple basal media, determining consistent and general trends becomes a considerable challenge. By extracting cell density and titer values from all of the reviewed studies, I was able to build a mixed-effect model capable of estimating the relative impact of additives, cell line, product type, basal medium, cultivation method (flask or reactor), and feeding strategy (batch or fed-batch). Overall, additives only accounted for 3% of the variation in cell density and 1% of the variation in titer. Similarly, the impact of basal media was also relatively modest, at 10% for cell density and 0% for titer. Cell line (10% and 13%), product type (9% and 33%), and feeding strategy (22% and 24%) were all found to have more impact on cell density and titer. These results emphasize the need for media studies to consider more factors to ensure that reported observations can be generalized and further developed.

## List of Abbreviations and Symbols Used

$\alpha$  Alpha.

$\gamma$  Gamma.

$\mu$  Micro.

.csv Comma-separated values.

**ADCC** Antibody dependent cellular cytotoxicity.

**AIC** Akaike information criterion.

**ANOVA** Analysis of Variance.

**Bcl-2** B-cell lymphoma 2.

**BSA** Bovine serum albumin.

**CDC** Complement dependent cell cytotoxicity.

**CHO** Chinese hamster ovary.

**CO<sub>2</sub>** Carbon dioxide.

**COS** African green monkey kidney.

**DHFR** Dihydrofolate reductase.

**DMSO** Dimethyl sulfoxide.

**DO** Dissolved oxygen.

**DOE** Design of Experiments.

**EGF** Epidermal growth factor.

**ELISA** Enzyme-linked immunosorbent assay.

**FAD** Flavin adenine dinucleotide.

**FAIM** Fas apoptosis inhibitory molecule.

**FBS** Fetal bovine serum.

**HCl** Hydrochloric acid.

**HPLC** High performance liquid chromatography.

**IFN- $\gamma$**  Human interferon- $\gamma$ .

**IGF-1** Insulin-like growth factor 1.

**IgG** Immunoglobulin G.

**ITS** Insulin-transferrin-selenium.

**KCl** Potassium chloride.

**LiCl** Lithium chloride.

**LPA** Lysophosphatidic acid.

**mAbs** Monoclonal antibodies.

**Man5** N-linked mannose-5 glycan.

**MFA** Metabolic flux analysis.

**MgCl<sub>2</sub>** Magnesium chloride.

**MTX** Methotrexate.

**NAC** N-acetylcysteine.

**NaCl** Sodium chloride.

**NAD** Nicotinamide adenine dinucleotide.



**NADP** Nicotinamide adenine dinucleotide phosphate.

**NBS** Newborn calf serum.

**NS0** Mouse myeloma.

**OFAT** One factor at a time.

**PBS** Phosphate buffered saline.

**RMSE** Root mean square error.

**SITE** Selenium-insulin-transferrin-ethanolamine.

**SMA** Spent media analysis.

**tPA** Tissue plasminogen activator.

**VCD** Viable cell density.

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# Chapter 1

## Introduction

Chinese hamster ovary (CHO) cell culture serves as one of the most important platforms for the production of recombinant proteins and monoclonal antibodies (mAbs) in particular — with over 80% of approved mAbs produced in CHO cells [1]. In 1987, tissue plasminogen activator (tPA) was the first approved recombinant protein produced in CHO cells [2]. Since then, the increasing approval of mAbs and CHO cell products [1] continues to highlight the importance of CHO cells in the biopharmaceutical industry. Overall, the biopharmaceutical industry has grown into a multi-billion dollar industry, with over 30 billion US dollars in sales for CHO cell products alone as of 2007 [2]. Recombinant proteins and mAbs are advantageous over chemically synthesized therapeutics because of their high specificity and success rate [3], so they are often used in treatments such as for cancer and arthritis [4]. However, the production of recombinant proteins is more expensive and more complicated by its cellular synthesis than chemically synthesized therapeutics, and their large size can prevent efficient target localization, where target localization refers to delivering a therapeutic to a particular location, for example, at a tumor site [3]. Although production has improved substantially since the 1980s [5], achieving higher product yields is still a cause for concern as there is demand for both more product and reduced prices [6, 7, 8]. As media optimization is generally perceived as a significant reason for the continuously increasing product titers [4, 5], this thesis aims to quantify the overall importance of media supplementation.

### 1.1 Cell Types

A variety of cell types have been used to produce protein therapeutics, ranging from prokaryotes to eukaryotes. Prokaryotic cells, such as *E. coli*, are associated with fast production, high yields, and low cost, but their post-translational modifications are not compatible with humans [9, 10]. For these reasons, *E. coli* is used to produce

about 30% of approved proteins, but these are mostly non-glycosylated proteins [9]. Despite slower production and greater expenses, mammalian cells are preferred because they are compatible with humans [10]. Popular mammalian cells include CHO, African green monkey kidney (COS), mouse myeloma (NS0) and hybridoma cells. However, COS cells are more appropriate for small-scale processes because they use transient expression and NS0 can be difficult to transfect [11]. To produce the desired large quantities of therapeutic proteins, stable expression systems are desired for easy scaling [12]. Therefore, CHO cells in particular are chosen because of their ability to grow in suspension, their scalability, and their inherent safety [13, 14]. In fact, most of the human pathogens tested do not replicate in CHO cells [2], improving the likelihood of regulatory approval. A general comparison of the types of cells possible for recombinant protein production can be seen in Table 1.1. Because mammalian cells are limited by the slower production rates, methods such as media development, process optimization and cell engineering have all been used to improve cell growth and production titer. And while overall protein yield is influenced by all of these factors, media optimization is generally highlighted as a significant reason for continuously increasing protein titers since the 1980s [5].

Table 1.1: Advantages and disadvantages of potential cell culture host cells [10].

<b>Host</b>	<b>Advantages</b>	<b>Disadvantages</b>
Prokaryotes	Rapid growth, low cost	Poor protein processing, release undesired by-products
Yeast	Rapid growth, secretes products to medium	Different glycosylation patterns
Insect Cells	Complex protein processing, no endotoxin contamination	Incapable of continuous fermentation
Mammalian Cells	Proper post-translational modifications	Expensive, contamination risk, slow growth

## 1.2 Cell Culture Media

Development of classical cell culture media began in the 1950s, with the earliest media containing only the minimum components for cell survival [15]. Over time, media has become more refined, with additional components ranging from carbohydrates and nitrogen sources to trace elements and vitamins. Although media was originally

designed for use with serum, this is no longer a requirement and most media is formulated without serum and animal-based products due to contamination potential [15]. Common media categories include serum-free, animal-origin-free or chemically defined media. While serum-free media may use animal based products or components of serum origin, such as albumin [16], animal-origin-free media replaces those components with non-animal based proteins (such as plants) [16]. As chemically defined media does not contain serum or proteins, its content is more predictable, safe, and meets regulation guidelines [17]. However, chemically defined media does not come without disadvantages: without the complex components, cells are more susceptible to shear stress and require extensive adaptations [16, 17]. Because of the variety of broad cell culture media categories and the desire to optimize cell culture on an individual basis, the continuous development and optimization of media has led to many available media formulations for each cell line. However, unlike classical media, many lab group formulations and especially commercial media formulations are undisclosed. The information available for these media is insufficient for many researchers [4], but Brunner [18] did produce a database of serum-free media available for consumers. The database was intended to simplify media selection and facilitate the exchange of information between researchers [18], but at this point in time the database is no longer available at the provided url. Furthermore, the database provides potential opportunities for improvement, such as expanding to other types of media, and including supplementation that may have further optimized media.

### **1.3 Research Motivation**

Media optimization has remained an important component of ongoing research, which aims to improve cell growth and product titer as new cell lines and products are developed. Indeed, Kuo et al. [14] found that media optimization studies account for approximately 25% of all the CHO bioprocessing literature published up to 2015. Given the continued importance of media optimization to recombinant protein production, this thesis aims to examine recently published media optimization studies in a more quantitative fashion than has yet been attempted. In addition, even with the serum-free media database [18], there is interest in easily accessible media formulations between lab groups [19]. Given the need for easily accessible information

and the quantity of research available for media supplementation, it is expected that a database of media supplementation would be beneficial. This database would easily compile the relevant supplementation information, and it will provide the ability to search for additives (and media) tested on a cell line as well as determine if the supplement had a positive effect.

While many individual reports focus on media supplementation, and some may even compare different types of additives, most studies do not compare the overall effect of additive supplementation. Furthermore, in the reviews concerned with cell culture media, the discussion tends to be qualitative in nature or focused on a small subset of media components. For example, a number of reviews serve primarily as introductions to CHO cell culture: providing the history of media development, typical components, and a general overview [15, 16]. These provide a basis for classical media design but limited discussion of specific outcomes in the form of cell concentration or protein titers. Other reviews have focused on specific media issues such as the removal of lactate and ammonium, using both media design and cell engineering [20, 21]. And in this context as well, there has been limited quantitative comparison between the articles reviewed. In some reviews, the media focus is only a small subsection of a larger review [4, 22]. These reviews typically serve to discuss general cell culture optimization and refer to media as a possible optimization method. In terms of media, the additives are mentioned in a general sense in terms of effectiveness, and do not perform a quantitative analysis. Reviews may not even address the quantitative aspects behind media supplementation, and instead choose to focus on the effect of supplements on product quality attributes [23, 24]. Furthermore, it has become an increasingly popular opinion that the next step in media optimization requires a more detailed understanding of the metabolic processes in order to improve media design [14, 25, 26, 27]. This project serves to address one component of this need by quantitatively analyzing the additives used in cell culture media to determine what trends exist and what improvements can be made.

The overall objectives of this research will be discussed in Chapter 2; Chapter 3 provides details of current research surrounding cell culture and media supplementation; Chapter 4 describes the approaches taken to record and analyse the research. Chapter 5 analyses the details recorded during the meta-analysis, while Chapter 6

attempts to quantify the effect of media additives.

## Chapter 2

### Objectives

This project aims to consolidate media supplementation research on CHO cell cultures and determine the effect of supplementation on therapeutic production. It is established that increased therapeutic production is necessary and can be influenced by many variables, including media. However, up to this point, there has been no quantification of the impact caused by these variables. The objective is to quantitatively determine the effect of media supplementation on the growth and productivity of recombinant therapeutics in CHO cells. Thereby, determining the overall importance of media optimization in comparison to other forms of cell culture optimization. Data selected from studies that focus on supplementation will be used as part of a meta-analysis that aims to:

- Outline current media optimization practices
- Identify specific additives beneficial to cell concentration or protein titer
- Quantify the overall impact of media optimization (on cell concentration/protein titer).

These objectives were obtained by creating a database of relevant media supplementation strategies and recording the maximum viable cell density (VCD) and titer responses. In addition, cell lines, recombinant products, basal media, media additives and the additive concentrations were collected for the analysis. The data collected was analysed using graphing techniques, case studies and statistical methods (mixed-effect modelling) to determine the quantitative impact of additives in comparison to the other variables recorded.



## Chapter 3

### Literature Review

#### 3.1 Standard Optimization Methods

The optimization of cell growth and production has been performed using cell engineering, process design, and media optimization. Cell engineering edits a cell's genetic material, using a range of different methods, to improve cell cultures: often in terms of cell growth, production, or product quality. Some common methods include overexpression, gene knockout, and gene knock-in. On the other hand, process design considers the physical or chemical aspects of cell cultures — varying from feeding strategy to culture conditions. In media design, the medium components are adjusted to suit the needs of the particular cell culture. The following subsections will discuss these optimization methods in more detail.

##### 3.1.1 Cell Engineering

The overexpression of a gene of interest can be used to enhance the efficiency of nutrient consumption, deter apoptosis, or enhance protein expression. Several studies have shown that the overexpression of genes can reduce the production of lactate and increase cell productivity [28, 29]. It is a common desire to lengthen cell culture duration, and the overexpression of anti-apoptotic genes is an effective way to extend cell life. This has been performed using a variety of different genes, such as Bcl-2 [30] or FAIM [31]. The overexpression of these genes increases the resistance to apoptosis in the mitochondrial apoptosis pathways or in the death receptor-mediated pathways, respectively [31]. Gene knockouts can be used to either delete or inactivate a gene [7], which has played a large role in cell culture developments by producing several high producing cell lines. The primary example of this is the dihydrofolate reductase (DHFR) deficient cell lines. DHFR produces important cofactors for DNA synthesis, so by knocking out this gene the cells need hypoxanthine and thymidine

to grow [32]. A vector containing both the gene of interest and DHFR is transfected into the cell, and when exposed to a medium without the proper additives only cells containing the gene can grow. Methotrexate (MTX) is used as a selection agent so that those producing more of the gene survive, thus creating higher producing cell lines [33]. Gene knock-ins are used to replace a small DNA fragment, which is selected when alterations to a specific gene are preferred over removing it completely [34]. Improvements to gene editing tools, such as CRISPR/Cas9, have led to overall improvements in gene editing and cell engineering [7]. In general, cell engineering is capable of altering cell production and improving product quality, but it can be a laborious task [35, 36], and once a cell clone has been established it still requires a suitable media to grow properly.

### 3.1.2 Process Design

When determining the feeding strategy, batch and fed-batch cultures are typically used for stable products, whereas the short residence time makes perfusion culture more suitable for labile products [37]. Batch is the simplest method as a stand-alone system, fed-batch processes have a fresh inlet of media, and perfusion cultures have both a steady inlet and outlet stream. Although batch processes are the simplest method, they are also the lowest producers and have the shortest culture duration around 6-8 days [5]. In a fed-batch system, reintroducing media or additives prevents important components from depleting in the media, thus extending culture lengths to 2-3 weeks and increasing overall yield [37]. A typical batch culture is expected to produce around 1 g/L while a fed-batch system may achieve titers of 1-10 g/L [38]. In general, the extended culture lengths are seen as the main reason for higher titers in fed-batch systems. In fact, Xu et al. [39] found that cultures run in different processes (batch, fed-batch, perfusion) had similar specific productivities, and it was the culture duration that ultimately affected the final yield. Similarly, Reinhart et al. [40] also found that feed supplementation improved cell density and product titer, with daily media exchange having a substantial effect on cell growth. The feed itself is not the only factor, as the timing of the feed can impact culture duration and production as well. A reduced number of feeds is able to maintain titer while reducing ammonia concentrations and osmolality [41]. In perfusion cultures the inlet

feed refreshes the media with necessary components, while the outlet removes spent media that may contain by-products, but the cells are retained within the system. While perfusion cultures have been around since the 90s, they still require media optimization to reduce overall media costs and maximize productivity [42]. Perfusion cultures are at a disadvantage in comparison to fed-batch as they produce products with less consistency, and generally take longer for approval [43].

The cell culture conditions, such as temperature, dissolved oxygen (DO) and osmolality, can also be altered to benefit production. Though the stress of temperature shifts or high osmolalities can accelerate cell death [22], it may also be used to improve productivity. In general CHO cell cultures are maintained at 37 °C, with 5% CO<sub>2</sub> to properly maintain pH of carbonate buffered media [44]. Temperature shifts have often been used to increase specific productivity, and have been successful when lowered to 33 °C [45], 32 °C [46], and 30 °C [47]. At reduced temperatures specific productivity has increased up to 4-fold [45]. However, temperature reduction is not always beneficial and can reduce viability or product quality [48]. Reinhart et al. [40] found that using sodium chloride feeds to raise the osmolality from 310 mOsmol/kg to 400 mOsmol/kg could improve productivity by as much as 30%. In addition, at a highly elevated osmolality, the use of osmoprotectants, such as glycine betaine, have been shown to improve titer over 40%, though improved productivity is dependent on the cell clone [49]. Other parameters such as DO, pH, and CO<sub>2</sub> levels can all impact the growth, yield and product quality and should also be considered during process design. In addition, agitation and aeration require careful consideration during the scale-up portion of design to ensure that the optimization results are comparable at the larger scale [22]. Process design must be done carefully to avoid cell death and product quality changes since the design changes can often rely on adding stress to the system to affect productivity.

### 3.1.3 Media Design

Despite the variety and availability of cell culture media, there is a large variability in cell culture responses. In fact, the impact of media optimization is rarely uniform — the cell line (and even clones within a single cell line) as well as the nature of the recombinant protein can impact the cells' response to a given medium formulation

[5]. Determining the medium of choice is a time-consuming process due to the sheer quantity of formulations and the need to distinguish its effectiveness on a cell clone. Depending on the method used, media optimization can take upwards of a year to complete [50]. Even then, media optimization studies occur frequently in literature because of its influence on growth, productivity, and product quality. A completely optimized cell culture requires clone selection, process design, and media development considerations to be made together. Highlighting the importance of media design is the knowledge that a high producing cell line still requires proper media to produce efficiently. The following section will discuss various methods of media optimization techniques that are seen frequently throughout literature. For the purpose of this project, media optimization is broken down into the following general categories: *ad hoc*, “design-of-experiments” (DOE), and metabolic studies.

The simplest form of *ad hoc* designs is “one factor at a time” (OFAT) where one or more variables are adjusted individually. OFAT designs neglect the interaction between the two (or more) variables, which can prevent the results from calculating the true optimum value. An example of OFAT designs is component titration. This traditional approach to media development adds media components in varying amounts to cell cultures and measures the cell line response [51]. To reduce the length of time spent optimizing, media blending can also be used. Instead of individual components, this approach blends existing media formulations together to find the best medium [51], where an individual medium is considered a single factor. In addition to the lack of interaction, OFAT processes are generally slow [52]. Despite the limitations, these often appear within research, likely because of their simplicity.

DOEs are a statistics based technique that systematically adjust factors to determine the effect of the factors on a given response variable. Screening style DOEs are often used as a preliminary test to determine the additives of interest in follow up optimization DOEs. Since screening does not consider interactions, it is important that an optimization design is also used to visualize both individual and interaction terms of a component. A Plackett-Burman design is often used as a preliminary design because it can screen a large number of variables. It is a special form of fractional factorial that is used when the number of experiments is a multiple of four,

where the number of experiments must be at least one greater than the total number of variables [53]. An example of its use is Zhang et al. [54] where a variety of additives including antioxidants, polyamines, and vitamins were tested in a single study. Using a statistical analysis of the experiments performed, Zhang et al. [54] was able to select three important components for further analysis in a central composite design. There is larger variation in the optimization style of DOEs seen in literature, which may include: central composite, factorial designs, and mixture designs. Statistical analysis of an optimization-based design can be used to calculate the optimum concentration of the additives of interest. This is often done using regression modelling, contour plots for multiple responses, and/or response surface plots [55]. Both central composite [54] and full factorial [56] designs have found improved titer results when optimum concentrations determined by their model were tested experimentally. The central composite design is advantageous because it includes five concentration levels, which can capture cubic responses, while mixture designs are seen as a simple and effective approach [57]. Though full factorials can have a large number of experiments, a fractional factorial can reduce the number of experiments while still including lower order interaction terms [58]. In general, the main advantage of DOEs is that it can consider interactions, where the interaction between components can have a substantial impact on the overall effectiveness. Mixture designs have often found that combinations of additives achieve higher cell growth and titers than the additives individually. For example, the combination of three nucleosides (deoxyuridine, thymidine, and deoxycytidine) was able to achieve up to 40% increase in cell density, and almost 60% increase in mAb concentrations over individual nucleosides [59]. Similarly with hydrolysates, it was found that wheat gluten hydrolysates were not effective individually, but in combination with soy hydrolysates cell growth improved 1.8-fold and antibody production improved 2-fold for a cell line [60]. In addition, DOEs are able to explore the entire design range with a minimum number of experiments, showing a general improvement over OFAT designs.

Recently there has been an increase in metabolic studies, where Kuo et al. [14] found that an additional 40% of the literature survey in 2015 included metabolic studies. In fact, many reviews consider these methods to be the next step in media development because the understanding of the metabolism can provide a unique view

of the necessary components and minimum concentrations [14, 25, 26, 61]. There are different approaches to understanding cell metabolism including spent media analysis (SMA) and metabolic flux analysis (MFA). SMA is typically used as a preliminary study to measure media content changes during an experiment. A comparison of fresh and spent media is evaluated and component concentrations are adjusted to prevent the depletion or accumulation of nutrients and metabolites [62]. SMA is often used to measure amino acid concentrations because they are main media components and easy to adjust [62]. For example, through SMA it was found that cysteine, asparagine, and tyrosine frequently deplete in cell cultures [62, 63]. MFA estimates the intracellular flux using rates of production and consumption of metabolites, thereby providing a better understanding of the cell metabolism [15, 64]. While using metabolic studies to design media has perceived benefits, studies are more frequently used to clarify cell behaviour than as a development strategy. For example, MFA has been used to develop an understanding of metabolic trends for varying cell lines under butyrate treatment [64], and the glucose and galactose metabolism [65].

### **3.2 Cell Culture Cultivation Methods**

Suspension cell cultures can be run in several different vessels including plates, flasks and bioreactors. The cultivation method is often dependent on the desired scale. Although multiwell plates have relatively small working volumes, they are easy to use when there are a large number of experiments. Due to plate sizing, they are more efficient than shake flasks to screen media, and it has even been found that with proper air exchange plates produce comparable results to flasks [66]. Although shake flasks were primarily developed for bacterial and fungal cultures, they have been used frequently for mammalian cells [67]. These are often used in small scale culturing because of their simplicity and effectiveness [68]. Similar in size, the spinner bottle is an alternative to shake flasks that provides improved gas exchange [69]. In addition, it is both cheaper and easier to use than bioreactors [67]. Bioreactors are used when it is necessary to produce large quantities of recombinant proteins. These vessels feature complex control systems improving both efficiency and reproducibility [44], but are more expensive than the previously mentioned vessels. Both plates and flasks require an external motion (ie. orbital shakers) to keep cells in suspension, while the

spinner bottles and bioreactors use impellers to stir the medium [44].

### 3.3 Media Additives

Additive selection varies heavily because of the number of available additives and the optimization goal. A typical medium can contain upwards of 50 core components, with the main ones consisting of: carbohydrates, a nitrogen source, amino acids, lipids and vitamins [15, 16, 17]. While the overall aim is to improve growth and production, additives may be selected to limit by-products [70, 71, 72, 73], due to perceived benefits to other cell lines and products [74, 75, 76], or as a small scale comparison of similar additives [77, 78]. While both by-products, lactate and ammonium, can inhibit cell growth and productivity, ammonium can also alter the glycosylation pattern of the product [79]. In addition, ammonium accumulation is a greater concern, as toxic effects occur at concentrations as low as at 2-10 mM as opposed to 20-40 mM for lactate [80]. Since by-product accumulation is due to the CHO cells' high consumption rate of glucose and glutamine [20], typically additive selection is limited to a few additive categories: specifically, alternative carbohydrates and glutamine replacements that are more efficiently metabolized [70]. On the other hand, previous experimentation or additive comparisons are not limited to particular additive categories. Therefore, the following examples will discuss the rationale for the additives that were selected more frequently in literature: hydrolysates, metals, organic acids, and antioxidants. In the mid-2000s, as media was moving away from serum containing media, optimization was dominated by the search for effective serum replacements. This often resulted in hydrolysate selection, as their nature has production advantages over simple amino acids [81]. For example, rapeseed peptides have been seen to have a 2.4-fold increase on VCD and a 5-fold increase in product titer [82]. Metals, or trace elements, appear frequently in literature due to their importance in regulating metabolic pathways, and enzyme and signal molecule activity [15]. Examples of the importance of metals are: copper reducing lactate accumulation [73, 83], zinc imitating the effects of insulin [84], and iron's roles in oxygen transfer and promoting cell growth [15, 54, 85]. Of the trace elements, copper has been seen to improve titer approximately 2-fold [73], and zinc has improved titer up to 6.5-fold [78]. However, high concentrations of trace metals can be toxic to cell health, and it has been shown

that 25 mg/L supplementation of zinc can lower maximum VCD up to 30% [86]. The most frequently used organic acid, sodium butyrate, is known for its ability to increase titer by improving specific productivity as a result of cell cycle arrest in the G1 phase [15]. While sodium butyrate is also cytotoxic and can lead to apoptotic cell death [87], it is often selected in studies because of the substantial improvements to productivity, with Mimura et al. [88] reporting up to 4-fold increases in mAb production. Finally, antioxidants and vitamins are selected to reduce the oxidative stress associated with *in vitro* conditions [89]. Compared to control cultures, Altamirano et al. [90] has shown that vitamins can increase cell density by about 13% and titers by about 6%. However, vitamins and antioxidants can be neglected in cell cultures, so it is debated whether the perceived benefits are a result of correcting a deficient medium rather than the additives themselves [91].

### 3.4 CHO Cells

While this project focuses specifically on CHO cells, there are still a variety of available CHO cell lines and clones. Theodore Puck isolated and immortalized the first CHO cell line in 1957 [2] from which all other CHO cell lines have been derived. These derived cell lines vary from CHO-K1 in 1968 [92] and CHO-S in the 1970s [93] to more recent daughter lines. In Figure 3.1 several of the commonly used cell lines within the data-set are summarized within a family tree to establish a basic understanding of the relationships between cell lines. As well as variation between the cell lines, there is clonal variation, which may be more prominent in cell lines that have been around for 60 years and endured more cloning. It should be noted that some of these groupings are more similar than others. For example, CHO-K1 and CHO-DG44 were formed from separate branches of the primary CHO cell line, while CHO-DXB11 (which is also referred to as CHO-DUK-XB11 among other synonyms) was derived from CHO-K1. However, it is unclear what impact the close genetic relationship between CHO-K1 and CHO-DXB11 [92] has on media optimization. In general, the clonal and cell line variations result in different responses to media in terms of both production and glycosylation. Lamotte et al. [94] found that by transfecting a cell line with  $\alpha$ 2,6-sialyltransferase human interferon- $\gamma$  (IFN- $\gamma$ ) sialylation could be doubled in comparison to its parental line. And while processes such as amplification can



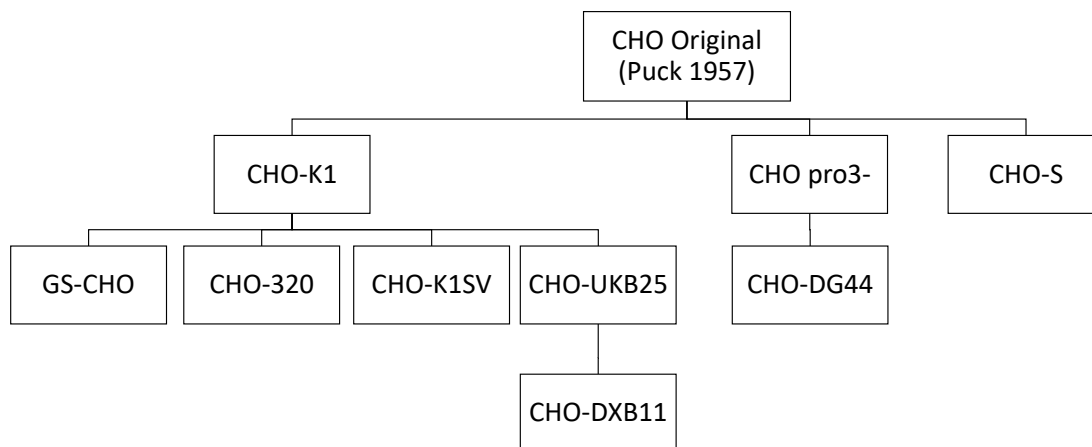


Figure 3.1: The family tree of major CHO cell lines. Information gathered from Lewis et al. [92] and Bairoch [95].

be used to select for higher producing cell lines, it is unknown how clonal variation impacts the general response to media or additives.

### 3.5 Product Quality Attributes

Although quantitative aspects are the primary focus of this research, qualitative attributes are addressed in relation to media supplementation. Mammalian cells, and CHO cells in particular, are chosen because the product glycoforms are compatible with humans, which indicates the importance of product quality in addition to quantity. Indeed, an immune response can be triggered if mAbs contain glycans not naturally present in humans [96]. The antibody glycosylation directly impacts potency, efficacy, and antibody dependent cellular cytotoxicity (ADCC) behaviour [97, 98]. For example, increases in sialylation often improve life span and efficacy [98]. On the other hand, increased mannosylation can lower the efficacy of an antibody and cause a loss in complement dependent cell cytotoxicity (CDC) activity [23, 96]. Although less predictable than genetic engineering, additives may still be used to alter product glycoforms. Examples of additives altering product profiles

include: uridine, manganese and galactose to increase galactosylation [63], lithium chloride or sodium butyrate to decrease overall sialic acid content [87, 99], and a variety of sugars (raffinose, mannose, palatinose, psicose, trehalose, and lactulose) that can increase N-linked mannose-5 glycan (Man5) [100]. Based on the effects of increased sialylation and mannosylation, it can be seen that lithium chloride, sodium butyrate, and the carbohydrates mentioned do not produce desired glycosylation results. A more comprehensive summary of glycosylation changes caused by additives can be seen in Ehret et al. [101].

## Chapter 4

### Methodology

#### 4.1 Defining Literature Review Criteria

Media supplementation has been practiced for many years, so to keep this research as up to date as possible while ensuring a large selection of studies, selected literature must have been published within the last 20 years. Studies from journal articles, supplementary material, and conference proceedings were included if they:

- Performed at least one study on CHO cells
- Compared the effects of media and its additives
- Recorded VCD and/or titer to facilitate comparisons between studies.

During the literature search, relevant studies that contained mammalian cell lines other than CHO cells were included in the database for reference only and were not included during the statistical analysis.

#### 4.2 Literature Search

The initial review began by using electronic databases such as Web of Science, PubMed Central, and Google scholar. Key word search terms included variations of: CHO cells, supplementation, media, and additives. These searches also included specific additive names, which were selected based on common media components or from previously selected studies. This review also considered the reference lists from studies pertaining to media supplementation, to ensure studies were not limited by the search terms. The last literature search was completed on Nov 1, 2019. All studies were reviewed and determined eligible based on the criteria in Section 4.1.

### 4.3 Data Extraction and Setup

Data from the studies was manually extracted into an Excel spreadsheet and saved into two separate comma-separated values (.csv) files. Where table data or exact values were available, maximum VCD and titer was copied directly. However, when only figure data was reported, values were read off the plot. The two .csv files were merged in R according to a “key”—the primary author and publication year of the paper with a letter associated with the individual experiments. The first file recorded general information about the study, while the second recorded the specific supplementation experiments. The general information includes potentially influential factors (cell, product, basal medium, cultivation method and feeding strategy), and experimental details (product purification and quantification methods, optimization strategy, consistency or depletion of background components, and whether VCD/titer are recorded). Within a paper, a new study was defined if any one of these factors changed. These changes may not be evident within the file since clones may be specified within the paper, which were grouped according to cell line in the data-set. The second file recorded the additive, as well as its category and concentration, according to an experiment number and matches it with the corresponding maximum VCD and maximum titer for the particular experiment. For consistency, units were converted where necessary so that all VCD measurements were recorded in terms of cells/mL and titer was recorded in g/L. Table 4.1 and Table 4.2 provide content descriptions for the data collected from the studies.

The number of VCD doublings between an experiment and the control is calculated by dividing the difference in cell density by  $\ln 2$  according to Equation 4.1. For titer, the percentage difference is calculated using Equation 4.2, where the average of the two titers is used to prevent errors when the control culture produced negligible titers.

$$D = \frac{\ln X_1 - \ln X_C}{\ln 2} \quad (4.1)$$

Where: D is the number of doublings, X is the VCD and C is the control.

$$Diff = \frac{|T_1 - T_C|}{(T_1 + T_C)/2} \times 100\% \quad (4.2)$$

Table 4.1: A description of all column names included in the Experiment.csv file for the data-set.

<b>Column Title</b>	<b>Description</b>
Paper	A simplified citation of the paper of interest
PID	PubMed ID, which is used as an alternative method to identify the article.
Cell	The cell line used in the study.
Product	The therapeutic product of interest in the study.
Basal	The starting medium prior to supplementation.
Purification	The method of product purification if it was specified within a study.
Quantification	The method used to measure titer.
Strategy	The optimization approach chosen to introduce additives.
Consistency	A simple way to record if nutrients within the system were held constant or allowed to deplete during the experiment.
Availability	Determines if both VCD and titer are recorded (not necessarily reported) within the study. "Null" represents a study that only recorded one of the metrics.
Feeding Strategy	Records if the process is batch or fed-batch.
Cultivation Method	The cell culture vessel.
Key	This is used to merge the .csv files. Letters are used to specify a single experiment, which is determined if all of the columns discussed above remain constant.

Table 4.2: A description of all column names included in the Conditions.csv file for the data-set

<b>Column Title</b>	<b>Description</b>
Key	As discussed for the Experiment.csv file in Table 4.1
Supplement	Used to distinguish the additives used in a single treatment of the overall experiment in a study.
Additive	The additive used within a treatment.
Category	One of 20 larger categories used to group additives together.
Concentration	The concentration of the additives used in the treatment.
Units	The additive concentration units.
VCD	The viable cell density reported in the study in cells/mL.
Titer	The titer reported in the study converted to g/L.
Titer (% Difference)	The percent difference calculated between the control and the treatment.
VCD Doubling	The number of doublings that occur in the viable cell density between the control and treatment.

Where: T is the titer, and C is the control.

#### 4.4 Statistical Analysis

Statistical analysis of this data-set was performed using the open-source R software. This software was chosen because of its frequent use in both statistics and biology because it is capable of processing large data-sets, producing quality graphics, and the variety of available packages [102]. For this research, ten different packages were used, starting with the “readr” to import the .csv files into R, and the “dplyr”, “tidyr” and “stringr” packages which are used for organizing the data for use. General plotting was performed using “ggplot2”, but the combination of multiple plots together was simplified using “cowplot”. The statistical analysis required “lme4” for modelling, and “moments” for the calculation of statistical moments. As well, the “cvms” and “groupdata2” packages were used to perform cross-validation of the models.

##### 4.4.1 Confidence Intervals

There was effort to record all relevant papers associated with media supplementation; however, it is acknowledged that some studies may have been unintentionally excluded. Therefore, this study provides sample estimates (instead of population), where it is necessary to include confidence intervals. Confidence intervals are calculated using a binomial estimate according to Equation 4.3.

$$CI = \pm z \sqrt{\frac{\hat{p}(1 - \hat{p})}{n}} \quad (4.3)$$

Where:  $\hat{p}$  is the proportion of studies with the desired statistic, n is the number of studies, and z is the z-score associated with the target error. Since the number of studies within the data-set is consistent, and the z-value is associated with a 95% confidence interval ( $z = 1.96$ ), only the  $\hat{p}$  will change the confidence intervals between values.

##### 4.4.2 Graphing Techniques and Modelling

Initial studies of the data were performed using a graphing analysis, where plots of the VCD and titer are arranged using the potentially influential factors to discern

overall patterns. The goal of this approach was to determine overall trends, such as additives that appear more frequently for high growth. Since the additives are variable, a case study of a single additive category was also used for this analysis to provide a simplified data-set. This data limits the number of additives and was used to investigate the effect of similar additives and the potential influence of other factors, such as cell line. However, these methods are limited and cannot be used definitively for a quantitative analysis. Therefore, a mixed-effect framework model was used to confirm the trends found graphically and quantify the effect of not just additives but all influential factors.

Traditionally, the effects in a model are considered fixed, but the definition of a fixed effect can vary depending on the source or research intent [103]. For the purpose of this thesis, an effect is considered fixed if it is constant among all individuals, while a random effect varies between groups of individuals [103]. The random factor therefore groups together elements that are assumed to be from the same distribution. For example, if cell line is considered a random factor then each cell line (e.g. CHO-K1 or CHO-320) is considered more closely related to each other than the cell line is to another factor (e.g. additives). Mixed-effect models consider both fixed and random effects, and are beneficial for complex data-sets that may have different grouping levels or are not independent. These models are used to account for the correlations between observations in a data-set, and they separate overall variation into differing coefficients for each factor level [104]. As with the definition of random effects, the general guidance for determining the type of effect varies based on the source. While some suggest that a random effect is dependent on the number of levels in a group, others suggest that all effects may be considered random because they extract more information than fixed effects, and they essentially revert to fixed effects if there is no group-level information [105]. Each random effect has its own coefficient representing the intercept for the individual factor. These can be compared using a coefficient plot, where the x-axis is representative of the conditional mode, which is the difference between the response of the fixed effect and the individual. Linear mixed effect models follow the assumptions associated with linear models. These assumptions include:

- The model is linear
- The residual errors are normally distributed

- The residuals have constant variance.

Modelling the VCD and titer using the data-set can be used to determine how much of the variation in data is associated with each effect. From the data-set, all recorded variables that may influence the VCD or titer were considered as an effect. Models of varying complexity and effects were considered, and the final model was selected based on the Akaike information criterion (AIC). The AIC value is calculated using the goodness of fit and number of parameters according to Equation 4.4 [106]. As seen in the equation, the AIC tends to penalize models with more effects. Since the lowest AIC is representative of the best model [107], the final model was selected by comparing the AIC values. While models can consider interactions between effects, these must be reviewed carefully to prevent confounding as the combination of effects can be representative of a single study. Due to the variation in data, combinations of factors were rarely repeated between studies. In another attempt to prevent confounding, any factor level that had less than three studies associated with it was removed from the model data. The residuals were also analysed to determine the model's prediction capability. The skewness is a measure of the distribution tails, and the kurtosis is a measure of how peaked a distribution is [108]. By reviewing the skewness and kurtosis of the model residuals it can be seen where the model best predicts results based on the distribution of the data.

$$AIC = -2 \ln L + 2k \tag{4.4}$$

Where:  $L$  is the maximum likelihood, and  $k$  is the number of parameters.



## Chapter 5

### General Overview

Based on the criteria mentioned in Section 4.1, a comprehensive and open-ended review of media supplementation literature identified 78 studies within the last 20 years that recorded VCD and/or titer. Although VCD and titer are not the only metrics of recombinant production, they do serve as a common quantitative output of media optimization (broadly representative of the general push for higher product yield) and serve as a point of comparison between the reviewed articles to determine relationships between recombinant production and media additives. 76 studies reported VCD and only 58 reported absolute titer despite 74 of the studies claiming to measure both VCD and titer. The following subsections will consider each of the observations mentioned in Section 4.3 in more detail.

#### 5.1 Optimization Strategies

Of the studies considered in this review, 37%<sup>1</sup> considered the impact of only one additive (consisting of one or more components held in a fixed composition), 37% considered two or more additives in an OFAT design, 6% modified multiple components at the same time, 15% considered multiple additives using a DOE approach, and the remainder performed a metabolic study. The prevalence of OFAT over DOE strategies is not particularly surprising, as OFAT experiments are considered to be the traditional approach to media design [51]. However, it does suggest that the interaction of multiple additives is rarely factored into experimental design or subsequent analysis. Infrequent use of DOE techniques may be reflective of broader engineering practice, with Lundkvist et al. [109] suggesting that limited statistical knowledge may be to blame.

It should also be noted that the vast majority of studies do not explicitly consider

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<sup>1</sup>As per Equation 4.3 discussed in Section 4.4.1, confidence intervals for the following statistics range from 5% to 11% for  $\hat{p}$  values between 0.95 and 0.6, respectively.

which media components or additives are limiting to growth or productivity. Only 13% of studies attempted to maintain the concentrations of basal components in some fashion (generally with a media replacement or addition strategy), with a further 3% maintaining the concentration of glucose alone. This is less of an issue for large DOE studies (which inherently consider a large number of media components at once), where half of the DOE studies began with an initial broad screening experiment that was followed by a more detailed design. While Coronel et al. [110] suggested that nutrient depletion was a likely factor for the decrease in productivity seen in their experiment, Gramer et al. [63] saw a direct correlation between amino acid depletion and the decline in antibody production rate, which shows that neglecting the other components in the media may limit the overall effects seen for a given test medium.

## 5.2 CHO Cell Lines

Overall, the studies considered in this review featured a large variety of different CHO cell lines. Although some of these were unique to specific laboratories or used non-standard names [93], seven general groupings were considered as part of this review: CHO-DG44, CHO-DXB11, CHO-K1, CHO TF 70R, CHO-DHFR, and CHO-320 along with a catch-all group for less common or unspecified CHO cells. Within the studies, CHO-DG44 was the most commonly used cell line, in 24% of studies, followed by CHO-DXB11 and CHO-K1 both in 22% of studies, the catch-all “CHO” group (10%), CHO-320 (9%), CHO-DHFR (7%), and CHO TF 70R (5%). The general relationship for many of these cell lines was specified in Figure 3.1; however, not all studies specified parental lines. Only 13% of articles considered an additives impact on different cell lines despite the high variability between CHO cell genomes [93]. To develop a more thorough understanding of cell line impact, the literature search would need to be expanded to include studies that focus primarily on the differences between cell lines instead of additives.

## 5.3 Cultivation Methods

Approximately 70% of the studies used shake flasks or spinner bottles for cell cultivation, 20% used bioreactors, and 10% used well plates. The frequent small scale

selection is likely due to the size of the experiments. While as many as 36 experimental runs were performed within a single study using flasks or plates, reactors were often used for less than three experimental runs (with a maximum of eight runs in a single study). In addition, although cell culture behaviour can vary between cultivation methods (in particular, between small scale vessels and bioreactors as discussed in Section 3.2), only about 15% of studies tested multiple scales. Batch growth made up 80% of the recorded data, with the remainder as fed-batch. Of the fed-batch processes, approximately one third were performed in bioreactors, while the remainder were performed in shake flasks. Given the difference in production titers for batch and fed-batch processes (discussed in Section 3.1.2), it is expected that fed-batch studies will stand out in the data-set. Specific productivities are more closely related than absolute titer for batch and fed-batch processes, thus providing an opportunity to compare additives with less influence from the cultivation method. Therefore, it is recommended that specific productivities are incorporated in future comparisons.

## 5.4 Products

The diversity among cell lines was matched by the diversity of recombinant products. The most popular recombinant products were mAbs (accounting for 44% of the studies); non-enzymatic proteins, such as cytokines, were 2nd in popularity (27% of studies), followed by enzymes (14%), and hormones and growth factors (10%), with the remainder of articles accounting for no product at all. It should be noted that only half of all unique recombinant products were considered in more than one study, with Immunoglobulin G (IgG) as the most common product (accounting for 22% of all the studies reviewed).

Product quantification methodology was found to be considerably less variable than the range of products themselves. The enzyme-linked immunosorbent assay (ELISA) is the most common method to determine product concentration (accounting for 54% of the studies with a specified method), and high performance liquid chromatography (HPLC) accounts for 17%. Of the articles that used an ELISA, three-quarters of the articles used a direct ELISA, where the product is immobilized to the surface of a well plate, and it is incubated with an antibody conjugated to an

enzyme that enhances the product signal [111, 112]. The remainder of ELISA articles used the sandwich ELISA, which uses two antibodies: one for capture and one for detection of the product [112]. It is suggested that ELISAs are often chosen because of their reasonable price along with high specificity and sensitivity [112]. HPLC and ELISA methods have been compared frequently for different substances, but the general consensus is that the two methods correlate well [113, 114] — meaning that improvements in product titer should be comparable between different studies.

## 5.5 Product Quality

In addition to product titer, a number of reviewed studies considered product quality attributes, with a predominant focus on glycosylation. Of the studies considered in this review, 26 articles measured the glycosylation profile of the product. Nine of the articles aimed to determine the effect of media additives on the glycosylation profile, while the rest aimed to keep the glycosylation profile constant while improving titer. Five of the articles concerned with product quality attributes tested carbohydrates and nucleosides, which Blondeel and Aucoin [23] discuss is common for those directly focusing on glycosylation profiles. Another five papers looked into the effect of sodium butyrate on glycosylation, while the majority, at 10 papers, looked at the effect of hydrolysates. With the exception of dextran sulfate [115], the additives that were tested in the glycosylation studies were commonly used additives to increase cell growth or titer. Overall, 15 studies reported no significant change to the glycosylation profile, and ten reported a change. The studies that discussed glycosylation characteristics are presented in Table 5.1. Six studies that reported a change sought to maintain a constant glycosylation profile, highlighting the importance of assessing product quality even when the primary goal is increasing product titer.

Table 5.1: A summary of the articles discussing glycosylation and whether or not additives affected the glycosylation profile.

<b>Article Goal</b>	<b>Glycosylation Change</b>	<b>Reference</b>
Remain Constant	No Change	[40, 46, 71, 88, 99, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124]
Remain Constant	Change	[56, 75, 81, 83, 87, 125]
Desire a Change	Change	[63, 100, 126, 127]

## 5.6 Media

Overall, the majority of studies did not reveal the composition of their basal media<sup>2</sup>, which makes it challenging to draw general conclusions about the effectiveness of specific additives or final additive concentrations. 40% of studies used a proprietary commercial medium as the starting point of their supplementation, 5% used an undefined in-house medium, and 25% of studies did not specify the starting media at all (without specifying whether it is an in-house or commercial formulation). Only 30% of the studies reported the full media composition. All the reported classic and commercial media used in the studies is presented in Table 5.2. While there is some repetition among media, 60% of recorded basal media was only used in one study.

Overall, I have identified 238 unique media components used as additives in this data-set — with additives often containing more than one of these components in fixed ratios. Approximately 70% of these 238 components appeared in only one study, significantly limiting robust comparison. To simplify identifying general trends, the additives were broken down into 20 subcategories, as presented in Table 5.3. For an exhaustive list of the additives with their respective articles see Appendix A. As with media, additive composition is not always known. 10% of the additives in the studies are “premade”, which is defined as a supplement containing multiple components. The majority of these premade additives are undefined, and are produced as commercial feeds for basal media. For example, CHO CD EfficientFeed is designed for use with the CD OptiCHO medium [123]. However, selenium-insulin-transferrin-ethanolamine (SITE), or insulin-transferrin-selenium (ITS), are two examples where both components and concentrations are known. Although the majority of the multi-component additives are grouped as premade, it was decided that additives with a specified supplement type would be placed in its respective supplement category (eg. lipid supplements Cell-Ess and Gibco 11905). Overall, the most common additives are hydrolysates (15% of studies), followed by metals (9%), and amino acids, antioxidants and organic acids (each at 8% of studies).

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<sup>2</sup>Where “basal” medium will be defined as the initial growth medium prior to supplementation regardless of whether this starting medium is complex in nature.

Table 5.2: Summary of commercial and classical basal media.

Medium	Count	Supplier
ActiCHO P	1	GE Healthcare
ActiPro	1	GE Healthcare
$\alpha$ -mem	3	Gibco
BalanCD	1	Irvine Scientific
BioGro-CHO	2	Biogro Technologies
BIOPRO1	4	Bio Whitaker Europe
BME	1	Gibco
CD CHO	5	Gibco, Invitrogen, Life Technologies
CD OptiCHO	4	Gibco, Life Technologies, Invitrogen
CHO-S-SFM II	1	Life Technologies
CDM4CHO	4	Hyclone
CDM4PERMAb	1	Hyclone
Cellvento CHO-100	1	EMD Millipore
CHO-III-A	1	Gibco
DMEM	1	Gibco
DMEM:F12	6	Invitrogen, Gibco, Inoclon, HiMedia Laboratories
Ex-Cell 325	1	SAFC Biosciences
Ex-Cell CD CHO	3	SAFC Bioscience, Sigma Aldrich
F-10	1	Gibco
Fisher's medium	1	Gibco
FMX-8	2	Dr. Messi Cell Culture Systems
FortiCHO	3	Thermo Life, Thermo Fischer, Life Technologies
Ham's F12	2	Dainippon, Gibco
HyQ PF-CHO	1	Hyclone
IMDM	5	Gibco, Invitrogen, Lonza
ISF-I	1	Biochrom
Keratinocyte SFM	1	Gibco
LG-SFSH	2	LG Life Science
McCoy's 5A	1	Gibco
NCTC 135	2	Sigma
PowerCHO2CD	4	Lonza
ProCHO5	3	PANBiotech, Lonza
RPMI1640	6	Gibco, Sigma, Inoclon, Lonza
SAFC	1	Sigma
SFM4CHO	2	HyClone
UC212	1	Nissui Pharmaceutical

Table 5.3: Summary of media additives categorized to facilitate analysis with more repetition among the additives.

Category	Additives
Amino Acid	The 21 common amino acids, citrulline, glycine betaine
Antibiotic	Penicillin, streptomycin
Antioxidant	$\alpha$ -ketoglutarate, ascorbic acid 2-phosphate, $\beta$ -mercaptoethanol, baicalein, catalase, citric acid, glutathione, NAC, sinapic acid, sodium selenate, sodium selenite, succinic acid, tropolone
Buffer	PBS
Carbohydrate	Fructose, galactose, glucose, lactose, lactulose, maltose, mannose, melezitose, palatinose, psicose, raffinose, sucrose, trehalose, turanose
Growth Factor	EGF, Hydrocortisone, IGF-1, insulin, LongR3 IGF-1
Hydrolysates	Animal, cotton, broadbean, pea, rapeseed, rice, soy, wheat, and yeast hydrolysates, Ex-Cell CD hydrolysate
Inorganic Acid	HCl
Inorganic Salt	KCl, MgCl <sub>2</sub> , NaCl
Lipid	Albumax, Cell-Ess, cholesterol, cod liver oil fatty acids, ethanolamine, Gibco 11905, linoleic acid BSA, LPA, oleic acid, phosphatidic acid, phosphatidylcholine
Metal	Calcium, cobalt, copper, iron, lithium, magnesium, manganese, molybdenum, nickel, vanadium, zinc
Nucleoside	Nucleobases (A,G,C,U), adenosine, cytidine, guanosine, and uridine phosphates, deoxycytidine, deoxyuridine, FAD, hypoxanthine, NAD(H), NADP(H), thymidine
Organic Acid	Butyric acid, pyruvic acid-Na, valeric acid, valproic acid, pyruvate, sodium butyrate, sodium lactate, sodium propionate, sodium pyruvate
Other	Aurintricarboxylic acid, DMSO, MTX
Polyamine	Putrescine, spermidine, spermine
Polysulfate	Dextran sulfate, polyvinyl sulfate, suramin
Premade	486F, Acticho Feed A & B, Cell Boost 7A & 7B, CHO CD EfficientFeed A & B, CHO Xtreme feed, FunctionMAX, Panexin NTS, Power feed, Sigma supplement, SITE/ITS, Xtreme feed
Serum	dFBS, FBS, NBS, newborn-calf serum
Surfactant	Pluronic F-68, Tween 80
Vitamin	Ascorbic acid, choline, cyanobalamin, D-a-tocopherol acetate, D-calcium pantothenate, folic acid, I-inositol, niacinamide, pyridoxine HCl, riboflavin, Sigma M6895, thiamine HCl, vitamin K1

## Chapter 6

### Results

#### 6.1 Overall Trends

The overall effect of the heterogeneity in cell line, product, and basal media across the various studies is that both VCD and titer observations range across approximately 6 orders of magnitude, with the impact of any one specific additive seen as relatively small in comparison. However, focusing analysis on the ranges of VCDs or titers observed in a given study, it becomes possible to visualize the effect of a given additive with less interference from factors such as cell line. For the studies that found positive changes in the VCD, cells doubled an average of approximately 0.6 times in comparison to the control; however, this could reach as high as 5 times in exceptional cases [128]. Figure 6.1A presents VCD observation from all reviewed studies as a function of publication year, while Figure 6.1B presents the corresponding interquartile range<sup>1</sup> VCD values, organized in order of increasing magnitude. Focusing on the VCD ranges makes it possible to dissociate the effect of additives from other factors such as basal media or cell line. The three studies with the largest ranges correspond to those of Reinhart et al. [123], Kim and Park [78], and Ho et al. [119]. Reinhart et al. [123] found that premade additives, EfficientFeed A and B, and FunctionMAX, can greatly increase cell concentrations, although there were substantial differences in the VCD depending on the basal media. Kim and Park [78] tested different concentrations of several trace elements, including copper, zinc, vanadium, manganese and molybdenum, and found that zinc in particular causes significant impact. Finally, Ho et al. [119] focused on hydrolysates, and found yeast provided the most consistent positive effects. However, it is important to highlight that both Kim and Park [78] and Ho et al. [119] found that the impact of specific additives varied considerably based on initial basal media and cell line. Additives found to be of interest with larger interquartile ranges include: premade additives (Cellboost 7A and B) [40], maltose

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<sup>1</sup>The range of observations excluding the top 25% and bottom 25% of the values.



(or glucose and maltose combinations) [71, 72], lipid supplements (Cell-Ess) [129], and long-term passaging with iron [130].

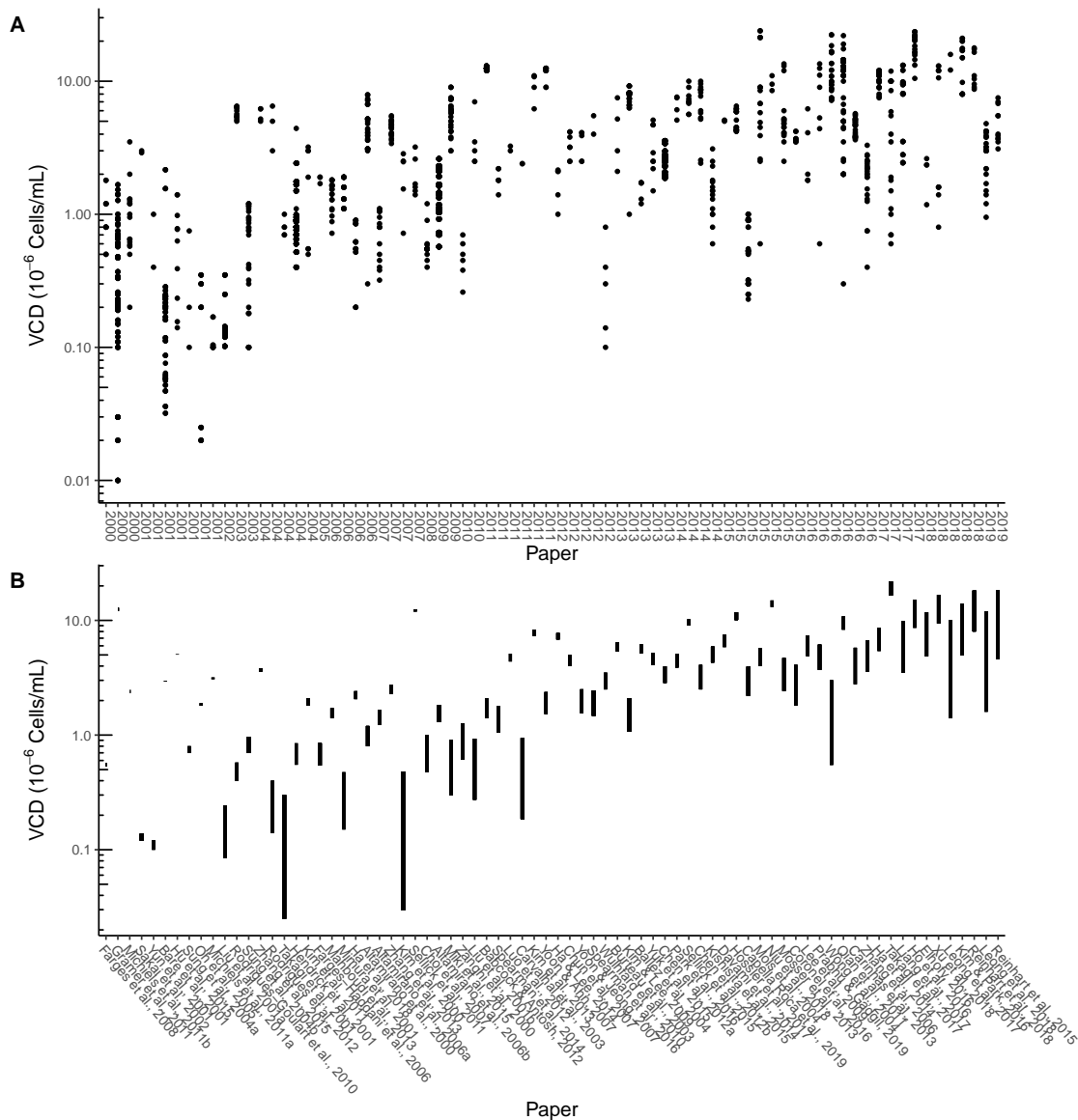


Figure 6.1: A summary of all recorded VCD observations organized by both **A** publication year and **B** interquartile VCD range.

For the titer, the percentage difference was calculated between the experiment and control to estimate the improvement (or lack thereof) within a study. For studies reporting an increase in titer, the mean percentage difference was 65% though it could reach as high as around 180% in extreme cases [128]. However, many of the studies that produced the highest percentage differences had low producing controls (in the

mg/L or  $\mu\text{g/L}$  range), which often produced changes less than a mg/L. Similarly to VCD, all reviewed titer ranges are presented in Figure 6.2, with Figure 6.2A data sorted by publication year and Figure 6.2B data sorted by observed titer range. As compared to VCD, the titer ranges observed in Figure 6.2 generally account for a smaller fraction of the overall observed range, suggesting that on the whole, specific additives have less influence on titer than VCD (as will be explored in more detail Section 6.1.1). On the other hand, reported titers appear to have a more pronounced increase year-over-year, a trend that has been noted frequently in literature [2, 5, 52]. It is therefore not surprising that the three articles reporting highest product titers were published within the last five years [59, 123, 126]. Of these, both Takagi et al. [59] and Niu [126] focused on nucleoside additives in their studies. It should be noted that not all studies that produce the highest titers are the same as those that produce the largest range, with Niu [126] as one such paper. Reinhart et al. [123] reported both a large titer range and a high maximum titer through the addition of premade supplements and commercial media as discussed for VCD previously. Larger interquartile titer ranges were observed for premade additives [40], and yeast hydrolysates [119] as with VCD above, but other additives of interest also included copper [83], lithium chloride [87], and antioxidants (baicalein) [131]. However, even more so than with VCD, there is evidence that factors other than media additives play a significant role in product titer.

### 6.1.1 Range Distributions

Whereas the discussion in Section 6.1 focused on a number of specific studies, it is worth considering the overall range of VCD and titer observations as a general benchmark for how much impact a specific additive is likely to have (with the caveat that increased cell death may also contribute to increased ranges). As individual observations may be liable to large variations such as unexpected cell death, interquartile ranges were calculated for each study and are presented in Figure 6.3. For VCD, the average difference achieved through media supplementation was found to be  $1.7 \cdot 10^6$  cells/mL with a median of  $0.8 \cdot 10^6$  cells/mL. On the other hand, the titer was found to have a mean difference of 0.24 g/L and a median of 0.02 g/L. The difference between average and median values was due to a small number of studies with very

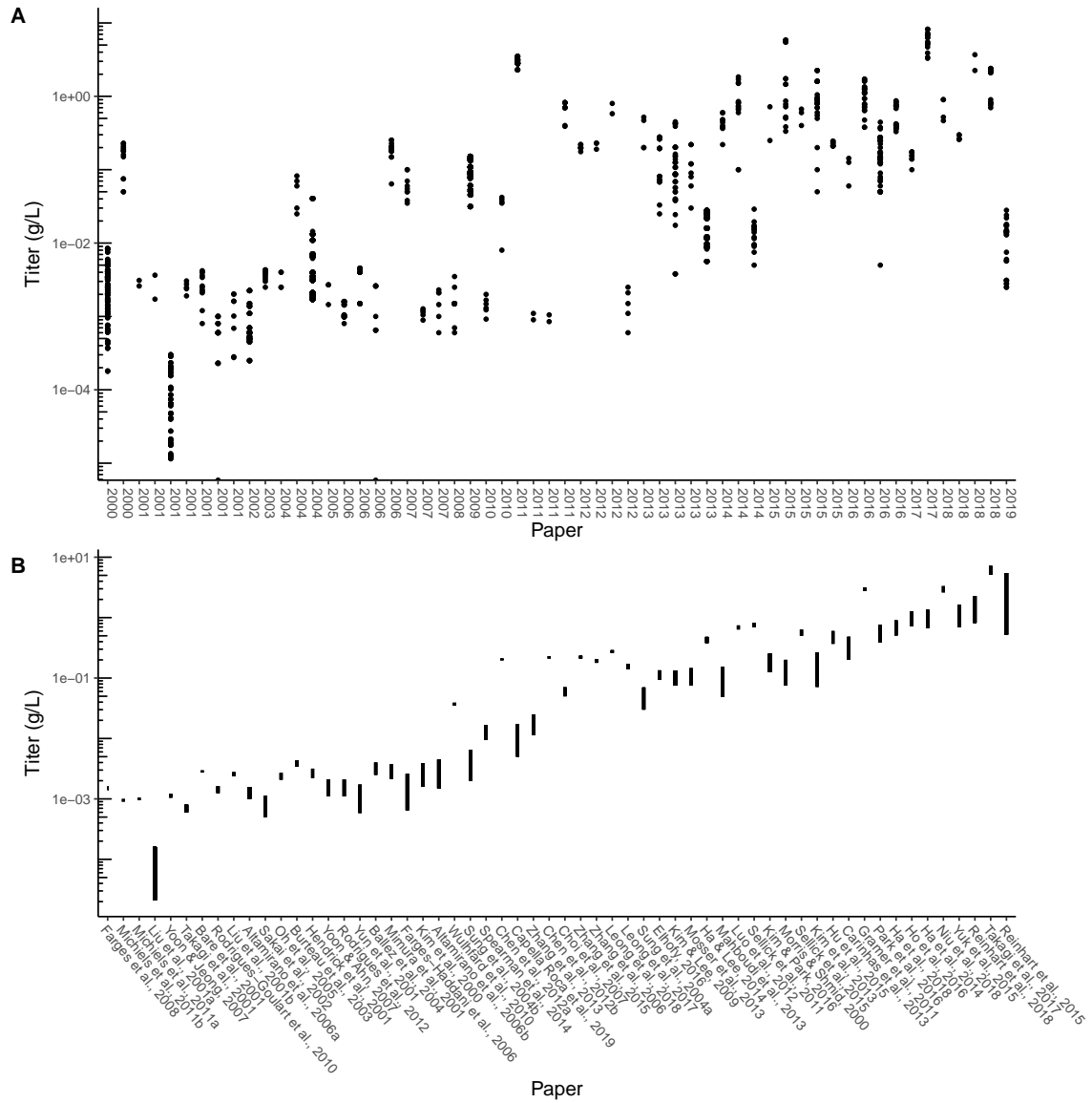


Figure 6.2: A summary of all recorded product titer observations organized by both **A** publication year and **B** interquartile titer range.

large ranges causing a positive skew in the distributions, which reinforces the idea that while some additives have a pronounced impact on VCD and titer, most outcomes are much more modest. For studies that reported both VCD and titer observations, I also determined an effective ratio of titer range vs VCD range by calculating the slope of titer versus VCD. The full distribution of these slopes is presented in Figure 6.3C. The slopes have a mean value of  $5.5 \cdot 10^{-5}$  g/ $10^6$  cells and a median of  $0.8 \cdot 10^{-5}$  g/ $10^6$ . Overall, it seems clear that additives that result in increased VCD have only very

modest impact on titers, meaning that VCD should not be used as a general proxy of additive impact if the overall goal is to actually increase productivity.

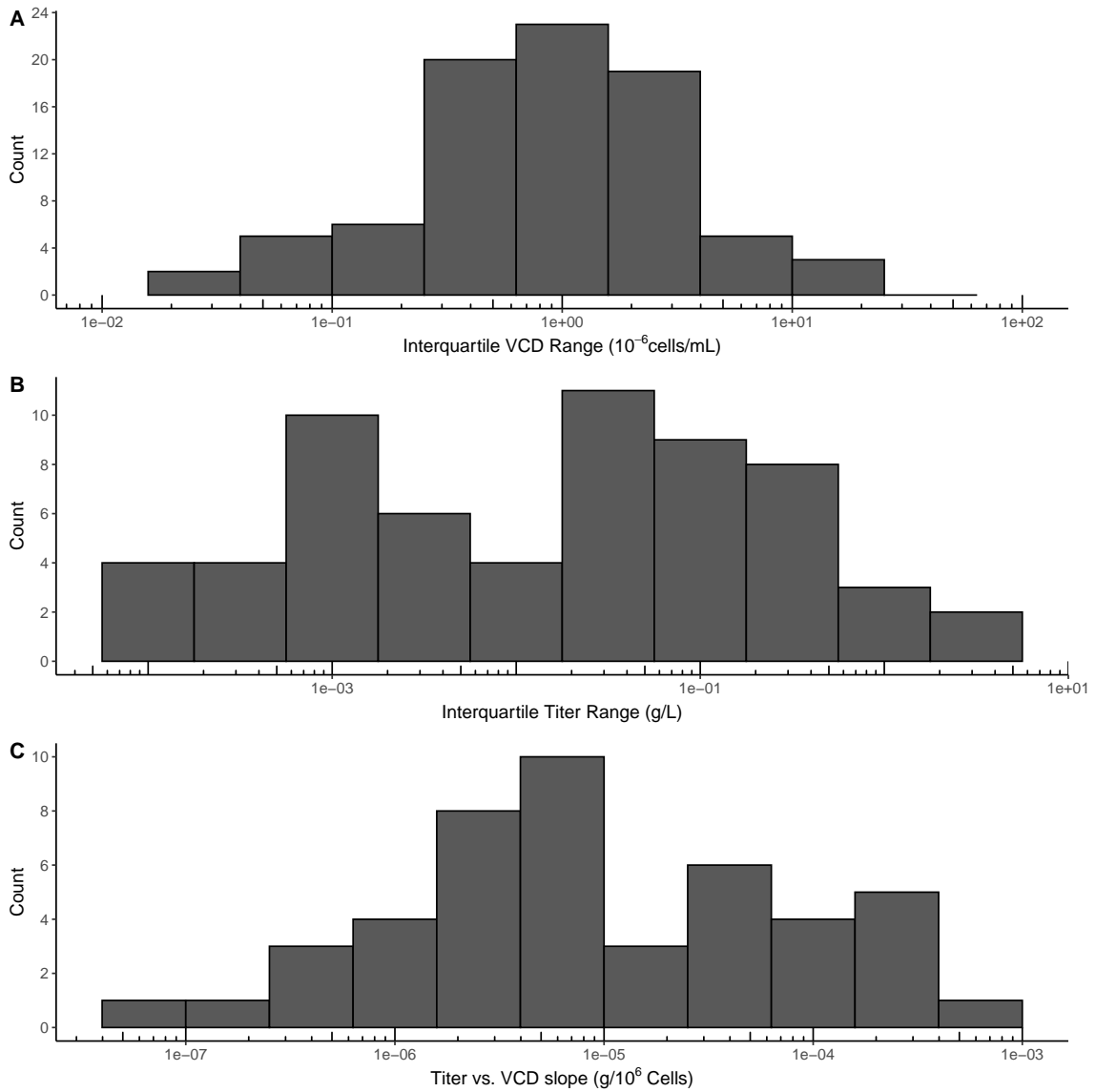


Figure 6.3: The distribution of **A** VCD interquartile ranges **B** titer interquartile ranges and **C** the slope of titer versus VCD. The slope distribution is limited to studies that recorded both VCD and titer, while the other distributions summarise all studies that recorded their respective variables.

## 6.2 Hydrolysates as a Case Study

Amid the plethora of additives listed in Appendix A, the single biggest group corresponds to hydrolysates (also referred to as peptides, peptones, and lysates), which were used in 15% of the studies within the literature survey. With their ability to increase both cell growth and titer, as well as protect against shear stress [17], hydrolysates are commonly seen as an effective replacement for serum as well as an effective additive for media optimization more generally [81]. Within the hydrolysate studies, approximately 76% used plant-based, 52% used yeast-based, and 28% used animal-based hydrolysates. In general, non-animal derived hydrolysates are preferred because they avoid the potential for contamination that can come from animal products [82]. Of the non-animal derived hydrolysate sources, yeast was the most common at 54% of studies, followed by soy (50%), wheat (46%), other (33%), and rice (21%). “Other” encompasses the less commonly used hydrolysates, such as rapeseed [82, 132], cotton [124], peas [132], and broadbeans [133].

Although hydrolysates represent the single most popular group of additives in this review, there is still a considerable degree of heterogeneity in hydrolysate products — of the 52 hydrolysate additives used across the reviewed studies, 32 are unique products. On top of differences due to source material (such as soy, yeast, or wheat), these complex additives are also known to suffer from batch variability [134]. And while ultrafiltration can be used to reduce some of this variability, only 13% of the hydrolysates in the reviewed studies were specifically labelled as ultrafiltered. The small number of relatively parallel comparisons that are possible underline the challenges of drawing general trends. For example, a series of studies examined the impact of adding rice peptones (HyPep 5115) to CHO-320 cells producing IFN- $\gamma$  and cultured in similar basal media [118, 116, 135]. In general, hydrolysates were able to improve both VCD and protein titer by approximately 30%, however, rice peptones from two different manufacturers resulted in dramatically different titers due to the presence of residual proteolytic enzymes [116]. In another example, a similar set of yeast, soy, and wheat gluten hydrolysates produced very different results for CHO-DG44 and CHO-DXB11 cell lines [99, 119].

## 6.3 Mixed Effect Modelling

### 6.3.1 Available Models

The data-set collected a wide variety of variables as stated in Section 4.3. To effectively explain the data, several models ranging from simple (limited variables) to complex (interactions) were considered. The following variables were considered in the models:

- Additive category
- Basal media
- Cell line
- Cultivation method
- Feeding strategy
- Product.

Given that variability of cell cultures, and the number of levels associated with each factor, the effects considered in the model are all considered random. Both basal media and additives are considered variables since the focus of this research is media supplementation. However, both variables were grouped to limit their diversity. For additives, the categories are those specified in Section 3.3, while the basal media was grouped according to classic, commercial, undisclosed, and known. A known media has a defined formulation, but it is not considered either classic or commercial. Interaction terms were initially considered, including the relationship between cell line and basal media, cell line and additive, and product and additive because it is known that the impact of media or additives can depend on the cell line or product [5]. The addition of interaction terms between factors often resulted in a better fit; however, this is because interaction terms often accounted for individual studies. To avoid confounding, interaction terms between factors were excluded from the final model.

The simplest model was used to determine the general effect of main variables on VCD and titer responses. This model (referred to as Model 1) contained only three variables — media, cell line, and product. Figure 6.4 presents the fit of Model 1 by

comparing the observed and predicted results for both VCD in Figure 6.4A and titer in Figure 6.4B. There is evidence of a linear trend for both variables, but there is large variation in the data — more so in the titer plot. It is interesting to note that these three variables are able to introduce a trend to the model; however, because of Model 1’s simplicity it is not surprising that the fit is limited.

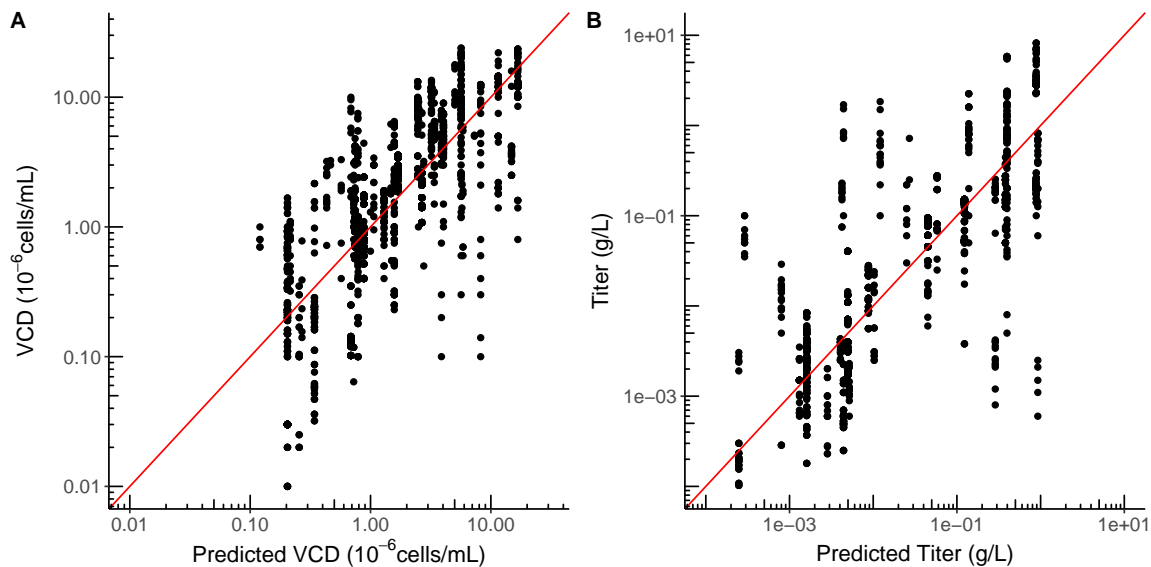


Figure 6.4: Determining the fit for Model 1 using a comparison of observed versus predicted values for **A** viable cell density and **B** titer.

The next model, henceforth referred to as Model 2, introduced the additive category as a possible variable. The fit for Models 2-5 can be seen in Figure 6.5 for the VCD and Figure 6.6 for the titer. Model 2, seen in Figure 6.5A and Figure 6.6A, was capable of predicting more VCD and titer values as there are more variables to distinguish between studies. The linear trend is still evident, with more clustered data than seen in Model 1. However, there are still deviations from the 45° line, and the additives did not affect the model fit as strongly as was hoped. In Model 3 (Figure 6.5B and Figure 6.6B), the addition of the cultivation variable was able to reduce deviation in the points above the 45° line, with higher titers predicted in the titer model as well. Despite specifying the cultivation method as the cell culture vessel, the variable inherently incorporates other factors including bias associated with lab equipment and experience level, and the feeding strategy. While some of these variables cannot be accounted for because they are not specified within the studies,

the feeding strategy can be included in the model separately. To determine the significance of the individual variables, Model 4 includes both cultivation and feeding strategy, while Model 5 replaced cultivation with feeding strategy. Model 4 (Figure 6.5C and Figure 6.6C) was capable of predicting higher VCD ranges than Model 3, indicating the relevance of fed-batch cultures to achieving higher cell density. On the other hand, smaller titer values (in the mg/L range) clustered more in Model 4 than Model 3. In Model 5, seen in Figure 6.5D and Figure 6.6D, the removal of cultivation coincided with an increase in deviation from the diagonal with less predicted values available. Although there are differences in all of the models, these can appear subtle in both Figure 6.5 and Figure 6.6.

As the interpretation of the fit through graphics is somewhat limited, an ANOVA and the AIC values are used to further compare the models. The results of the ANOVA can be seen in Table 6.1. The model with the lowest AIC value for both VCD and titer is Model 4, where all six factors (cell type, product, basal medium, additive category, cultivation method, and feeding strategy) are considered significant. A factor was considered significant if the ANOVA showed a significant difference between models with and without the factor of interest and the AIC was lower for the model with the factor. The predicted VCD and titer are treated as a sum of the six factors, with a unique effect calculated for each factor component. While Figures 6.4-6.6 provide a general indication of improved fit between Model 1 and Model 4, the AIC values are able to confirm that Model 4 is best suited for the data-set.

Table 6.1: The main mixed-effect models and the corresponding AIC values for both VCD and titer models.

<b>Model</b>	<b>VCD AIC</b>	<b>Titer AIC</b>
Model 1	6043	5954
Model 2	5917	5894
Model 3	5806	5750
Model 4	5654	5525
Model 5	5749	5645

In Figure 6.7 the mean of the predicted values is plotted on top of the observed values for VCD in Figure 6.7A and titer in Figure 6.7B. In general, the model shows reasonable predictions for most studies, though the residuals will be covered in more detail in Section 6.3.4. The evaluation of model predictions is performed using a



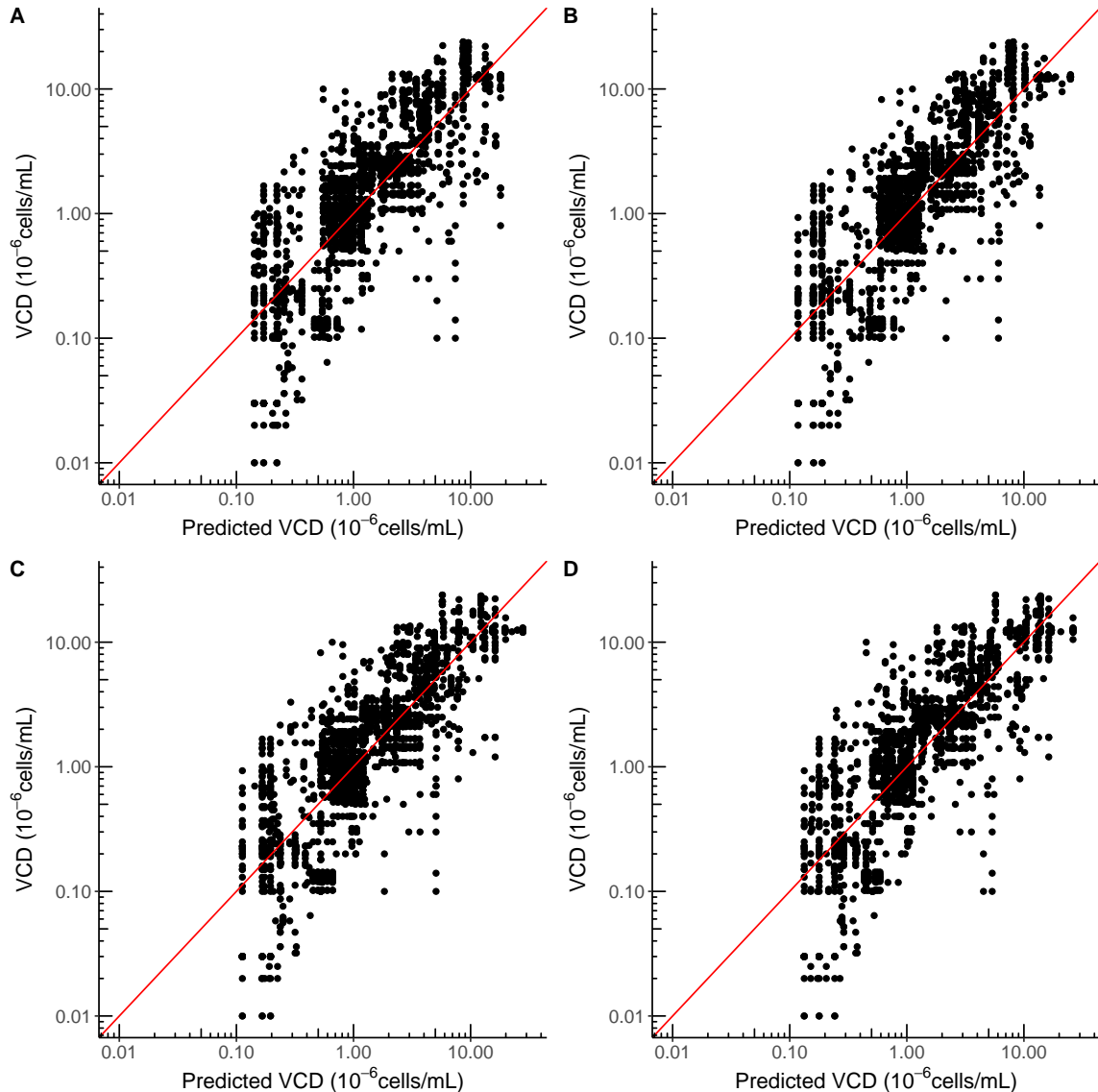


Figure 6.5: Determining the fit for VCD models using a comparison of observed versus predicted values for **A** Model 2, **B** Model 3, **C** Model 4, **D** Model 5.

k-fold cross-validation procedure, which splits the overall data-set into several training and test sets for model fitting and evaluation, respectively. The cross-validation is performed using 10 folds because it lowers the variance in the error estimate by limiting the correlation associated with using repeated data while also limiting the bias [136]. The relative root mean squared error (RMSE) is calculated during the cross-validation as a measure of error between the predicted and observed values, which can be used to determine the quality of the model. For the VCD model, the

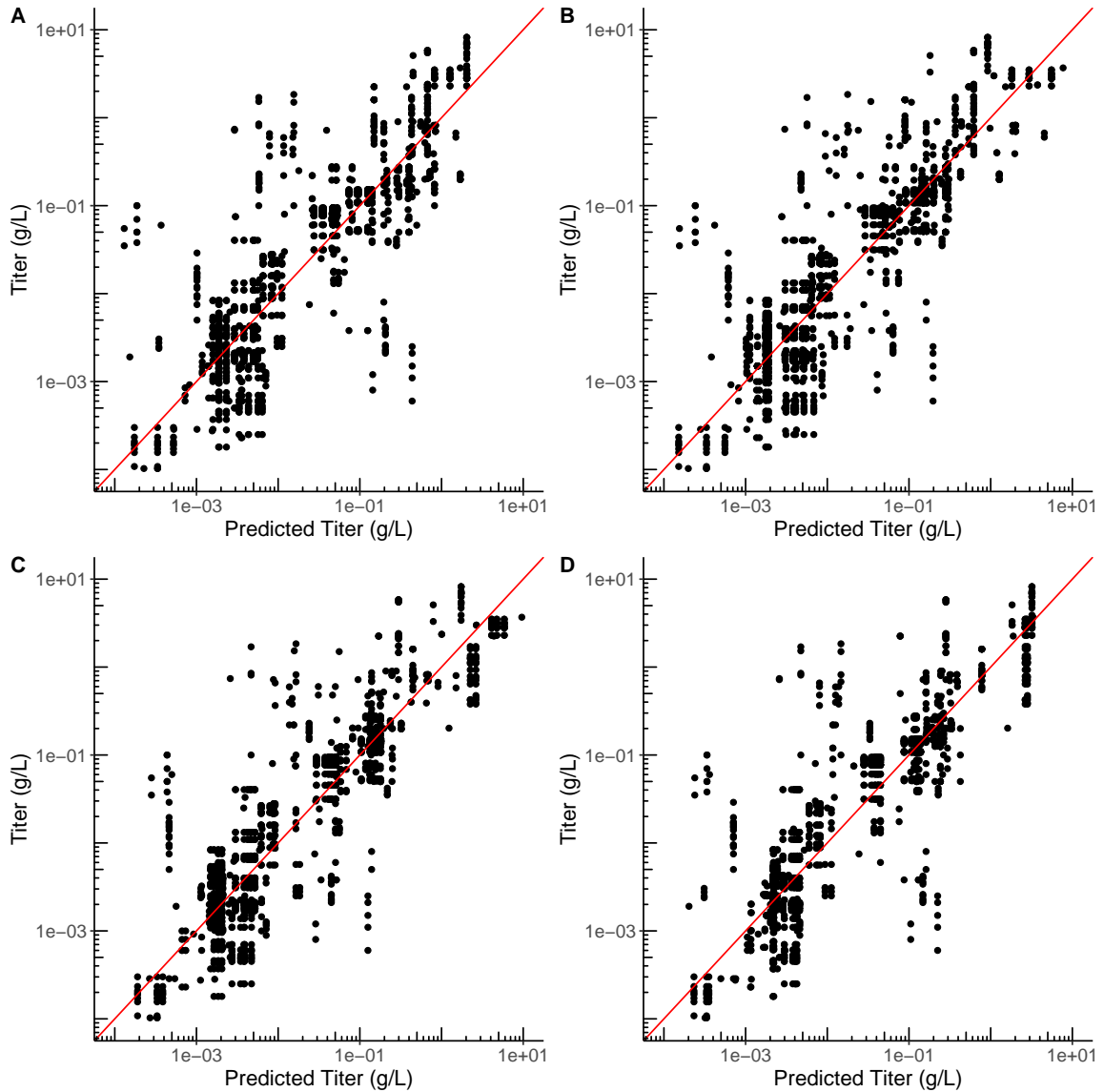


Figure 6.6: Determining the fit for titer models using a comparison of observed versus predicted values for **A** Model 2, **B** Model 3, **C** Model 4, **D** Model 5.

relative RMSE is less than 10%. For the titer model, the relative RMSE is approximately 30%. While the titer model has a larger error associated with predictions, these models are not intended for predictions outside of the data-set, but instead to determine the contributions to data variance. Since the predictions are a sum of the effects, it is likely that errors appear larger for studies where a random effect was eliminated to prevent confounding. This has a greater effect on the titer model than the VCD model because the smaller number of studies resulted in the removal of more



focus of this project, and the papers included in the data-set, is to test the impact of additives, less than 5% of the variance accounts for the additives for both VCD and titer. This supports the trends that have been discussed in Section 6.1, where additives may have significant impacts on an individual study, but the overall impact is rarely consistent. Media as a whole only accounts for 10% of the VCD variance and is practically negligible for titer. It appears that while an optimized commercial formulation is likely to outperform a classical formulation for a specific cell line or product type, this impact is unlikely to be consistent with a different cell line and a different product type. While media appears to have a minimal impact, its greatest impact appears in terms of feeding strategy, i.e., batch or fed-batch cultivation. In fact, the feeding strategy is the highest source of variance for VCD and second highest for titer. Although it is well known that fed-batch cultivation is more productive than batch [37], it is nonetheless interesting to note how much more impact it has than the choice of basal medium (at least for the studies reviewed here). The impact of cultivation in comparison to both media and additives indicates that the prevention of nutrient depletion (and the choice of additive) may be of greater importance than simple media addition. In addition, the product type has the greatest overall effect on titer. Therapeutic proteins have differing expression levels and may be more difficult to express than others [137], which even includes sub-classes of the same mAb [138].

Table 6.2: Percent variance of the main random effect variables used to create the prediction model.

<b>Random Effects</b>	<b>VCD</b>	<b>Titer</b>
Additive Category	3%	1%
Product	9%	33%
Basal Medium	10%	0%
Cell	10%	13%
Cultivation Method	14%	14%
Feeding Strategy	22%	24%
Residual	30%	15%

### 6.3.3 Model Coefficients

Each factor that is included in the model can be broken down into sub-factors (or levels) which each have a corresponding coefficient determined by the model. The coefficients are represented in Figure 6.8, the coefficients for both VCD, in Figure 6.8A, and titer, in Figure 6.8B, are displayed in terms of their deviation from the overall effect (where a large positive value can be interpreted as a large positive effect on the final VCD or titer). Overall, the production of mAbs was the single biggest predictor of high VCD and titer. And while it is possible that this stems from lower metabolic burden of mAb production when compared to other products, it is also likely that mAb-producing cell lines have been subject to additional selection pressure due to the general popularity and high adoption of mAb production. Following mAbs, the next biggest impact is fed-batch cultivation, which has a pronounced impact on both VCD and titer. The cell lines display a wide range of effect, and the most positive for titer include CHO-DHFR, CHO-DG44, and CHO-K1. Both CHO-DHFR and CHO-DG44 are deficient in the DHFR gene, and the CHO-K1 cell line includes its daughter line GS-CHO, which is deficient in glutamine synthetase (GS). The knock-out and amplification process associated with these cell lines, better equips them to be higher producers. A number of additives can be seen as having a general positive effect, although these results should be considered within the context that additives as a category do not account for a particularly large fraction of the overall variance. The additives that appear to have a more positive impact include vitamins, metals and premade additives for VCD and serum, nucleosides and premade additives for titer. The metals, premade additives, and nucleosides were all seen in Section 6.1 previously. And while the basal media also has a minimal impact on the overall variance, it should be noted that classical media tended to have a negative effect, whereas undisclosed (for VCD) and commercial media were more inclined to positive effects.

### 6.3.4 Model Residuals

While this model can cover a large portion of the variance, there are residuals in the data. The VCD residuals have a slightly negative skew, with a residual skewness of -0.44. Therefore, while the model captures a greater portion of the high VCD values in the positive region, it is less able to predict the lower VCD values. However, I am

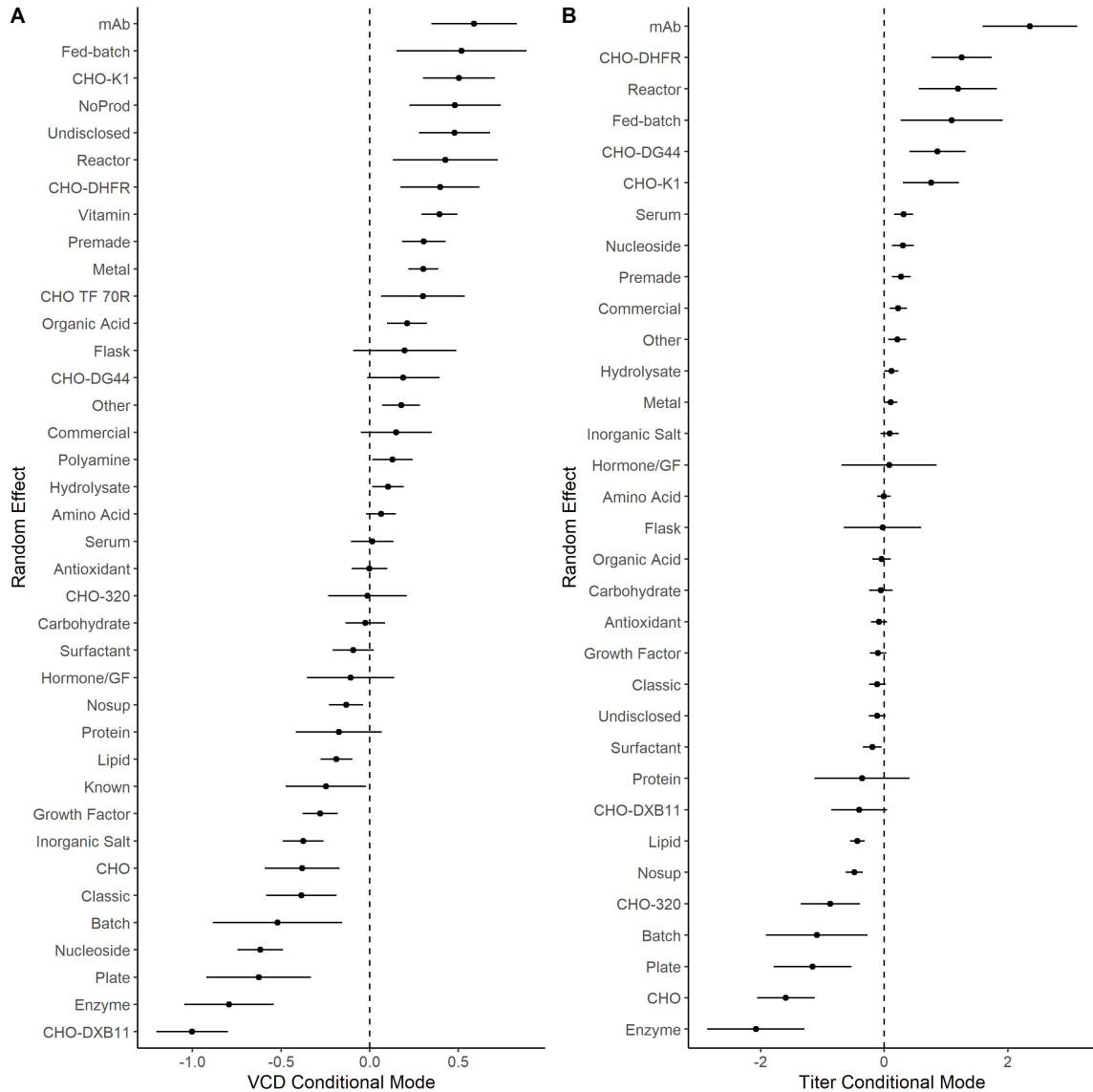


Figure 6.8: Summary of the model coefficients for **A** VCD and **B** titer, with error bars spanning  $\pm 1$  standard deviation.

less concerned with the lower VCD values as these are more likely categorized by cell death, which are inherently less predictable than the intended cell growth. While the residuals are leptokurtic (represented by long tails), many of the large residuals are negative as described by the skewness. The three articles with the highest positive VCD residuals were Ha et al. [87], Spearman et al. [139], and Kim et al. [49]; however, there is no common trend among these three studies that the model may be consistently underestimating. Ha et al. [87] used lithium chloride (LiCl), which

at low concentrations extended the culture viability, while Spearman et al. [139] tested a variety of fractions and types of hydrolysates and found varying levels of improved VCD responses. However, Kim et al. [49] had both some of the most positive and most negative residuals, where the highest VCDs were associated with the non-supplemented medium and the lowest VCDs associated with a hyperosmolar medium.

The titer residuals have a slightly positive skew (residual skewness of 0.64). These residuals are also leptokurtic; however, the positive skewness indicates larger positive residuals. The model is more easily able to predict the lower titer values, which is due to the frequency that mg/L results were achieved within the data-set. The higher g/L changes are less common and are treated as more of an anomaly within the model. The highest positive titer residuals are associated with Ha et al. [87] and Choi et al. [140]. In both studies, higher titers were achieved due to extended culture lengths. These two studies are an example of the benefits of high productivity and long culture, but these are not the only studies that used this approach. Within the data-set, many additives were selected based on their ability to improve productivity although it was often at the expense of cell growth.

### **6.3.5 Testing Model Assumptions**

#### **6.3.5.1 Linearity**

As discussed in Section 4.4.2, there are a few different assumptions that are made in relation to the mixed effect model. The first assumption of linearity can be determined by looking at a residual plot. In this plot, the residuals are plotted against the observed values, where random points indicate that a linear trend is reasonable. The residual plots for both the VCD in Figure 6.9A and titer in Figure 6.9B. Though there is some randomness to the data, there also appears to be some linearity in the residuals. Both vertical and diagonal lines that are visible are a result of individual studies. For the vertical lines, any data point with the same observed value may have different factors and result in a variety of residual values for a given VCD or titer response. This often occurs for studies that use multiple additives within the same experiment. The diagonal lines that appear are associated with the additives in an individual study. If between several experiments the only change is the additive

concentration (which is not considered in the model), the predicted value will be constant despite the differing response value, which creates a slight trend in the residuals. However, if the paper is included as a factor in the model, the trend is not removed from the residual plot, and it creates confounding in the coefficients due to the large influx of coefficients from the studies. The plot does not show a pattern indicating that the data is non-linear, but the slight trend in the lines mentioned may be an indication of a missing factor. This factor is likely an interaction with additives, which cannot be included due to confounding. Although this is a limitation of the model, to solve this issue a data-set with more overlap in factors between studies is required.

### 6.3.5.2 Residual Distribution and Variance

A quantile-quantile (q-q) plot can be used to determine if the residuals follow a normal distribution. The q-q plot for the VCD is presented in Figure 6.10A and the titer is presented in Figure 6.10B. As well the Chi-square test for normality can be used, where the null hypothesis is that the residuals are normally distributed. For both VCD and titer, residuals have a p value less than 0.05, so the null hypothesis is rejected and there is sufficient evidence the residuals are not normal. This can also be seen in the q-q plots, where deviation in the tails (which is more extreme for titer) indicate values deviating from normality, though, the center of the distribution does approximately follow the 45° line. For the VCD, the deviations are on the left side of the data due to the negative skew in the residuals. On the other hand, the titer model more heavily deviates on the right side due to the positive skew in titer residuals. The deviations in the titer residuals indicate that the model is not able to capture the higher positive titer values, which, as discussed in Section 6.3.4 is likely due to how infrequently these values are produced in literature. Since most studies produced titers in the  $\mu\text{g/L}$  to  $\text{mg/L}$  range, and factors in these studies will overlap with those producing  $\text{g/L}$  titers, it is expected that the model will predict a smaller titer on average for higher producing studies. Despite the deviations, the model is able to capture a large portion of the data; however, there should be careful consideration of studies producing highly positive titers. Mixed-effect models have been found to be quite robust for models that do not fully observe the distribution assumptions [141],



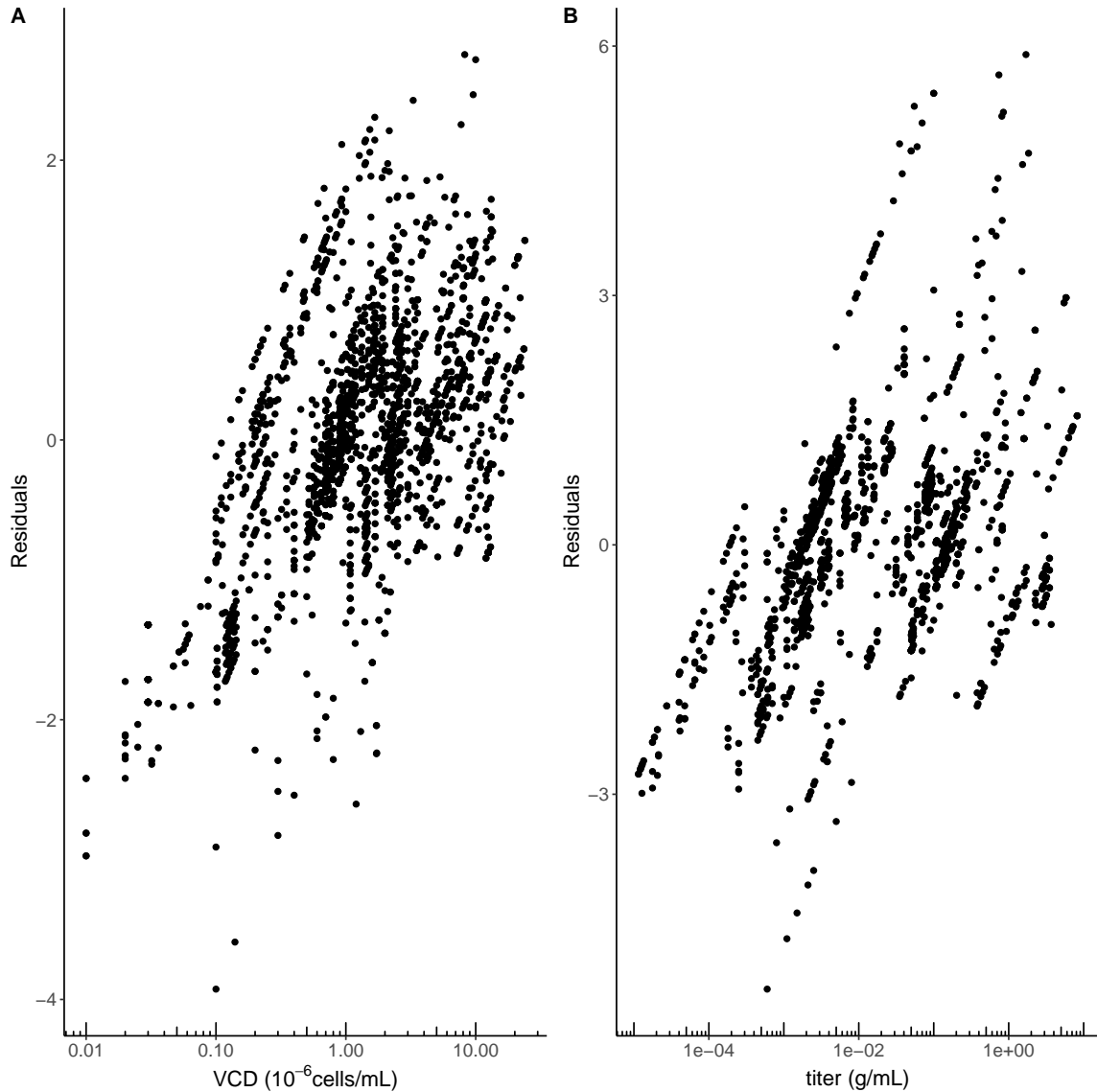


Figure 6.9: Residual plot of the final model (Model 4) for **A** VCD and **B** titer.

so these models can be used even with the deviations from normal. The deviation from normality does not affect the random effect estimates, but it can impact their standard errors [142]. Due to the likely inaccuracy of the error estimates, confidence intervals have not been provided for the random effects.

The Levine's test can be used to determine the heterogeneity of the residual variance by testing the null hypothesis that the variance is equal [143]. To perform this test in R first the absolute value of the residuals is squared and a linear model of the squared residuals and response variables is compared using an ANOVA. For the VCD

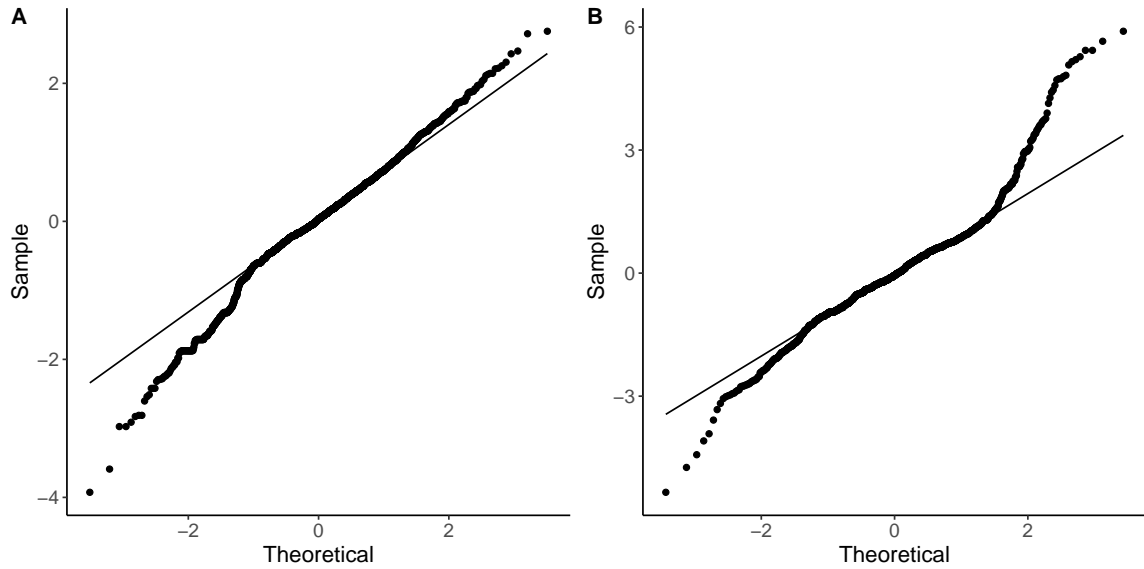


Figure 6.10: Quantile-Quantile plot of **A** VCD and **B** titer to visualize the distribution of the model residuals.

model the p value is less than 0.05, so the null hypothesis is rejected and there is sufficient evidence that the variances are not equal. On the other hand, the titer ANOVA produces a p value greater than 0.05, so the null hypothesis cannot be rejected. While the VCD model does not meet the variance homogeneity assumption, this is not a large concern due to the general robustness of mixed effect modelling, where the affect of variance violations on bias and error appear minimal [141]. Since the residual assumptions are not met (with the exception of titer residual homogeneity), residuals were considered in more detail in Section 6.3.4.

## Chapter 7

### Conclusion

A comprehensive review of the CHO cell media optimization literature identified 238 unique media components that have been supplemented over the last 20 years. Among these additives, zinc stood out as having a positive impact on VCD and nucleosides show potential for increasing titer, while both showed further promise with the addition of premade commercial supplements. However, systematic analysis also identified a number of serious gaps that make it very challenging to draw general conclusions from the available literature. Of the 238 unique media components, approximately 70% appear in only one study. Furthermore, only 30% of the studies revealed the composition of the basal media that the additives are supplementing. Finally, approximately 70% of the studies tested only a single additive or multiple additives in a one-factor-at-a-time design, meaning that the interaction of various factors is often ignored. Drawing general conclusions is likely to remain challenging unless more studies consider the impact of additives on multiple cell lines and multiple basal media (ideally including some with a published composition) using experimental designs that can account for additive interaction. Despite the aforementioned challenges, a mixed-effect modelling framework was able to tease out some potentially interesting trends. Based on the available data, both additives and media on the whole contribute to a relatively low fraction of overall variability in both VCD and titer. In contrast, fed-batch cultivation appears to have a consistent positive effect, meaning that the impact of additives should be considered within the context of cultivation mode. Both the nature of recombinant product and specific cell line were also found to have a significant and much more consistent impact than media as a whole, making cell line development the more attractive target for achieving large titers. While media optimization may also contribute to this end, generalizing the results of media optimization to other cell lines or product types may be a challenge. In this context, it may be more fruitful to focus media optimization on targets other than raw titer,

such as increasing batch-to-batch consistency, facilitating downstream purification, and improving product quality through glycosylation or other metrics.

Due to the focus of the literature included in this data-set, only broad conclusions can be made about factors such as cell line. In the future, this can be addressed by expanding the literature search to include studies that focus on comparisons between the cell lines as opposed to just media supplementation. The additional recording of specific productivity may provide a source of production changes more relevant than VCD. In its current state, this project was able to determine the general impact of media additives, though it was determined to be minimal in comparison to other factors. It also provides a database of available literature on media supplementation in CHO cells that may be used as a guideline for those looking to optimize their cell line.

## Bibliography

- [1] Gary Walsh. Biopharmaceutical benchmarks 2018. *Nature Biotechnology*, 36(12):1136–1145, 2018.
- [2] K P Jayapal, Katie F. Wlaschin, Wei-Shou Hu, and Miranda Yap. Recombinant protein therapeutics from CHO cells-20 years and counting. *Chemical Engineering Progress*, 103:40–47, 2007.
- [3] Paul J. Carter. Introduction to current and future protein therapeutics: A protein engineering perspective. *Experimental Cell Research*, 317(9):1261–1269, 2011.
- [4] Evan Wells and Anne S. Robinson. Cellular engineering for therapeutic protein production: Product quality, host modification, and process improvement, 2017.
- [5] Florian Wurm. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nature Biotechnology*, 22(11):1393–1398, 2004.
- [6] Stefanie Dietmair, Lars K. Nielsen, and Nicholas E. Timmins. Engineering a mammalian super producer. *Journal of Chemical Technology & Biotechnology*, 86(7):905–914, 2011.
- [7] Simon Fischer, René Handrick, and Kerstin Otte. The art of CHO cell engineering: A comprehensive retrospect and future perspectives. *Biotechnology Advances*, 33:1878–1896, 2015.
- [8] Catherine B. Matthews, Chapman Wright, Angel Kuo, Noelle Colant, Matthew Westoby, and J. Christopher Love. Reexamining opportunities for therapeutic protein production in eukaryotic microorganisms. *Biotechnology and Bioengineering*, 114(11):2432–2444, 2017.
- [9] M N Baeshen, Ahmed M Al-Hejin, Roop S Bora, M M M Ahmed, H A Ramadan, K S Saini, N A Baeshen, and E M Redwan. Production of biopharmaceuticals in E. coli: current scenario and future perspectives. *Journal of microbiology and biotechnology*, 25(7):953–962, 2015.
- [10] Jiechao Yin, Guangxing Li, Xiaofeng Ren, and Georg Herrler. Select what you need: A comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. *Journal of Biotechnology*, 127(3):335–347, 2007.
- [11] Kishwar Hayat Khan. Gene expression in mammalian cells and its applications. *Advanced Pharmaceutical Bulletin*, 3(2):257–263, 2013.

- [12] David Gray. Overview of Protein Expression by Mammalian Cells. *Current Protocols in Protein Science*, 10(1), 1997.
- [13] Jee Yon Kim, Yeon-Gu Kim, and Gyun Min Lee. CHO cells in biotechnology for production of recombinant proteins: Current state and further potential. *Applied Microbiology and Biotechnology*, 93:917–930, 2012.
- [14] Chih Chung Kuo, Austin WT Chiang, Isaac Shamie, Mojtaba Samoudi, Jahir M. Gutierrez, and Nathan E Lewis. The emerging role of systems biology for engineering protein production in CHO cells. *Current Opinion in Biotechnology*, 51:64–69, 2018.
- [15] Frank V. Ritacco, Yongqi Wu, and Anurag Khetan. Cell culture media for recombinant protein expression in Chinese hamster ovary (CHO) cells: History, key components, and optimization strategies. *Biotechnology Progress*, 34(6):1407–1426, 2018.
- [16] Paul J. Price. Best practices for media selection for mammalian cells. *In Vitro Cellular & Developmental Biology - Animal*, 53:673–681, 2017.
- [17] Wai Lam Ling. Development of protein-free medium for therapeutic protein production in mammalian cells: recent advances and perspectives. *Pharmaceutical Bioprocessing*, 3(3):215–226, 2015.
- [18] D. Brunner, J Frank, Helmut Appl, Harald Shoffl, Walter Pfaller, and G. Gstraunthaler. Serum-free cell Culture: The Serum-free media interactive online database. *ALTEX*, 27(1):53–62, 2010.
- [19] J. van Der Valk, D. Brunner, K. De Smet, Å Fex Svenningsen, P. Honegger, L. E. Knudsen, T. Lindl, J. Noraberg, A. Price, M. L. Scarino, and G. Gstraunthaler. Optimization of chemically defined cell culture media - Replacing fetal bovine serum in mammalian in vitro methods. *Toxicology in Vitro*, 24(4):1053–1063, 2010.
- [20] C Altamirano, J Berrios, M Vergara, and S Becerra. Advances in improving mammalian cells metabolism for recombinant protein production. *Electronic Journal of Biotechnology*, 16(3), 2013.
- [21] Neil Templeton and Jamey D. Young. Biochemical and metabolic engineering approaches to enhance production of therapeutic proteins in animal cell cultures. *Biochemical Engineering Journal*, 136:40–50, 2018.
- [22] Feng Li, Natarajan Vijayasankaran, Amy Shen, Robert Kiss, and Ashraf Amanullah. Cell culture processes for monoclonal antibody production. *mAbs*, 2(5):466–477, 2010.

- [23] Eric J.M. Blondeel and Marc G. Aucoin. Supplementing glycosylation: A review of applying nucleotide-sugar precursors to growth medium to affect therapeutic recombinant protein glycoform distributions. *Biotechnology Advances*, 36:1505–1523, 2018.
- [24] David Brühlmann, Martin Jordan, Jürgen Hemberger, Markus Sauer, Matthieu Stettler, and Hervé Broly. Tailoring recombinant protein quality by rational media design. *Biotechnology Progress*, 31(3):615–629, 2015.
- [25] Zhuangrong Huang, Dong-Yup Lee, and Seongkyu Yoon. Quantitative intracellular flux modeling and applications in biotherapeutic development and production using CHO cell cultures. *Biotechnology and Bioengineering*, 114:2717–2728, 2017.
- [26] Paul S Kelly, Antonio Alarcon Miguez, Christina Alves, and Niall Barron. From media to mitochondria–rewiring cellular energy metabolism of Chinese hamster ovary cells for the enhanced production of biopharmaceuticals. *Current Opinion in Chemical Engineering*, 22:71–80, 2018.
- [27] Gino Stolfa, Matthew T. Smonskey, Ryan Boniface, Anna-Barbara Hachmann, Paul Gulde, Atul D. Joshi, Anson P. Pierce, Scott J. Jacobia, and Andrew Campbell. CHO-Omics Review: The Impact of Current and Emerging Technologies on Chinese Hamster Ovary Based Bioproduction. *Biotechnology Journal*, 13(3):1700227, 2018.
- [28] Mariela Bollati Fogolín, Roland Wagner, Marina Etcheverrigaray, and Ricardo Kratje. Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells. *Journal of Biotechnology*, 109(1-2):179–191, 2004.
- [29] Sung Hyun Kim and Gyun Min Lee. Functional expression of human pyruvate carboxylase for reduced lactic acid formation of Chinese hamster ovary cells (DG44). *Applied Microbiology and Biotechnology*, 76(3):659–665, 2007.
- [30] Jae Seong Lee, Tae Kwang Ha, Jin Hyoung Park, and Gyun Min Lee. Anti-cell death engineering of CHO cells: Co-overexpression of Bcl-2 for apoptosis inhibition, Beclin-1 for autophagy induction. *Biotechnology and Bioengineering*, 110(8):2195–2207, 2013.
- [31] Danny Chee Fung Wong, Kathy Tin Kam Wong, Peter Morin Nissom, Chew Kiat Heng, and Miranda Gek Sim Yap. Targeting early apoptotic genes in batch and fed-batch CHO cell cultures. *Biotechnology and Bioengineering*, 95(3):350–361, 2006.
- [32] Gail Urlaub and Lawrence A Chasin. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc Natl Acad Sci USA*, 77(7):4216–4220, 1980.

- [33] Hongwen Li, Kaiming Chen, Zhe Wang, Dong Li, Jiannan Lin, Chao Yu, Fang Yu, Xin Wang, Lei Huang, Cizhong Jiang, Hua Gu, and Jianmin Fang. Genetic analysis of the clonal stability of Chinese hamster ovary cells for recombinant protein production. *Molecular BioSystems*, 12(1):102–109, 2016.
- [34] Jeremy W Dale, Malcolm von Shantz, and Nicholas Plant. *From genes to genomes: Concepts and applications of dna technology*. John Wiley and Sons, Chichester, West Sussex, 3 edition, 2012.
- [35] S. C. Huhn, Y. Ou, A. Kumar, R. Liu, and Z. Du. High throughput, efficacious gene editing & genome surveillance in Chinese hamster ovary cells. *PLoS ONE*, 14(12), 2019.
- [36] Mattia Matasci, David L. Hacker, Lucia Baldi, and Florian M. Wurm. Recombinant therapeutic protein production in cultivated mammalian cells: current status and future prospects. *Drug Discov Today Technol*, 5(2-3):37–42, 2008.
- [37] A Kantardjieff and W Zhou. Mammalian Cell Cultures for Biologics Manufacturing. In W Zhou and A Kantardjieff, editors, *Advances in Biochemical Engineering/Biotechnology*. Springer, Berlin, Heidelberg, 2013.
- [38] Renate Kunert and David Reinhart. Advances in recombinant antibody manufacturing. *Applied Microbiology and Biotechnology*, 100(8):3451–3461, 2016.
- [39] Sen Xu, John Gavin, Ruben Jiang, and Hao Chen. Bioreactor productivity and media cost comparison for different intensified cell culture processes. *Biotechnology Progress*, 33(4):867–878, 2017.
- [40] David Reinhart, Lukas Damjanovic, Andreas Castan, Wolfgang Ernst, and Renate Kunert. Differential gene expression of a feed-spiked super-producing CHO cell line. *Journal of Biotechnology*, 285:23–37, 2018.
- [41] Nadine Kochanowski, Gaetan Siriez, Sarah Roosens, and Laetitia Malphettes. Medium and feed optimization for fed-batch production of a monoclonal antibody in CHO cells. *BMC proceedings*, 5 Suppl 8(Suppl 8):P75, 2011.
- [42] Jean Marc Bielser, Moritz Wolf, Jonathan Souquet, Hervé Broly, and Massimo Morbidelli. Perfusion mammalian cell culture for recombinant protein manufacturing – A critical review. *Biotechnology Advances*, 36(4):1328–1340, 2018.
- [43] S Abu-Absi, S Xu, H Graham, N Dalal, M Boyer, and K Dave. Cell culture process operations for recombinant protein production. *Adv Biochem Eng Biotechnol*, 139:35–68, 2014.
- [44] Ulrike Rasche. Bioreactors and Fermentors-Powerful Tools for Resolving Cultivation Bottlenecks. Technical report, Eppendorf, 2019.



- [45] Sung Kwan Yoon, Ji Yong Song, and Gyun Min Lee. Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. *Biotechnology and Bioengineering*, 82(3):289–298, 2003.
- [46] V. Hendrick, P. Winnepeninckx, C. Abdelkafi, O. Vandeputte, M. Cherlet, T. Marique, G. Renemann, A. Loa, G. Kretzmer, and J. Werenne. Increased productivity of recombinant tissular plasminogen activator (t-PA) by butyrate and shift of temperature: a cell cycle phases analysis. *Cytotechnology*, 36(1-3):71–83, 2001.
- [47] Hitto Kaufmann, Xenia Mazur, Martin Fussenegger, and James E. Bailey. Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells. *Biotechnology and Bioengineering*, 63(5):573–582, 1999.
- [48] Amy Farrell, N McLoughlin, John J. Milne, Ian W. Marison, and Jonathan Bones. Application of multi-omics techniques for bioprocess design and optimization in Chinese hamster ovary cells. *Journal of Proteome Research*, 13(7):3144–3159, 2014.
- [49] Tae Kyung Kim, Joon Soo Ryu, Joo Young Chung, Min Soo Kim, and Gyun Min Lee. Osmoprotective effect of glycine betaine on thrombopoietin production in hyperosmotic Chinese hamster ovary cell culture: Clonal variations. *Biotechnology Progress*, 16:775–781, 2000.
- [50] Zhihua Xiao, Michelle Sabourin, Graziella Piras, and Stephen F. Gorfien. Screening and Optimization of Chemically Defined Media and Feeds with Integrated and Statistical Approaches. In Ralf Portner, editor, *Animal Cell Biotechnology: Methods in Molecular Biology (Methods and Protocols)*, pages 117–135. Humana Press, Totowa, NJ, 3rd edition, 2014.
- [51] T Fletcher. Designing Culture Media for Recombinant Protein Production A Rational Approach. *Bioprocess International*, 3:2–6, 2005.
- [52] Martin Jordan, Damien Voisard, Antoine Berthoud, Laetitia Tercier, Beate Kleuser, Gianni Baer, and Hervé Broly. Cell culture medium improvement by rigorous shuffling of components using media blending. *Cytotechnology*, 65:31–40, 2013.
- [53] Analytical Methods Committee. Experimental design and optimisation (4): Plackett–Burman designs. *Analytical Methods*, 5(8):1901–1903, 2013.
- [54] Huifeng Zhang, Haibin Wang, Mei Liu, Tao Zhang, Ji Zhang, Xiangjing Wang, and Wensheng Xiang. Rational development of a serum-free medium and fed-batch process for a GS-CHO cell line expressing recombinant antibody. *Cytotechnology*, 65(3):363–378, 2013.

- [55] Riccardo Leardi. Experimental design in chemistry: A tutorial. *Analytica Chimica Acta*, 652:161–172, 2009.
- [56] Han Kyu Oh, Moon Kyoung So, Jihye Yang, Ho Chul Yoon, Ji Soo Ahn, Jong Min Lee, Ji Tai Kim, Ji Uk Yoo, and Tae Ho Byun. Effect of N-Acetylcystein on Butyrate-Treated Chinese Hamster Ovary Cells To Improve the Production of Recombinant Human Interferon- $\beta$ -1a. *Biotechnology Progress*, 21:1154–1164, 2005.
- [57] Carl-Fredrik Mandenius and Anders Brundin. Bioprocess optimization using design-of-experiments methodology. *Biotechnology Progress*, 24(6):1191–1203, 2008.
- [58] Derek Toms, Rob Deardon, and Mark Ungrin. Climbing the mountain: Experimental design for the efficient optimization of stem cell bioprocessing. *Journal of Biological Engineering*, 11(35):1–10, 2017.
- [59] Yasuhiro Takagi, Takuya Kikuchi, Ryuta Wada, and Takeshi Omasa. The enhancement of antibody concentration and achievement of high cell density CHO cell cultivation by adding nucleoside. *Cytotechnology*, 69:511–521, 2017.
- [60] Sung Hyun Kim and Gyun Min Lee. Development of serum-free medium supplemented with hydrolysates for the production of therapeutic antibodies in CHO cell cultures using design of experiments. *Applied Microbiology and Biotechnology*, 83:639–648, 2009.
- [61] Jahir M. Gutierrez and Nathan E Lewis. Optimizing eukaryotic cell hosts for protein production through systems biotechnology and genome-scale modeling. *Biotechnology Journal*, 10(7):939–949, 2015.
- [62] Shohei Kishishita, Satoshi Katayama, Kunihiko Kodaira, Yoshinori Takagi, Hiroki Matsuda, Hiroshi Okamoto, Shinya Takuma, Chikashi Hirashima, and Hideki Aoyagi. Optimization of chemically defined feed media for monoclonal antibody production in Chinese hamster ovary cells. *Journal of Bioscience and Bioengineering*, 120(1):78–84, 2015.
- [63] Michael J. Gramer, Jackie J. Eckblad, Ruth Donahue, Joseph Brown, Carrie Shultz, Kent Vickerman, Patrick Priem, Ewald T.J. van den Bremer, Jolanda Gerritsen, and Patrick H.C. van Berkel. Modulation of antibody galactosylation through feeding of uridine, manganese chloride, and galactose. *Biotechnology and Bioengineering*, 108(7):1591–1602, 2011.
- [64] Nuno Carinhas, Tiago M. Duarte, Laura C. Barreiro, M. J. T. Carrondo, Paula M. Alves, and Ana P. Teixeira. Metabolic signatures of GS-CHO cell clones associated with butyrate treatment and culture phase transition. *Biotechnology and Bioengineering*, 110(12):3244–3257, 2013.

- [65] C Altamirano, A Illanes, S Becerra, J J Cairó, and F Gòdia. Considerations on the lactate consumption by CHO cells in the presence of galactose. *Journal of Biotechnology*, 125(4):547–556, 2006.
- [66] Kirti Chaturvedi, Susan Y. Sun, Thomas O’Brien, Yan J. Liu, and James W. Brooks. Comparison of the behavior of CHO cells during cultivation in 24-square deep well microplates and conventional shake flask systems. *Biotechnology Reports*, 1-2:22–26, 2014.
- [67] Natalie Muller, Philippe Girard, David L. Hacker, Martin Jordan, and Florian M. Wurm. Orbital shaker technology for the cultivation of mammalian cells in suspension. *Biotechnology and Bioengineering*, 89(4):400–406, 2005.
- [68] G. Jänicke, C. Sauter, R. Bux, and J. Haas. Characterisation of Shake Flasks for Cultivation of Animal Cell Cultures. In R Smith, editor, *Cell Technology for Cell Products: Proceedings of the 19th ESACT Meeting, Harrogate, UK, June 5-8, 2005*, pages 727–731. Springer Netherlands, Dordrecht, 2007.
- [69] Marques Starks. *Immunology and Animal Biotechnology*. ED Tech Press, United Kingdom, 2019.
- [70] C Altamirano, C Paredes, J J Cairó, and F Godia. Improvement of CHO Cell Culture Medium Formulation: Simultaneous Substitution of Glucose and Glutamine. *Biotechnology Progress*, 16:69–75, 2000.
- [71] Dawn Sow Zong Leong, Brian Kah Hui Teo, Janice Gek Ling Tan, Hayati Kamari, Yuan Sheng Yang, Peiqing Zhang, and Say Kong Ng. Application of maltose as energy source in protein-free CHO-K1 culture to improve the production of recombinant monoclonal antibody. *Scientific Reports*, 8, 2018.
- [72] Dawn Sow Zong Leong, Janice Gek Ling Tan, Christine Lin Chin, Shi Ya Mak, Ying Swan Ho, and Say Kong Ng. Evaluation and use of disaccharides as energy source in protein-free mammalian cell cultures. *Scientific Reports*, 7, 2017.
- [73] Yueming Qian, Sarwat F. Khattak, Zizhuo Xing, Aiqing He, Paul S. Kayne, Nan Xin Qian, Shih-Hsie Pan, and Zheng Jian Li. Cell culture and gene transcription effects of copper sulfate on Chinese hamster ovary cells. *Biotechnology Progress*, 27(4):1190–1194, 2011.
- [74] Herbert Rodrigues Goulart, Fernanda dos Santos Arthuso, Marcos Vinicius Nucci Capone, Taís Lima de Oliveira, Paolo Bartolini, and Carlos Roberto Jorge Soares. Enhancement of human prolactin synthesis by sodium butyrate addition to serum-free CHO cell culture. *Journal of Biomedicine & Biotechnology*, 2010, 2010.

- [75] Y. H. Sung, Yeon Jung Song, Seung Wook Lim, Joo Young Chung, and Gyun Min Lee. Effect of sodium butyrate on the production, heterogeneity and biological activity of human thrombopoietin by recombinant Chinese hamster ovary cells. *Journal of Biotechnology*, 112:323–335, 2004.
- [76] Sarah Wulhfard, Lucia Baldi, David L. Hacker, and Florian Wurm. Valproic acid enhances recombinant mRNA and protein levels in transiently transfected Chinese hamster ovary cells. *Journal of Biotechnology*, 148:128–132, 2010.
- [77] James F Babcock and Amy Antosh. Partial Replacement of Chemically Defined CHO Media with Plant-Derived Protein Hydrolysates. In N Jenkins, N Barron, and P Alves, editors, *Proceedings of the 21st Annual Meeting of the European Society for Animal Cell Technology (ESACT), Dublin, Ireland, June 7-10, 2009*, pages 295–298. Springer Netherlands, Dordrecht, 2012.
- [78] Bong Gyun Kim and Hong Woo Park. High zinc ion supplementation of more than 30  $\mu\text{M}$  can increase monoclonal antibody production in recombinant Chinese hamster ovary DG44 cell culture. *Applied Microbiology and Biotechnology*, 100:2163–2170, 2016.
- [79] Bhanu Chandra Mulukutla, Jaitashree Kale, Taylor Kalomeris, Michaela Jacobs, and Gregory W. Hiller. Identification and control of novel growth inhibitors in fed-batch cultures of Chinese hamster ovary cells. *Biotechnology and Bioengineering*, 114(8):1779–1790, 2017.
- [80] Nathaniel W. Freund and Matthew S. Croughan. A simple method to reduce both lactic acid and ammonium production in industrial animal cell culture. *International Journal of Molecular Sciences*, 19(2), 2018.
- [81] Maureen Spearman, Carly Lodewyks, Meika Richmond, and Michael Butler. The Bioactivity and Fractionation of Peptide Hydrolysates in Cultures of CHO Cells. *Biotechnology Progress*, 30(3):584–593, 2014.
- [82] B. Farges, S. Chenu, A. Marc, and J. L. Goergen. Kinetics of IFN- $\gamma$  producing CHO cells and other industrially relevant cell lines in rapeseed-supplemented batch cultures. *Process Biochemistry*, 43:945–953, 2008.
- [83] Inn H. Yuk, Stephen Russell, Yun Tang, Wei-Ting Hsu, Jacob B. Mauger, Rigzen P. S. Aulakh, Jun Luo, Martin Gawlitzek, and John C. Joly. Effects of copper on CHO cells: Cellular requirements and product quality considerations. *Biotechnology Progress*, 31(1):226–238, 2015.
- [84] Victor V.T. Wong, Kah Wye Ho, and Miranda Yap. Evaluation of insulin-mimetic trace metals as insulin replacements in mammalian cell cultures. *Cytotechnology*, 45:107–115, 2004.

- [85] Y Bai, C Wu, J Zhao, Y H Liu, W Ding, and W L Ling. Role of iron and sodium citrate in animal protein-free CHO cell culture medium on cell growth and monoclonal antibody production. *Biotechnology Progress*, 27(1):209–219, 2011.
- [86] Berta Capella Roca, Antonio Alarcón Miguez, Joanne Keenan, Srinivas Suda, Niall Barron, Donal O’Gorman, Pdraig Doolan, and Martin Clynes. Zinc supplementation increases protein titer of recombinant CHO cells. *Cytotechnology*, 71(5):915–924, 2019.
- [87] Tae Kwang Ha, Yeon-Gu Kim, and Gyun Min Lee. Effect of lithium chloride on the production and sialylation of Fc-fusion protein in Chinese hamster ovary cell culture. *Applied Microbiology and Biotechnology*, 98:9239–9248, 2014.
- [88] Yusuke Mimura, John Lund, Stephen Church, Sucui Dong, Jun Li, Margaret Goodall, and Roy Jefferis. Butyrate increases production of human chimeric IgG in CHO-K1 cells whilst maintaining function and glycoform profile. *Journal of Immunological Methods*, 247:205–216, 2001.
- [89] B. Halliwell. Oxidative stress in cell culture: An under-appreciated problem? *FEBS Letters*, 540(1-3):3–6, 2003.
- [90] C Altamirano, A Illanes, R Canessa, and S Becerra. Specific nutrient supplementation of defined serum-free medium for the improvement of CHO cells growth and t-PA production. *Electronic Journal of Biotechnology*, 9(1):61–67, 2006.
- [91] B. Halliwell. Cell culture, oxidative stress, and antioxidants: Avoiding pitfalls. *Biomedical Journal*, 37(3):99–105, 2014.
- [92] Nathan E Lewis, Xin Liu, Yuxiang Li, Harish Nagarajan, George Yerganian, Edward O’Brien, Aarash Bordbar, Anne M Roth, Jeffrey Rosenbloom, Chao Bian, Min Xie, Wenbin Chen, Ning Li, Deniz Baycin-Hizal, Haythem Latif, Jochen Forster, Michael J Betenbaugh, Iman Famili, Xun Xu, Jun Wang, and Bernhard O Palsson. Genomic landscapes of Chinese hamster ovary cell lines as revealed by the *Cricetulus griseus* draft genome. *Nature Biotechnology*, 31(8):759–765, 2013.
- [93] Florian Wurm and Maria Wurm. Cloning of CHO Cells, Productivity and Genetic Stability—A Discussion. *Processes*, 5(2):20, 2017.
- [94] Damien Lamotte, Lorraine Buckberry, Lucia Monaco, Marco Soria, Nigel Jenkins, Jean-marc Engasser, and A. Marc. Na-butyrate increases the production and  $\alpha$ 2,6-sialylation of recombinant interferon- $\gamma$  expressed by  $\alpha$ 2,6-sialyltransferase engineered CHO cells. *Cytotechnology*, 29:55–64, 1999.
- [95] Amos Bairoch. The cellosaurus, a cell-line knowledge resource. *Journal of Biomolecular Techniques*, 29(2):25–38, 2018.

- [96] Liming Liu. Antibody glycosylation and its impact on the pharmacokinetics and pharmacodynamics of monoclonal antibodies and Fc-fusion proteins. *Journal of Pharmaceutical Sciences*, 104(6):1866–1884, 2015.
- [97] R Anthony, F Nimmerjahn, D Ashline, V Reinhold, J Paulson, and J Ravetch. Recapitulation of IVIG Anti-Inflammatory Activity with a Recombinant IgG Fc. *Science*, 320(5874):373–376, 2008.
- [98] Qiong Wang, Cheng-Yu Chung, Sandra Chough, and Michael J. Betenbaugh. Antibody glycoengineering strategies in mammalian cells, 2018.
- [99] Y. H. Sung, S. W. Lim, J. Y. Chung, and G. M. Lee. Yeast hydrolysate as a low-cost additive to serum-free medium for the production of human thrombopoietin in suspension cultures of Chinese hamster ovary cells. *Applied Microbiology and Biotechnology*, 63:527–536, 2004.
- [100] Patrick Hossler, Chris Racicot, Chris Chumsae, Sean McDermott, and Keith Cochran. Cell culture media supplementation of infrequently used sugars for the targeted shifting of protein glycosylation profiles. *Biotechnology Progress*, 33(2), 2017.
- [101] Janike Ehret, Martina Zimmermann, Thomas Eichhorn, and Aline Zimmer. Impact of cell culture media additives on IgG glycosylation produced in Chinese hamster ovary cells. *Biotechnology and Bioengineering*, 116:816–830, 2019.
- [102] Gregg Hartvigsen. *A Primer in Biological Data Analysis and Visualization Using R*. Columbia University Press, New York, 2014.
- [103] Andrew Gelman, Tue Tjur, Peter McCullagh, Joop Hox, Herbert Hoijtink, and Alan M. Zaslavsky. Analysis of Variance - Why it is more important than ever. *Annals of Statistics*, 33(1):1–53, 2005.
- [104] Andrzej Galleki and Tomasz Burzykowski. *Linear Mixed-Effects Models Using R: A step-by-step approach*. Springer, New York, 2013.
- [105] Andrew Gelman and Jennifer Hill. *Data Analysis Using Regression and Multi-level/Hierarchical Models*. Cambridge University Press, 2007.
- [106] H Akaike. A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, 19(6):716–723, 1974.
- [107] Emad A. Mohammed, Christopher Naugler, and Behrouz H. Far. Emerging Business Intelligence Framework for a Clinical Laboratory Through Big Data Analytics. *Emerging Trends in Computational Biology, Bioinformatics, and Systems Biology: Algorithms and Software Tool*, pages 577–602, 2015.
- [108] Geoffrey Norman and David Streiner. *Biostatistics: The Bare Essentials*. BC Decker, Hamilton, 3 edition, 2008.

- [109] Peder Lundkvist, Bjarne Bergquist, and Erik Vanhatalo. Statistical methods - still ignored? The testimony of Swedish alumni. *Total Quality Management & Business Excellence*, 31(3-4):245–262, 2020.
- [110] Juliana Coronel, Sandra Klausning, Christoph Heinrich, Thomas Noll, Alvio Figueredo-Cardero, and Leda R. Castilho. Valeric acid supplementation combined to mild hypothermia increases productivity in CHO cell cultivations. *Biochemical Engineering Journal*, 114:101–109, 2016.
- [111] Limor Cohen and David R. Walt. Highly Sensitive and Multiplexed Protein Measurements. *Chemical Reviews*, 119:293–321, 2019.
- [112] Harleen Kaur, Sonali R Bhagwat, Tarun Kumar Sharma, and Amit Kumar. Analytical techniques for characterization of biological molecules – proteins and aptamers/oligonucleotides, 2019.
- [113] Vlastimil Dohnal, Vladimír Dvořák, František Malíř, Vladimír Ostrý, and Tomáš Roubal. A comparison of ELISA and HPLC methods for determination of ochratoxin A in human blood serum in the Czech Republic. *Food and Chemical Toxicology*, 62:427–431, 2013.
- [114] Fernando Rubio, Linda Veldhuis, B Clegg, James Fleeker, and J Hall. Comparison of a Direct ELISA and an HPLC Method for Glyphosate Determinations in Water. *Journal of Agricultural and Food Chemistry*, 51:691–696, 2003.
- [115] Jin Hyoung Park, Myung Sin Lim, Ju Rang Woo, Jong Won Kim, and Gyun Min Lee. The molecular weight and concentration of dextran sulfate affect cell growth and antibody production in CHO cell cultures. *Biotechnology Progress*, 32(5):1113–1122, 2016.
- [116] J Mols, C. Peeters-Joris, S. Agathos, and Y.-J. Schneider. Origin of rice protein hydrolysates added to protein-free media alters secretion and extracellular proteolysis of recombinant interferon- $\gamma$  as well as CHO-320 cell growth. *Biotechnology Letters*, 26:1043–1046, 2004.
- [117] J. S. Ballez, J Mols, C. Burteau, S. N. Agathos, and Y. J. Schneider. Plant protein hydrolysates support CHO-320 cells proliferation and recombinant IFN- $\gamma$  production in suspension and inside microcarriers in protein-free media. *Cytotechnology*, 44(3):103–114, 2004.
- [118] C. Burteau, Francis Verhoeve, J Mols, J. S. Ballez, S. Agathos, and Yves-Jacques Schnieder. Fortification of a protein-free cell culture medium with plant peptones improves cultivation and productivity of an interferon- $\gamma$ -producing CHO cell line. *In Vitro Cellular & Developmental Biology - Animal*, 39:291–296, 2003.

- [119] Steven C.L. Ho, Rui Nian, Susanto Woen, Jake Chng, Peiqing Zhang, and Yuansheng Yang. Impact of hydrolysates on monoclonal antibody productivity, purification and quality in Chinese hamster ovary cells. *Journal of Bioscience and Bioengineering*, 122(4):499–506, 2016.
- [120] Dongdong Hu, Yating Sun, Xuping Liu, Jintao Liu, Xintao Zhang, Liang Zhao, Haibin Wang, Wen-Song Tan, and Li Fan. Understanding the intracellular effects of yeast extract on the enhancement of Fc-fusion protein production in Chinese hamster ovary cell culture. *Applied Microbiology and Biotechnology*, 99, 2015.
- [121] J.-F. Michiels, J. Barbau, S. De Boel, S. Dessy, S N Agathos, and Y.-J. Schneider. Characterisation of beneficial and detrimental effects of a soy peptone, as an additive for CHO cell cultivation. *Process Biochemistry*, 46:671–681, 2011.
- [122] J.-F. Michiels, S. Sart, Y.-J. Schneider, and S. Agathos. Effects of a soy peptone on  $\gamma$ -IFN production steps in CHO-320 cells. *Process Biochemistry*, 46:1759–1766, 2011.
- [123] David Reinhart, Lukas Damjanovic, Christian Kaisermayer, and Renate Kunert. Benchmarking of commercially available CHO cell culture media for antibody production. *Applied Microbiology and Biotechnology*, 99:4645–4657, 2015.
- [124] Do Yun Kim, Muhammad Arshad Chaudhry, Malcolm L. Kennard, Mario A. Jardon, Katrin Braasch, Ben Dionne, Michael Butler, and James M. Piret. Fed-batch CHO cell t-PA production and feed glutamine replacement to reduce ammonia production. *Biotechnology Progress*, 29(1):165–175, 2013.
- [125] Tae Kwang Ha and Gyun Min Lee. Effect of glutamine substitution by TCA cycle intermediates on the production and sialylation of Fc-fusion protein in Chinese hamster ovary cell culture. *Journal of Biotechnology*, 180:23–29, 2014.
- [126] Huijie Niu, Jiaqi Wang, Mengjuan Liu, Miaomiao Chai, Liang Zhao, Xuping Liu, Li Fan, and Wen-Song Tan. Uridine modulates monoclonal antibody charge heterogeneity in Chinese hamster ovary cell fed-batch cultures. *Bioresources and Bioprocessing*, 5(42), 2018.
- [127] Anuja Prabhu and Mugdha Gadgil. Nickel and cobalt affect galactosylation of recombinant IgG expressed in CHO cells. *BioMetals*, 32:11–19, 2019.
- [128] Chi-Hsien Liu, I-Ming Chu, and Shiaw-Min Hwang. Factorial designs combined with the steepest ascent method to optimize serum-free media for CHO cells. *Enzyme and Microbial Technology*, 28:314–321, 2001.



- [129] Adam Elhofy. Novel Cell-Ess supplement used as a feed or as an initial boost to CHO serum free media results in a significant increase in protein yield and production. *Computational and Structural Biotechnology Journal*, 14:319–324, 2016.
- [130] Jianlin Xu, Matthew S. Rehmann, Xuankuo Xu, Chao Huang, Jun Tian, Nan Xin Qian, and Zheng Jian Li. Improving titer while maintaining quality of final formulated drug substance via optimization of CHO cell culture conditions in low-iron chemically defined media. *mAbs*, 10(3):488–499, 2018.
- [131] Tae Kwang Ha, Anders Holmgaard Hansen, Stefan Kol, Helene Faustrup Kildegaard, and Gyun Min Lee. Baicalein Reduces Oxidative Stress in CHO Cell Cultures and Improves Recombinant Antibody Productivity. *Biotechnology Journal*, 13, 2018.
- [132] B. Farges-Haddani, B. Tessier, S. Chenu, I. Chevalot, C. Harscoat, I. Marc, J.L. Goergen, and A. Marc. Peptide fractions of rapeseed hydrolysates as an alternative to animal proteins in CHO cell culture media. *Process Biochemistry*, 41:2297–2304, 2006.
- [133] Ji Young Lee, Bok-Hwan Chun, Yong Kwon Lee, Jang Han Lee, Jason Ahn, and Namhyun Chung. Influence of Mixed Protein Hydrolysates on the Growth and Viability of Chinese Hamster Ovary Cells. *Journal of the Korean Society for Applied Biological Chemistry*, 52(6):607–612, 2009.
- [134] Ying Luo and Guoxiang Chen. Combined approach of NMR and chemometrics for screening peptones used in the cell culture medium for the production of a recombinant therapeutic protein. *Biotechnology and Bioengineering*, 97(6):1654–1659, 2007.
- [135] G. Baré, H. Charlier, L. De Nijs, F. Verhoeye, Yves-Jacques Schneider, S. Agathos, and P. Thonart. Effects of a Rice Protein Hydrolysate on Growth of CHO Cells and Production of Human Interferon- $\gamma$  in a Serum-Free Medium. In E Lindner-Olsson, N Chatzissavidou, and E Lullau, editors, *Animal Cell Technology: From Target to Market*, pages 217–219, Dordrecht, 2001. Springer.
- [136] Max Kuhn and Kjell Johnson. *Applied Predictive Modeling*. Springer-Verlag, New York, 2013.
- [137] Lena Thoring, Srujan K. Dondapati, Marlitt Stech, Doreen A. Wüstenhagen, and Stefan Kubick. High-yield production of "difficult-to-express" proteins in a continuous exchange cell-free system based on CHO cell lysates. *Scientific Reports*, 7(1), 2017.
- [138] Valérie Le Fourn, Pierre Alain Girod, Montse Buceta, Alexandre Regamey, and Nicolas Mermoud. CHO cell engineering to prevent polypeptide aggregation and improve therapeutic protein secretion. *Metabolic Engineering*, 21:91–102, 2014.

- [139] Maureen Spearman, Sarah Chan, Vince Jung, Vanessa Kowbel, Meg Mendoza, Vivian Miranda, and M Butler. Components of yeast (*Sacchromyces cervisiae*) extract as defined media additives that support the growth and productivity of CHO cells. *Journal of Biotechnology*, 233:129–142, 2016.
- [140] Yeon Sook Choi, Doo Young Lee, Ick Young Kim, Hong Jin Kim, Hong Woo Park, Tae Boo Choe, and Ik-Hwan Kim. Enhancement of Erythropoietin Production in Recombinant Chinese Hamster Ovary Cells by Sodium Lactate Addition. *Biotechnology and Bioprocess Engineering*, 12:60–72, 2007.
- [141] Holger Schielzeth, Niels J. Dingemanse, Shinichi Nakagawa, David F. Westneat, Hassen Allegue, Céline Teplitsky, Denis Réale, Ned.A. Dochtermann, László Z. Garamszegi, and Yimen G. Araya-Ajoy. Robustness of linear mixed-effects models to violations of distributional assumptions. *Methods in Ecology and Evolution*, 2020.
- [142] Cora J.M. Maas and Joop J. Hox. The influence of violations of assumptions on multilevel parameter estimates and their standard errors. *Computational Statistics and Data Analysis*, 46:427–440, 2004.
- [143] R. E. Glaser. Levene’s Robust Test of Homogeneity of Variances. In S Kotz, Balakrishnan N, B Vidakovic, and N L Johnson, editors, *Encyclopedia of Statistical Sciences*. John Wiley & Sons, New York, 2006.
- [144] Xintao Zhang, Hongping Tang, Ya-Ting Sun, Xu-Ping Liu, Wen-Song Tan, and Li Fan. Elucidating the effects of arginine and lysine on a monoclonal antibody C-terminal lysine variation in CHO cell cultures. *Applied Microbiology and Biotechnology*, 99:6643–6652, 2015.
- [145] Christopher A. Sellick, Alexandra S. Croxford, Arfa R. Maqsood, Gill Stephens, Hans V. Westerhoff, Royston Goodacre, and Alan J. Dickson. Metabolite profiling of recombinant CHO cells: Designing tailored feeding regimes that enhance recombinant antibody production. *Biotechnology and Bioengineering*, 108(12):3025–3031, 2011.
- [146] Fereidoun Mahboudi, Mohammad Reza Abolhassan, Armita Azarpanah, Hamideh Aghajani-Lazarjani, Mohammad Amin Sadeghi-Haskoo, Shaian Maleknia, and Behrouz Vaziri. The Role of Different Supplements in Expression Level of Monoclonal Antibody against Human CD20. *Avicenna Journal of Medical Biotechnology*, 5(3), 2013.
- [147] Mutsumi Takagi, Hui Ching Hia, Jin Ho Jang, and Toshiomi Yoshida. Effects of High Concentrations of Energy Sources and Metabolites on Suspension Culture of Chinese Hamster Ovary Cells Producing Tissue Plasminogen Activator. *Journal of Bioscience and Bioengineering*, 91(5):515–521, 2001.

- [148] Victor V.T. Wong, Peter M. Nissom, Siow Leng Sim, Jessna H.M. Yeo, Song Hui Chuah, and Miranda Yap. Zinc as an insulin replacement in hybridoma cultures. *Biotechnology and Bioengineering*, 93(3):553–563, 2006.
- [149] Zhanyou Yun, Mutsumi Takagi, and Toshiomi Yoshida. Effect of antioxidants on the apoptosis of CHO cells and production of tissue plasminogen activator in suspension culture. *Journal of Bioscience and Bioengineering*, 91(6):581–585, 2001.
- [150] Jinyou Zhang, David Robinson, and Peter Salmon. A novel function for selenium in biological system: Selenite as a highly effective iron carrier for Chinese hamster ovary cell growth and monoclonal antibody production. *Biotechnology and Bioengineering*, 95(6):1188–1197, 2006.
- [151] Do Yun Kim, Joon Chul Lee, Ho Nam Chang, and Duk Jae Oh. Development of serum-free media for a recombinant CHO cell line producing recombinant antibody. *Enzyme and Microbial Technology*, 39, 2006.
- [152] Kentaro Sakai, Toshio Matsunaga, Chisa Hayashi, Hideki Yamaji, and Hideki Fukuda. Effects of phosphatidic acid on recombinant protein production by Chinese hamster ovary cells in serum-free culture. *Biochemical Engineering Journal*, 10:85–92, 2002.
- [153] Hideo Miki and Mutsumi Takagi. Design of serum-free medium for suspension culture of CHO cells on the basis of general commercial media. *Cytotechnology*, 67:689–97, 2015.
- [154] A.E. Morris and J. Schmid. Effects of Insulin and LongR3 on Serum-Free Chinese Hamster Ovary Cell Cultures Expressing Two Recombinant Proteins. *Biotechnology Progress*, 16:693–697, 2000.
- [155] James A Zanghi, Wolfgang A Renner, James E Bailey, and Martin Fussenegger. The Growth Factor Inhibitor Suramin Reduces Apoptosis and Cell Aggregation in Protein-Free CHO Cell Batch Cultures. *Biotechnology Progress*, 16:319–325, 2000.
- [156] Paula Castro, Andrew P. Ison, Paul M. Hayter, and Alan Bull. CHO cell growth and recombinant interferon- $\gamma$  production: Effects of BSA, Pluronic and lipids. *Cytotechnology*, 19:27–36, 1995.
- [157] Fatemeh Davami, Lucia Baldi, Yashas Rajendra, and Florian M Wurm. Peptone Supplementation of Culture Medium Has Variable Effects on the Productivity of CHO Cells. *International Journal of Molecular and Cellular Medicine*, 3(3):146–56, 2014.

- [158] Bok Hwan Chun, Jong Hwang Kim, Ho Joung Lee, and Namhyun Chung. Usability of size-excluded fractions of soy protein hydrolysates for growth and viability of Chinese hamster ovary cells in protein-free suspension culture. *Biore-source Technology*, 98(5):1000–1005, 2007.
- [159] Jee Yon Kim, Yeon-Gu Kim, Young Kue Han, Hyun Soo Choi, Young Hwan Kim, and Gyun Min Lee. Proteomic understanding of intracellular responses of recombinant Chinese hamster ovary cells cultivated in serum-free medium supplemented with hydrolysates. *Applied Microbiology and Biotechnology*, 89:1917–1928, 2011.
- [160] Mathilde Mosser, Isabelle Chevalot, Eric Olmos, Fabrice Blanchard, Romain Kapel, Eric Oriol, I. Marc, and A. Marc. Combination of yeast hydrolysates to improve CHO cell growth and IgG production. *Cytotechnology*, 65:629–41, 2013.
- [161] Sung Kwan Yoon and Myeong Hyeon Jeong. Adaptation of Chinese Hamster Ovary Cells to High Potassium Ion-Containing Medium for Enhancement of Follicle-Stimulating Hormone Production. *Biotechnology and Bioprocess Engineering*, 12:399–403, 2007.
- [162] Hong Liang, Baojin Fei, Hu Shen, Tao Lei, Chunlei Song, Xiaojiao Li, Yantao Nie, and Liheng Wu. Improve the production of recombinant human factor ix in CHO cells by adding metal ions. *Biomedical Research*, 28(5):2095–2098, 2017.
- [163] Jun Luo, Natarajan Vijayasankaran, Jennifer Autsen, Rodell Santuray, Terry Hudson, Ashraf Amanullah, and Feng Li. Comparative metabolite analysis to understand lactate metabolism shift in Chinese hamster ovary cell culture process. *Biotechnology and Bioengineering*, 109(1):146–156, 2012.
- [164] A. V. Carvalhal, S. SÁ Santos, Jose Calado, Matthias Haury, and M. J. T. Carrondo. Cell Growth Arrest by Nucleotides as a Tool for Improved Production of Recombinant Proteins. *Biotechnology Progress*, 19(1):69–83, 2003.
- [165] Fei Chen, Zhaoyang Ye, Liang Zhao, Xu-Ping Liu, Li Fan, and Wen-Song Tan. Biphasic addition strategy of hypoxanthine and thymidine for improving monoclonal antibody production. *Journal of Bioscience and Bioengineering*, 114(3):347–352, 2012.
- [166] Fei Chen, Li Fan, Jiaqi Wang, Yan Zhou, Zhaoyang Ye, Liang Zhao, and Wen-Song Tan. Insight into the roles of hypoxanthine and thymidine on cultivating antibody-producing CHO cells: cell growth, antibody production and long-term stability. *Applied Microbiology and Biotechnology*, 93:169–178, 2012.
- [167] Sung Kwan Yoon and Yong-Ho Ahn. Application of sodium propionate to the suspension culture of chinese hamster ovary cells for enhanced production of follicle-stimulating hormone. *Biotechnology and Bioprocess Engineering*, 12:497–501, 2007.

- [168] Chi Hsien Liu, I-Ming Chu, and Shiaw-Min Hwang. Enhanced expression of various exogenous genes in recombinant chinese hamster ovary cells in presence of dimethyl sulfoxide. *Biotechnology Letters*, 23:1641–1645, 2001.
- [169] Christopher A. Sellick, Alexandra S. Croxford, Arfa R. Maqsood, Gill M. Stephens, Hans V. Westerhoff, Royston Goodacre, and Alan J. Dickson. Metabolite profiling of CHO cells: Molecular reflections of bioprocessing effectiveness. *Biotechnology Journal*, 10:1434–1445, 2015.

## Appendix A

### Media Additives

Category	Additives	Paper
Amino Acid	Alanine	[82], [99]
	Arginine	[82], [144]
	Asparagine	[90], [65], [82], [62], [145]
	Aspartate	[145]
	Aspartic Acid	[82]
	Citrulline	[139]
	Cysteine	[60], [62]
	Essential Amino Acid	[146]
	Glutamate	[70], [65], [145]
	Glutamic Acid	[82], [125]
	Glutamine	[70], [82], [125], [60], [124], [62]
	Glycine	[82]
	Glycine betaine	[49]
	Histidine	[82]
	Isoleucine	[82]
	Leucine	[82], [99]
	Lysine	[82], [144]
	Lysine HCl	[99]
	Methionine	[82], [60]
	Non-essential Amino Acid	[146]
	Ornithine	[139]
	Phenylalanine	[82]
	Proline	[90], [65], [82]
Serine	[90], [65], [82], [62]	

Category	Additives	Paper
	Threonine	[82], [60]
	Tryptophan	[99]
	Tyrosine	[82], [62]
	Valine	[82]
Antibiotic	Penicillin	[147], [148]
	Streptomycin	[147], [148]
Antioxidant	$\alpha$ - ketoglutarate	[125]
	Ascorbic Acid 2- Phosphate	[149]
	$\beta$ - mercaptoethanol	[148]
	Baicalein	[131]
	Catalase	[128]
	Citric Acid	[125]
	Glutathione	[54], [149]
	$\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$	[60], [99]
	NAC	[56]
	Sinapic Acid	[82]
	Sodium Selenate	[150]
	Sodium Selenite	[118], [78], [151], [152], [150], [54]
	Succinic acid	[125]
	Tropolone	[150]
Buffer	PBS	[77]
Carbohydrate	Fructose	[70]
	Galactose	[70], [65], [63]
	Glucose	[70], [65], [72], [71], [145]
	Lactose	[100], [72]
	Lactulose	[100]
	Maltose	[72], [71]
	Mannose	[70], [100]
	Melezitose	[100]

Category	Additives	Paper
Growth Factor	Palatinose	[100]
	Psicose	[100]
	Raffinose	[100]
	Sucrose	[72]
	Trehalose	[100], [72]
	Turanose	[100]
	EGF	[153]
	Hydrocortisone	[60], [128], [54]
	IGF-1	[153]
	Insulin	[132], [60], [153], [154], [152], [99], [84], [148], [155]
Hydrolysate	LongR3 IGF-1	[151], [154]
	BSA	[156], [132], [128]
	Casein Peptone Plus	[157]
	Human Serum Albumin	[146]
	Peptone	[128], [146]
	Primatone	[81], [139]
	Transferrin	[132], [152]
	Tryptone N1	[157]
	Cottonseed Hydrolysate	[77]
	Cottonseed Hydrolysate (7504)	[124]
	animal component-free hydrolysate	[151]
	Ex-Cell CD Hydrolysate	[119]
	Lucratone Broadbean	[133]
	Pea Peptone (7401)	[118]
Rapeseed Peptide	[132], [82]	
SR3	[151]	



<b>Category</b>	<b>Additives</b>	<b>Paper</b>
	Rice hydrolysate	[158]
	Rice hydrolysate (CWBI)	[116]
	Rice Peptone (5115)	[135], [118], [116], [99]
	Bacto Soytone	[158]
	Glycine max Soybean	[119]
	Peptone	
	Hy-Soy	[121], [122]
	Hy-Soy UF	[158], [99]
	HyQ Soy hydrolysate UF	[158]
	Phytone Peptone	[158], [81]
	Lucratone Soy F	[133]
	Lucratone Soy P	[133]
	Select soytone	[158]
	Soy Hydrolysate	[158],[159], [133]
	Soy Hydrolysate (1510)	[60], [151], [124], [81]
	Soy Hydrolysate UF	[158]
	Soy Peptone E110	[157]
	Soy Peptone UF	[81]
	Soy Plus UF	[81]
	Wheat-rice Hydrolysate	[158]
	Wheat Gluten Hydrolysate	[158], [159]
	Wheat Hydrolysate	[158], [133]
	Wheat Hydrolysate (4601)	[119], [60], [124], [81], [99]
	Wheat Hydrolysate (4602)	[151]
	Wheat Hydrolysate (4605)	[117], [118]
	Wheat Peptone 2a	[81]
	Wheat Peptone 2b	[81]
	Yeast extract	[158], [120], [128], [146], [160], [139]

Category	Additives	Paper
	Yeast extract (7455)	[151]
	Yeast extract UF	[158], [81]
	Yeast hydrolysate	[158], [133]
	Yeast hydrolysate UF	[119]
	Yeast lysate	[139]
	Yeast lysate (3KF)	[139]
	Yeast lysate (3KR)	[139]
	Yeast Peptone	[160]
	Yeastolate	[158], [60], [159]
	Yeastolate UF	[81],[99]
Inorganic Acid	HCl	[154]
Inorganic Salt	KCl	[161]
	MgCl <sub>2</sub>	[63]
	NaCl	[49], [40], [140]
Lipid	Albumax	[128]
	Cell-Ess	[129]
	Cholesterol	[156],[128], [54]
	Cod liver oil fatty acids	[54]
	Ethanolamine	[156], [60], [151], [152], [99], [148], [54]
	Gibco 11905	[65]
	Linoleic acid BSA	[156], [128]
	Lipid Concentrate	[90], [128]
	Lipid mixture	[156]
	Lipid supplement	[146]
	LPA	[153]
	Oleic acid	[156], [128]
	Phosphatidic acid	[152]
	Phosphatidylcholine	[60], [151], [99]
Metal	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O	[78]

Category	Additives	Paper
	Ca <sup>2+</sup>	[162]
	Cobalt Chloride	[127]
	Copper	[163], [83]
	CuCl <sub>2</sub>	[60], [151], [99]
	CuSO <sub>4</sub>	[73]
	CuSO <sub>4</sub> ·5H <sub>2</sub> O	[78], [150]
	Fe	[130]
	Fe(NO <sub>3</sub> ) <sub>3</sub> ·9H <sub>2</sub> O	[99], [151]
	Ferric chloride	[150]
	Ferric citrate	[118], [60], [151], [99], [150]
	Ferrous Sulfate	[150]
	Lithium Chloride	[87]
	Mg <sup>2+</sup>	[162]
	MnSO <sub>4</sub> ·H <sub>2</sub> O	[78]
	NH <sub>4</sub> VO <sub>3</sub>	[78]
	Nickel Chloride	[127]
	ZnCl <sub>2</sub>	[150]
	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	[86], [60], [78], [151], [99], [84], [148]
Nucleoside	Adenine	[164]
	Adenosine	[164]
	ADP	[164]
	AMP	[164]
	ATP	[164]
	CDP	[164]
	CMP	[164]
	CTP	[164]
	Cytidine	[164]
	Cytosine	[164]

Category	Additives	Paper
	Deoxycytidine	[59]
	Deoxyuridine	[59]
	FAD	[164]
	GDP	[164]
	GMP	[164]
	GTP	[164]
	Guanine	[164]
	Guanosine	[164]
	Hypoxanthine	[165], [166]
	NAD	[164]
	NADH	[164]
	NADP	[164]
	NADPH	[164]
	Thymidine	[165], [166], [59]
	UDP	[164]
	UMP	[164]
	Uracil	[164]
	Uridine	[164], [63], [126]
	UTP	[164]
Organic Acid	Butyric Acid	[110]
	Pyruvate	[145]
	Pyruvic acid-Na	[60]
	Sodium Butyrate	[64], [46], [94], [88], [56], [74], [75]
	Sodium Lactate	[140]
	Sodium Propionate	[167]
	Sodium Pyruvate	[124], [54]
	Valeric acid	[110]
	Valproic acid	[76]
Other	Aurintricarboxylic acid	[86], [153]

Category	Additives	Paper
	DMSO	[131], [168]
	MTX	[49], [162], [147]
Polyamine	Putrescine	[60], [128], [139], [99], [54]
	Spermidine	[139]
	Spermine	[139]
Polysulfate	Dextran Sulfate	[60], [155], [115]
	Polyvinyl Sulfate	[155]
	Suramin	[155]
Premade	486F	[151]
	ActiCHO Feed A	[123]
	ActiCHO Feed B	[123]
	Cell Boost 7A	[40]
	Cell Boost 7B	[40]
	CHO CD EfficientFeed A	[119], [124], [123], [169]
	CHO CD EfficientFeed B	[119], [169]
	CHO Xtreme feed	[119]
	FunctionMAX	[123]
	ITS	[146]
	Panexin NTS	[146]
	Power feed	[146]
	Sigma Supplement	[146]
	SITE	[128]
	Xtreme feed	[146]
Serum	dFBS	[49],[99]
	FBS	[118]
	NBS	[149]
	NCS	[147]
	Serum	[151]

<b>Category</b>	<b>Additives</b>	<b>Paper</b>
Surfactant	Pluronic F-68	[118], [156], [60], [151], [152], [99], [148]
	Tween 80	[54], [152]
Vitamin	Ascorbic Acid	[60], [54]
	Choline	[156]
	Choline Chloride	[54]
	Cyanobalamin	[54]
	D-a-tocopherol acetate	[54]
	D-Calcium Pantothenate	[54]
	Folic Acid	[54]
	I-inositol	[54]
	Niacinamide	[54]
	Pyridoxine HCl	[54]
	Riboflavin	[54]
	Sigma M6895	[90], [65]
	Thiamine HCl	[54]
V-3FB	[90]	
Vitamin K1	[162]	